Antioxidant capacity of human saliva and periodontal screening assessment in healthy adults.

Running title: Saliva and periodontal status.

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Highlights

- Saliva antioxidant levels (SAL) are reduced in patients with periodontal disease
- Recently, a biochemical test for SAL was proposed
- The relationship between clinical evidence and SAL measures was evaluated.
- The SAL test had 84.6% sensitivity and 36% specificity
- The SAL test may detect pre-clinical conditions predisposing to periodontal disease

Abstract

Objective
Saliva plays a pivotal role as an antioxidant system, and saliva antioxidant levels are reduced in patients with periodontal disease. Recently, a biochemical test able to determine saliva antioxidant levels was proposed as predictive for oral cavity diseases, but it was not clinically tested. In this preliminary study, we evaluated the relationships between Periodontal Screening and Recordings characteristics of patients and saliva antioxidant levels measures.

Design
Thirty-nine patients (12 men, 27 women; mean age, 46 years, SD 17) patients attending the dental hygiene unit of a Private Clinic underwent a Periodontal Screening and Recordings examination and a saliva antioxidant levels measurement using a biochemical commercial test. The results of the clinical periodontal examination were compared to those obtained by the saliva test.

Results
Approximately 70% of patients showed a low saliva antioxidant levels value, while the other patients had Optimal/Normal values. Thirteen patients (33%) resulted positive to Periodontal Screening and Recordings test. Using Periodontal Screening and Recordings values as gold standard, the saliva antioxidant levels test correctly classified 52.6% of patients; sensitivity was 84.6%, specificity was 36%.

Conclusions
The saliva antioxidant levels test had a good sensitivity when compared to the gold standard; this finding corroborates the hypothesis that alterations of the oral antioxidant levels are related to periodontal disease. The reduced specificity shows that saliva antioxidant levels test could detect alterations predisposing to periodontal disease before clinically evident aspects.

Key-words: Periodontal conditions; Saliva; Oxidative stress; Saliva antioxidant levels; Periodontal Screening and Recordings
**Introduction**

Periodontal disease (PD), outlined as “An infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment and bone loss” from the American Academy of Periodontology Task Force (2015), is one of the most prevalent bacterial-induced chronic diseases affecting the majority of adults (Dye, 2012; Zare Javid, Seal, Heasman, & Moynihan, 2014). One of its forms, severe periodontitis, with dental attachment loss and periodontal depth larger than 6 mm, was the sixth-most prevalent disease condition, affecting 10.8% or 743 million people worldwide (Kassebaum, Bernabé, Dahiya, Bhandari, Murray, & Marcenes, 2014).

Consequence of untreated PD is tooth loss. The patient-reported outcome measures of tooth loss are reduced functional capacity (e.g. chewing or biting), reduced self-esteem and social relationships, thus resulting in reduced people life quality (Peterson & Ogawa, 2012). Moreover, inflammatory periodontal diseases may be a risk factor for different multiple systemic conditions like metabolic syndrome-MetS (Watanabe & Cho, 2014; Torumtay, Kirzıoğlu, Öztürk Tonguç, Kale, Calapoğlu, & Orhan, 2016) and various chronic diseases (Otomo-Corgel, Pucher, Rethman, & Reynolds, 2012). Interestingly, evidence that mobile oral microbiome should be a reservoir for extra-oral infections and systemic dissemination of pathogenic toxins is increasing, although studies linking oral bacteria to extra-oral infections are still at the stage of association (Han & Wan, 2013). At the same time, recent genome-wide association studies found some suggestive genetic polymorphisms associated with specific phenotypes of chronic periodontitis, thus posing the bases for future investigations about the pathways leading to this disease (Shaffer, Polk, Wang, Feingold, Weeks, Lee, Cuenco, Weyant, Crout, McNeil, & Marazita, 2014).

In terms of clinical recommendations, maintenance of good oral hygiene, especially for immune-compromised patients, is crucial for controlling total bacterial load to prevent bacterial dissemination. Progression of periodontal disease is dependent on the host immune response and individual susceptibility (Kornmann, 2008; Muniz, Nogueira, Mendes, Rösing, Moreira, de Andrade, & Carvalho, 2015). Oxidative stress (OS), due to imbalance between oxidants load and antioxidant capacity, is a potential mediator of the progression of different seemingly unrelated diseases including cancer, Parkinson’s and Alzheimer’s diseases, atherosclerosis, myocardial infarction as well as periodontal disease (Avezov, Reznick, & Aizenbud, 2015; Torumtay et al., 2016).

OS is one of the most important causative factors for the induction of cell apoptosis, bringing the cells into a state similar to senescence called stress-induced premature senescence. In vitro experimental studies show that OS is possibly correlated with the development of periodontal diseases (Baňasová, Kamodyová, Janšáková, Tóthová, Stanko, Turňa, & Celec, 2015; Kiyoshima, Enoki, Kobayashi, Sakai, Nagata, Wada, Fujiwara, Ookuma, & Sakai, 2012). It was demonstrated that OS affects gingival fibroblasts by inhibiting cell viability and proliferation, and affecting cell morphology, therefore
influencing their ability for extracellular matrix remodeling in the periodontal tissues (Colombo, Dalle-Donne, Orioli, Giustarini, Rossi, Clerici, Regazzoni, Aldini, Milzani, Butterfield, & Gagliano, 2012). In the inflammatory process generated from the microbial plaque that accumulates around the gingival margin, polymorphonuclear leucocytes are activated, and reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced as a result of bacterial destruction. In predisposed persons that have an aberrantly exaggerated inflammatory/immune response, the production of ROS and RNS can result in inadvertent or collateral host tissue damage (Chapple, Brock, Eftimiadi, & Matthews, 2002; Zare Javid et al., 2014).

Not only OS but also the redox balance may be relevant in pathological conditions. Decreased antioxidant ability might render the cells more vulnerable to OS in the oral cavity and might impair the reparative and regenerative potential of gingival tissues (Bergstrom, 2004; Cattaneo, Getta, Rota, Vezzoni, Rota, Gallanti, Boratto, & Poggi, 2000; Poggi, Rota, & Boratto, 2002). Saliva plays a pivotal role as an antioxidant system, including various molecules and enzymes, of which the most important are the uric acid molecule and the peroxidase enzyme (Nagler, Klein, Zarzhevskey, Drigues, & Reznick, 2002). Decreased salivary antioxidant ability to reduce OS was related to alterations of the oral mucosa, including oral cancer (Dayan, Hirshberg, Kaplan, Rotem, & Bodner, 1997; Giebultowicz, Wroczynski, & Samolczyk-Wanyura, 2011; Nishioka, Nish, & Kyokane, 1981) and lichen planus (Batu, Ofluoglu, Ergun, Warnakulasuriya, Uslu, Guven, & Tanyeri, 2015; Shirzad, Pouramir, Seyedmajidi, Jenabian, Bijani, & Motallebnejad, 2014).

Saliva antioxidant levels (SAL) are significantly lower in chronic periodontitis patients when compared to periodontally healthy individuals (Baňasová et al., 2015), and it was demonstrated that PD is associated with reduced salivary antioxidant status and increased oxidative damage in the oral cavity (Sculley & Langley-Evans, 2003). Therefore, SAL and PD seem to be associated with one another, leading to increased oxidative damages in the oral environment (Brock, Butterworth, Matthews, & Chapple, 2004).

Interestingly, the composition of saliva varies in different local and systemic diseases, and salivary antioxidant potential may reflect many pathophysiological states (Eisenberg, Shtahl, Geller, Reznick, Sharf, Ravbinovich, Erenreich, & Nagler, 2008; Nagler et al., 2003; Reznick, Hershkovich, & Nagler, 2004; Zloczower, Reznick, Zouby, & Nagler, 2007). The removal and control of ROS and RNS is important in preventing destruction of periodontal tissue (Chapple et al., 2002), and it is currently successfully treated by removing the supra- and subgingival biofilm by scaling and root planing combined with adequate periodontal support maintenance (Ramfjord, Knowles, Nissle, Burgett, & Shick, 1975; Torumtay et al., 2016). The early identification of predisposed patients able to develop PD and/or its severe form is very interesting in both clinical and socio-economical terms for researcher and dental professionals. Thus, on the wave of recent research suggestions on molecular aspects of
pathogenesis of periodontitis (Meyle & Chapple, 2015), it is important, first of all, to understand if SAL measures could be representative of patient incipient dysbiosis.

Benedetti, Primiterra, Finco, Canestrari, and Cornelli (2014) recently validated a biochemical test able to determine SAL in a simple, fast and economical way, and proposed its use as a predictive test for both local oral cavity and general body diseases. To the best of our knowledge, no clinical investigation compared the actual results of SAL assessments to some recognized gold standard for PD. Therefore, the aim of the present preliminary investigation is to evaluate a clinical gold standard assessment for PD, Periodontal Screening and Recordings, (PSR); in comparison to SAL measures in terms of detection of periodontal problems. The vast majority of academicians and professionals consider PSR beneficial in terms of disease detection, record keeping, cost effectiveness and patient education (Diamanti-Kipioti et al. 1993; Landry & Jean, 2002). Furthermore, PSR is a dental community appreciated index (Primal, Esther, & Boehm, 2014).

In the present study, the PSR characteristics of a group of adult patients attending a private practice dental clinic were analyzed and compared to SAL measures taken in the same occasion (Benedetti et al., 2014). The use of quantitative clinical evaluations can reduce inter-operator variability, accelerate the diagnosis of PD, and promote patient’s motivation (Wang, Schipper, Velly, Mohit, & Gornitsky, 2015).

Material and methods

Patient selection

From the beginning to the end of September 2015, 39 Caucasian patients (12 men and 27 women; mean age, 46 years, SD 16.9 years; age range, 18 to 80 years) were selected from patients attending the dental hygiene unit of a Private Clinic in Segrate, Milan (Italy). The patients underwent a Periodontal Screening and Recordings examination (PSR) and a saliva sample collection to perform a SAL measurement (Benedetti et al., 2014). The procedures were performed by two dental hygienists and a dentist; each professional made only one of the two examinations on each patient, and was blind to the results of the other test. Patients were randomly allocated to the professionals.

Overall, the Study Inclusion Criteria were to enroll patients of both genders, older than 18 years, with a moderate-good oral health condition, encountering a combination of the following points:

1. With 28 teeth at least
2. DMFT index ≤50
3. Tooth mobility Modified Miller index ≤ 1 (Wasserman, Geiger, & Turgeon, 1973)
4. Plaque index ≤25% (O’Leary, Drake, & Naylor, 1972)
5. Absence of active caries (Gomez, Tellez, Pretty, Ellwood, & Ismail, 2013)
6. Motivation to maintenance program
7. No dental treatment during the preceding two months period.

The patients were asked to update their personal and medical history in particular about alcohol and tobacco use; “drinkers” were all patients who declared to had been consuming more than half a liter/day of generic alcoholic beverages continuously for at least one year, while “smokers” were all patients who declared to be habitual tobacco users. These last ones were excluded. All subjects signed a written consent form given thorough explanation regarding the saliva collection procedures to be performed. The principles were in accordance with Helsinki declaration and Italian Law. The Institutional Review Board (IRB02-2015 Doc. MQ 03 AL 02-34) approved the procedures.

The Exclusion Criteria were as follow:

1. Presence of acute mouth infection or the necessity of anti-inflammatory or antimicrobial therapy within the previous 3 months
2. Pregnancy
3. Current orthodontic treatment
4. Poor motivation to accept a customized oral hygiene maintenance program
5. Irradiation of the head or neck region or chemotherapy within the past 60 months
6. Undiagnosed abnormal morphological variations of the oral soft and hard tissues
7. Consensus neglection
8. Smoke (as detailed above).

A preliminary screening was performed using clinical examination. Patients were informed about the study, and agreed to be a part of the investigation. Overall, they formed a convenience series. The two assessments were made one after the other by two different professionals.

Periodontal Screening and Recordings examination (PSR)

The PSR system is not alternative for a comprehensive periodontal examination but is a time saving screening of periodontal status. The mouth is divided into sextants; each tooth and implant is evaluated using six measurements. A color-coded (3.5 to 5.5 mm from the tip) 0.5 mm ball-tipped plastic or metal probe is used.

After each tooth in the sextant has been examined, only the highest score for each sextant is recorded. PSR grade scale is between zero to four. “Zero” is indicative of a healthy periodontal status, while “four” indicates a depth on probing of at least 6 mm.

In the present study, a cut-off of 2 was set as indicative of periodontal detailed examinations and treatment needs (Primal et al., 2014).

Assessment of Saliva Antioxidant Levels (SAL)
A saliva sample was collected for each patient to perform the Saliva Antioxidant Test (SAT) according to the procedure detailed by Benedetti et al. (2014). In brief, using a cotton square rolled in the mouth for 1 minute, unstimulated saliva samples of 1.1-1.4 mL were collected into a 20 mL sterile polypropylene container. These values are considered the optimal saliva flow (mL/minute) enough to have a constant level of uric acid, the main antioxidant agent in saliva. Saliva samples were collected from buccal molar area indifferently from the upper left or upper right quadrant. No attempt was made to specifically select sites with PSR ≥ 3. Site-specific differences were not analyzed. Saliva samples were analyzed as described by Benedetti et al. (2014), and SAT test values were categorized as reported in Table 1.

Reliability of SAT
The SAT test has been attentively validated against conventional standards (µmol/L of Vitamin C, reference standard used as ferrous-reducing agent). All the relevant specific information is reported by Benedetti et al. (2014). In particular, one important advantage of SAT test is its capability to exclude influences of interferences (in particular phosphates) in the determination of antioxidant levels (Benedetti et al., 2014). In fact, it has been reported that the high concentration of phosphates in the saliva can produce false positives: the SAT test avoids this limitation.

A training session for the operators occurred prior to start the data collection with one of the study author (GMT). Three operators (one dentist and two dental hygienists) participated in the repeatability and reproducibility measure trial. Three randomly selected patients were measured for SAT by the three operators participating in the study and each measurement was repeated 2 times with one hour interval.

Statistical analysis
The statistical analysis was performed using the statistical computer package Stata release 13.1 (Stata Comp, College Station, TX, USA). Intraclass correlation coefficients (ICCs) for the same observer (intra operator) and different observers (inter operators) were calculated using multilevel mixed model.

An independent Student’s T test was used to compare age between patients with a low SAT value and patients with a normal value. Fisher exact test was used to evaluate the relationship between PSR and SAT, and between gender and SAT.

Sensitivity and Specificity and their 95% CIs were calculated considering PSR as the gold standard. A p value <0.05 was considered statistically significant.

Results
The intra-operator and inter-operators assessment of repeatability and reproducibility of SAT measures were good, with intra rater ICC of 0.89 and inter rater ICC of 0.76.

SAT TEST results ranged from 329 to 2305 µmol/L. Table 1 shows the distribution of SAT results: approximately 70% of patients showed a low value, while the other patients had Optimal or Normal values. Only one patient had a borderline result. Thirteen patients out of 39 (33%) resulted positive to PSR test.

To evaluate the relationship between SAT test and PSR findings, and between SAT test results and gender, SAT test data were categorized in two classes: Normal (Normal value, Optimal value) and Subnormal (“severe shortage values”, according to Benedetti et al., 2014). The only patient with borderline values was excluded from this analysis.

The prevalence of PSR positive patients was higher in the Subnormal SAT group than in the Normal one but this difference was not statistically significant (Table 2). Women had a somewhat larger percentage of Subnormal values, but the difference did not reach statistical significance. Using PSR values as gold standard, SAT correctly classified 52.6% of patients; sensitivity was 84.6% (95% CI, 54.5-98.1) and specificity was 36% (95% CI, 18.0-57.5).

Considering the actual number of patients, the test allows to estimate the calculated sensitivity (85%) with a 95% CI with a precision of 14%.

Discussion

Among the various clinical indices that can be used to assess periodontal status, the Periodontal Screening and Recording or PSR Index has been reported to be reproducible, reliable, and fast (Landry and Jean, 2002). PSR possess a high predictive potential (81-93% for chronic periodontitis, gingivitis and periodontal health), that make it a useful screening and follow-up instrument for dental patients (Primal et al., 2014).

The actual cause of PD is still debated, and one of the conditions that may predispose and maintain the periodontal inflammation is the increased OS (Avezov et al., 2015; Baňasová et al., 2015; Kiyoshima et al., 2012; Muniz et al., 2015). Its assessment, therefore, could offer new insights for the early treatment of the single patient and for the definition of the actual etiology and pathogenesis of the disorder (Wang et al., 2015). Recently, the importance of a salivary antioxidant defense system was demonstrated (Nagler et al., 2002), suggesting that the assessment of salivary antioxidant potential could be relevant in the pathogenesis of PD. The recent introduction on the market of simple and low-cost tests of SAL (Benedetti et al., 2014) promoted the present preliminary study: our aim was to assess if subjects with a positive PSR evaluation had a SAL measure indicating oral inflammation and increased saliva oxidant levels, according with the objective of implementing standardized practice in this field (Wang et al, 2015). Strict inclusion patient criteria, saliva collection methodology and confidence intervals were
reported as considered essentials in the above established guidelines. This approach is able to give the level of measurements variability to compare the SAT test with a well-established and consolidated gold standard in the periodontal practice with attention to research cost and its management.

One third of the analyzed patients (13/39) resulted positive to the PSR test, with scores higher than 2. Their clinical condition seems therefore better than that reported by Primal et al. (2014) for a community-based study performed on patients of comparable age. Indeed, we selected patients with a moderate-good oral health condition, since our goal was to test if the proposed SAL test was sufficiently sensitive to screen early alterations in the oral environment. Statistical comparison of the two evaluations showed that SAL test had a good sensitivity (more than 80%) when compared to the selected gold standard. This finding seems to corroborate the hypothesis that oral alterations of the antioxidant levels are somehow related to PD, even if our single assessment cannot indicate if the reduced SAL was the cause or the result of periodontal inflammation.

In contrast, the reduced specificity of SAL test (lower than 40%) may indicate two different situations: first, oral and body conditions other than PD may possibly influence saliva composition; second, SAL test may possibly detect saliva alterations predisposing to PD, that has not reached a clinically evident aspect. Only a longitudinal assessment of the analyzed patients could offer an answer to this question, therefore a mid-term (6 months) recall examination has been programmed for all patients with a negative PSR and/or a positive SAL test.

Another factor that may have influenced the results of SAL test is the actual location of the PD in the mouth. Indeed, no attempt was made to specifically sample sextants with PSR ≥ 3, and site-specific differences were not taken into account. The antioxidant power of saliva and crevicular fluid has been found to be different (Brock et al., 2014; Chapple et al., 2002), but significant effects of PD have been reported even for saliva samples (Baňasová et al., 2015), that are easier to collect and standardize.

Among the limitations of the current study there is the exclusion of smoker patients. Indeed, as recently underlined (Avezov et al., 2015; Brock et al., 2014), smoking is a well-known source of ROS, and a recognized risk factor for PD. The actual results of SAL test in these patients are therefore unknown.

We are well aware that these preliminary results and the scientific evidence of this cohort study do not still permit clinical conclusions but, in accordance with the established guidelines (Wang et al., 2005), they provide to minimize the variability in redox biomarker discovery based on human saliva. They are the essential basis to move a step forward encouraging future research implementing the number of subjects involved (and consequently the economic cost) and activating larger multi-centric studies. This fundamental step is essential in order to better establish the SAT test limitations as an adjuvant tool in the periodontal screening of our patients.
In conclusion, standardized clinical evaluations can accelerate the diagnosis of periodontal conditions (Wang et al., 2015), and allow a more efficient follow-up of the single patient because of an easier communication among the clinicians (dentists, dental hygienists). Additionally, quantitative clinical records can be very useful in patient’s motivation, favoring the communication at all stages of treatment. The use of validated biochemical tests similar to that used in the current investigation may additionally reduce inter-operator variability in PD screening (Primal et al., 2014), and simplify operators’ training. To further reduce the economical and biological costs, the test may also be hypothetically performed directly by the patients at home, thus making the diagnostic process faster. Further investigations on this aspect are being planned.

Conflict of interest and source of funding statement
All authors have no conflict of interest relevant to the content of the submission and did not receive funding for this study.

References


oxidative stress and periodontal outcomes: A systematic review. *Archives of Oral Biology, 60*, 1203-1214.


### Tables

**Table 1.** SAT test distribution according to Benedetti et al. (2014).

<table>
<thead>
<tr>
<th>Range</th>
<th>Description</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1000 µmol/L</td>
<td>Severe shortage</td>
<td>27</td>
<td>69.2</td>
</tr>
<tr>
<td>1000 -1500 µmol/L</td>
<td>Optimum values</td>
<td>10</td>
<td>25.6</td>
</tr>
<tr>
<td>1500-2000 µmol/L</td>
<td>Normal values</td>
<td>1</td>
<td>2.6</td>
</tr>
<tr>
<td>2000- 2500 µmol/L</td>
<td>Borderline values</td>
<td>1</td>
<td>2.6</td>
</tr>
<tr>
<td>&gt;=2500</td>
<td>Possible inflammatory processes in course</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Table 2.** Comparison between patients with Subnormal SAT and Normal SAT values.

<table>
<thead>
<tr>
<th>Gender (n,%)</th>
<th>Normal</th>
<th>Subnormal</th>
<th>Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>9</td>
<td>17</td>
<td>63.0 Fisher</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>10</td>
<td>37.0</td>
</tr>
<tr>
<td>age (mean, SD; years)</td>
<td>46</td>
<td>46</td>
<td>16.9 Independent T 0.993</td>
</tr>
<tr>
<td>PSR (n, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
<td>16</td>
<td>59.3 Fisher</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>11</td>
<td>40.7</td>
</tr>
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