Received: 14 August 2019 | Revised: 12 October 2019 | Accepted: 1 November 2019 | Published online: 15 February 2020

DOI: 10.1002/dad2.12000

Alzheimer's & Dementia
Diagnosis, Assessment & Disease Monitoring

DIAGNOSTIC ASSESSMENT & PROGNOSIS

Classifying dementia progression using microbial profiling of saliva

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Abstract

Introduction: There is increasing evidence linking periodontal infections to Alzheimer's disease (AD). Saliva sampling can reveal information about the host and pathogen interactions that can inform about physiological and pathological brain states.

Methods: A cross-sectional cohort of age-matched participants (78) was segmented according to their chemosensory (University of Pennsylvania Smell Identification Test; UPSIT) and cognitive scores (Mini-Mental State Exam; MMSE and clinical dementia rating; CDR). Mid-morning saliva was sampled from each participant and processed for microbiome composition and cytokine analysis. Linear discriminant analysis (LDA) was used to unravel specific changes in microbial and immunological signatures and logistic regression analysis (LRA) was employed to identify taxa that varied in abundance among patient groups.

Results: Using olfaction we distinguish in the cognitively normal population a segment with high chemosensory scores (CNh, 27) and another segment with chemosensory scores (CNr, 16) as low as mild cognitive impairment (MCI, 21) but higher than the AD group (17). We could identify stage-specific microbial signatures changes but no clearly distinct cytokine profiles. Periodontal pathogen species as *Filifactor villosus* decline with the increasing severity of AD, whereas opportunistic oral bacteria such as *Leptotrichia wadei* show a significant enrichment in MCI.

Conclusions: The salivary microbiome indicates stage-dependent changes in oral bacteria favoring opportunistic species at the expense of periodontal bacteria, whereas the inflammatory profiles remain mainly unchanged in the sampled population.

KEYWORDS

Alzheimer's disease, cytokines, olfaction, oral microbiome

1 | BACKGROUND

A plethora of studies has accumulated evidence about a dysbiosis of microbial pathogens colonizing the head and neck area and their dissemination to the brain in the progression of AD.[1] In particular, *Porphyromonas gingivalis (P. ginigivalis)*, a keystone periodontal pathogen, and its antigens gingipains were found in the brain specimen from AD patients and its abundance correlated positively with the pathological progression of the disease.[2] Interestingly, levels of *P. gingivalis* in saliva were inversely correlated to CSF in the AD subjects examined

suggesting a potential evasion of the periodontal pathogens from the oral compartment to the brain. In this paper, we have used olfactory and cognitive scores to behaviorally segment the population, which was sampled for microbial and inflammatory salivary titers. Olfactory decline is an overarching preclinical sign of dementia and a strong predictor of impending neurodegenerative processes during aging.[3,4] Chemosensory testings are often implemented in the first medical screening along with memory testing. The UPSIT called also "scratch and smell test" measures olfactory discrimination and identification, relying on central neural processing from the olfactory bulb to the olfactory cortices.[5] The olfactory circuitry is one of the first structures displaying a pronounced tauopathy already in healthy aging,[6] and is one of the two central nervous system structures with direct access to the external environment representing an anatomical port of entry for neurotoxic species that can disseminate to the brain.[4] In support of the infectious hypothesis of AD, the present salivary microbiome composition analysis indicates stage-specific changes, but cytokine profiling shows only subtle variations in pro-inflammatory chemokines. The present study supports the use of saliva as a monitoring biofluid for tracking the periodontal oral health in connection with brain aging.

RESEARCH IN CONTEXT

1. Systematic review: A bibliographic research on PubMed revealed 26 original manuscripts investigating salivary biomarkers for Alzheimer's disease (AD) but no report about the microbial salivary content.
2. Interpretation: We observe specific changes in oral microbiome composition with the onset of olfactory deficits, progressing into mild cognitive impairment (MCI) and AD.
3. Future direction: Expanding the sampling to a larger multicentric cross-sectional and a longitudinal cohort with matched cerebrospinal fluid (CSF) or brain imaging data will confirm how oral microbial signatures and inflammatory responses change in the progression of AD.

2 | MATERIALS AND METHODS

The study was approved by the Swiss Ethics Board of the Canton of Vaud and Fribourg under the protocol n. CER-VD 2016-01627.

2.1 | Study participants

Participants were recruited at the Cantonal Hospital of Fribourg Memory Clinic. At the visit, patients with cognitive impairment and their accompanying partner, serving as controls, provided written consent, and all data collection was in compliance with the clinical protocol CER-VD 2016-01627 (selection criteria in Table S1). All participants were asked not to ingest food for at least 2 hours before the mid-morning visit (9–11 am). Before starting the test, they rinsed their mouth with an antiseptic solution followed by 4 washes with tap water and then underwent cognitive testing (MMSE) and chemosensory probing (UPSIT; 16 odors[7]) (Sensonic International) taking typically 30 minutes all together. CDR assessment was done retrospectively based on the medical records or the interview with the certified clinical nurse (Table 1). After behavioral testing, about 2 mL of whole unstimulated saliva was sampled and processed as described previously.[8]

2.2 | APOE genotyping and 16S amplicon sequencing

Purified DNA from saliva (Norgen Biotech) was genotyped for apolipoprotein E (*APOE*) variants ($\varepsilon 2$, $\varepsilon 3$, $\varepsilon 4$) using multiplex tetraprimer amplification according to a published protocol.[9] For the microbiome composition, V3-V4 16S ribosomal RNA (rRNA) amplicon sequencing was performed on an Illumina MiSeq platform using standard protocols. The raw sequence data are available on request. The estimation of bacterial diversity within and between samples was performed with qiime2-2019.4.[10] The Illumina BaseSpace 16S metagenomics tool was used to characterize the bacterial species, and their abundance in the patients' samples (Supplementary File 1).

2.3 | Bead-based immunoassay

Fifteen microliters of saliva supernatant were probed using an inflammatory cytokines detection kit (Human Inflammation 16-Plex Panel, Aimplex). Samples were run on the BD LSRII flow cytometer (BD Biosciences). The data were evaluated using the open access flow cytometry data analysis software (http://flowingsoftware.btk.fi/). Outliers were excluded in the final analysis (Supplementary File 2).

TABLE 1 Classification of the cross-sectional study cohort

Variable	CNh	CNr	MCI	AD
N (F)	27 (16)	15 (6)	21 (13)	17 (11)
Age	67.0 ± 9.2	68.1 ± 10.0	73.2 ± 8.1	71.1 ± 6.6
BMI	26.3 ± 5.0	25.2 ± 4.6	24.6 ± 4.6	26.0 ± 4.1
MMSE	28.4 ± 1.1	28.4 ± 1.5	22.5 ± 1.5‡	14.2 ± 4.3‡
CDR	0.0 ± 0.2	0.1 ± 0.2	0.8 ± 0.3‡	1.4 ± 0.6‡
UPSIT(%)	0.7 ± 0.1	0.5 ± 0.1‡	0.5 ± 0.2‡	0.3 ± 0.2‡,#
APOEε4	10	5	10	8
Education*	1.5 ± 0.6	2.1 ± 0.8	1.6 ± 0.7	1.7 ± 0.7

Values are means ± SD.
F, female; M, male, MMSE, Mini-Mental Status Exam; UPSIT, University of Pennsylvania Smell Identification Test.
*Is for level of education: 1 = <12 years of school; 2 = 12–15 years of school.
‡$P < 0.01$ from pairwise comparison with CNh using Student's t test.
#$P = 0.05$ from pairwise comparison with MCI using Student's t test.

3 | RESULTS

3.1 | Clinical diagnosis

Participants are of European origin, representative of both sexes, age-matched, with comparable body mass index (BMI) and the same level of education. In the cohorts, the *APOE* $\varepsilon 4$ allele distribution is aligned with the *APOE* $\varepsilon 4$ prevalence in Europeans ($\chi^2 = 1.01$, df = 1, $P = 0.5$).[11] The subjects were segmented in groups according to their cognitive (MMSE and CDR) and olfactory scores (UPSIT; Table 1), of which MMSE and UPSIT are positively correlated (Figure S1A). In the cognitively normal group, we identified two populations, one with high olfactory scores (CNh = cognitively normal healthy) and one other with hyposmia (CNr = cognitively normal at risk; $t = 7.6$, $P < 0.001$) (Figure S1B). Patients with MCI identified on the basis of their MMSE/CDR scores (Table 1) also reported hyposmia ($t = 6.7$, $P < 0.001$). Patients with an MMSE below 20 and CDR greater than 1 are classified as AD and display the most severe olfactory impairment of all groups ($t = 8.9$, $P < 0.001$).

3.2 | Oral microbiome analysis

Bacterial diversity does not differ among groups (Figure S1C) Nevertheless, linear discriminant analysis (LDA) applied to the oral bacterial content, reveals group-specific microbiome signatures (Figure 1A) but no clear inflammatory profiles, based on the sampling of 16 cytokines of the innate and adaptive immunity (Figure 1B and Table S2). The strength of the LDA model in identifying stage-specific profiles was tested for both microbiome and cytokines. The LDA applied on the microbiome shows an accuracy of 0.94 (95% CI: 0.92, 0.95; $P < 0.001$), in contrast to the LDA applied to cytokines displaying an accuracy of 0.52 (95% CI: 0.48, 0.55; $P < 0.001$). Furthermore, the cladograms (Figure S2A, C, and E) represent the phylogenetic relationship between the bacterial taxa, which vary in abundance in the pairwise comparison between the CNh group and the CNr, MCI, and AD groups. The linear discriminant analysis effect size (LEfSe) shows a depletion of bacterial taxa in MCI as compared to the other conditions (Figure S2B, D, and F) and a systematic difference in *Filifactor* species between CNh and the other groups. Indeed, *Filifactor villosus* (*F. villosus*) decreases progressively from CNr to AD ($F_{3,74} = 3.5$, $P < 0.05$), whereas *Leptotrichia wadei* (*L. wadei*) increases in abundance from CNr to MCI and then decays in AD ($F_{3,74} = 5.1$, $P < 0.01$) Figure 1C. In addition, *Porphyromonas gingivalis* (*P. gingivalis*) declines in MCI as compared to CNh ($U = 376$, $P < 0.001$) (Figure 1D). Species being depleted in MCI as compared to CNh are *Prevotella tannerae* (*P. tannerae*; $U = 358$, $P = 0.05$), *Filifactor alocis* (*F. alocis*; $U = 399$, $P = 0.05$), while *Cardiobacterium valvarum* (*C. valvarum*; $U = 177$, $P = 0.05$) (Figure 1E) is increased. Correlation analysis conducted for each group show the positive interactions among *P. gingivalis* and *Filifactor* species (CNh, MCI, and AD) and between *P. gingivalis* and *L. wadei* (CNr) (Figure S3). Among the cytokine measured in saliva, IL-1α and IL-8 levels tend to be higher in CNr as compared to CNh but not to a significant extent within the population sampled (IL-1α, $H = 2.1$, $P = 0.15$; IL-8, $H = 1.86$, $P = 0.178$). Only in CNr but not in the other conditions, the interaction between IL-1α and IL-8 is moderately high ($r_s = 0.71$, $P < 0.01$). In MCI, IL-6 levels and MIP-1α show a higher but not significant trend as compared to CNh (IL-6, $H = 0.8$, $P = 0.3$; MIP-1α, $H = 1.59$, $P = 0.2$). Interestingly, IL-6 and MIP-1α expression are highly correlated in all conditions supporting a synergistic interaction ($r_s > 0.8$), with the strongest association in CNr ($r_s = 0.98$, $P < 0.001$) and MCI ($r_s = 0.94$, $P < 0.001$).

3.3 | Predictive value of microbial signatures models

Logistic regression analysis test the predictive values of microbial species that differentiate alone or in aggregate the clinical groups (CNr, MCI, AD) as opposed to controls (CNh), considering MMSE as the dependent variable. In the distinction CNh versus CNr, *F. alocis* and *F. villosus* are the best differentiators of the conditions (Figure 1F), with highest sensitivity (0.76 and 0.62) but modest specificity (0.44 and 0.55) and surpass the aggregate species model (Figure 1F and Figure S4). In the distinction CNh versus MCI, *F. villosus* and *L. wadei* are the best differentiators (Figure 1F′), with moderate sensitivity (0.68 and 0.62) and specificity (0.55 and 0.59), whereas the aggregate model of all species shows the best discriminating capacity with the highest sensitivity (0.72) and specificity (0.66). In the comparison, CNh versus AD, *F. villosus* and *P. tannerae* are the best differentiators (Figure 1F″), with a moderate sensitivity (0.63 and 0.65) and moderate specificity (0.56 and 0.53). Also in this case, the aggregate model of all species shows the best discriminating capacity, with moderate sensitivity (0.67) and specificity (0.60). The mixed model of *L. wadei* combined with UPSIT strongly differentiate CNr, MCI, and AD as compared to CNh (Figure 1G-G″) with comparable sensitivity (0.76, 0.75, and 0.78) and moderately high specificity (0.63, 0.68, and 0.66), respectively.

4 | DISCUSSION

Olfactory deficit is an overarching preclinical sign of dementia.[3,4] In our study, olfactory phenotyping helped to identify subjects at risk, who retained high cognitive performance but were differentiated based on their chemosensory scores. These participants displayed an olfactory deficit comparable to MCI, whereas AD cases showed the strongest smell deficit, likely aggravated by their cognitive impairment.[12] Our operating framework supports that CNr is likely an asymptomatic clinical condition preceding MCI and AD. Along with the behavioral phenotyping, in an effort to discover novel non-invasive diagnostic methods, we have employed salivary microbiome profiling with cytokine fingerprinting to assess pathogen-host interactions differentiating and staging AD. Previous work has shown that saliva can be employed as an accessible biofluid for biomarkers that are diagnostic of neurological conditions. A most recent study has indicated that in addition to amyloid β (Aβ)[13] and phosphorylated tau species,[14] which are detectable in the saliva of severe patients, the antimicrobial humoral factor, lactoferrin, declines with the progression of AD.[15] These data strongly

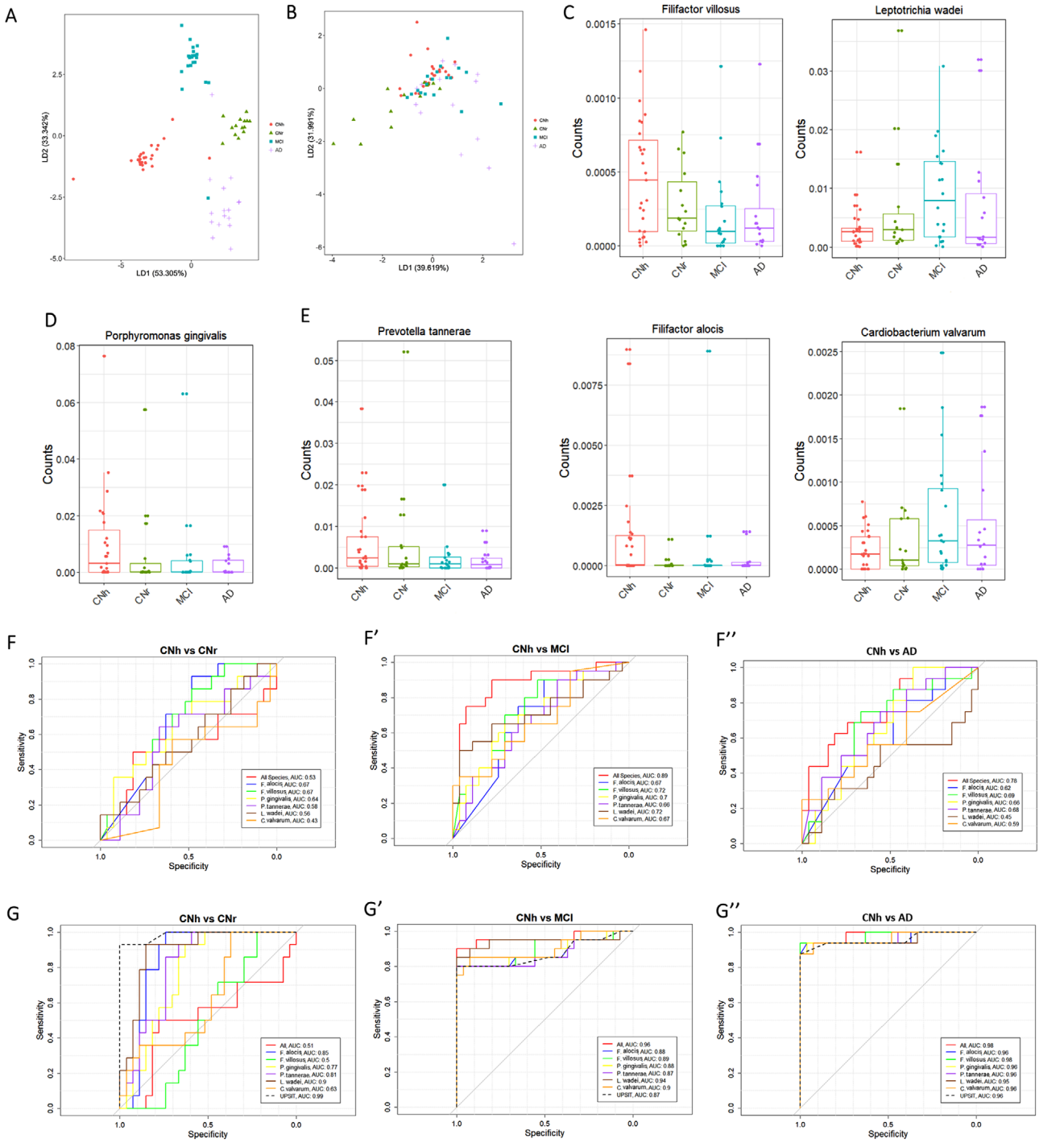

FIGURE 1 Microbiome and inflammation profiling of saliva. A) LDA plots indicate that the oral microbiome composition is stage-specific. B) LDA analysis does not reveal any stage-specific inflammatory signatures. C) *L. wadei* and *F. villosus* diverge significantly in CNr, MCI, and AD as compared to CNh. D) *P. gingivalis* is reduced in MCI as compared to CNh. E) *P. tannerae*, *F. alocis*, and *C. valvarum* are significantly changed in AD as compared to CNh and tend to diminish in CNr and MCI. F) Logistic regression using species alone or together shows the discriminating power of DE species for CNh versus the other conditions. G) Logistic regression using a mixed model (DE species + UPSIT, and all DE species + UPSIT = all) improves the differentiating capacity. DE, differentially expressed; LDA, linear discriminant analysis

suggest a microbial dysbiosis with dementia progression. Our study using LDA confirms that despite taxa diversity remains unchanged, there are specific changes in oral bacterial composition, which are stage-dependent. LEfSe shows the strongest depletion in periodontal bacteria in MCI, while *Leptorichia* species tends to increase from CNr to MCI. Leptorichia are opportunistic bacteria found in oral biofilm and saliva. Increased amounts of *L. wadei* are associated with rheumatoid arthritis via stimulating the release of antimicrobial chemokines and

proinflammatory mediators, IL-6 and IL-8.[16] Interestingly, our analysis indicates a synergistic increase in the upper quartile for IL-8 (2.7 folds) and IL-1α (2 folds) in CNr and IL-6 (2.6 folds) and MIP-1α (2.3 folds) in MCI, likely reflecting an innate immune response to the opportunistic microbes in these conditions. On the other hand, the progressive decline in anaerobic periodontal *Filifactor* species[17] is accompanied by a concomitant depletion of the keystone periodontopathic pathogens *P. gingivalis* and *P. tannerae*. A decrease in *P. gingivalis* counts was previously observed in the saliva from AD patients.[2] The depletion in periodontal taxa suggests either a reduction in number of teeth, due to earlier periodontal disease, or an oral dysbiosis with the advancement of opportunistic pathogens in patients with AD. The differentially expressed oral bacterial species alone or in association with UPSIT holds a moderate to strong predictive value in differentiating between clinical conditions (CNr, MCI, and AD) as compared to healthy controls (area under the curve [AUC] = 0.67–0.96). The differential expression of microbial flora in the progression of AD is influenced by changes in oral hygiene, which has been shown to deteriorate with the onset of dementia.[18] While this important variable is not examined in this study and will need to be integrated in the future, the progressive changes of the microbial species start at CNr, when cognition is intact, questioning to some extent this confounder. Overall, this pilot work indicates that oral microbial signatures may be employed in larger studies as non-invasive classifiers of dementia alone or in addition to olfactory phenotyping.

ACKNOWLEDGMENTS

This research was supported by the Swiss National Foundation (SNF-163470; LA) and Bundes-Exzellenz-Stipendien für ausländische Forschende (EKSAS) Fellowship from the Swiss Ministry of Education (ESKAS No. 2017.0480; PB). We thank Muriel Jaquet, Romain Badel and Eva Martin for technical assistance. We acknowledge the help of the undergraduate students who contributed to this project: Martina Minoli, Giulia Casal, and Laura Leuenberger.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Bathini P, Foucras S, Dupanloup I, et al. Classifying dementia progression using microbial profiling of saliva. *Alzheimer's Dement*. 2020;12:e12000. https://doi.org/10.1002/dad2.12000