Bacterial rhamnolipids (RLs) in saliva of Alzheimer's disease and Mild Cognitive Impairment patients and correlation with neuroinflammation and cognitive state

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Abstract

Alzheimer's disease (AD) is increasingly affecting the aging population and the estimated prevalence reaches 50 million people worldwide. The need for the discovery of new biomarkers for AD diagnosis is urgent and especially in biological fluids other than cerebrospinal fluid (CSF), as its collection is invasive. Arguments are numerous that chronic bacterial infections might be considered as one of the possible causes of AD. Rhamnolipids (RLs) are bacterial virulence factors, suspicious for dysfunctions and disorders including AD. The aim of this pilot trial was to investigate RLs levels in saliva of Mild Cognitive Impairment (MCI) and AD patients with indirect ELISA. Specifically, salivary RLs were determined in 30 AD patients, 24 MCI patients and 15 cognitively healthy individuals and were found elevated in AD and MCI patients compared to those of the control group. The established biomarkers of AD, tau and Aβ42 amyloid, and the inflammatory markers cyclooxygenases (COX-1 and COX-2) were also determined, to evaluate their possible interdependence from RLs levels. Levels of RLs positively correlate with COX-2 levels and negatively with the mental state according to Mini–Mental State Examination (MMSE) score of donors. Multilinear regression verified the tight interrelation of RLs with COX-2 in saliva of MCI and AD patients. The results of this study stand by the hypothesis of inflammatory involvement in AD and indicate that RLs could be suggested as eventual biomarkers for AD diagnosis using saliva as biological fluid.

Keywords: Biomarkers; Alzheimer's disease; Mild Cognitive Impairment; Saliva; Rhamnolipids; Inflammation

1. Introduction

The need for new biomarkers in other fluids than cerebrospinal fluid (CSF) is of the highest priority for accurate and specific diagnosis of AD and Mild Cognitive Impairment (MCI). Definitive diagnosis of AD, in exclusion of other types of dementia, currently relies on postmortem clinical assessment and pathological evidence [1]. Additionally, AD pathogenic pathways seem to set on course decades before the actual dementia onset, so biomarkers correlating timely with the gradual cognitive deterioration, should be discovered. Furthermore, MCI is considered a possible early phase of several dementia-related disorders (including AD), so better molecular criteria for its study and diagnosis are yet to be defined [2].

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Current biomarkers (usually Aβ42 and tau protein) are measured mainly in the CSF of patients after lumbar puncture, a rather demanding sampling procedure with possible implications on the patient's physical and psychological health and sample integrity [2, 3]. Saliva sampling has been lately highlighted as a promising alternative to lumbar puncture or blood sampling [4–7]. Saliva contains a variety of molecular and microbial analytes, which could constitute effective indicators of both local and systemic disorders. Salivary glands are highly permeable and enveloped by capillaries, allowing the free exchange of blood biomarkers into the saliva-producing cells [8]. Inflammation and oral/periodontal microbiome are highly implicated in AD pathology [9–14]. Lately salivary biomarkers gain the scientific attentiveness while they promise an early AD diagnosis through a non-invasive and of easy collection [2, 6, 15–17].

Inflammation plays a key role in AD, and microbial virulence factors like lipopolysaccharides (LPSs) have been implicated extensively in AD pathology [18–20]. Taking into consideration our previous work that another virulence factor such RLs, were found increased in the CSF and blood serum of AD and MCI patients [21], we focused to investigate the presence of RLs in saliva of AD and MCI patients, aiming to acquire insights for possible association.

Rhamnolipids (RLs) are bacterial glycolipids with virulence-associated and surfactant abilities, mainly known from *Pseudomonas aeruginosa* and from other bacterial species [22–24]. The early infiltration promotion of primary human airway epithelia by *P. aeruginosa* [25], the cytotoxicity induced on fibroblastic cell line [26], the induction of cytogenetic damage in human lymphocytes [27] and the cytotoxic effects on MCF-7 human breast cancer cells [28] of RLs are indicative of their pathogenicity. Cyclooxygenases (COX), known as prostaglandin H synthases, are highly associated with neuroinflammation and AD, especially with the constitutively expressed isoform COX-1 and the inducible isoform COX-2 [29, 30]. Additionally, various extracellular and intracellular stimuli, including LPSs, are able to rapidly induce COX-2 [31–33].

In this study, we firstly examined the levels of virulence factors RLs in saliva of AD and MCI patients in comparison with cognitively healthy individuals, to evaluate whether there is any association with the disease, and if they could constitute potential biomarkers for AD or MCI. Furthermore, we investigated whether RLs are correlated with the inflammatory biomarkers COX-1/2, the established in AD neurodegenerative biomarkers Aβ42 and tau or the mental state of the patients.

### 2. Material and methods

#### 2.1. Materials

The antibody used against RLs was prepared in Laboratory of Biochemistry, Department of Chemistry of the Aristotle University of Thessaloniki, Greece, as previously described [34]. Polyclonal antibodies against COX-1 and COX-2 were produced in rabbit host (#160108 and #160107, respectively) and were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-Aβ42 (#sc-28356) and anti-tau (#AH0042) monoclonal antibodies produced in mouse host were purchased from Santa Cruz (Dallas, TX, USA) and Invitrogen (Waltham, MA, USA), respectively. As secondary antibodies we were used Goat anti-rabbit IgG (#A3687) and goat anti-mouse IgG (#A2429) both bound to alkaline phosphatase (Sigma Aldrich, St. Louis, USA). 96-wells ELISA PS plates (Microlon® high-binding) were purchased from Greiner Bio-One (Kremsmünster, Austria). All other chemicals and solvents used were of great analytical purity.

#### 2.2. Manipulation of saliva samples

The present study has been approved by the 66th Meeting of The Bioethical Committee of the Greek Association of Alzheimer’s disease and Related Disorders (Pr Nr.: 105/2020 AI). Saliva samples from 30 AD and 22 MCI patients and from 18 cognitively healthy individuals were collected after the signed consent of the patients or their legal representatives.

Saliva was collected in morning hours by passive drooling, without using any stimulant, after patients washing their mouth thoroughly with water. Care was given to harvest saliva samples from individuals not suffering from any form of periodontal disease or had active lesions. All samples were immediately centrifuged at 13,500 rpm for 20 min to remove insoluble material, cell debris, bacteria, and possible food remnants and the supernatants have been collected and stored in centrifuge tubes. Finally, samples have been vacuum-dried overnight and re-suspended in sterile dd H₂O containing 0.01% (v/v) Proteases Inhibitor Cocktail (P-8849/Sigma-Aldrich), to receive 15-times concentrated samples. All samples were stored at -80 °C until analysis.

The samples were kindly offered by the 1st Neurological Clinic of the University Hospital of Thessaloniki “AHEPA”, in Thessaloniki, Greece. The demographics characteristics of the survey participants are summarized in Table 1, where
the values displayed represent mean values ± standard deviations (SD). MCI or AD patients were diagnosed based on Petersen and the NINCDS - ADRDA criteria, respectively, using the necessary neurological, neuropsychological, blood, CSF and, neuroimaging examination according the accepted guidelines [35, 36].

Table 1 Demographic and clinical characteristics of AD and MCI patients and healthy controls

<table>
<thead>
<tr>
<th>Demographics</th>
<th>AD</th>
<th>MCI</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants Number (N)</td>
<td>30</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Gender (Male/ Female)</td>
<td>15/15</td>
<td>11/12</td>
<td>6/12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>73.4 ± 3.3</td>
<td>74.2 ± 6.9</td>
<td>68.9 ± 7.8</td>
</tr>
<tr>
<td>Education (years)</td>
<td>7.3 ± 3.9</td>
<td>8.6 ± 5.0</td>
<td>9.2 ± 3.6</td>
</tr>
<tr>
<td>MMSE</td>
<td>16.9 ± 6.6  (^a,b)</td>
<td>24.1 ± 5.0  (^a)</td>
<td>27.94 ± 2.2</td>
</tr>
</tbody>
</table>

Statistical analysis for differences between groups was performed with usage of Graph Pad Prism 8 statistical package. Statistically significant differences (p < 0.05) when comparing with: \(^a\)Control, or \(^b\)MCI. AD: Alzheimer’s disease; MCI: Mild Cognitive Impairment; MMSE: Mini Mental State Exam.

Patients enrolled in the study were chosen strictly from a bigger cohort, setting as criteria during the design of the research the narrow age range of 60-76 years old. Also, the three groups employed showed no statistically significant differences in terms of gender and educational level of the participants.

2.3. ELISA method

The investigation of RLs levels in saliva samples was performed by the method of indirect ELISA, as previously described [21] and under sterile conditions. Concentrated saliva samples were diluted 50-times with PBS, prior to analysis. Diluted samples were coated in 96-well ELISA plates overnight, at room temperature in the hood. Plates were then washed with PBS and blocked with 1% (w/v) bovine serum albumin in PBS for 2 hours, 37°C. Plates were washed again with PBS and diluted primary antibody was added for 2 hours, 37°C. Wells were washed with PBS-0.05% Tween-20 and then incubated with diluted secondary antibody for 2 hours, 37°C. Finally, wells were washed with PBS-0.05% Tween-20 and diethanolamine buffer and incubated with 1 mg/mL p-nitro-phenyl-phosphate in diethanolamine buffer for 2 hours. Reaction was terminated after adding NaOH 0.5 M in the same volume and the absorbance at 405 nm (A405) of the produced color was measured in an ELISA-plate reader. Optimal dilutions for antigens, primary and secondary antibodies were decided after chessboard titration, setting blanks’ low background (A405 < 0.1) as the main selective criterion, as well as linearity between antigen dilution and received A405 value.

2.4. Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc.) has been employed for the statistical analysis and graphs’ construction. Statistical analysis for differences in age, education, or Mini Mental State Examination (MMSE) score was done with Kruskal-Wallis test, followed by Dunn’s multiple comparison Post hoc test, while for gender, Chi-Squared analysis with Yates’ correction was used and the corresponding p values are given in Table 2.

Table 2 Statistical analysis for determination of demographics’ differences (gender, age, education in years and MMSE score) of Table 1, between AD and MCI patients and neurologically healthy individuals, who donated saliva samples.

<table>
<thead>
<tr>
<th>Saliva Donors</th>
<th>AD vs. MCI</th>
<th>AD vs. Control</th>
<th>MCI vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (Male/ Female)</td>
<td>0.9043</td>
<td>0.4086</td>
<td>0.5383</td>
</tr>
<tr>
<td>Age</td>
<td>&gt;0.9999</td>
<td>0.1470</td>
<td>0.0934</td>
</tr>
<tr>
<td>Education (years)</td>
<td>0.9535</td>
<td>0.3271</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>MMSE</td>
<td>0.0021</td>
<td>&lt;0.0001</td>
<td>0.0227</td>
</tr>
</tbody>
</table>

For age, education and MMSE score, Kruskal-Wallis test followed by Dunn’s multiple comparison Post hoc tests were performed, while for gender, Chi-Squared analysis with Yates’ correction was used. Statistical analysis was performed with usage of Graph Pad Prism 8 statistical package. Ns: non-significant; \(^\ast\): p < 0.05, \(^\ast\ast\): p < 0.01, \(^\ast\ast\ast\): p < 0.001, \(^\ast\ast\ast\ast\): p < 0.0001. AD: Alzheimer’s disease; MCI: Mild Cognitive Impairment; MMSE: Mini Mental State Examination.
To evaluate the discrimination between the groups in terms of the studied biomarkers, Mann-Whitney non-parametric test has been used and results are represented as means ± SD. Additionally, ROC curve analysis was performed, to evaluate the possibility of using RLs as biomarkers for MCI or AD in saliva, and ROC curve graphs were plotted. ROC curve graphs were produced by plotting sensitivity (true positive rate - TPR) on the y-axis against 1-specificity (false positive rate - FPR) on the x-axis. The area under the ROC curve (AUC) is generally accepted that measures the ability of a test to discriminate the presence or not of a specific condition [37] and is given the results with 95% confidence interval and the acquired p values. AUC over the value 0.8 means that the test shows a good discriminating ability.

Correlation analysis with Spearman’s test has been used for determining the relationships among analyzed variables, and multilinear regression with backward elimination for evaluating the best model for the determinants of RLs in saliva. COX-1, COX-2, Aβ42 and tau were also analyzed in all saliva samples (data not included). Data were used to proceed with the correlation and the multilinear regression analysis of all these biomarkers with RLs.

For statistical significance to be reached, a value of p < 0.05 has been demanded in all cases.

3. Results

3.1. RLs levels are increased in saliva of AD compared to MCI patients and cognitively healthy individuals.

RLs were analyzed in all saliva samples and were found to be increased in MCI and AD patients, in comparison with cognitively healthy individuals (Fig. 1a). ROC analysis curves were graphed to examine the potential of exploiting RLs as biomarkers in MCI or AD diagnosis and are given in Fig. 1b-c. RLs can discriminate in a good, and significant manner, AD patients from cognitively healthy individuals.

Figure 1 Levels of RLs in (a) saliva of MCI or AD patients and of cognitively healthy donors (control), as analysed with indirect ELISA (A405 nm). Results are given with individual values scatter plots. Black lines present mean values ± standard deviation. (b, c) ROC curve analyses of saliva RLs between the groups that showed statistically significant differences in levels of RLs and their corresponding AUC values. Sensitivity vs 1-Specificity values are graphed for different possible cut-off values discriminating between groups, and AUC values were calculated. Statistical analysis was performed with Graph Pad Prism 8.0 statistical software. AD: Alzheimer’s disease; MCI: Mild Cognitive Impairment; ns: non-significant, *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.000; AUC: Area under the ROC curve. 95% CI: 95% Confidence Interval.

3.2. Correlation of RLs levels with COX-1, COX-2, Aβ42 and tau levels and the mental state of patients

Possible correlations of RLs levels in saliva with COX-1, COX-2, Aβ42 and tau levels and the mental state of the patients were investigated and evaluated for the whole cohort and separately for the groups tested and are given in Fig. 2 and Table 3. RLs are positively correlated in a statistically significant manner with COX-2, suggesting that increase of virulence factor affect the inflammatory enzyme levels COX-2, and negatively with the mental state of the participants, meaning that the augmented RLs levels are linked with worse mental state of the patients. In cognitively healthy
patients, RLs correlate negatively with tau and the mental state of donors. In MCI patients, RLs correlate significantly with COX-2 in a positive manner, while in AD patients no significant correlations were established.

**Figure 2** Correlation analysis of saliva RLs levels with COX-1, COX-2, Aβ42, tau and MMSE score in patients employed in the study. The correlations were evaluated using Spearman’s rank correlation coefficients (r) and their corresponding p values. Statistical analysis was performed with Graph Pad Prism 8.0 statistical software. MMSE: Mini-Mental State Examination.

**Table 3** Correlation analyses of saliva RLs levels with COX-1, COX-2, Aβ42, and tau levels, and MMSE score, in patients with MCI or AD and in cognitively healthy (Control) individuals.

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Whole Cohort</th>
<th>Control</th>
<th>MCI</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>COX-1</td>
<td>-0.069</td>
<td>ns</td>
<td>-0.189</td>
<td>ns</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.581</td>
<td>****</td>
<td>0.477</td>
<td>ns</td>
</tr>
<tr>
<td>Aβ42</td>
<td>0.230</td>
<td>ns</td>
<td>0.139</td>
<td>ns</td>
</tr>
<tr>
<td>tau</td>
<td>0.177</td>
<td>ns</td>
<td>-0.603</td>
<td>*</td>
</tr>
<tr>
<td>MMSE</td>
<td>-0.322</td>
<td>**</td>
<td>-0.503</td>
<td>*</td>
</tr>
</tbody>
</table>

Correlation analysis has been performed with Graph Pad Prism 8.0 statistical package and rank correlation coefficients (r) and their corresponding p values were evaluated using Spearman’s test. MCI: Mild Cognitive Impairment; AD: Alzheimer’s disease; MMSE: Mini Mental State Examination score. Ns: non-significant; *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

3.3. **Multilinear regression of RLs levels with COX-1, COX-2, Aβ42 and tau level and the mental state of patients**

Multilinear regression was carried out both for the whole cohort and separately for the groups tested and results are shown in Table 4. Analysis yields that COX-2 is the main determinant of RLs levels in saliva of MCI and AD patients, as well as for the whole studied cohort. Other biomarkers did not seem to interrelate directly with levels of RLs.
Table 4 Multilinear regression of saliva RLs levels against COX-1, COX-2, Aβ42 and tau levels, and MMSE score, in patients with MCI or AD and in cognitively healthy (Control) individuals.

<table>
<thead>
<tr>
<th>Saliva RLs multilinear regression</th>
<th>Whole Cohort</th>
<th>Control</th>
<th>MCI</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biomarkers</strong></td>
<td><strong>β</strong></td>
<td><strong>p</strong></td>
<td><strong>β</strong></td>
<td><strong>p</strong></td>
</tr>
<tr>
<td>COX-1</td>
<td>-0.0055</td>
<td>ns</td>
<td>-0.0090</td>
<td>ns</td>
</tr>
<tr>
<td>COX-2</td>
<td>0.3132</td>
<td>****</td>
<td>-0.0127</td>
<td>ns</td>
</tr>
<tr>
<td>Aβ42</td>
<td>0.0840</td>
<td>ns</td>
<td>-0.0076</td>
<td>ns</td>
</tr>
<tr>
<td>tau</td>
<td>0.1182</td>
<td>ns</td>
<td>-0.5646</td>
<td>ns</td>
</tr>
<tr>
<td>MMSE</td>
<td>&lt;0.0001</td>
<td>ns</td>
<td>-0.0036</td>
<td>ns</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0015</td>
<td>ns</td>
<td>3.402</td>
<td>**</td>
</tr>
<tr>
<td>R squared</td>
<td>0.3693</td>
<td>****</td>
<td>0.1752</td>
<td>**</td>
</tr>
</tbody>
</table>

Multilinear regression has been performed with Graph Pad Prism 8.0 statistical package. β coefficient degree of change, p values, intercept and R squared values were determined by backward elimination process, for each cohort, until most significantly important model has been achieved. MCI: Mild Cognitive Impairment; AD: Alzheimer’s disease; MMSE: Mini Mental State Examination score. ns: non-significant, *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

A graph for the best model describing levels of RLs in the whole cohort is given in Fig. 3a. Especially for MCI patients, levels of RLs could be modelled in a good, significant manner, and the corresponding graph is given in Fig. 3b.

**Figure 3** Scatter plots of actual RLs values against predicted RLs values by model deriving from multilinear regression analysis for (a) the whole studied cohort, and (b) MCI patients, as affected by COX-1, COX-2, Aβ42, tau or MMSE score. Multilinear regression analysis with stepwise elimination has been performed for the evaluation of the individual effect of COX-1, COX-2, Aβ42, tau and MMSE score. R squared values of the proposed models; p values and the corresponding equation are given in the plot. Statistical analysis was performed with Graph Pad Prism 8.0 statistical software. MMSE: Mini-Mental State Examination; R: R squared values.

4. Discussion

To the best of our knowledge, bacterial RLs have been studied in saliva of patients with MCI or AD and cognitively healthy subjects for the first time in the current study. We have previously studied extensively RLs on blood and CSF. A specific antibody for RLs has been synthesized and characterized [34] and elevated titers of RLs have been verified and
measured in the blood and CSF of patients with MCI and AD [21]. Additionally, RLs levels were previously found in saliva of cystic fibrosis patients chronically infected with *P. aeruginosa*, and scientists proved that augmented RLs levels correlate with worst disease outcome [38]. Production of RLs has been recognized by several bacterial species, some of which are present in the physiological human microflora [24]. Oral microbiome alterations and gut microflora dysbiosis is a well-established matter in MCI and AD patients [39, 40]. Patients with periodontal disease were previously found to present significant deterioration of mental state in comparison with healthy individuals [41]. Additionally, bacteria of the nasal and oral cavities, were previously detected almost exclusively in brains of patients with AD, including *Chlamydia pneumoniae* [42], *Borrelia burgdorferi* [43], and *Porphyromonas gingivalis* [44]. Bacteria colonize human tissues via biofilm, forming a symbiotic structure which enables strong attachment and long proliferation. Components produced by microbes can withstand immune responses and may cross gut or blood-brain barriers [39]. RLs are indeed crucial bacterial products involved in biofilm development, remodeling and disruption, possessing surfactant abilities and enabling the translocation of bacteria to other sites [45, 46]. Increased levels of salivary RLs found here, in line with previous results for blood and CSF [21], indicate disruption of the physiological microflora, either with the presence of non-commensal infectious agents (systemically or centrally), or by altered composition and dysbiosis of the commensal bacteria. RLs over-production by these agents would constitute either a stress reaction or lysis due to unfavorable environment, or even an orchestrated attack against the human tissues.

Several studies have proven the cytotoxic effect of RLs. RLs of *P. aeruginosa* supernatants have been previously recognized to impair polymorphonuclear leukocytes recruitment, leading to damage of plasma membranes, rapid death, and disintegration. Additionally, leukocytes of lung-infected mice with *P. aeruginosa* could not be established in the infected region, due to the necrotic effects of RLs [47]. Additionally, RLs induce permeabilization of cell membranes and β-structure formation and aggregation, by α-synuclein – a crucial amyloid in Parkinson's disease patients. Interestingly enough, RLs presence was crucial for the seeding of further fibrillation [48].

The current study also proved that RLs correlate significantly with COX-2 in saliva and that COX-2 is the main determinant of the levels of RLs - especially in patients with MCI or AD. COX-2 expression is induced by bacterial virulence factors, including LPSs [49] and its found upregulated in AD brain [50, 51]. COX-2 activity has also been linked with β2 aggregation and tau phosphorylation [52, 53]. Increased levels of RLs found in patients of MCI and AD, in cooperation with other virulence factors, could thus assist the aggregation of amyloids, tau phosphorylation and drive neurotoxic effects in brain. Especially in MCI patients, there seems to be a significant interrelation of microbial-inflammatory condition, with - mainly - COX-2 being able to determine in a statistically significant way RLs levels in these patients. Multilinear regression also proved that levels of RLs could better be linked with MCI pathology. It is thus possible that microbial implication is an early event during cognitive deterioration, which is followed by an early upregulation of COX-2. However, this effect seems to silence during the progression to AD, as no correlations between RLs and COX-2 were established in AD patients’ saliva. Indeed, it was previously reported that COX-2 upregulation is an early event in dementia, and the enzyme is later downregulated, in mild and severe AD [50].

These results verify the interrelation of RLs and COX-2, as no other AD-related molecular component seems to affect in an immediate manner the levels of RLs. It is however interesting to assess in the future whether the release of RLs from the co-residing microbiota induces the upregulation of COX-2 or the already-increased COX-2 levels could possibly alter the physiology of the natural microbiota and thus induce a stress-related release of RLs. Also, RLs possess amyloidogenic activity, inducing fibrillation of *Pseudomonas* FapC protein – a bacterial natural amyloid [54]. This points out to a possible mechanism for early fibrillation-neuroinflammation induction in MCI or AD. RLs could induce, amongst other possible alterations, aggregation of Aβ42. Aβ42 aggregates – mainly oligomers, are known inducers of inflammation in AD, binding to a number of inflammatory receptors (including toll-like receptors, which also recognize bacterial components), and then would erupt a cascade of pro-inflammatory mediators that lead to the induction of COX-2 and early neurodegeneration [55, 56].

It is worth mentioned that RLs are correlated in a negative manner with the mental state of patients (as expressed by the MMSE score), an effect mainly driven by cognitively healthy individuals. In these donors, tau levels also correlate significantly, and in a negative manner with RLs. These results imply that the state of microbial flora is implicated in cognition skills and brain status. Commensal microbes produce neuromodulating agents, able to alter neurotransmission, indirectly affect innate immune system and circulating levels of pro- and anti-inflammatory cytokines and so affect brain function and shape human behavior [57].

5. Conclusion
This study addresses for the first time the opportunity of employing salivary microbial products as possible biomarkers in MCI and AD. RLs were found increased in saliva of MCI and AD patients and could be sufficiently employed as a
biomarker for discriminating AD patients from cognitively healthy individuals. RLs also correlate with inducible inflammatory marker COX-2, and negatively with the mental state of participants, underlining the significance of the microbial/ inflammatory etiology of dementia. As the present study is cross-sectional, more research should be conducted on the matter, to verify the effect of RLs on neurodegeneration, employing larger patient cohorts, as well as sampling in several point intervals, during the course of the disease.

**Compliance with ethical standards**

**Acknowledgments**

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The authors thank D. Giagkas for the preparation of the antibody against RLs in a previous study.

**Disclosure of conflict of interest**

The authors declare that they have no conflict of interest.

**Statement of ethical approval**

All procedures performed in studies involving human participants were approved by the 66th Meeting of The Bioethical Committee of the Greek Association of Alzheimer’s disease and Related Disorders (Pr Nr.: 105/2020 AI) and are in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Statement of informed consent**

Informed consent was obtained from all individual participants included in the study and/or their relatives or legal representatives.

**Author Contributions**

EA and GK performed the experiments and wrote the manuscript. AP and MT supervised and organized the research. MT kindly provided the biological fluids and the diagnosis of patients based on Petersen and NINCDS - ADRDA criteria. AP conceptualization; provided resources and corrected the final draft.

**References**


