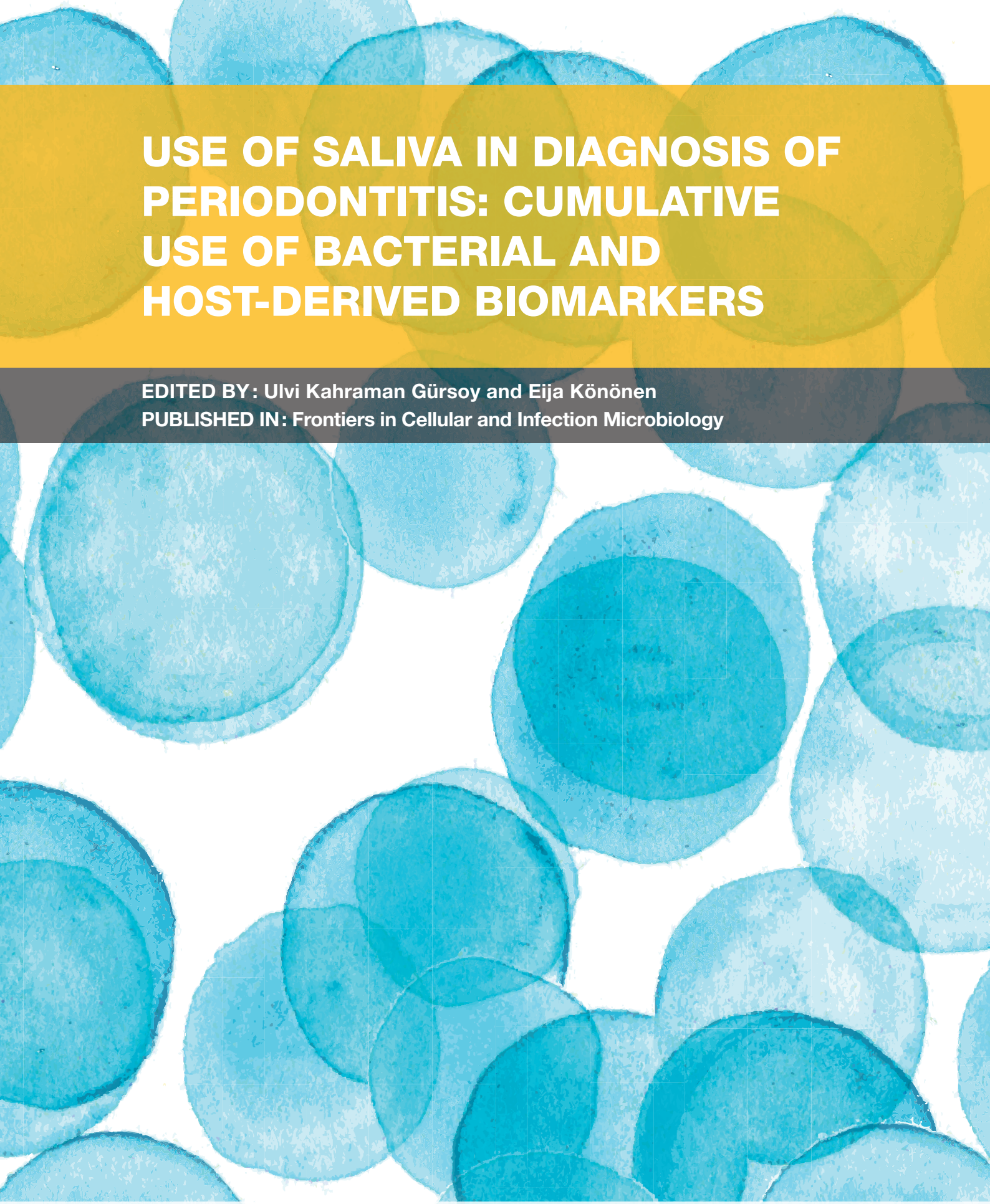
USE OF SALIVA IN DIAGNOSIS OF PERIODONTITIS: CUMULATIVE USE OF BACTERIAL AND HOST-DERIVED BIOMARKERS

EDITED BY: Ulvi Kahraman Gürsoy and Eija Könönen
PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-124-1
DOI 10.3389/978-2-88945-124-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

USE OF SALIVA IN DIAGNOSIS OF PERIODONTITIS: CUMULATIVE USE OF BACTERIAL AND HOST-DERIVED BIOMARKERS

Topic Editors:
Ulvi Kahraman Gürsoy, University of Turku, Finland
Eija Könönen, University of Turku, Finland & Oral Health Care, Welfare Division, Finland

Periodontitis is an infection-induced inflammatory disease of the tooth supporting tissues. Treatment of periodontal diseases and regeneration of the effected tissues can be possible only in the early diagnosis of the disease. If left undiagnosed or untreated, periodontitis leads to irreversible soft and hard tissue destruction and finally to tooth loss. Saliva is known to contain inflammatory mediators, host tissue and cell degradation products as well as microbial metabolites and enzymes, reflecting the health status of the oral cavity. In this topic, in collaboration with the well-known scientists working on the field of salivary diagnostics, we demonstrate evidence on monitoring periodontitis by salivary analysis.

Citation: Gürsoy, U. K., Könönen, E., eds. (2017). Use of Saliva in Diagnosis of Periodontitis: Cumulative Use of Bacterial and Host-Derived Biomarkers. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88945-124-1

Table of Contents

frontiers in Cellular and Infection Microbiology

EDITORIAL
published: 22 December 2016
doi: 10.3389/fcimb.2016.00196

Editorial: Use of Saliva in Diagnosis of Periodontitis: Cumulative Use of Bacterial and Host-Derived Biomarkers

Ulvi K. Gursoy[1]* *and Eija Könönen*[1,2]

[1] *Department of Periodontology, Institute of Dentistry, University of Turku, Turku, Finland,* [2] *Oral Health Care, Welfare Division, Turku, Finland*

Keywords: saliva, biomarker discovery, periodontal diseases, dentistry, oral health

Editorial on the Research Topic

Use of Saliva in Diagnosis of Periodontitis: Cumulative Use of Bacterial and Host-Derived Biomarkers

Oral cavity is the gate of the gastrointestinal tract, where the digestion process begins. Due to a wide range of nutritional intake, the oral cavity is constantly prone to physical and chemical stress. Mouth with its different types of surfaces and a warm and humid atmosphere offers a comfortable environment for a myriad of microbes to survive and replicate easily. Oral microbiota is an essential part of the host-tissue homeostasis; it contributes to the innate immune response and nitric oxide synthesis (Sintim and Gürsoy, 2016). However, bacterial colonization on non-shedding tooth surfaces allows the formation and maturation of biofilms where bacterial cells are well-protected from various host response activities. When pathogenic bacteria reside in these complex microbial communities, it may result in dysbiotic biofilms that get benefit from inflammation. Two wide-spread diseases of the oral cavity, connected to dysbiosis, are tooth decay and periodontitis.

In periodontitis, a degradative process occurs in tooth-supporting tissues, i.e., gingiva, periodontal ligament, and alveolar bone. The disease is initiated by bacteria; however, the main reason behind the tissue degradation is uncontrolled immune response. It often proceeds without any symptoms and, therefore, patients may seek professional help "at the point of no return" and experience loss of teeth. It is estimated that about 10–15% of the adult populations suffer from advanced periodontitis (Petersen and Ogawa, 2012). The prevalence and severity of periodontitis is increased in individuals with low socioeconomic status, tobacco use, excessive alcohol consumption, and certain systemic diseases, such as diabetes mellitus. According to the available data, the economic impact of dental diseases was 442 billion US$ worldwide in 2010 (Listl et al., 2015). Out of that sum, 298 billion US$ was the direct treatment costs, and 144 billion US$ was the indirect costs due to dental diseases (Petersen and Ogawa, 2012).

On one hand, a dentist can readily detect periodontal disease with conventional methods using a periodontal probe and, in case of clinical signs of periodontitis, radiographs. On the other hand, this type of diagnostics is economically costly and time-consuming and even not feasible to perform in large-scale studies. To use resources in an economic way, a cost-effective method to identify people suffering from periodontal disease is warranted.

Analysis of saliva for setting periodontal diagnosis is not a new idea. Information and experience on salivary diagnostics has improved tremendously during the past two decades. Saliva as a diagnostic tool offers several advantages; for example, by analyzing an array of constituents present in saliva, it is possible to monitor the disease onset and progression. Moreover, sufficient

OPEN ACCESS

Edited by:
Yousef Abu Kwaik,
University of Louisville, USA

Reviewed by:
Ashu Sharma,
University at Buffalo, USA

***Correspondence:**
Ulvi K. Gursoy
ulvi.gursoy@utu.fi

Received: *09 November 2016*
Accepted: *13 December 2016*
Published: *22 December 2016*

Citation:
Gursoy UK and Könönen E (2016) Editorial: Use of Saliva in Diagnosis of Periodontitis: Cumulative Use of Bacterial and Host-Derived Biomarkers. Front. Cell. Infect. Microbiol. 6:196. doi: 10.3389/fcimb.2016.00196

quantities of saliva can be collected in a non-invasive and simple way. Nevertheless, there are also shortcomings in salivary diagnostics. As an example, periodontitis has exacerbation and remission periods and the fluctuation in disease progression affects the diagnostic power of the salivary biomarker selected. Furthermore, systemic diseases (e.g., diabetes) and conditions (e.g., pregnancy), and local factors aggravating the progression of periodontitis (e.g., smoking) affect the levels of salivary biomarkers. To overcome these limitations, it has been proposed that the simultaneous detection of bacterial and host-derived biomarkers in saliva improves considerably the accuracy of periodontal diagnosis (Gursoy et al., 2011; Sorsa et al., 2016).

The present issue with its research topic "Use of saliva in diagnosis of periodontitis: cumulative use of bacterial and host-derived biomarkers" is composed of nine articles with altogether 49 authors, aiming to bring new information and aspects on the combinational use of biomarkers. In their mini-review, Ji and Choi discussed the challenges of point-of-care diagnostics of periodontitis and proposed an organization, International Consortium for Biomarkers of Periodontitis, to be built. Combinational analyses of salivary concentrations of several bacterial and host inflammatory markers were tested in two independent studies where the authors successfully demonstrated that these types of combinational analyses bring additional power to detect periodontitis (Ebersole et al.; Salminen et al.). Aboodi et al. followed the changes at an early phase of periodontal disease, performing proteome analyses in an experimental gingivitis model. Use of oxidative stress markers present in saliva for detecting periodontitis was discussed in a review Tóthová et al., while the study by Zhang et al. indicated a dysregulated immune response of being associated with antioxidant capacity in periodontitis patients. A combination of salivary nitrite and nitrate levels was found as a potential biomarker for phenytoin-induced gingival overgrowth, but this was not applicable for other drugs Sukuroglu et al. The role of antimicrobial peptides, small cationic peptides, in innate immune response and their usability as potential salivary markers of periodontitis were discussed in a review by Güncü et al. Finally, systems biology approach was used, up to our knowledge for the first time, to define putative host-derived biomarkers of periodontitis in saliva Zeidán-Chuliá et al.

We hope that the information brought together by the authors of the present issue will encourage researchers in the field of salivary diagnostics to have a new perspective to overcome the current limitations. The development of sophisticated methods will bring laboratory to clinics and the public.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Gursoy, U. K., Könönen, E., Pussinen, P. J., Tervahartiala, T., Hyvärinen, K., Suominen, A. L., et al. (2011). Use of host- and bacteria-derived salivary markers in detection of periodontitis: a cumulative approach. *Dis. Markers* 30, 299–305. doi: 10.1155/2011/621484

Listl, S., Galloway, J., Mossey, P. A., and Marcenes, W. (2015). Global economic impact of dental diseases. *J. Dent. Res.* 94, 1355–1361. doi: 10.1177/0022034515602879

Petersen, P. E., and Ogawa, H. (2012). The global burden of periodontal disease: towards integration with chronic disease prevention and control. *Periodontol 2000* 60, 15–39. doi: 10.1111/j.1600-0757.2011.00425.x

Sintim, H. O., and Gürsoy, U. K. (2016). Biofilms as "connectors" for oral and systems medicine: a new opportunity for biomarkers, molecular targets, and bacterial eradication. *OMICS* 20, 3–11. doi: 10.1089/omi.2015.0146

Sorsa, T., Gursoy, U. K., Nwhator, S., Hernandez, M., Tervahartiala, T., Leppilahti, J., et al. (2016). Analysis of matrix metalloproteinases, especially MMP-8, in gingival creviclular fluid, mouthrinse and saliva for monitoring periodontal diseases. *Periodontol. 2000* 70, 142–163. doi: 10.1111/prd.12101

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 03 September 2015
doi: 10.3389/fcimb.2015.00065

Point-of-care diagnosis of periodontitis using saliva: technically feasible but still a challenge

Suk Ji[1] *and Youngnim Choi*[2]*

[1] Department of Periodontology, Anam Hospital, Korea University, Seoul, South Korea, [2] Department of Oral Microbiology and Immunology, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, South Korea

Periodontitis is a chronic inflammation of the periodontium caused by persistent bacterial infection that leads to the breakdown of connective tissue and bone. Because the ability to reconstruct the periodontium is limited after alveolar bone loss, early diagnosis and intervention should be the primary goals of periodontal treatment. However, periodontitis often progresses without noticeable symptoms, and many patients do not seek professional dental care until the periodontal destruction progresses to the point of no return. Furthermore, the current diagnosis of periodontitis depends on time-consuming clinical measurements. Therefore, there is an unmet need for near-patient testing to diagnose periodontitis. Saliva is an optimal biological fluid to serve as a near-patient diagnostic tool for periodontitis. Recent developments in point-of-care (POC) testing indicate that a diagnostic test for periodontitis using saliva is now technically feasible. A number of promising salivary biomarkers associated with periodontitis have been reported. A panel of optimal biomarkers must be carefully selected based on the pathogenesis of periodontitis. The biggest hurdle for the POC diagnosis of periodontitis using saliva may be the process of validation in a large, diverse patient population. Therefore, we propose the organization of an International Consortium for Biomarkers of Periodontitis, which will gather efforts to identify, select, and validate salivary biomarkers for the diagnosis of periodontitis.

Keywords: periodontitis, point-of care testing, saliva, bacteria-derived biomarkers, host-derived biomarkers

OPEN ACCESS

Edited by:
Ulvi Kahraman Gürsoy, University of Turku, Finland

Reviewed by:
Nick Stephen Jakubovics, Newcastle University, UK
Sinem Esra Sahingur, Virginia Commonwealth University, USA

***Correspondence:**
Youngnim Choi, Laboratory of Infection and Immunity, Department of Oral Microbiology and Immunology, School of Dentistry, Seoul National University, 101 Daehak-ro, Jongno-gu, Seoul 110-744, South Korea
youngnim@snu.ac.kr

Received: *15 June 2015*
Accepted: *21 August 2015*
Published: *03 September 2015*

Citation:
Ji S and Choi Y (2015) Point-of-care diagnosis of periodontitis using saliva: technically feasible but still a challenge. Front. Cell. Infect. Microbiol. 5:65. doi: 10.3389/fcimb.2015.00065

Usefulness of Salivary Diagnostics for Periodontitis

Periodontitis is a chronic inflammation of the periodontium caused by persistent bacterial infection that leads to the breakdown of connective tissue and bone (Ji et al., 2014). Due to its chronic nature, periodontitis progresses without causing severe discomfort in the oral cavity, and patients often seek professional care only after the periodontium is considerably destroyed. Thus, there is a need to diagnose periodontitis in its initial stages using an easy, safe, and easily accessible method. Periodontitis is currently diagnosed using radiography and clinical measurements of probing pocket depth (PD), bleeding on probing (BOP), and clinical attachment level (CAL) (Salvi et al., 2008). However, these traditional clinical measurements are time-consuming and yield limited information because they are indicators of previous periodontal disease rather than present disease activity. Moreover, they are inadequate for predicting susceptible individuals who might be at risk of periodontitis in the future. The best predictor of gingival

inflammation to date is BOP, but there are too many false positives associated with this method (Lang et al., 1990). There is an unmet need for near-patient testing to diagnose periodontitis.

Saliva is an optimal biological fluid to serve as the diagnostic tool for periodontitis. The collection of saliva is safe, non-invasive, and simple, and saliva can be collected repeatedly with minimum discomfort to the patient. A number of promising biomarkers have been already identified in saliva that correlate with the clinical parameters of periodontitis (Miller et al., 2010; AlMoharib et al., 2014; Taylor, 2014). Saliva contains locally produced proteins, genetic/genomic biomarkers such as DNA and mRNA, and various metabolites that originate from the host and the bacteria (Cuevas-Córdoba and Santiago-García, 2014). However, the diagnosis of periodontitis using saliva has a limitation in detecting disease activity at each individual tooth site; traditional clinical measurements are required in order to accomplish this. In this respect, the diagnosis of periodontitis using saliva must be realized as a point-of-care (POC) testing. POC testing is defined as medical testing conducted outside of a laboratory at or near the site of patient care, including the patient's bedside, the doctor's office, and the patient's home (Song et al., 2014). If periodontitis is diagnosed by a POC device using saliva, patients could easily diagnose their periodontitis at home and visit dental clinics at a suitable time. In dental clinics, current disease activity and responses to treatment can be easily monitored at a chair-side. A POC device for diagnosing periodontitis would also assist medical doctors in assessing the periodontal status of their patients because periodontitis is associated with many systemic diseases, such as atherosclerosis, coronary heart disease, diabetes mellitus, and rheumatoid arthritis (Scannapieco, 2005; Kobayashi and Yoshie, 2015). When medical doctors prescribe bisphosphonate or other medicines associated with medication-related osteonecrosis of the jaw (MRONJ), they can consider the periodontal status of their patients in advance to prevent the development of MRONJ, a common complication of medication combined with tooth extraction (Katsarelis et al., 2015). Recent developments in POC testing indicate that the diagnosis of periodontitis using saliva is now technically feasible.

POC Technologies for Molecular Diagnostics

Technologies for detecting biomarker signals in biofluids have advanced significantly. In particular, the combination of microfluidic and lab-on-a-chip technologies allows for real-time monitoring of biomarkers in a small volume of a bodily fluid at POC sites (Sackmann et al., 2014). Lab-on-a-chip approaches integrate processing steps such as sampling, sample preparation, detection, and data analysis into one small device (Su et al., 2015). Microfluidics-based devices can analyze diverse clinical samples, including blood, saliva, nasal aspirate, and urine (Su et al., 2015).

Diagnostic targets detected by POC technologies include nucleic acids, proteins, metabolites and other small molecules (Song et al., 2014; Su et al., 2015). For example, nucleic acid can be amplified by on-chip PCR (non-isothermal) or on-chip isothermal amplification techniques (Su et al., 2015). Many PCR-based POC devices for the detection of pathogens such as influenza, RSV, HIV, Methicillin-resistant *Staphylococcus aureus*, *Clostridium difficile*, and malaria are already commercially available (Su et al., 2015). POC DNA tests have also been developed to detect genetic mutations associated with various cancers (Yang et al., 2014). The microfluidic detection of protein biomarkers generally relies on antibody-based immunoassays. Aptamers, DNA, or RNA oligonucleotides designed to bind to various biomolecules with high specificity and sensitivity are an alternative to antibodies (Toh et al., 2015). The simple lateral flow assay is rapid and specific but not sensitive or quantitative. Diverse new technologies have been developed to improve sensitivities and to allow for quantitative measurements of multiplex protein biomarkers (Gaster et al., 2009; Warsinke, 2009; Rissin et al., 2010; de la Rica and Stevens, 2012). Glucose is the best-known metabolite targeted by POC testing, with a long history of use (Wilkins and Atanasov, 1996). Now a much wider range of analytes can be quantified using POC technology (Sia and Chin, 2011). For example, the i-STAT POC device, millions of which are sold annually, electrochemically measures blood gas (pH, PCO_2, PO_2, TCO_2, HCO_3, base excess, and sO_2), electrolyte (sodium, potassium, chloride, TCO_2, anion gap, ionized calcium, glucose, urea nitrogen, creatinine, and lactate), and hematology (hematocrit and hemoglobin) parameters (Lauks, 1998). A microfluidic device that measures nitric oxide has also been developed (Halpin and Spence, 2010).

Various approaches have been developed to detect the target molecules, but optical detection and electrochemical detection are the ones most commonly adopted. Optical detection methods implemented in POC devices include absorbance colorimetry, chemiluminescence, fluorescence, surface-enhanced Raman scattering spectroscopy, and surface plasmon resonance (Gubala et al., 2012; Su et al., 2015). Electrochemical detection methods include amperometric, potentiometric, and impedimetric measurements (Su et al., 2015).

Salivary Biomarkers of Periodontitis

Ideal biomarkers of periodontitis must be able to (1) diagnose the presence of periodontal disease, (2) reflect the severity of the disease (3) monitor the response of the disease to treatment, and (4) predict the prognosis/progress of the disease. A number of biomarkers that satisfy at least one of the four requirements have been identified in saliva (**Tables 1–4**). Salivary biomarkers of periodontal disease can originate from both bacteria and the host. As periodontitis progresses, gingival inflammation, soft tissue destruction, and bone destruction occur sequentially and release associated proteins or metabolites into the saliva. Therefore, host-derived biomarkers are categorized according to whether they reflect inflammation, soft tissue destruction, or bone destruction. The biomarkers that satisfy three of the four requirements in at least three separate studies are classified as strong (S) biomarkers. When the number of studies that reported no difference or contradictory results is equal or greater than those with supporting results, the biomarkers are classified as

TABLE 1 | Bacteria-derived salivary biomarkers.

	Salivary biomarkers	Supporting reports		No difference or contradictory reports		Strength
		References	Study size†	References	Study size	
DNA	*Porphyromonas gingivalis*	von Troil-Lindén et al., 1995 (C); Sawamoto et al., 2005 (I)*; Ramseier et al., 2009 (C); Saygun et al., 2011 (C); Nomura et al., 2012 (L); Pereira et al., 2012 (I)	512			S
	Prevotella intermedia	von Troil-Lindén et al., 1995 (C); Ramseier et al., 2009 (C); Saygun et al., 2011 (C); Nomura et al., 2012 (L); Pereira et al., 2012 (I)	463			S
	Tannerella forsythia	Sawamoto et al., 2005 (C)*; Ramseier et al., 2009 (C); Saygun et al., 2011 (C); Pereira et al., 2012 (I)	387	Nomura et al., 2012 (L)	85	S
	Treponema denticola	Ramseier et al., 2009 (C); Pereira et al., 2012 (I)	188			P
	Campylobacter rectus	von Troil-Lindén et al., 1995 (C); Ramseier et al., 2009 (C); Saygun et al., 2011 (C)	289	Pereira et al., 2012 (I)	89	P
	Pseudomonas aeruginosa + *Acinetobacter* spp.	Souto et al., 2014 (C)	224			P
	Peptostreptococcus micros	von Troil-Lindén et al., 1995 (C)	40			P
	Fusobacterium nucleatum	Saygun et al., 2011 (C)	150	Ramseier et al., 2009 (C)	99	Q
	Aggregatibacter actinomycetemcomitans	von Troil-Lindén et al., 1995 (C); Saygun et al., 2011 (C)	190	Sawamoto et al., 2005 (C); Pereira et al., 2012 (I)	138	Q
Proteins	Dipeptidyl peptidase	Aemaimanan et al., 2009 (C)*	90			P

†*Combined from multiple studies, C, cross-sectional study; L, longitudinal study; I, interventional study; *, correlation with clinical parameters; S, strong; P, potential; Q, questionable.*

questionable (Q). The remaining biomarkers are classified as potential (P).

Bacteria-derived Biomarkers

Bacteria-derived biomarkers include DNA and proteins. The levels of well-known pathogenic bacteria, such as *Aggregatibacter actinomycetemcomitans*, the three red complex species, and several species of the orange complex in saliva were determined by targeting a specific area of the 16S rRNA gene (**Table 1**). Among them, only *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Tannerella forsythia* have been proved by multiple studies as strong biomarkers of periodontitis. Recent studies using high-throughput sequencing of the 16S rRNA gene have identified new species/phylotypes that are associated with periodontitis (Griffen et al., 2012; Göhler et al., 2014). Given the complexity of dental biofilm, the potential of the newly identified species/phylotypes to serve as salivary biomarkers of periodontitis needs to be investigated. The activity of dipeptidyl peptidase IV in saliva has been shown to be associated with periodontitis and the presence of *P. gingivalis* (Aemaimanan et al., 2009). Dipeptidyl peptidase IV is a serine protease that cleaves X-Pro dipeptide from the N-terminus of polypeptide chains, thus contributing to collagen degradation (Banbula et al., 2000). DPP4 in saliva may originate from both the host and bacteria, including *P. gingivalis* (Aemaimanan et al., 2009).

Host-derived Inflammatory Biomarkers

Periodontitis begins with inflammation of the gingival tissue in response to dental biofilm. As inflammatory biomarkers in saliva, diverse enzymes (arginase, dipeptidyl peptidase IV, β-glucuronidase, and myeloperoxidase), anti-microbial proteins (lactoferrin and calprotectin), inflammatory cytokines (IL-1β, IL-6, IL-18, IFN-γ, and MIP-1α), and proteins that mediate inflammation (chemerin, CRP, TLR4, soluble CD14, and procalcitonin) have been studied (**Table 2**). Particularly, IL-1β, MIP-1α, and arginase are strong biomarkers that correlate with inflammatory parameters of periodontitis, such as the gingival index or BOP (Miller et al., 2006; Gheren et al., 2008; Al-Sabbagh et al., 2012; Rathnayake et al., 2013). In addition to protein biomarkers, nitric oxide, 8-hydroxydeoxyguanosine, platelet activating factor and fatty acid metabolites (neopterin, docosapentaenoate, linoleate, and arachidonate) have been identified as inflammation-associated biomarkers in saliva (**Table 2**).

Host-derived Biomarkers Associated with Soft Tissue Destruction

As periodontitis progresses, soft tissues are destroyed, releasing several enzymes and proteins that are involved in tissue destruction into the saliva. Among them, MMP-8, MMP-9, HGF, lactate dehydrogenase, aspartate aminotransferase, and TIMP-2 are strong or potential biomarkers of periodontitis (**Table 3**). In addition, a recent metabolomic profiling of saliva revealed increased amounts of metabolites originated from macromolecular degradation, including dipeptides (proteins), oligo/mono-saccharides (polysaccharides), lysolipids, fatty acids, and monoacylglycerol (glycerophospholipid and triacylglycerol), and uridine (DNA/RNA) in periodontitis (**Table 3**).

TABLE 2 | Host-derived salivary biomarkers associated with inflammation.

	Salivary biomarkers	Supporting reports		No difference or contradictory reports		Strength
		References	Study size†	References	Study size	
Proteins	IL-1β	Miller et al., 2006 (C)*; Ng et al., 2007 (C)*; Christodoulides et al., 2007 (C); Scannapieco et al., 2007 (L); Tobon-Arroyave et al., 2008 (C)*; Fine et al., 2009 (L)*; Gursoy et al., 2009 (C); Mirrielees et al., 2010 (C); Yoon et al., 2012 (C); Rathnayake et al., 2013 (C)*; Ebersole et al., 2013 (C); Fine et al., 2014 (L)	1396	Teles et al., 2009 (C); Ramseier et al., 2009 (C)	217	S
	MIP-1α	Fine et al., 2009 (L)*; Al-Sabbagh et al., 2012 (C)*; Fine et al., 2014 (L)	198			S
	Arginase	Ozmeriç et al., 2000 (C); Gheren et al., 2008 (I)*; Pereira et al., 2012 (I)	160			S
	soluble CD14	Isaza-Guzmán et al., 2008 (C)*; Prakasam and Srinivasan, 2014 (I)	110			P
	IFN-γ and IFN-γ/IL-22 ratio	Isaza-Guzmán et al., 2015 (C)*	149			P
	Lactoferrin	Fine et al., 2002 (C); Jentsch et al., 2004 (I); Glimvall et al., 2012 (C)*	79	Groenink et al., 1999 (C)	39	P
	Dipeptidyl peptidase	Aemaimanan et al., 2009 (C)*	90			P
	Chemerin	Özcan et al., 2015 (C)*	72			P
	Procalcitonin	Hendek et al., 2015 (C)*	72			P
	Calprotectin	Ramseier et al., 2009 (C)	99			P
	Myeloperoxidase	Meschiari et al., 2013 (C)	72			P
	IL-18	Banu et al., 2015 (C)	60			P
	TLR4	Banu et al., 2015 (C)	60			P
	β-glucuronidase	Lamster et al., 2003 (C)*; Yoon et al., 2012 (C)	497	Pietruska et al., 2006 (I)	16	P
	CRP	Pederson et al., 1995 (C); Christodoulides et al., 2007 (C); Shojaee et al., 2013 (C)*	186	Aurer et al., 2005 (C)	51	P
	IL-6	Costa et al., 2010 (C); Ebersole et al., 2013 (C); Prakasam and Srinivasan, 2014 (C)	210	Teles et al., 2009 (C); Gursoy et al., 2009 (C); Ramseier et al., 2009 (C); Rathnayake et al., 2013 (C); Khalaf et al., 2014 (C)	873	Q
	IL-8	Fine et al., 2014 (L)	70	Teles et al., 2009 (C); Rathnayake et al., 2013 (C)*; Khalaf et al., 2014 (C)	609	Q
	TNFα	Frodge et al., 2008 (C)	42	Teles et al., 2009 (C); Ramseier et al., 2009 (C); Gursoy et al., 2009 (C); Mirrielees et al., 2010 (C); Ebersole et al., 2013 (C)	567	Q
Metabolites	Nitric oxide	Reher et al., 2007 (C)*; Khorsavi Samani et al., 2012 (C); Parwani et al., 2012 (I)*; Han et al., 2013 (C); Sundar et al., 2013 (C)	466			S
	8-hydroxydeoxyguanosine	Sugano et al., 2003 (I); Sawamoto et al., 2005 (I); Takane et al., 2005 (C); Canakçi et al., 2009a (C); Canakçi et al., 2009b (C); Sezer et al., 2012 (C)*	297			S
	Platelet activating factor	McManus and Pinckard, 2000 (C)	165			P
	Neopterin	Ozmeriç et al., 2002 (C)	30			P
	ω-3 (docosapentaenoate) and ω-6 (linoleate and arachidonate) fatty acids	Barnes et al., 2014 (C)	80			P

† *Combined from multiple studies, C, cross-sectional study; L, longitudinal study; I, interventional study; *, correlation with clinical parameters; S, strong; P, potential; Q, questionable.*

TABLE 3 | Host-derived salivary biomarkers associated with soft tissue destruction.

	Salivary biomarkers	Supporting reports		No difference or contradictory reports		Strength
		References	Study size†	References	Study size	
Protein	MMP-8	Gorska and Nedzi-Gora, 2006(I); Miller et al., 2006 (C)*; Christodoulides et al., 2007 (C); Ramseier et al., 2009 (C); Mirrielees et al., 2010 (C); Costa et al., 2010 (C); Gursoy et al., 2010 (C); Gursoy et al., 2013 (C); Ebersole et al., 2013 (C); Meschiari et al., 2013 (I); Rathnayake et al., 2013 (C)*	1371			S
	HGF	Wilczynska-Borawska et al., 2006 (C)*; Scannapieco et al., 2007 (L); Rudrakshi et al., 2011 (C)*; Lönn et al., 2014 (C)	222			S
	Aspartate aminotransferase	Nomura et al., 2006 (C); Totan et al., 2006 (C); Nomura et al., 2012 (L); Banu et al., 2015 (C)	382			P
	Lactate dehydrogenase	de la Peña et al., 2005 (I); Nomura et al., 2006 (C); Kugahara et al., 2008 (C); Nomura et al., 2012 (L)	568	Gursoy et al., 2009 (I)	165	P
	MMP-9	Ramseier et al., 2009 (C); Isaza-Guzmán et al., 2011 (C)*; Gursoy et al., 2013 (C)	452	Gorska and Nedzi-Gora, 2006 (I)	40	P
	TIMP-2	Meschiari et al., 2013 (I)	72			P
	Alanine aminotransferase	Nomura et al., 2012 (L); Banu et al., 2015 (C)	145	Nomura et al., 2006 (C); Totan et al., 2006 (C)	237	Q
	TIMP-1	Gursoy et al., 2010 (C); Isaza-Guzmán et al., 2011 (C)*	288	Hayakawa et al., 1994 (C); Gorska and Nedzi-Gora, 2006 (I); Meschiari et al., 2013 (I); Rathnayake et al., 2013 (C)*	573	Q
Metabolites	Purine degradation metabolites (e.g., guanosine and inosine)	Barnes et al., 2014 (C)	80			P
	Dipeptide, amino acid, carbohydrate, lipids, and nucleotide metabolites	Barnes et al., 2011 (C)	68			P

†*Combined from multiple studies, C, cross-sectional study; L, longitudinal study; I, interventional study; *, correlation with clinical parameters; S, strong; P, potential; Q, questionable.*

Host-derived Biomarkers Associated with Bone Destruction

Salivary biomarkers of bone remodeling can be used as indicators of bone destruction in periodontitis. These include alkaline phosphatase, osteonectin, RANKL, and calcium (**Table 4**). A positive correlation between the levels of salivary calcium and CAL has been reported (Sutej et al., 2012).

Further Considerations

Among the various salivary biomarkers listed, *P. gingivalis* has been shown to satisfy all four requirements of ideal biomarkers for periodontitis in at least one study for each requirement. However, single biomarker detection may not be effective enough for accurate diagnoses without false-positive or false-negative results. Periodontitis is a disease that involves complex interactions between bacteria and the host immune system. The combination of the host-derived biomarkers, which reflect inflammation, soft tissue destruction, and bone destruction together with bacteria-derived biomarkers, may be useful to diagnose not only the presence of periodontitis but also the degree of progression and the response to therapy. A number of host-derived biomarkers have already shown strong associations with periodontitis.

The fact that the concentration of biomarkers can be affected by the saliva flow rate, circadian rhythm, age, the physiological status of the patients, and other factors raises concern over the accuracy and reproducibility of diagnoses using salivary biomarkers (Nový, 2014). Although within-subject correlations between unstimulated and stimulated samples and over time have been reported for some salivary proteins (Rudney et al., 1985), such studies have not been done for all salivary proteomes or metabolomes. Nevertheless, many biomarkers, in numerous studies, have shown consistent associations with periodontitis. For example, significantly higher levels of salivary MMP-8 in periodontitis than in healthy controls were observed in six studies that used the non-stimulatory whole saliva samples (Miller et al., 2006; Christodoulides et al., 2007; Ramseier et al., 2009; Costa et al., 2010; Mirrielees et al., 2010; Ebersole et al.,

TABLE 4 | Host-derived salivary biomarkers associated with hard tissue destruction.

	Salivary biomarkers	Supporting reports		No difference or contradictory reports		Strength
		References	Study size†	References	Study size	
Protein	Alkaline phosphatase	Totan et al., 2006 (C); Kugahara et al., 2008 (C); Dabra and Singh, 2012 (C)	331	Nomura et al., 2012 (L)	85	P
	Osteonectin	Scannapieco et al., 2007 (L); Ng et al., 2007 (C)*	80			P
	RANKL	Buduneli et al., 2008 (C); Tobón-Arroyave et al., 2012 (C)*	195	Frodge et al., 2008 (C)	42	P
	Osteoprotegerin	Ramseier et al., 2009 (C); Tobón-Arroyave et al., 2012 (C)*; Hassan et al., 2015 (I)	269	Miller et al., 2006 (C)*; Buduneli et al., 2008 (C); Costa et al., 2010 (C); Al-Sabbagh et al., 2012 (C)*	282	Q
Metabolites	Calcium	Acharya et al., 2011 (C); Sutej et al., 2012 (C)*	67			P
	Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen	Gursoy et al., 2010 (C)	165	Frodge et al., 2008 (C); Ramseier et al., 2009 (C); Al-Sabbagh et al., 2012 (C); Gursoy et al., 2013 (C)	386	Q

†*Combined from multiple studies, C, cross-sectional study; L, longitudinal study; I, interventional study; *, correlation with clinical parameters; S, strong; P, potential; Q, questionable.*

2013) and in five studies that used stimulated whole saliva samples (Gorska and Nedzi-Gora, 2006; Gursoy et al., 2010, 2013; Meschiari et al., 2013; Rathnayake et al., 2013). These findings suggest that within-subject variations in the concentration of salivary biomarkers can be overcome for diagnostic purposes if a biomarker with substantial inter-group differences is chosen.

POC Devices in Periodontology

A few POC devices have been developed for the salivary diagnosis of periodontitis. A device called the Integrated Microfluidic Platform for Oral Diagnostics (IMPOD) was able to detect salivary proteins with a low sample volume requirements (10 μL) and considerable sensitivity (nM-pM) by integrating sample pretreatment (filtering, enrichment, mixing) with electrophoretic immunoassays and a laser-induced fluorescence detection system. Using this device, rapid (<10 min) measurements of MMP-8, TNF-α, IL-6, and CRP in saliva were performed (Herr et al., 2007a,b). However, validation in the clinical setting has not yet been reported.

A group at the University of Texas at Austin developed a lab-on-a-chip (LOC) system that integrates microfluidics and a fluorescence-based optical system in which sandwich immunoassays are performed on chemically sensitized beads. They reported the application of the LOC system for the multiplex measurement of three salivary biomarkers, C-reactive protein, MMP-8, and IL-1β, which are related to the clinical expression of periodontitis. The LOC approach yielded a limit of detection five orders of magnitude lower than that of a standard ELISA, and the results obtained by the LOC approach were consistent with ELISA results (Christodoulides et al., 2007). Whether the POC device using this LOC approach can accurately measure levels of the salivary biomarker MMP-8 and thus indicate if a patient has periodontal health, gingivitis or periodontal disease is currently being studied in a clinical trial (NCT02403297 at ClinicalTrials.gov).

Suggestions for Organizing the International Consortium for Salivary Biomarkers of Periodontitis

For the success of POC diagnostics of periodontitis using saliva, it is important to validate the candidate biomarkers with large populations which suitably account for diversity such as those related to race, region, gender, and age. In periodontal research, full-mouth or selected teeth have been examined to diagnose periodontitis, and different criteria have been also used to classify the severity of periodontitis. Such variations in the classifications of patients make it difficult to integrate the results of different studies. Furthermore, variations in saliva sampling methods (e.g., resting vs. stimulatory, whole vs. individual gland sampling), target biomarkers, and detection methods of salivary biomarkers prevent direct comparisons of the data obtained from different studies or centers. We propose the organization of an International Consortium for Salivary Biomarkers of Periodontitis (ICSBP). The ICSBP can put forth collaborative efforts to create standardized protocols for clinical research, including a uniform method for clinical diagnoses of periodontitis. In addition, the ICSBP can accelerate the validation of biomarkers and the implementation of salivary diagnostics by sharing clinical samples and experience.

Funding

This work was supported by the National Research Foundation of Korea (Daejun, Korea) Grant 2014050477 funded by the Korean Government through the Oromaxillofacial Dysfunction Research

Center for the Elderly at Seoul National University and by Basic Science Research Program through the National Research Foundation grants 2013R1A1A3005669 and 2014R1A1A10 05507.

References

Acharya, A., Kharadi, M. D., Dhavale, R., Deshmukh, V. L., and Sontakke, A. N. (2011). High salivary calcium level associated with periodontal disease in Indian subjects–a pilot study. *Oral Health Prev. Dent.* 9, 195–200.

Aemaimanan, P., Sattayasai, N., Wara-aswapati, N., Pitiphat, W., Suwannarong, W., Prajaneh, S., et al. (2009). Alanine aminopeptidase and dipeptidyl peptidase IV in saliva of chronic periodontitis patients. *J. Periodontol.* 80, 1809–1814. doi: 10.1902/jop.2009.090233

AlMoharib, H. S., AlMubarak, A., AlRowis, R., Geevarghese, A., Preethanath, R. S., and Anil, S. (2014). Oral fluid based biomarkers in periodontal disease: part 1. Saliva. *J. Int. Oral Health* 6, 95–103.

Al-Sabbagh, M., Alladah, A., Lin, Y., Kryscio, R. J., Thomas, M. V., Ebersole, J. L., et al. (2012). Bone remodeling-associated salivary biomarker MIP-1α distinguishes periodontal disease from health. *J. Periodontal Res.* 47, 389–395. doi: 10.1111/j.1600-0765.2011.01445.x

Aurer, A., Jorgic-Srdjak, K., Plancak, D., Stavljenic-Rukavina, A., and Aurer-Kozelj, J. (2005). Proinflammatory factors in saliva as possible markers for periodontal disease. *Coll. Antropol.* 29, 435–439.

Banbula, A., Bugno, M., Goldstein, J., Yen, J., Nelson, D., Travis, J., et al. (2000). Emerging family of proline-specific peptidases of *Porphyromonas gingivalis*: purification and characterization of serine dipeptidyl peptidase, a structural and functional homologue of mammalian prolyl dipeptidyl peptidase IV. *Infect. Immun.* 68, 1176–1182. doi: 10.1128/IAI.68.3.1176-1182.2000

Banu, S., Jabir, N. R., Mohan, R., Manjunath, N. C., Kamal, M. A., Kumar, K. R., et al. (2015). Correlation of Toll-like receptor 4, interleukin-18, transaminases, and uric acid in patients with chronic periodontitis and healthy adults. *J. Periodontol.* 86, 431–439. doi: 10.1902/jop.2014.140414

Barnes, V. M., Ciancio, S. G., Shibly, O., Xu, T., Devizio, W., Trivedi, H. M., et al. (2011). Metabolomics reveals elevated macromolecular degradation in periodontal disease. *J. Dent. Res.* 90, 1293–1297. doi: 10.1177/0022034511416240

Barnes, V. M., Kennedy, A. D., Panagakos, F., Devizio, W., Trivedi, H. M., Jönsson, T., et al. (2014). Global metabolomic analysis of human saliva and plasma from healthy and diabetic subjects, with and without periodontal disease. *PLoS ONE* 18:e105181. doi: 10.1371/journal.pone.0105181

Buduneli, N., Biyikoglu, B., Sherrabeh, S., and Lappin, D. F. (2008). Saliva concentrations of RANKL and osteoprotegerin in smoker versus non-smoker chronic periodontitis patients. *J. Clin. Periodontol.* 35, 846–852. doi: 10.1111/j.1600-051X.2008.01310.x

Canakçi, C. F., Canakçi, V., Tatar, A., Eltas, A., Sezer, U., Ciçek, Y., et al. (2009a). Increased salivary level of 8-hydroxydeoxyguanosine is a marker of premature oxidative mitochondrial DNA damage in gingival tissue of patients with periodontitis. *Arch. Immunol. Ther. Exp.* 57, 205–211. doi: 10.1007/s00005-009-0026-9

Canakçi, C. F., Cicek, Y., Yildirim, A., Sezer, U., and Canakci, V. (2009b). Increased levels of 8-hydroxydeoxyguanosine and malondialdehyde and its relationship with antioxidant enzymes in saliva of periodontitis patients. *Eur. J. Dent.* 3, 100–106.

Christodoulides, N., Floriano, P. N., Miller, C. S., Ebersole, J. L., Mohanty, S., Dharshan, P., et al. (2007). Lab-on-a-chip methods for point-of-care measurements of salivary biomarkers of periodontitis. *Ann. N. Y. Acad. Sci.* 1098, 411–428. doi: 10.1196/annals.1384.035

Costa, P. P., Trevisan, G. L., Macedo, G. O., Palioto, D. B., Souza, S. L., Grisi, M. F., et al. (2010). Salivary interleukin-6, matrix metalloproteinase-8, and osteoprotegerin in patients with periodontitis and diabetes. *J. Periodontol.* 81, 384–391. doi: 10.1902/jop.2009.090510

Cuevas-Córdoba, B., and Santiago-García, J. (2014). Saliva: a fluid of study for OMICS. *OMICS* 18, 87–97. doi: 10.1089/omi.2013.0064

Dabra, S., and Singh, P. (2012). Evaluating the levels of salivary alkaline and acid phosphatase activities as biochemical markers for periodontal disease: a case series. *Dent. Res. J.* 9, 41–45. doi: 10.4103/1735-3327.92942

de la Peña, V. A., Dios, P. D., Rodríguez-Nuñez, I., and Rodríguez-Segade, S. (2005). Effect of ultrasonic scaling on salivary lactate dehydrogenase. *Am. J. Dent.* 18, 113–115.

de la Rica, R., and Stevens, M. M. (2012). Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye. *Nat. Nanotechnol.* 7, 821–824. doi: 10.1038/nnano.2012

Ebersole, J. L., Schuster, J. L., Stevens, J., Dawson, D. III., Kryscio, R. J., Lin, Y., et al. (2013). Patterns of salivary analytes provide diagnostic capacity for distinguishing chronic adult periodontitis from health. *J. Clin. Immunol.* 33, 271–279. doi: 10.1007/s10875-012-9771-3

Fine, D. H., Furgang, D., and Beydouin, F. (2002). Lactoferrin iron levels are reduced in saliva of patients with localized aggressive periodontitis. *J. Periodontol.* 73, 624–630. doi: 10.1902/jop.2002.73.6.624

Fine, D. H., Markowitz, K., Fairlie, K., Tischio-Bereski, D., Ferrandiz, J., Godboley, D., et al. (2014). Macrophage inflammatory protein-1α shows predictive value as a risk marker for subjects and sites vulnerable to bone loss in a longitudinal model of aggressive periodontitis. *PLoS ONE* 5:e98541. doi: 10.1371/journal.pone.0098541

Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., et al. (2009). Macrophage inflammatory protein-1alpha: a salivary biomarker of bone loss in a longitudinal cohort study of children at risk for aggressive periodontal disease? *J. Periodontol.* 80, 106–113. doi: 10.1902/jop.2009.080296

Frodge, B. D., Ebersole, J. L., Kryscio, R. J., Thomas, M. V., and Miller, C. S. (2008). Bone remodeling biomarkers of periodontal disease in saliva. *J. Periodontol.* 79, 1913–1919. doi: 10.1902/jop.2008.080070

Gaster, R. S., Hall, D. A., Nielsen, C. H., Osterfeld, S. J., Yu, H., Mach, K. E., et al. (2009). Matrix-insensitive protein assays push the limits of biosensors in medicine. *Nat. Med.* 15, 1327–1332. doi: 10.1038/nm.2032

Gheren, L. W., Cortelli, J. R., Rodrigues, E., Holzhausen, M., and Saad, W. A. (2008). Periodontal therapy reduces arginase activity in saliva of patients with chronic periodontitis. *Clin. Oral Investig.* 12, 67–72. doi: 10.1007/s00784-007-0146-8

Glimvall, P., Wickström, C., and Jansson, H. (2012). Elevated levels of salivary lactoferrin, a marker for chronic periodontitis? *J. Periodontal Res.* 47, 655–660. doi: 10.1111/j.1600-0765.2012.01479.x

Göhler, A., Hetzer, A., Holtfreter, B., Geisel, M. H., Schmidt, C. O., Steinmetz, I., et al. (2014). Quantitative molecular detection of putative periodontal pathogens in clinically healthy and periodontally diseased subjects. *PLoS ONE* 9:e99244. doi: 10.1371/journal.pone.0099244

Górska, R., and Nedzi-Góra, M. (2006). The effects of the initial treatment phase and of adjunctive low-dose doxycycline therapy on clinical parameters and MMP-8, MMP-9, and TIMP-1 levels in the saliva and peripheral blood of patients with chronic periodontitis. *Arch. Immunol. Ther. Exp.* 54, 419–426. doi: 10.1007/s00005-006-0047-6

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., et al. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J.* 6, 1176–1185. doi: 10.1038/ismej.2011.191

Groenink, J., Walgreen-Weterings, E., Nazmi, K., Bolscher, J. G., Veerman, E. C., van Winkelhoff, A. J., et al. (1999). Salivary lactoferrin and low-Mr mucin MG2 in Actinobacillus actinomycetemcomitans-associated periodontitis. *J. Clin. Periodontol.* 26, 269–275. doi: 10.1034/j.1600-051X.1999.260501.x

Gubala, V., Harris, L. F., Ricco, A. J., Tan, M. X., and Williams, D. E. (2012). Point of care diagnostics: status and future. anal chem. point of care diagnostics: status and future. *Anal. Chem.* 84, 487–515. doi: 10.1021/ac2030199

Gursoy, U. K., Könönen, E., Huumonen, S., Tervahartiala, T., Pussinen, P. J., Suominen, A. L., et al. (2013). Salivary type I collagen degradation end-products and related matrix metalloproteinases in periodontitis. *J. Clin. Periodontol.* 40, 18–25. doi: 10.1111/jcpe.12020

Gursoy, U. K., Könönen, E., Pradhan-Palikhe, P., Tervahartiala, T., Pussinen, P. J., Suominen-Taipale, L., et al. (2010). Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *J. Clin. Periodontol.* 37, 487–493. doi: 10.1111/j.1600-051X.2010.01563.x

Gursoy, U. K., Könönen, E., Uitto, V. J., Pussinen, P. J., Hyvärinen, K., Suominen-Taipale, L., et al. (2009). Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis. *J. Clin. Periodontol.* 36, 922–927. doi: 10.1111/j.1600-051X.2009.01480.x

Halpin, S. T., and Spence, D. M. (2010). Direct plate-reader measurement of nitric oxide released from hypoxic erythrocytes flowing through a microfluidic device. *Anal. Chem.* 82, 7492–7497. doi: 10.1021/ac101130s

Han, D. H., Kim, M. S., Shin, H. S., Park, K. P., and Kim, H. D. (2013). Association between periodontitis and salivary nitric oxide metabolites among community elderly Koreans. *J. Periodontol.* 84, 776–784. doi: 10.1902/jop.2012.120237

Hassan, S. H., El-Refai, M. I., Ghallab, N. A., Kasem, R. F., and Shaker, O. G. (2015). Effect of periodontal surgery on osteoprotegerin levels in gingival crevicular fluid, saliva, and gingival tissues of chronic periodontitis patients. *Dis. Markers* 2015:341259. doi: 10.1155/2015/341259

Hayakawa, H., Yamashita, K., Ohwaki, K., Sawa, M., Noguchi, T., Iwata, K., et al. (1994). Collagenase activity and tissue inhibitor of metalloproteinases-1 (TIMP-1) content in human whole saliva from clinically healthy and periodontally diseased subjects. *J. Periodontal Res.* 29, 305–308. doi: 10.1111/j.1600-0765.1994.tb01226.x

Hendek, M. K., Erdemir, E. O., and Kisa, U. (2015). Evaluation of salivary procalcitonin levels in different periodontal diseases. *J. Periodontol.* 86, 820–826. doi: 10.1902/jop.2015.130751

Herr, A. E., Hatch, A. V., Giannobile, W. V., Throckmorton, D. J., Tran, H. M., Brennan, J. S., et al. (2007a). Integrated microfluidic platform for oral diagnostics. *Ann. N.Y. Acad. Sci.* 1098, 362–374. doi: 10.1196/annals.1384.004

Herr, A. E., Hatch, A. V., Throckmorton, D. J., Tran, H. M., Brennan, J. S., Giannobile, W. V., et al. (2007b). Microfluidic immunoassays as rapid saliva-based clinical diagnostics. *Proc. Natl. Acad. Sci. U.S.A.* 27, 5268–5273. doi: 10.1073/pnas.0607254104

Isaza-Guzmán, D. M., Arias-Osorio, C., Martínez-Pabón, M. C., and Tobón-Arroyave, S. I. (2011). Salivary levels of matrix metalloproteinase (MMP)-9 and tissue inhibitor of matrix metalloproteinase (TIMP)-1: a pilot study about the relationship with periodontal status and MMP-9(-1562C/T) gene promoter polymorphism. *Arch. Oral Biol.* 56, 401–411. doi: 10.1016/j.archoralbio.2010.10.021

Isaza-Guzmán, D. M., Aristizábal-Cardona, D., Martínez-Pabón, M. C., Velásquez-Echeverri, H., and Tobón-Arroyave, S. I. (2008). Estimation of sCD14 levels in saliva obtained from patients with various periodontal conditions. *Oral Dis.* 14, 450–456. doi: 10.1111/j.1601-0825.2007.01400.x

Isaza-Guzmán, D. M., Cardona-Vélez, N., Gaviria-Correa, D. E., Martínez-Pabón, M. C., Castaño-Granada, M. C., and Tobón-Arroyave, S. I. (2015). Association study between salivary levels of interferon (IFN)-gamma, interleukin (IL)-17, IL-21, and IL-22 with chronic periodontitis. *Arch. Oral Biol.* 60, 91–99. doi: 10.1016/j.archoralbio.2014.09.002

Jentsch, H., Sievert, Y., and Göcke, R. (2004). Lactoferrin and other markers from gingival crevicular fluid and saliva before and after periodontal treatment. *J. Clin. Periodontol.* 31, 511–514. doi: 10.1111/j.1600-051X.2004.00512.x

Ji, S., Choi, Y. S., and Choi, Y. (2014). Bacterial invasion and persistence: critical events in the pathogenesis of periodontitis? *J. Periodontal Res.* doi: 10.1111/jre.12248. [Epub ahead of print].

Katsarelis, H., Shah, N. P., Dhariwal, D. K., and Pazianas, M. (2015). Infection and medication-related osteonecrosis of the jaw. *J. Dent. Res.* 94, 534–539. doi: 10.1177/0022034515572021

Khalaf, H., Lönn, J., and Bengtsson, T. (2014). Cytokines and chemokines are differentially expressed in patients with periodontitis: possible role for TGF-β1 as a marker for disease progression. *Cytokine* 67, 29–35. doi: 10.1016/j.cyto.2014.02.007

Khorsavi Samani, M., Poorsattar Bejeh Mir, A., Kashiri, M., and Gujeq, D. (2012). Introducing cut-points for salivary nitric oxide to distinguish periodontitis from the normal periodontium. *Minerva Stomatol.* 61, 443–448.

Kobayashi, T., and Yoshie, H. (2015). Host responses in the link between periodontitis and rheumatoid arthritis. *Curr. Oral Health Rep.* 2, 1–8. doi: 10.1007/s40496-014-0039-2

Kugahara, T., Shosenji, Y., and Ohashi, K. (2008). Screening for periodontitis in pregnant women with salivary enzymes. *J. Obstet. Gynaecol. Res.* 34, 40–46. doi: 10.1111/j.1447-0756.2007.00681.x

Lamster, I. B., Kaufman, E., Grbic, J. T., Winston, L. J., and Singer, R. E. (2003). β-glucuronidase activity in saliva: relationship to clinical periodontal parameters. *J. Periodontol.* 74, 353–359. doi: 10.1902/jop.2003.74.3.353

Lang, N. P., Adler, R., Joss, A., and Nyman, S. (1990). Absence of bleeding on probing. An indicator of periodontal stability. *J. Clin. Periodontol.* 17, 714–721. doi: 10.1111/j.1600-051X.1990.tb01059.x

Lauks, I. R. (1998). Microfabricated biosensors and microanalytical systems for blood analysis. *Acc. Chem. Res.* 31, 317–324. doi: 10.1021/ar9700670

Lönn, J., Johansson, C. S., Nakka, S., Palm, E., Bengtsson, T., Nayeri, F., et al. (2014). High concentration but low activity of hepatocyte growth factor in periodontitis. *J. Periodontol.* 85, 113–122. doi: 10.1902/jop.2013.130003

McManus, L. M., and Pinckard, R. N. (2000). PAF, a putative mediator of oral inflammation. *Crit. Rev. Oral Biol. Med.* 11, 240–258. doi: 10.1177/10454411000110020701

Meschiari, C. A., Marcaccini, A. M., Santos Moura, B. C., Zuardi, L. R., Tanus-Santos, J. E., and Gerlach, R. F. (2013). Salivary MMPs, TIMPs, and MPO levels in periodontal disease patients and controls. *Clin. Chim. Acta.* 5, 140–146. doi: 10.1016/j.cca.2013.03.008

Miller, C. S., Foley, J. D., Bailey, A. L., Campell, C. L., Humphries, R. L., Christodoulides, N., et al. (2010). Current developments in salivary diagnostics. *Biomark. Med.* 4, 171–189. doi: 10.2217/bmm.09.68

Miller, C. S., King, C. P. Jr., Langub, M. C., Kryscio, R. J., and Thomas, M. V. (2006). Salivary biomarkers of existing periodontal disease: a cross-sectional study. *J. Am. Dent. Assoc.* 137, 322–329. doi: 10.14219/jada.archive.2006.0181

Mirrielees, J., Crofford, L. J., Lin, Y., Kryscio, R. J., Dawson, D. R. III, Ebersole, J. L., et al. (2010). Rheumatoid arthritis and salivary biomarkers of periodontal disease. *J. Clin. Periodontol.* 37, 1068–1074. doi: 10.1111/j.1600-051X.2010.01625.x

Ng, P. Y., Donley, M., Hausmann, E., Hutson, A. D., Rossomando, E. F., and Scannapieco, F. A. (2007). Candidate salivary biomarkers associated with alveolar bone loss: cross-sectional and *in vitro* studies. *FEMS Immunol. Med. Microbiol.* 49, 252–260. doi: 10.1111/j.1574-695X.2006.00187.x

Nomura, Y., Shimada, Y., Hanada, N., Numabe, Y., Kamoi, K., Sato, T., et al. (2012). Salivary biomarkers for predicting the progression of chronic periodontitis. *Arch. Oral Biol.* 57, 413–420. doi: 10.1016/j.archoralbio.2011.09.011

Nomura, Y., Tamaki, Y., Tanaka, T., Arakawa, H., Tsurumoto, A., Kirimura, K., et al. (2006). Screening of periodontitis with salivary enzyme tests. *J. Oral Sci.* 48, 177–183. doi: 10.2334/josnusd.48.177

Nový, B. B. (2014). Saliva and biofilm-based diagnostics: a critical review of the literature concerning sialochemistry. *J. Evid. Based Dent. Pract.* 14(Suppl.), 27–32. doi: 10.1016/j.jebdp.2014.04.004

Özcan, E., Saygun, N. I., Serdar, M. A., and Kurt, N. (2015). Evaluation of the salivary levels of visfatin, chemerin, and progranulin in periodontal inflammation. *Clin. Oral Investig.* 19, 921–928. doi: 10.1007/s00784-014-1308-0

Ozmeriç, N., Elgün, S., and Uraz, A. (2000). Salivary arginase in patients with adult periodontitis. *Clin. Oral Investig.* 4, 21–24. doi: 10.1007/s007840050108

Ozmeriç, N., Baydar, T., Bodur, A., Engin, A. B., Uraz, A., Eren, K., et al. (2002). Level of neopterin, a marker of immune cell activation in gingival crevicular fluid, saliva, and urine in patients with aggressive periodontitis. *J. Periodontol.* 73, 720–725. doi: 10.1902/jop.2002.73.7.720

Parwani, S. R., Chitnis, P. J., and Parwani, R. N. (2012). Salivary nitric oxide levels in inflammatory periodontal disease - a case-control and interventional study. *Int. J. Dent. Hyg.* 10, 67–73. doi: 10.1111/j.1601-5037.2011.00508.x

Pederson, E. D., Stanke, S. R., Whitener, S. J., Sebastiani, P. T., Lamberts, B. L., and Turner, D. W. (1995). Salivary levels of alpha 2-macroglobulin, alpha 1-antitrypsin, C-reactive protein, cathepsin G and elastase in humans with or without destructive periodontal disease. *Arch. Oral Biol.* 40, 1151–1155. doi: 10.1016/0003-9969(95)00089-5

Pereira, A. L., Cortelli, S. C., Aquino, D. R., Franco, G. C., Cogo, K., Rodrigues, E., et al. (2012). Reduction of salivary arginine catabolic activity through periodontal therapy. *Quintessence Int.* 43, 777–787.

Pietruska, M., Bernaczyk, A., Knas, M., Pietruski, J., and Zwierz, K. (2006). Assessment of salivary levels of the chosen exoglycosidases in patients with aggressive periodontitis after treatment with doxycycline. *Adv. Med. Sci.* 51, 158–161.

Prakasam, S., and Srinivasan, M. (2014). Evaluation of salivary biomarker profiles following non-surgical management of chronic periodontitis. *Oral Dis.* 20, 171–177. doi: 10.1111/odi.12085

Ramseier, C. A., Kinney, J. S., Herr, A. E., Braun, T., Sugai, J. V., Shelburne, C. A., et al. (2009). Identification of pathogen and host-response markers correlated with periodontal disease. *J. Periodontol.* 80, 436–446. doi: 10.1902/jop.2009.080480

Rathnayake, N., Akerman, S., Klinge, B., Lundegren, N., Jansson, H., Tryselius, Y., et al. (2013). Salivary biomarkers of oral health: a cross-sectional study. *J. Clin. Periodontol.* 40, 140–147. doi: 10.1111/jcpe.12038

Reher, V. G., Zenóbio, E. G., Costa, F. O., Reher, P., and Soares, R. V. (2007). Nitric oxide levels in saliva increase with severity of chronic periodontitis. *J. Oral Sci.* 49, 271–276. doi: 10.2334/josnusd.49.271

Rissin, D. M., Kan, C. W., Campbell, T. G., Howes, S. C., Fournier, D. R., Song, L., et al. (2010). Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. *Nat. Biotechnol.* 28, 595–599. doi: 10.1038/nbt.1641

Rudney, J. D., Kajander, K. C., and Smith, Q. T. (1985). Correlations between human salivary levels of lysozyme, lactoferrin, salivary peroxidase and secretory immunoglobulin A with different stimulatory states and over time. *Arch. Oral Biol.* 30, 765–771. doi: 10.1016/0003-9969(85)90129-3

Rudrakshi, C., Srinivas, N., and Mehta, D. S. (2011). A comparative evaluation of hepatocyte growth factor levels in gingival crevicular fluid and saliva and its correlation with clinical parameters in patients with and without chronic periodontitis: a clinico-biochemical study. *J. Indian Soc. Periodontol.* 15, 147–151. doi: 10.4103/0972-124X.84384

Sackmann, E. K., Fulton, A. L., and Beebe, D. J. (2014). The present and future role of microfluidics in biomedical research. *Nature* 13, 181–189. doi: 10.1038/nature13118

Salvi, G. E., Lindhe, J., and Lang, N. P. (2008). "Examination of patients with periodontal diseases," in *Clinical Periodontology and Implant Dentistry*, eds N. P. Lang and J. Lindhe (Oxford: Munksgaard), 573–586.

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Saygun, I., Nizam, N., Keskiner, I., Bal, V., Kubar, A., Açikel, C., et al. (2011). Salivary infectious agents and periodontal disease status. *J. Periodontal Res.* 46, 235–239. doi: 10.1111/j.1600-0765.2010.01335.x

Scannapieco, F. A. (2005). Systemic effects of periodontal diseases. *Dent. Clin. North Am.* 49, 533–550. doi: 10.1016/j.cden.2005.03.002

Scannapieco, F. A., Ng, P., Hovey, K., Hausmann, E., Hutson, A., and Wactawski-Wende, J. (2007). Salivary biomarkers associated with alveolar bone loss. *Ann. N.Y. Acad. Sci.* 1098, 496–497. doi: 10.1196/annals.1384.034

Sezer, U., Ciçek, Y., and Canakçi, C. F. (2012). Increased salivary levels of 8-hydroxydeoxyguanosine may be a marker for disease activity for periodontitis. *Dis. Markers* 32, 165–172. doi: 10.3233/DMA-2011-0876

Shojaee, M., Fereydooni Golpasha, M., Maliji, G., Bijani, A., Aghajanpour Mir, S. M., and Mousavi Kani, S. N. (2013). C – reactive protein levels in patients with periodontal disease and normal subjects. *Int. J. Mol. Cell. Med.* 2, 151–155.

Sia, S. K., and Chin, C. D. (2011). Analytical chemistry: sweet solution to sensing. *Nature Chem.* 3, 659–660. doi: 10.1038/nchem.1119

Song, Y., Huang, Y. Y., Liu, X., Zhang, X., Ferrari, M., and Qin, L. (2014). Point-of-care technologies for molecular diagnostics using a drop of blood. *Trends. Biotechnol.* 32, 132–139. doi: 10.1016/j.tibtech.2014.01.003

Souto, R., Silva-Boghossian, C. M., and Colombo, A. P. (2014). Prevalence of *Pseudomonas aeruginosa* and Acinetobacter spp. in subgingival biofilm and saliva of subjects with chronic periodontal infection. *Braz. J. Microbiol.* 45, 495–501. doi: 10.1590/S1517-83822014000200017

Su, W., Gao, X., Jiang, L., and Qin, J. (2015). Microfluidic platform towards point-of-care diagnostics in infectious diseases. *J. Chromatogr. A* 16, 13–26. doi: 10.1016/j.chroma.2014.12.041

Sugano, N., Yokoyama, K., Oshikawa, M., Kumagai, K., Takane, M., Tanaka, H., et al. (2003). Detection of *Streptococcus anginosus* and 8-hydroxydeoxyguanosine in saliva. *J. Oral Sci.* 45, 181–184. doi: 10.2334/josnusd.45.181

Sundar, N. M., Krishnan, V., Krishnaraj, S., Hemalatha, V. T., and Alam, M. N. (2013). Comparison of the salivary and the serum nitric oxide levels in chronic and aggressive periodontitis: a biochemical study. *J. Clin. Diagn. Res.* 7, 1223–1227. doi: 10.7860/JCDR/2013/5386.3068

Sutej, I., Peros, K., Benutic, A., Capak, K., Basic, K., and Rosin-Grget, K. (2012). Salivary calcium concentration and periodontal health of young adults in relation to tobacco smoking. *Oral Health Prev. Dent.* 10, 397–403.

Takane, M., Sugano, N., Ezawa, T., Uchiyama, T., and Ito, K. (2005). A marker of oxidative stress in saliva: association with periodontally-involved teeth of a hopeless prognosis. *J. Oral Sci.* 47, 53–57. doi: 10.2334/josnusd.47.53

Taylor, J. J. (2014). Protein biomarkers of periodontitis in saliva. *ISRN Inflamm.* 22, 593151. doi: 10.1155/2014/593151

Teles, R. P., Likhari, V., Socransky, S. S., and Haffajee, A. D. (2009). Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: a cross-sectional study. *J. Periodontal Res.* 44, 411–417. doi: 10.1111/j.1600-0765.2008.01119.x

Tobón-Arroyave, S. I., Isaza-Guzmán, D. M., Restrepo-Cadavid, E. M., Zapata-Molina, S. M., and Martínez-Pabón, M. C. (2012). Association of salivary levels of the bone remodelling regulators sRANKL and OPG with periodontal clinical status. *J. Clin. Periodontol.* 39, 1132–1140. doi: 10.1111/jcpe.12012

Tobon-Arroyave, S. I., Jaramillo-Gonzalez, P. E., and Isaza-Guzman, D. M. (2008). Correlation between salivary IL-1β levels and periodontal clinical status. *Arch. Oral Biol.* 53, 346–352. doi: 10.1016/j.archoralbio.2007.11.005

Toh, S. Y., Citartan, M., Gopinath, S. C., and Tang, T. H. (2015). Aptamers as a replacement for antibodies in enzyme-linked immunosorbent assay. *Biosens. Bioelectron.* 64, 392–403. doi: 10.1016/j.bios.2014.09.026

Totan, A., Greabu, M., Totan, C., and Spinu, T. (2006). Salivary aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase: possible markers in periodontal diseases? *Clin. Chem. Lab. Med.* 44, 612–615. doi: 10.1515/cclm.2006.096

von Troil-Lindén, B., Torkko, H., Alaluusua, S., Jousimies-Somer, H., and Asikainen, S. (1995). Salivary levels of suspected periodontal pathogens in relation to periodontal status and treatment. *J. Dent. Res.* 74, 1789–1795. doi: 10.1177/00220345950740111201

Warsinke, A. (2009). Point-of-care testing of proteins. *Anal. Bioanal. Chem.* 393, 1393–1405. doi: 10.1007/s00216-008-2572-0

Wilczynska-Borawska, M., Borawski, J., Kovalchuk, O., Chyczewski, L., and Stokowska, W. (2006). Hepatocyte growth factor in saliva is a potential marker of symptomatic periodontal disease. *J. Oral Sci.* 48, 47–50. doi: 10.2334/josnusd.48.47

Wilkins, E., and Atanasov, P. (1996). Glucose monitoring: state of the art and future possibilities. *Med. Eng. Phys.* 18, 273–288. doi: 10.1016/1350-4533(95)00046-1

Yang, J., Wei, F., Schafer, C., and Wong, D. T. (2014). Detection of tumor cell-specific mRNA and protein in exosome-like microvesicles from blood and saliva. *PLoS ONE* 14:e110641. doi: 10.1371/journal.pone.0110641

Yoon, A. J., Cheng, B., Philipone, E., Turner, R., and Lamster, I. B. (2012). Inflammatory biomarkers in saliva: assessing the strength of association of diabetes mellitus and periodontal status with the oral inflammatory burden. *J. Clin. Periodontol.* 39, 434–440. doi: 10.1111/j.1600-051X.2012.01866.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 19 August 2015
doi: 10.3389/fcimb.2015.00062

Targeted salivary biomarkers for discrimination of periodontal health and disease(s)

Jeffrey L. Ebersole[1]*, *Radhakrishnan Nagarajan*[2], *David Akers*[3] and *Craig S. Miller*[1,4]

[1] Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, KY, USA, [2] Division of Biomedical Informatics, College of Public Health, University of Kentucky, Lexington, KY, USA, [3] Department of Statistics, College of Arts and Sciences, University of Kentucky, Lexington, KY, USA, [4] Division of Oral Diagnosis, Oral Medicine and Oral Radiology, College of Dentistry, University of Kentucky, Lexington, KY, USA

Generally, clinical parameters are used in dental practice for periodontal disease, yet several drawbacks exist with the clinical standards for addressing the needs of the public at large in determining the current status/progression of the disease, and requiring a significant amount of damage before these parameters can document disease. Therefore, a quick, easy and reliable method of assessing and monitoring periodontal disease should provide important diagnostic information that improves and speeds treatment decisions and moves the field closer to individualized point-of-care diagnostics.

Objective: This report provides results for a saliva-based diagnostic approach for periodontal health and disease based upon the abundance of salivary analytes coincident with disease, and the significant progress already made in the identification of discriminatory salivary biomarkers of periodontitis.

Methods: We evaluated biomarkers representing various phases of periodontitis initiation and progression (IL-1ß, IL-6, MMP-8, MIP-1α) in whole saliva from 209 subjects categorized with periodontal health, gingivitis, and periodontitis.

Results: Evaluation of the salivary analytes demonstrated utility for individual biomarkers to differentiate periodontitis from health. Inclusion of gingivitis patients into the analyses provided a more robust basis to estimate the value of each of these analytes. Various clinical and statistical approaches showed that pairs or panels of the analytes were able to increase the sensitivity and specificity for the identification of disease.

Conclusions: Salivary concentrations of IL-1ß, IL-6, MMP-8, MIP-1α alone and in combination are able to distinguish health from gingivitis and periodontitis. The data clearly demonstrated a heterogeneity in response profiles of these analytes that supports the need for refinement of the standard clinical classifications if we are to move toward precision/personalized dentistry for the twenty-first century.

Keywords: periodontitis, saliva, MMP-8, cytokines, personalized medicine

OPEN ACCESS

Edited by:
Ulvi Kahraman Gürsoy, University of Turku, Finland

Reviewed by:
Gill Diamond, University of Florida, USA
Georgios N. Belibasakis, University of Zürich, Switzerland
Gülnur Emingil, Ege University, Turkey

***Correspondence:**
Jeffrey L. Ebersole, Center for Oral Health Research, College of Dentistry, HSRB Rm. 422, 1095 VA Drive, Lexington, KY 4-536, USA
jleber2@uky.edu

Received: *16 June 2015*
Accepted: *03 August 2015*
Published: *19 August 2015*

Citation:
Ebersole JL, Nagarajan R, Akers D and Miller CS (2015) Targeted salivary biomarkers for discrimination of periodontal health and disease(s). Front. Cell. Infect. Microbiol. 5:62. doi: 10.3389/fcimb.2015.00062

Introduction

Historically, the health of the periodontium has been defined by clinical parameters that describe a lack of gingival inflammation bleeding on probing (BOP), changes in the epithelial barrier relationship to the cementoenamel junction clinical attachment level (CAL), and loss of underlying alveolar bone architecture probing pocket depth (PPD) (Armitage and Robertson, 2009; Armitage and Cullinan, 2010). However, coincident with these macro-clinical measures, histological studies have clearly identified some local tissue inflammatory response to the juxtaposed supra- and subgingival microbial ecology, even in "clinically healthy" sites (Page and Schroeder, 1976; Armitage et al., 1977; Brecx et al., 1986). This type of mucosal "physiologic inflammation" has also been described in gastrointestinal mucosal tissues and is considered important in the normal host-bacterial interactions to maintain tissue homeostasis (Silva et al., 2007; Rabinowitz and Mayer, 2012). These histological findings of the periodontium are coincident with data describing detectable levels of a select group of host response molecules in healthy tissues, that are generally considered to increase significantly in magnitude and expand in an array of responses with gingivitis (Offenbacher et al., 2009, 2010; Jönsson et al., 2011; Leishman et al., 2013) and periodontitis (Kim et al., 2006; Beikler et al., 2008; Kebschull et al., 2013, 2014). However, these findings have not been effectively extrapolated into creating a paradigm that integrates biological and clinical measures of health of the gingival tissues as an important, and potentially crucial, biomedical informatics approaches for assessing disease presence, prognosis, and progression.

Gingivitis is an often-overlooked disease, despite being the "Gateway" to periodontitis for a significant portion of the population (Page, 1986; Albandar et al., 1998; Schätzle et al., 2003, 2004, 2009; Lang et al., 2009). This issue has persisted for years, in part because clinical parameters along with pre-defined thresholds of inflammation and measurable tissue destruction have been used as the "gold standard" for discerning health/gingivitis from periodontitis. While very helpful, they neither provide insights into patient-specific variations within these inexact disease groups, nor do they help predict non-responders and those who "at risk" for disease progression. Thus, novel methods for identifying those "at risk persons" are needed.

Differential host responses are thought to contribute to various susceptibilities that play an important role in determining progression of the inflammatory lesion (Kornman et al., 1997; Trombelli, 2004; Van Dyke and Sheilesh, 2005; Grigoriadou et al., 2010; Ebersole et al., 2013). At the cellular level, exposure to bacterial products and lipopolysaccharide (LPS) elicit activation of monocytes/macrophages that promote secretion of cytokines and inflammatory mediators such as IL-1β, IL-6, and TNFα that results in the release of matrix metalloproteinases (MMPs) that undermine the integrity of the gingival tissues (Yucel-Lindberg and Båge, 2013). Many of these inflammatory molecules have been detected in oral fluids (Sorsa et al., 1999; Miller et al., 2006), which has allowed saliva to emerge as an important and easily accessible biological fluid that can provide important diagnostic information regarding oral health and disease (Henskens et al., 1993; Fine et al., 2009; Giannobile et al., 2009; Miller et al., 2010; Kinney et al., 2011; Shaila et al., 2013; Prakasam and Srinivasan, 2014) Consistent with this, recent data from our lab and others indicate that salivary concentrations of IL-6, IL-8, albumin, calprotectin, PGE_2, MMP-8, and MIP-1α are elevated in patients who have gingivitis (Lee et al., 2012; Syndergaard et al., 2014).

Use of salivary biomarkers in conjunction with the expanded panel of potential biomarkers from recent investigations using proteomic and transcriptomic analyses could help dentistry move toward the era of personalized medicine. However, advances will require studies that analyze biospecimens and compare biomarkers from patients exhibiting the full spectrum of disease (health, gingivitis, and periodontitis), as studies regarding this spectrum of disease have been lacking. This report addresses this gap and describes an approach that reflects Phase I standards that are articulated regarding the discovery, validation, and utility assessment of biomarkers for disease detection (Pepe et al., 2001).

Periodontal disease is a chronic inflammatory and destructive condition that affects an estimated 80% of U.S. adults that can have significant systemic consequences. Customary clinical parameters are used in dental practice because of their ease of use, relative non-invasiveness and reliability. Yet several drawbacks exist with the current standards for addressing the needs of the public at large. First, a highly trained clinician and assistant are needed to record the findings. Second, collection of this diagnostic information includes the use of expensive radiographic equipment that makes the procedure time and labor intensive, as well as imposing significant financial costs to the consumer. Third, even in the hands of experts, several of these readings are somewhat subjective by the evaluator and tend to vary in accuracy not only from one evaluator to the next, but by the best of examiners. Equally important is the fact that these clinical parameters cannot determine current status of the disease, and a significant amount of damage must occur before these diagnostic parameters are able to detect a sufficient level of disease. Our hypothesis was that a combination of salivary analytes that relate to the biological processes of periodontitis will effectively discriminate this destructive disease from gingival inflammation and periodontal health. Therefore, the possibility of a quick, easy and reliable method of assessing and monitoring periodontal disease should provide important diagnostic information that improves and speeds treatment decisions and moves the field closer to individualized point-of-care diagnostics.

Materials and Methods

These case-control studies were conducted at the University of Kentucky College of Dentistry from 2009 through 2013. The protocols were approved by the Institutional Review Board at the University of Kentucky (12-0673-F2L; 04-0339-F1V; 10-0615-F6A; 07-0780-F6A). Participants were recruited from the general clinic and student populations of the College of Dentistry. Two hundred and nine persons participated some of whom have been described in previous reports (Thomas et al., 2009; Al-Sabbagh

et al., 2012; Syndergaard et al., 2014). Each participant was given verbal and written information that described the nature of the study, and each signed informed consent prior to enrollment of the study. Inclusion criteria included subjects older than 18 years of age who were in good general health (excluding the case definition) and had a minimum of 20 teeth.

Individuals were excluded from either group if there was evidence of alcoholism, liver, kidney, or salivary gland dysfunction, inflammatory bowel disease, granulomatous diseases, diabetes, undergoing or had undergone organ transplant or cancer therapy, had a periodontal abscess or had previous treatment for periodontal disease or aggressive periodontitis. Pregnancy or lactation, use of antibiotics or immunosuppressant medication within the last 1 month, need for antibiotics for infective endocarditis prophylaxis during dental procedures, symptoms of acute illness (i.e., fever, sore throat, body aches, and diarrhea), removable prosthodontic or orthodontic appliances or presence of an oral mucosal inflammatory condition (e.g., aphthous, lichen planus, leukoplakia, and oral cancer) also were exclusion criteria.

Clinical Evaluation

All subjects received a full mouth periodontal examination. The medical and dental history was obtained and reviewed along with exclusion criteria prior to the periodontal examination. Findings from the head, neck, and oral examination were recorded as being normal or abnormal. All clinical findings were recorded on data collection worksheets. PPD was measured at six locations per tooth (mesial-buccal, mid-buccal, distal-buccal, mesial lingual, mid-lingual, and distal-lingual) using a PCP-UNC 15 probe. After the measurement of PPDs, all sites were observed for BOP (Thomas et al., 2009; Sexton et al., 2011; Al-Sabbagh et al., 2012; Syndergaard et al., 2014). CAL was also determined at all six locations per tooth. The percentage of sites affected with BOP and PPD were calculated by taking the number of sites affected divided by the total number of sites present for each subject. Healthy patients were categorized by BOP at $\leq$10% of sites (6 sites per tooth), <3% of sites with PPD $\geq$ 4 mm, and no sites with clinical attachment loss (CAL) $\geq$ 2 mm. Subjects in the gingivitis group were defined as BOP at $\geq$20% of sites, <3% of sites with PPD $\geq$ 4 mm, and no sites with CAL $\geq$ 2 mm. The periodontitis group had BOP at >10% of sites, with >5% of sites with PPD $\geq$ 4 mm and CAL $\geq$ 2 mm.

Salivary Samples

Saliva samples were collected from both groups prior to clinical evaluation. All subjects rinsed with tap water (10 mL) for 30 s and expectorated prior to saliva collection. Unstimulated whole saliva was collected according to a modification of the method described by Navazesh (1993). Subjects were asked to avoid oral hygiene measures (i.e., flossing, brushing, and mouth rinses), eating, drinking, or gum chewing 1 h prior to saliva collection. Subjects then expectorated at least 5 mL of unstimulated whole saliva into sterile tubes containing lyophilized protease inhibitor solution (SIGMAFast). Saliva samples were collected on ice. Aliquots were prepared and samples were frozen at -80°C until analysis.

Salivary Molecular Biomarkers

The MILLIPLEX MAP Kit (EMD Millipore, Billerica, MA, USA) was used to detect IL-1β, IL-6, MMP-8, and MIP-1α. This kit was used to analyze individual saliva samples for the four analytes using a Luminex 100IS instrument (EMD Millipore) according to the manufacturer's instructions. All analyses were performed in duplicate within 6 months of obtaining the sample. Standards were included on all runs, and all results are reported within the linearity of the assays.

Statistical Analyses

Descriptive statistics were calculated for the demographic data and individual salivary anaytes. An ANOVA was used to evaluate differences in levels across the three groups with Tukey's *post-hoc* testing (SigmaStat v3.5, San Jose, CA). A Pearson Correlation analysis was conducted to relate levels of the salivary analytes to clinical features of the population. Finally, Chi-square test and Relative Risk ratio was determined using individual analytes to discriminate periodontitis from health or gingivitis (MedCalc, v14.12, Ostend, Belgium).

To determine the optimal cut points for distinguishing between the periodontitis and non-periodontitis patients (healthy and gingivitis) the analytes were used individually as predictors in a linear regression model (SAS v9.4, Cary, NC). The intersection of the sensitivity and specificity was used as the optimal cut point for the predictors. The AUC was also calculated for each individual analyte.

Four different classification techniques namely [Linear Discriminant Analysis (LDA), Quadratic Discriminant Analysis (QDA), Naïve Bayes Classifier (NB), Support Vector Machine (SVM)] were used to discern gingivitis from periodontitis and health from periodontitis using the salivary markers. Classification performance measures (sensitivity, specificity and accuracy) were estimated using the clinical labels of the samples as the ground truth and leave-ten-out cross-validation where 10 samples are set aside as the test set with the remaining samples as the training set. The mean values of the classification performance measures were estimated across ($N = 100$) independent realizations by randomly assigning the samples to the test and training sets. Classification was repeated using all possible combinations of markers (pairs, triplets, and all four markers).

Results

Distribution of Analytes in Saliva

Included in the cohort were 65 subjects in the healthy group, 43 subjects in the gingivitis group and 101 subjects in the periodontitis group, with some differences in age, gender, race/ethnicity, and smoking across the groups (**Table 1**).

The profiles of salivary analytes IL-1ß, IL-6, MMP-8, and MIP-1α in the cohort are displayed in **Figure 1**. IL-1β concentrations were significantly higher in the periodontitis group (102.3 $\pm$ 10.1 SEM pg/mL) compared to levels in the gingivitis (28.7 $\pm$ 7.3) and healthy (14.6 $\pm$ 2.6) subjects. A minimal difference was noted in the cytokine comparing the overall gingivitis group to the healthy individuals. Similarly, a significantly elevated concentration of

TABLE 1 | Demographics of the population.

	Healthy ($n = 65$)	Gingivitis ($n = 43$)	Periodontitis ($n = 101$)
Age (years; mean ± SD)	28.2 ± 5.9[a]	27.8 ± 4.5[a]	42.0 ± 10.4[a]
Female (%)	60.0[a]	48.8[a]	32.7[a]
White (%)	90.0[a]	95.4[a]	41.60[a]
Hispanic (%)	2.5	2.3	34.7
African American (%)	0	2.3	17.8
Asian/Other (%)	7.5	0	6.9
Current tobacco use (%)	0[a]	0[a]	28.0[a]
No. of teeth	27.6 (range 20–32)	27.6 (range 24–28)	27.1 (range 20–28)
PERIODONTAL INDICES (%SITES; MEAN ± SD)			
BOP sites	4.26 ± 3.4[a,b]	25.4 ± 5.9[a,b]	57.4 ± 24.2[a]
PD ≥ 4 mm sites	0.6 ± 1.1[a]	0.6 ± 2.0[a]	27.3 ± 14.7[a]
PD ≥ 5 mm sites	0.1 ± 0.3[a]	0.1 ± 0.2[a]	16.6 ± 11.2[a]

[a] *Significantly different from periodontitis group at least at $p < 0.05$.*
[b] *Significantly different from healthy group at least at $p < 0.05$.*

salivary IL-6 was found in the periodontitis group (22.8 ± 3.7 pg/mL) compared to both the gingivitis (6.3 ± 2.7) and healthy (3.7 ± 0.5) subjects. Consistent with this, IL-6 levels were below the level of detection in the assay (0.64 pg/mL) in approximately 2% of the periodontitis patients, compared with nearly 20% of both gingivitis and healthy subjects. MMP-8 levels were also significantly elevated in the periodontitis group (314.1 ± 25.5 ng/mL) compared to both gingivitis (199.0 ± 29.1) and healthy (130.7 ± 14.5) subjects, although there appeared to be a greater overlap across the groups with this analytes vs. the others that were examined. Finally, MIP-1α was significantly increased in the periodontitis group (16.2 ± 2.2 pg/mL) compared to both gingivitis (12.0 ± 2.2) and health (3.2 ± 1.0) groups.

Salivary Analytes and Disease Measures

The relationship of these salivary biomarkers with the clinical parameters of oral disease was examined in an attempt to identify any clinical and biological relationships. Across the spectrum of patents, IL-1ß, IL-6, and MMP-8 levels were significantly positively correlated with BOP frequency in the population, while both IL-1ß and MMP-8 were significantly correlated with percent sites with advanced PPDs across the population (**Figure 2**). **Table 2** shows the results describing that only IL-1ß and MMP-8 levels were significantly correlated with PPD levels across the population and that this relationship was also observed in the periodontitis patients to a similar degree with the extent of disease (i.e., %sites with ≥4 mm or ≥5 mm PPD) and the severity of disease (i.e., mouth mean PPD).

Discriminatory Analytes and Periodontal Disease

Data were then used to explore the capacity of these four analytes to effectively discriminate periodontitis from gingivitis and health. Three strategies were used in this approach. First threshold cutoff values for each analyte were based upon the population distribution for IL-1ß (≥28 pg/mL), IL-6 (≥5.5 pg/mL), MMP-8 (≥140 ng/mL), and MIP-1α (≥5 pg/mL) and selected to optimize sensitivity for detection of periodontitis. Based upon these threshold values, the results in **Table 3** show that for elevated concentrations of IL-1ß, IL-6, and MMP-8 significantly categorized periodontitis patients compared with both healthy and gingivitis groups. These data also provided an assessment of the relative risk for a patient to be clinically classified as periodontitis based upon the individual biomarkers, with each marker showing a very high level of significance and a 2 to 4-fold relative risk when the concentration was above the threshold.

We developed ROC curves and determined the AUC (c-statistic) for each analyte individually (**Figure 3**) by comparing the levels in the periodontitis group vs. those identified in both health and gingivitis. The summary results in **Table 4** show the highest sensitivity and specificity for periodontitis with both IL-1ß and IL-6, paralleling the results found using our earlier approaches to stratify the analyte levels with disease.

Finally, in recognizing the capacity of ROC analyses to potentially overfit the classification properties of the data (Baker, 2003), we utilized four classification techniques (LDA, QDA, NB, SVM) to evaluate the capacity of various groupings of analytes to effectively categorize the periodontitis patients. **Table 5** provides the classification performance measures (sensitivity, specificity, accuracy) using the LDA approach. Outcomes from QDA, NB, and SVM were similar hence not shown. The LDA results were consistent with the ROC analyses and clearly showed that combinations of these biomarkers improved the sensitivity, specificity, and accuracy of the identification of periodontitis vs. either health or gingivitis subjects when compared to the performance of the individual biomarkers. As might be expected, the greatest sensitivity, specificity, and accuracy were identified in comparing health with periodontitis patients. The classification performance measures using the pair (IL-1ß, IL-6) was especially pronounced indicating that these molecular markers play a critical role in discerning periodontitis from gingivitis and health.

Discussion

Studies over the last two decades have provided an array of targets for detection of various substances in saliva (Desai and Mathews, 2014), including drugs of abuse (Moore and Crouch, 2013), alcohol (Swift, 2003), estradiol (Lewis, 2006), cotinine (Scheidweiler et al., 2011), cortisol (van Andel et al., 2014), and HIV antibody (Pant Pai et al., 2012) as examples. Based upon the potential value of saliva as a non-invasive screening tool for oral disease(s), this study focused on the quantification of a group of analytes that may act as biomarkers for periodontitis and aid in the development of personalized approaches for periodontal risk assessment. Movement toward an era of personalized medicine and individualized clinical decisions in periodontology requires significant improvement in our ability to define risk and predict disease progression. While the medical field routinely makes clinical diagnoses based on signs and symptoms (e.g., pneumonia, diarrhea), decisions on patient management and treatment do not stop here. Modern medicine integrates these clinical descriptors with biological assessments that enable the physician to focus on the specific disease etiology and unique features of the patient in finalizing a treatment strategy. However,

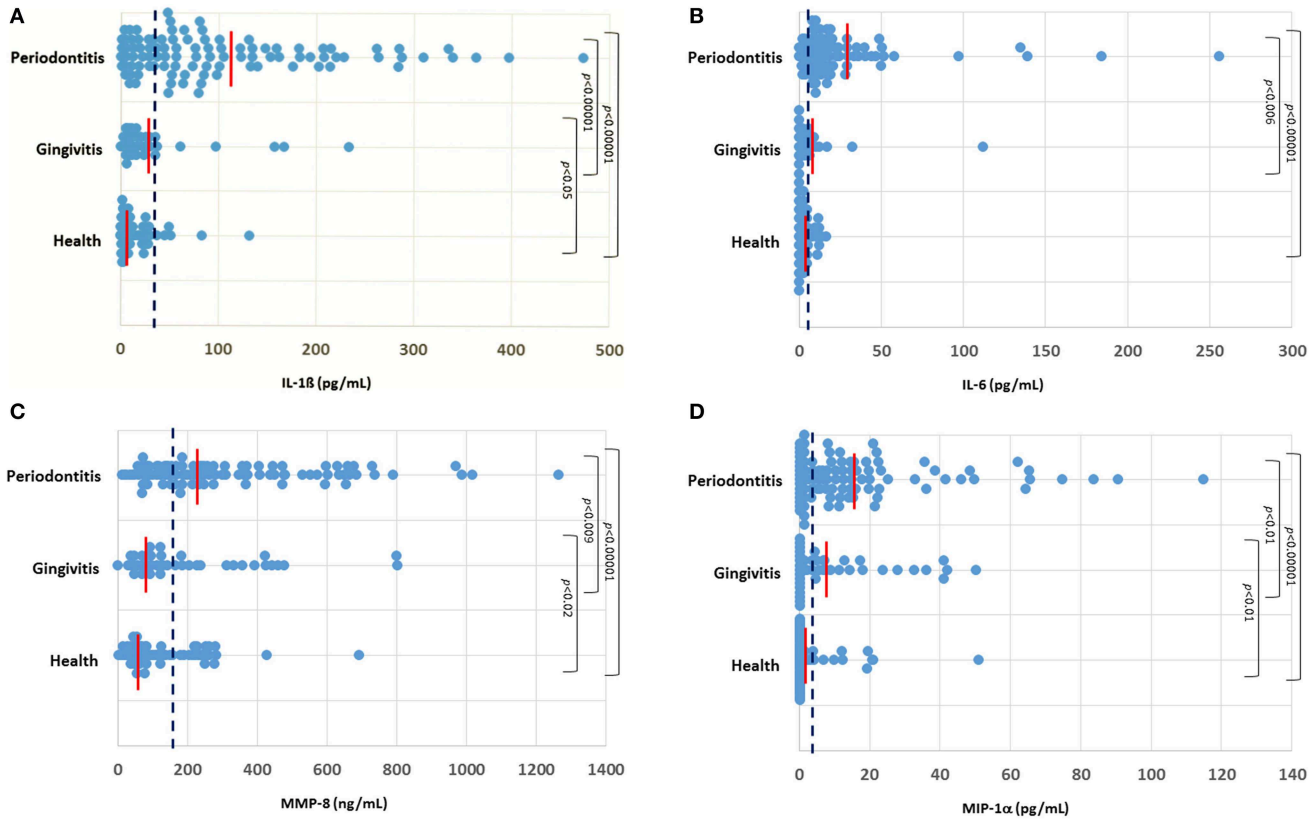


FIGURE 1 | Distribution of levels of IL-1ß (A), IL-6 (B), MMP-8 (C), and MIP-1α (D) in the three groups of subjects. Each point denotes the analyte value for a patient. The vertical red line denotes the group mean and the vertical blue dashed line denotes the threshold cutoff for each analyte (IL-1ß ≥ 28 pg/mL; IL-6 ≥ 5.5 pg/mL; MMP-8 ≥ 140 ng/mL; MIP-1α ≥ 5 pg/mL).

it is clear the clinical measures alone do not provide sufficient information to determine which patients will/won't progress, and what therapy should be provided to those at risk. Recent investigations using proteomic and transcriptomic analyses have dramatically expanded the potential panel of biomarkers for gingivitis; however, generally these studies have been limited to comparisons of gingivitis with health, or periodontitis with health (Offenbacher et al., 2009; Jönsson et al., 2011). No studies have been published that extrapolate from these reports analytes in gingival tissues, crevicular fluid, or saliva that discriminate gingivitis from periodontitis.

Periodontitis represents a persistent inflammatory response to chronic biofilms inhabiting the subgingival crevice (Hajishengallis, 2014; Nibali et al., 2014). The current paradigm suggests that variations in the quantity and quality of the oral microbial ecology at health, gingivitis, and periodontitis sites results in a dysregulated inflammatory response that causes release of a variety of host biomolecules that lead to the clinical features of periodontitis. These biomolecules can represent the various stages of progression of the destructive inflammatory response, including IL-1ß as a proinflammatory cytokine that has effects on coupling processes in bone biology (Nakashima and Takayanagi, 2009; Braun and Schett, 2012), IL-6 as a pleiotropic cytokine that communicates inflammatory signals with a number of cell types, and can elicit bone resorptive processes (Huang et al., 2001; Braun and Schett, 2012), MMP-8 a primary collagenase effective on both types I and III collagen and released by neutrophils that alters the integrity of soft tissues in the periodontium (Salminen et al., 2014), and MIP-1α (also known as CCL3), a chemokine macrophage inflammatory protein that binds to CCR1, CCR4, and CCR5 receptors frequently on the surface of immune cells, recruiting them into sites of inflammation (Kabashima et al., 2001). This chemokine has also been found to activate osteoclasts, particularly related to bone resorption in multiple myeloma through these receptors (Terpos et al., 2005). Importantly, many of these biomarkers have been detected in saliva and correlate with periodontal disease. While it is clear that these biomolecules contribute to the inflammatory and tissue destructive processes of periodontitis, a number of them have also been detected in serum associated with chronic inflammation related to systemic diseases (Fain, 2006; Zakynthinos and Pappa, 2009; Wu et al., 2010; Cierny et al., 2014). We have also evaluated some of these in saliva and while they can be elevated to some degree with systemic inflammation vs. control individuals, the levels of these analytes in saliva with periodontitis are significantly increased compared to any of the systemic conditions (Mirrielees et al., 2010; Miller et al., 2014). Nevertheless, a clinical medical history should be taken into

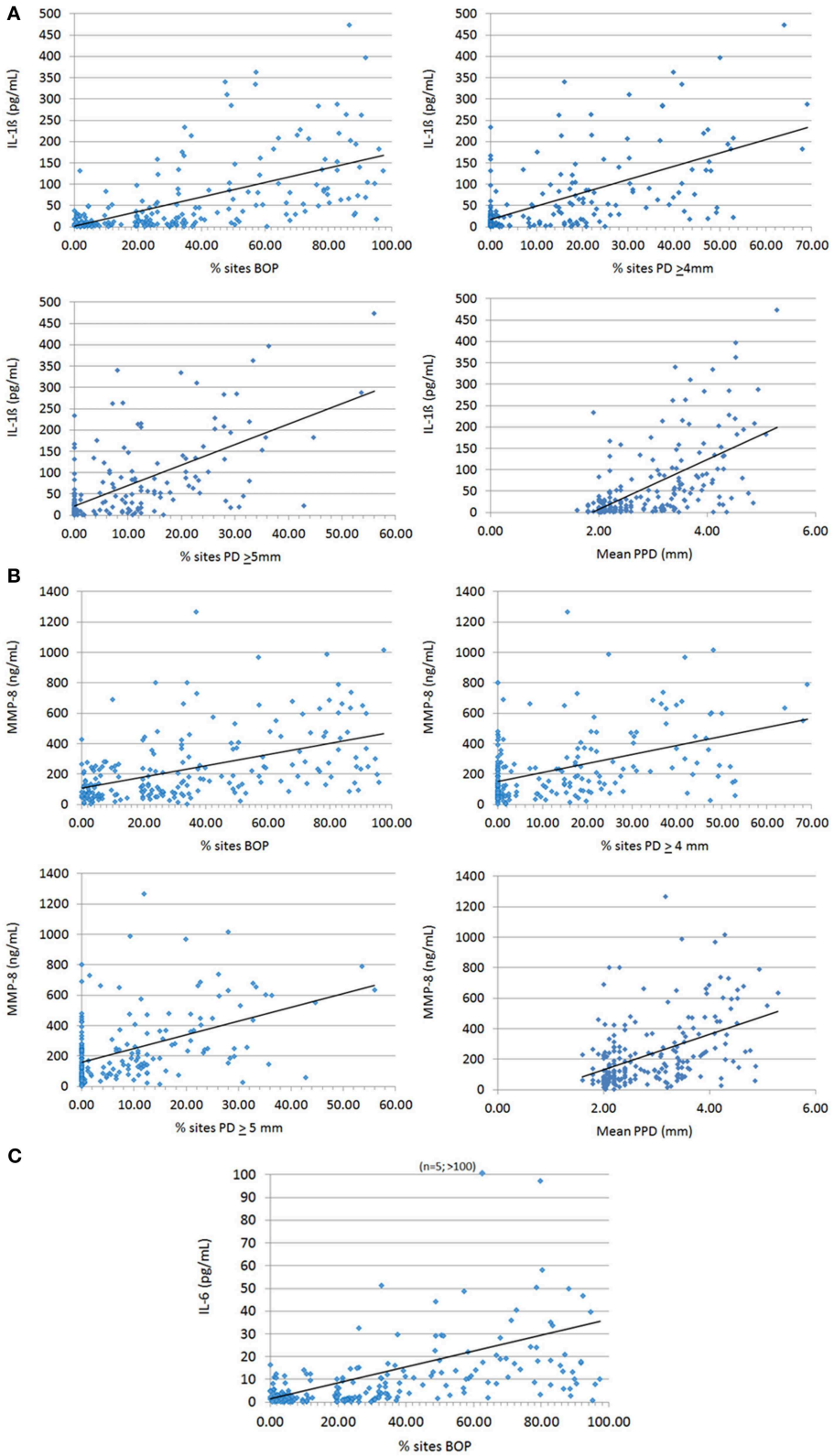


FIGURE 2 | Correlation analyses of biomarkers IL-1ß (A), MMP-8 (B), and IL-6 (C) significantly related to clinical indices of periodontitis. Each point denotes a patient in the population ($n = 209$) and the line denotes the correlation trendline.

account to minimize false-positive responses in periodontally healthy subjects.

IL-1ß has been identified in gingival crevicular fluid (GCF; Faizuddin et al., 2003; Kinney et al., 2014) and saliva (Miller et al., 2006; Yoon et al., 2012; Salminen et al., 2014) in elevated levels in numerous investigations of periodontitis. Some of these studies have also shown that the levels are related to disease extent/severity (Tobón-Arroyave et al., 2008) and decrease with therapy (Sexton et al., 2011). Moreover, while it is somewhat controversial at this time, data exist suggesting that polymorphisms in this gene impose a risk for periodontitis (Lang et al., 2000; Lee et al., 2012; Diehl et al., 2015). We have previously identified elevated levels of IL-1ß in saliva from periodontitis patients compared to orally healthy individuals (Miller et al., 2006, 2014; Frodge et al., 2008), and have shown a relatively stable level of this analyte in whole saliva of healthy subjects over time (Thomas et al., 2009; Syndergaard et al., 2014). The findings in this study extended these results by inclusion of gingivitis patients and demonstrating significant elevation in periodontitis saliva and positive correlations of salivary IL-1ß levels with BOP and measures of pocket PPD. Determining a threshold for positive response at ≥28 pg/mL showed a significant discriminatory power in periodontitis for this analyte, with a RR = 4.2772 for periodontitis with elevated salivary IL-1ß. This finding is consistent with previous results reported by us and others; however, these reports generally compared periodontitis to oral health (Miller et al., 2006; Gursoy et al., 2009; Kaushik et al., 2011; Kinney et al., 2014; Salminen et al., 2014).

Increased IL-6 levels have also been found in GCF (Fujita et al., 2012; Javed et al., 2012) and saliva (Costa et al., 2010; Gümüs et al., 2014; Javed et al., 2014) from periodontitis patients vs. health controls. A limited number of studies have also identified increases in IL-6 in periodontitis tissues (Duarte et al., 2012), although a meta-analysis by Song et al. (2013) suggested genetic polymorphisms for this cytokine may be limited in their relationship to periodontitis across various populations. Treatment studies have also documented decreases in IL-6 in GCF following non-surgical periodontal therapy (Kardesler et al., 2011; de Lima Oliveira et al., 2012). The overall levels of this cytokine were substantially lower than IL-1ß, and nearly 25% of the healthy/gingivitis groups showed no detectable IL-6 in saliva and only 2/101 periodontitis patients. As such, the levels were significantly elevated in the periodontitis group. Minimal correlations in IL-6 levels across the population were observed, appearing only related to BOP levels. However, using a threshold value of =5.5 pg/mL, showed a significant increase in positive responses in the periodontitis patients with a highly significant RR = 4.1703 for elevated salivary IL-6 levels commensurate with periodontitis.

MMP-8, a major factor produced by neutrophils at sites of inflammation has a robust literature demonstrating elevations in GCF (Tervahartiala et al., 2000; Mäntylä et al., 2006; Kinney et al., 2014) and saliva (Javed et al., 2014; Salminen et al., 2014) in periodontitis. Sorsa et al. (1999) have demonstrated the value of detection of elevations in this analyte for diagnosing periodontitis and following therapeutic intervention for the disease (Kinane et al., 2003; Sexton et al., 2011). We had confirmed these types of findings in periodontitis and healthy groups (Miller et al., 2006), but demonstrate in this study differences also between periodontitis and gingivitis patients. Interesting aspects of the distribution of MMP concentrations was the large range in values detected in saliva from each of the groups, and that the gingivitis group showed significantly higher levels than

TABLE 2 | Correlation of individual salivary biomarkers with bleeding on probing and frequency of probing pocket depths in the population.

Clinical measure	Analyte	Total	Health	Gingivitis	Periodontitis
%BOP > 0	**IL-1ß**	**0.6144**	−0.0311	−0.0719	**0.4669**
%PPD ≥ 4 mm		**0.6429**	−0.1219	−0.0006	**0.5167**
%PPD ≥ 5 mm		**0.5836**	0.0356	0.2142	**0.4068**
Mean PPD		**0.6009**	0.0757	−0.1269	**0.4596**
%BOP > 0	**IL−6**	**0.3622**	0.0156	−0.0936	0.2098
%PPD ≥ 4 mm		0.2887	0.0848	−0.0136	0.0611
%PPD ≥ 5 mm		0.3163	−0.0146	0.0835	0.1305
Mean PPD		0.3247	0.1312	0.3292	0.0861
%BOP > 0	**MMP−8**	**0.4841**	0.1657	0.2583	**0.3827**
%PPD ≥ 4 mm		**0.4502**	−0.1248	−0.00794	**0.3613**
%PPD ≥ 5 mm		**0.4590**	−0.1182	−0.1395	**0.3746**
Mean PPD		**0.4629**	0.0733	−0.0602	**0.3862**
%BOP > 0	**MIP−1α**	0.3280	−0.0308	−0.1141	0.1641
%PPD ≥ 4 mm		0.2821	−0.1540	−0.0136	0.1179
%PPD ≥ 5 mm		0.2883	−0.0958	−0.0691	0.1449
Mean PPD		0.2942	−0.3365	0.1428	0.1130

Figures in bold denote significant correlation at least at p < 0.05. Total denotes all 3 subgroups comprising the entire cohort.

TABLE 3 | Discriminatory power of individual analytes using thresholds based on responses across the population.

Analyte	Healthy (*n* = 65)	Gingivitis (*n* = 43)	Periodontitis (*n* = 101)	Periodontitis vs. health	Periodontitis vs. gingivitis	Relative risk
IL-1ß	9[a]	10	76	$X^2 = 57.24$ $P < 0.0001$	$X^2 = 31.76$ $P < 0.0001$	4.2772 (2.8013–6.5309) $P < 0.0001$
IL-6	10	10	78	$X^2 = 58.26$ $P < 0.0001$	$X^2 = 34.73$ $P < 0.0001$	4.1703 (2.7689–6.2810) $P < 0.0001$
MMP-8	22	18	72	$X^2 = 21.07$ $P < 0.0001$	$X^2 = 9.92$ $P = 0.0016$	1.9440 (1.4722–2.5583) $P < 0.001$
MIP-1α	7	12	42	$X^2 = 34.6$ $P < 0.0001$	$X^2 = 2.15$ NS	2.3637 (1.4787–3.7785) $P = 0.0003$

[a]*Denotes number of patient samples above the thresholds of: IL-1ß—28 pg/mL, IL-6—5.5 pg/mL, MMP-8—140 ng/mL, MIP-1α—5 pg/mL.*

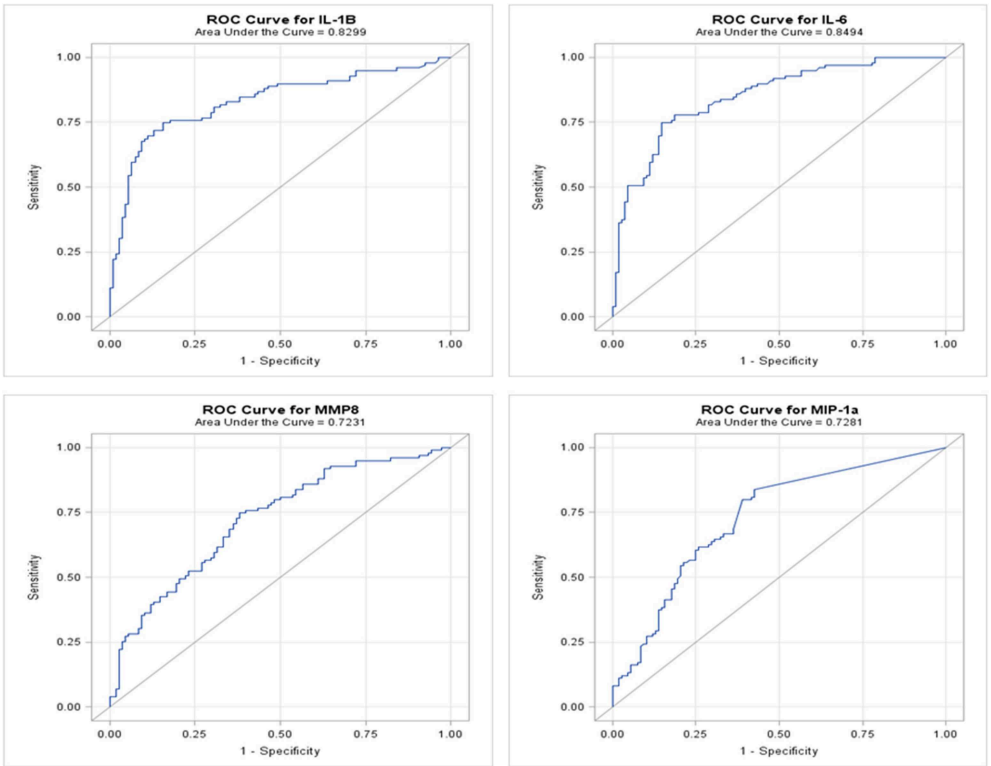


FIGURE 3 | ROC analysis of individual salivary analytes in the entire population, comparing levels in periodontitis to those in "not periodontitis" (i.e., health and gingivitis).

TABLE 4 | Results from ROC logistic regression analysis of individual salivary biomarker levels comparing periodontitis group to "not periodontitis" (i.e., health and gingivitis) group.

Analyte	Optimal cut*	Sensitivity	Specificity	PPV	NPV	AUC
IL-1β	24.00 pg/mL	0.752	0.759	0.745	0.766	0.830
IL-6	5.11 pg/mL	0.780	0.787	0.772	0.794	0.849
MMP8	165.92 ng/mL	0.653	0.667	0.647	0.673	0.728
MIP-1α	3.28 pg/mL	0.663	0.676	0.657	0.682	0.723

**The optimal cut was selected based on the intersection of the specificity and sensitivity plots.*

healthy subjects. This was not totally unexpected due to the inflammation in the gingivitis patients. This finding was also supported by the significant positive correlation of MMP-8 levels with all of the clinical measures. Identifying a threshold of ≥140 ng/mL demonstrated a significantly greater frequency of positive responses in periodontitis with an RR = 1.9440.

Of the analytes targeted in this study, MIP-1α has a least amount of information regarding its distribution in periodontitis. MIP-1α (CCL3) is a member of the cysteine-cysteine chemokine family which is secreted by macrophages, neutrophils, basophils, dendritic cells, lymphocytes and epithelial cells and mediates granulocyte migration and adhesion (Kabashima et al., 2001, 2002; Glass et al., 2003). It is an upstream signaling protein that stimulates monocytes and/or osteoclast progenitor cells to become active osteoclasts in a RANK/RANKL and dose-dependent manner (Giuliani et al., 2004). MIP-1α has been detected at higher salivary levels (50-fold) in a longitudinal study of seven adolescents who had aggressive periodontitis compared with controls (Fine et al., 2009), and appeared to increase prediction of disease progression. We had shown previously that MIP-1α was significantly higher in periodontitis subjects compared to healthy individuals and decreased following periodontal therapy (Sexton et al., 2011; Al-Sabbagh et al., 2012). These findings suggested that the salivary level of MIP-1α could have clinical utility as a screening tool for moderate to severe periodontal disease. However, as we noted its utility for discriminating between intermediate levels of disease (gingivitis, mild periodontitis) and health was indeterminant. This study expanded the target population and identified additional critical features of this salivary analyte. First, concentrations in periodontitis were significantly increased compared to both health and gingivitis groups. Second, correlation with the various clinical parameters was observed with the entire population. Finally using a threshold of ≥5 pg/mL demonstrated a significantly increased frequency of being classified as periodontitis with an RR = 2.3637.

While each of these analytes appeared useful in discriminating periodontitis from health and gingivitis, the combination of sensitivity, specificity, and accuracy was improved by exploring combinations of the biomarkers. Our data showed that concentrations above a defined threshold for any three of the biomarkers identified 67.3% of periodontitis, 18.6% of gingivitis,

TABLE 5 | Average Classification performance measures (Sensitivity, Specificity, Accuracy) estimated across all possible combinations of markers using leave-10-out cross-validation and 100 independent realizations.

	IL-1ß IL-6	IL-1ß MMP-8	IL-1ß MIP-1α	IL-6 MMP-8	IL-6 MIP-1 α	MMP-8 MIP-1 α	IL-1ß IL-6 MMP-8	IL-1ß IL-6 MIP-1 α	IL-6 MMP-8 MIP-1 α	IL-1ß IL-6 MMP-8 MIP-1 α
HEALTH vs. PERIODONTITIS										
Sensitivity	0.8115	0.7798	0.7965	0.8103	0.7878	0.7010	0.7780	0.8055	0.7948	0.7868
Specificity	0.7718	0.7675	0.7520	0.7283	0.7720	0.7728	0.7708	0.7868	0.7430	0.7820
Accuracy	0.7916	0.7736	0.7743	0.7693	0.7799	0.7369	0.7744	0.7961	0.7689	0.7844
GINGIVITIS vs. PERIODONTITIS										
Sensitivity	0.7755	0.7630	0.7545	0.7843	0.8073	0.6473	0.7653	0.7830	0.7260	0.7803
Specificity	0.7565	0.7323	0.7865	0.7115	0.7073	0.6190	0.7538	0.7905	0.7340	0.7810
Accuracy	0.7660	0.7476	0.7705	0.7479	0.7573	0.6331	0.7595	0.7868	0.7300	0.7806

and 6.2% of healthy subjects. We then identified that pairs of markers, including IL-1ß/IL-6, IL-1ß/MMP-8, and IL-6/MMP-8, provided an increase in diagnostic ability by demonstrating sensitivity and specificity values approximating 0.8. We also identified that inclusion of three biomarkers into the biologic diagnostic model, IL-1ß/IL-6/MMP-8, yielded a small increase in the sensitivity, specificity, and accuracy values. Using MIP-1α in these various groupings provided little improvement in the discriminatory characteristics to identify periodontitis patients. Finally, our diverse analyses allowed us to provide potential thresholds to discriminate periodontitis (i.e., IL-1ß: 24–28 pg/mL; IL-6: 5.11–5.5 pg/mL; MIP-1α: 3.28–5 pg/mL, and MMP-8: 140–165.9 ng/mL).

While these analytes have been evaluated as single biomarkers in saliva for periodontitis in other studies, generally these previous studies have not evaluated combinations of biomarkers representing the various disease processes that occur in periodontitis, nor have they included patients with gingivitis to elucidate the effect of gingival inflammation on these analytes. This cross-sectional study enabled a "head-to-head" comparison of these salivary analytes in destructive (periodontitis) and non-destructive reversible (gingivitis) gingival inflammation. Thus, we can conclude from these studies that select biomarkers, particularly in combination provide enhanced sensitivity and specificity for identification of periodontitis in the population. Also, of important note, as can be seen in the salivary analyte distribution graphs, there was a subset of gingivitis patients and even a few healthy subjects that demonstrated elevated levels of one or more of these salivary analytes. Generally, when these elevated responses occurred, there were multiple analyte elevations in the same individuals. Historically, evaluation of salivary biomarker data was structured to "force" the patients into a specific clinical group and accept that this "within group heterogeneity" would be reflected in the variation in analyte levels contributing to group differences. However, in this era of personalized and precision medicine (Hood et al., 2012; Mirnezami et al., 2012; Flores et al., 2013; Schmidt, 2014), we submit that there is substantive value in identifying these subsets of individuals within the larger clinical groupings and document unique features of their disease trajectory and/or patient specific responses that could characterize risk or resistance to disease, and/or response to therapy.

Acknowledgments

This study was supported by grants NIH U01 DE017793, M01-RR02602, P20 GM103538 (P20 RR020145) and UL1TR000117 from the National Center for Applied and Translational Sciences (NCATS). We thank Jason Stevens and Michelle Steffen for technical support in analysis of the salivary analytes. We also thank clinical collaborators, Drs. D. Dawson, J. Schuster, A. Aldallal, M. Al-Sabbagh, and B. Syndergaard for providing patient samples and the members of the Delta Dental Clinical Research Center for coordinating patient assessment and sample collection.

References

Albandar, J. M., Kingman, A., Brown, L. J., and Löe, H. (1998). Gingival inflammation and subgingival calculus as determinants of disease progression in early-onset periodontitis. *J. Clin. Periodontol.* 25, 231–237. doi: 10.1111/j.1600-051X.1998.tb02433.x

Al-Sabbagh, M., Alladah, A., Lin, Y., Kryscio, R. J., Thomas, M. V., Ebersole, J. L., et al. (2012). Bone remodeling-associated salivary biomarker MIP-1α distinguishes periodontal disease from health. *J. Periodont. Res.* 47, 389–395. doi: 10.1111/j.1600-0765.2011.01445.x

Armitage, G. C., and Cullinan, M. P. (2010). Comparison of the clinical features of chronic and aggressive periodontitis. *Periodontol. 2000* 53, 12–27. doi: 10.1111/j.1600-0757.2010.00353.x

Armitage, G. C., and Robertson, P. B. (2009). The biology, prevention, diagnosis and treatment of periodontal diseases: scientific advances in the United States. *J. Am. Dent. Assoc.* 140(Suppl. 1), 36S–43S. doi: 10.14219/jada.archive.2009.0356

Armitage, G. C., Svanberg, G. K., and Löe, H. (1977). Microscopic evaluation of clinical measurements of connective tissue attachment levels. *J. Clin. Periodontol.* 4, 173–190. doi: 10.1111/j.1600-051X.1977.tb02271.x

Baker, S. G. (2003). The central role of receiver operating characteristic (ROC) curves in evaluating tests for the early detection of cancer. *J. Natl. Cancer Inst.* 95, 511–515. doi: 10.1093/jnci/95.7.511

Beikler, T., Peters, U., Prior, K., Eisenacher, M., and Flemmig, T. F. (2008). Gene expression in periodontal tissues following treatment. *BMC Med. Genomics* 1:30. doi: 10.1186/1755-8794-1-30

Braun, T., and Schett, G. (2012). Pathways for bone loss in inflammatory disease. *Curr. Osteoporos. Rep.* 10, 101–108. doi: 10.1007/s11914-012-0104-5

Brecx, M. C., Nalbandian, J., Kornman, K. S., and Robertson, P. B. (1986). Morphological studies on periodontal disease in the cynomolgus monkey. III. Electron microscopic observations. *J. Periodont. Res.* 21, 137–153. doi: 10.1111/j.1600-0765.1986.tb01446.x

Cierny, J. T., Unal, E. R., Flood, P., Rhee, K. Y., Praktish, A., Olson, T. H., et al. (2014). Maternal inflammatory markers and term labor performance. *Am. J. Obstet. Gynecol.* 210, 447.e1–6. doi: 10.1016/j.ajog.2013.11.038

Costa, P. P., Trevisan, G. L., Macedo, G. O., Palioto, D. B., Souza, S. L., Grisi, M. F., et al. (2010). Salivary interleukin-6, matrix metalloproteinase-8, and osteoprotegerin in patients with periodontitis and diabetes. *J. Periodontol.* 81, 384–391. doi: 10.1902/jop.2009.090510

de Lima Oliveira, A. P., de Faveri, M., Gursky, L. C., Mestnik, M. J., Feres, M., Haffajee, A. D., et al. (2012). Effects of periodontal therapy on GCF cytokines in generalized aggressive periodontitis subjects. *J. Clin. Periodontol.* 39, 295–302. doi: 10.1111/j.1600-051X.2011.01817.x

Desai, G. S., and Mathews, S. T. (2014). Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance. *World J. Diabetes* 5, 730–738. doi: 10.4239/wjd.v5.i6.730

Diehl, S. R., Kuo, F., and Hart, T. C. (2015). Interleukin 1 genetic tests provide no support for reduction of preventive dental care. *J. Am. Dent. Assoc.* 146, 164–173.e4. doi: 10.1016/j.adaj.2014.12.018

Duarte, P. M., Miranda, T. S., Lima, J. A., Dias Goncalves, T. E., Santos, V. R., Bastos, M. F., et al. (2012). Expression of immune-inflammatory markers in sites of chronic periodontitis in patients with type 2 diabetes. *J. Periodontol.* 83, 426–434. doi: 10.1902/jop.2011.110324

Ebersole, J. L., Dawson, D. R. III, Morford, L. A., Peyyala, R., Miller, C. S., and Gonzaléz, O. A. (2013). Periodontal disease immunology: 'double indemnity' in protecting the host. *Periodontol. 2000* 62, 163–202. doi: 10.1111/prd.12005

Fain, J. N. (2006). Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells. *Vitam. Horm.* 74, 443–477. doi: 10.1016/S0083-6729(06)74018-3

Faizuddin, M., Bharathi, S. H., and Rohini, N. V. (2003). Estimation of interleukin-1β levels in the gingival crevicular fluid in health and in inflammatory periodontal disease. *J. Periodont. Res.* 38, 111–114. doi: 10.1034/j.1600-0765.2003.01649.x

Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., et al. (2009). Macrophage inflammatory protein-1α: a salivary biomarker of bone loss in a longitudinal cohort study of children at risk for aggressive periodontal disease? *J. Periodontol.* 80, 106–113. doi: 10.1902/jop.2009.080296

Flores, M., Glusman, G., Brogaard, K., Price, N. D., and Hood, L. (2013). P4 medicine: how systems medicine will transform the healthcare sector and society. *Per. Med.* 10, 565–576. doi: 10.2217/pme.13.57

Frodge, B. D., Ebersole, J. L., Kryscio, R. J., Thomas, M. V., and Miller, C. S. (2008). Bone remodeling biomarkers of periodontal disease in saliva. *J. Periodontol.* 79, 1913–1919. doi: 10.1902/jop.2008.080070

Fujita, Y., Ito, H., Sekino, S., and Numabe, Y. (2012). Correlations between pentraxin 3 or cytokine levels in gingival crevicular fluid and clinical parameters of chronic periodontitis. *Odontology* 100, 215–221. doi: 10.1007/s10266-011-0042-1

Giannobile, W. V., Beikler, T., Kinney, J. S., Ramseier, C. A., Morelli, T., and Wong, D. T. (2009). Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol. 2000* 50, 52–64. doi: 10.1111/j.1600-0757.2008.00288.x

Giuliani, N., Colla, S., and Rizzoli, V. (2004). New insight in the mechanism of osteoclast activation and formation in multiple myeloma: focus on the receptor activator of NF-kappaB ligand (RANKL). *Exp. Hematol.* 32, 685–691. doi: 10.1016/j.exphem.2004.03.015

Glass, W. G., Rosenberg, H. F., and Murphy, P. M. (2003). Chemokine regulation of inflammation during acute viral infection. *Curr. Opin. Allergy Clin. Immunol.* 3, 467–473. doi: 10.1097/00130832-200312000-00008

Grigoriadou, M. E., Koutayas, S. O., Madianos, P. N., and Strub, J. R. (2010). Interleukin-1 as a genetic marker for periodontitis: review of the literature. *Quintessence Int. (Berl.)* 41, 517–525.

Gümüs, P., Nizam, N., Lappin, D. F., and Buduneli, N. (2014). Saliva and serum levels of B-cell activating factors and tumor necrosis factor-α in patients with periodontitis. *J. Periodontol.* 85, 270–280. doi: 10.1902/jop.2013.130117

Gursoy, U. K., Könönen, E., Uitto, V. J., Pussinen, P. J., Hyvärinen, K., Suominen-Taipale, L., et al. (2009). Salivary interleukin-1β concentration and the presence of multiple pathogens in periodontitis. *J. Clin. Periodontol.* 36, 922–927. doi: 10.1111/j.1600-051X.2009.01480.x

Hajishengallis, G. (2014). The inflammophilic character of the periodontitis-associated microbiota. *Mol. Oral Microbiol.* 29, 248–257. doi: 10.1111/omi.12065

Henskens, Y. M., van der Velden, U., Veerman, E. C., and Nieuw Amerongen, A. V. (1993). Protein, albumin and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis or periodontitis. *J. Periodont. Res.* 28, 43–48. doi: 10.1111/j.1600-0765.1993.tb01049.x

Hood, L., Balling, R., and Auffray, C. (2012). Revolutionizing medicine in the 21st century through systems approaches. *Biotechnol. J.* 7, 992–1001. doi: 10.1002/biot.201100306

Huang, G. T., Do, M., Wingard, M., Park, J. S., and Chugal, N. (2001). Effect of interleukin-6 deficiency on the formation of periapical lesions after pulp exposure in mice. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 92, 83–88. doi: 10.1067/moe.2001.115025

Javed, F., Ahmed, H. B., Saeed, A., Mehmood, A., and Bain, C. (2014). Whole salivary interleukin-6 and matrix metalloproteinase-8 levels in patients with chronic periodontitis with and without prediabetes. *J. Periodontol.* 85, e130–e135. doi: 10.1902/jop.2013.130514

Javed, F., Al-Askar, M., and Al-Hezaimi, K. (2012). Cytokine profile in the gingival crevicular fluid of periodontitis patients with and without type 2 diabetes: a literature review. *J. Periodontol.* 83, 156–161. doi: 10.1902/jop.2011.110207

Jönsson, D., Ramberg, P., Demmer, R. T., Kebschull, M., Dahlén, G., and Papapanou, P. N. (2011). Gingival tissue transcriptomes in experimental gingivitis. *J. Clin. Periodontol.* 38, 599–611. doi: 10.1111/j.1600-051X.2011.01719.x

Kabashima, H., Yoneda, M., Nagata, K., Hirofuji, T., and Maeda, K. (2002). The presence of chemokine (MCP-1, MIP-1α, MIP-1β, IP-10, RANTES)-positive cells and chemokine receptor (CCR5, CXCR3)-positive cells in inflamed human gingival tissues. *Cytokine* 20, 70–77. doi: 10.1006/cyto.2002.1985

Kabashima, H., Yoneda, M., Nagata, K., Hirofuji, T., Ishihara, Y., Yamashita, M., et al. (2001). The presence of chemokine receptor (CCR5, CXCR3, CCR3)-positive cells and chemokine (MCP1, MIP-1α, MIP-1β, IP-10)-positive cells in human periapical granulomas. *Cytokine* 16, 62–66. doi: 10.1006/cyto.2001.0947

Kardesler, L., Buduneli, N., Cetinkalp, S., Lappin, D., and Kinane, D. F. (2011). Gingival crevicular fluid IL-6, tPA, PAI-2, albumin levels following initial periodontal treatment in chronic periodontitis patients with or without type 2 diabetes. *Inflamm. Res.* 60, 143–151. doi: 10.1007/s00011-010-0248-7

Kaushik, R., Yeltiwar, R. K., and Pushpanshu, K. (2011). Salivary Interleukin-1β levels in chronic periodontitis patients before and after periodontal phase I therapy and healthy controls: a case-control study. *J. Periodontol.* 82, 1353–1359. doi: 10.1902/jop.2011.100472

Kebschull, M., Demmer, R. T., Grün, B., Guarnieri, P., Pavlidis, P., and Papapanou, P. N. (2014). Gingival tissue transcriptomes identify distinct periodontitis phenotypes. *J. Dent. Res.* 93, 459–468. doi: 10.1177/0022034514527288

Kebschull, M., Guarnieri, P., Demmer, R. T., Boulesteix, A. L., Pavlidis, P., and Papapanou, P. N. (2013). Molecular differences between chronic and aggressive periodontitis. *J. Dent. Res.* 92, 1081–1088. doi: 10.1177/0022034513506011

Kim, D. M., Ramoni, M. F., Nevins, M., and Fiorellini, J. P. (2006). The gene expression profile in refractory periodontitis patients. *J. Periodontol.* 77, 1043–1050. doi: 10.1902/jop.2006.050254

Kinane, D. F., Darby, I. B., Said, S., Luoto, H., Sorsa, T., Tikanoja, S., et al. (2003). Changes in gingival crevicular fluid matrix metalloproteinase-8 levels during periodontal treatment and maintenance. *J. Periodont. Res.* 38, 400–404. doi: 10.1034/j.1600-0765.2003.00663.x

Kinney, J. S., Morelli, T., Braun, T., Ramseier, C. A., Herr, A. E., Sugai, J. V., et al. (2011). Saliva/pathogen biomarker signatures and periodontal disease progression. *J. Dent. Res.* 90, 752–758. doi: 10.1177/0022034511399908

Kinney, J. S., Morelli, T., Oh, M., Braun, T. M., Ramseier, C. A., Sugai, J. V., et al. (2014). Crevicular fluid biomarkers and periodontal disease progression. *J. Clin. Periodontol.* 41, 113–120. doi: 10.1111/jcpe.12194

Kornman, K. S., Page, R. C., and Tonetti, M. S. (1997). The host response to the microbial challenge in periodontitis: assembling the players. *Periodontol. 2000* 14, 33–53. doi: 10.1111/j.1600-0757.1997.tb00191.x

Lang, N. P., Schatzle, M. A., and Loe, H. (2009). Gingivitis as a risk factor in periodontal disease. *J. Clin. Periodontol.* 36, 3–8. doi: 10.1111/j.1600-051X.2009.01415.x

Lang, N. P., Tonetti, M. S., Suter, J., Sorrell, J., Duff, G. W., and Kornman, K. S. (2000). Effect of interleukin-1 gene polymorphisms on gingival inflammation assessed by bleeding on probing in a periodontal maintenance population. *J. Periodontal Res.* 35, 102–107. doi: 10.1034/j.1600-0765.2000.03500 2102.x

Lee, A., Ghaname, C. B., Braun, T. M., Sugai, J. V., Teles, R. P., Loesche, W. J., et al. (2012). Bacterial and salivary biomarkers predict the gingival inflammatory profile. *J. Periodontol.* 83, 79–89. doi: 10.1902/jop.2011.110060

Leishman, S. J., Seymour, G. J., and Ford, P. J. (2013). Local and systemic inflammatory responses to experimentally induced gingivitis. *Dis. Markers* 35, 543–549. doi: 10.1155/2013/948569

Lewis, J. G. (2006). Steroid analysis in saliva: an overview. *Clin. Biochem. Rev.* 27, 139–146.

Mäntylä, P., Stenman, M., Kinane, D., Salo, T., Suomalainen, K., Tikanoja, S., et al. (2006). Monitoring periodontal disease status in smokers and nonsmokers using a gingival crevicular fluid matrix metalloproteinase-8-specific chair-side test. *J. Periodont. Res.* 41, 503–512. doi: 10.1111/j.1600-0765.2006.00897.x

Miller, C. S., Foley, J. D. III, Floriano, P. N., Christodoulides, N., Ebersole, J. L., Campbell, C. L., et al. (2014). Utility of salivary biomarkers for demonstrating acute myocardial infarction. *J. Dent. Res.* 93(7 Suppl.), 72S–79S. doi: 10.1177/0022034514537522

Miller, C. S., Foley, J. D., Bailey, A. L., Campbell, C. L., Humphries, R. L., Christodoulides, N., et al. (2010). Current developments in salivary diagnostics. *Biomark. Med.* 4, 1–18. doi: 10.2217/bmm.09.68

Miller, C. S., King, C. P. Jr., Langub, M. C., Kryscio, R. J., and Thomas, M. V. (2006). Salivary biomarkers of existing periodontal disease: a cross-sectional study. *J. Am. Dent. Assoc.* 137, 322–329. doi: 10.14219/jada.archive.2006.0181

Mirnezami, R., Nicholson, J., and Darzi, A. (2012). Preparing for precision medicine. *N. Engl. J. Med.* 366, 489–491. doi: 10.1056/NEJMp1114866

Mirrielees, J., Crofford, L. J., Lin, Y., Kryscio, R. J., Dawson, D. R. III, Ebersole, J. L., et al. (2010). Rheumatoid arthritis and salivary biomarkers of periodontal disease. *J. Clin. Periodontol.* 37, 1068–1074. doi: 10.1111/j.1600-051X.2010.01625.x

Moore, C., and Crouch, D. (2013). Oral fluid for the detection of drugs of abuse using immunoassay and LC-MS/MS. *Bioanalysis* 5, 1555–1569. doi: 10.4155/bio.13.115

Nakashima, T., and Takayanagi, H. (2009). Osteoimmunology: crosstalk between the immune and bone systems. *J. Clin. Immunol.* 29, 555–567. doi: 10.1007/s10875-009-9316-6

Navazesh, M. (1993). Methods for collecting saliva. *Ann. N.Y. Acad. Sci.* 694, 72–77. doi: 10.1111/j.1749-6632.1993.tb18343.x

Nibali, L., Henderson, B., Sadiq, S. T., and Donos, N. (2014). Genetic dysbiosis: the role of microbial insults in chronic inflammatory diseases. *J. Oral Microbiol.* 6. doi: 10.3402/jom.v6.22962

Offenbacher, S., Barros, S. P., Paquette, D. W., Winston, J. L., Biesbrock, A. R., Thomason, R. G., et al. (2009). Gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans. *J. Periodontol.* 80, 1963–1982. doi: 10.1902/jop.2009.080645

Offenbacher, S., Barros, S., Mendoza, L., Mauriello, S., Preisser, J., Moss, K., et al. (2010). Changes in gingival crevicular fluid inflammatory mediator levels during the induction and resolution of experimental gingivitis in humans. *J. Clin. Periodontol.* 37, 324–333. doi: 10.1111/j.1600-051X.2010.01543.x

Page, R. C. (1986). Gingivitis. *J. Clin. Periodontol.* 13, 345–359. doi: 10.1111/j.1600-051X.1986.tb01471.x

Page, R. C., and Schroeder, H. E. (1976). Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab. Invest.* 34, 235–249.

Pant Pai, N., Balram, B., Shivkumar, S., Martinez-Cajas, J. L., Claessens, C., Lambert, G., et al. (2012). Head-to-head comparison of accuracy of a rapid point-of-care HIV test with oral versus whole-blood specimens: a systematic review and meta-analysis. *Lancet Infect. Dis.* 12, 373–380. doi: 10.1016/S1473-3099(11)70368-1

Pepe, M. S., Etzioni, R., Feng, Z., Potter, J. D., Thompson, M. L., Thornquist, M., et al. (2001). Phases of biomarker development for early detection of cancer. *J. Natl. Cancer Inst.* 93, 1054–1061. doi: 10.1093/jnci/93.14.1054

Prakasam, S., and Srinivasan, M. (2014). Evaluation of salivary biomarker profiles following non-surgical management of chronic periodontitis. *Oral Dis.* 20, 171–177. doi: 10.1111/odi.12085

Rabinowitz, K., and Mayer, L. (2012). Working out mechanisms of controlled/physiologic inflammation in the GI tract. *Immunol. Res.* 54, 14–24. doi: 10.1007/s12026-012-8315-5

Salminen, A., Gursoy, U. K., Paju, S., Hyvärinen, K., Mäntylä, P., Buhlin, K., et al. (2014). Salivary biomarkers of bacterial burden, inflammatory response, and tissue destruction in periodontitis. *J. Clin. Periodontol.* 41, 442–450. doi: 10.1111/jcpe.12234

Schätzle, M., Faddy, M. J., Cullinan, M. P., Seymour, G. J., Lang, N. P., Bürgin, W., et al. (2009). The clinical course of chronic periodontitis: V. Predictive factors in periodontal disease. *J. Clin. Periodontol.* 36, 365–371. doi: 10.1111/j.1600-051X.2009.01391.x

Schätzle, M., Löe, H., Burgin, W., Anerud, A., Boysen, H., and Lang, N. P. (2003). Clinical course of chronic periodontitis. I. Role of gingivitis. *J. Clin. Periodontol.* 30, 887–901. doi: 10.1034/j.1600-051X.2003.00414.x

Schätzle, M., Löe, H., Lang, N. P., Bürgin, W., Anerud, A., and Boysen, H. (2004). The clinical course of chronic periodontitis. *J. Clin. Periodontol.* 31, 1122–1127. doi: 10.1111/j.1600-051X.2004.00634.x

Scheidweiler, K. B., Marrone, G. F., Shakleya, D. M., Singleton, E. G., Heishman, S. J., and Huestis, M. A. (2011). Oral fluid nicotine markers to assess smoking status and recency of use. *Ther. Drug Monit.* 33, 609–618. doi: 10.1097/ftd.0b013e318228ba39

Schmidt, C. (2014). Leroy Hood looks forward to P4 medicine: predictive, personalized, preventive, and participatory. *J. Natl. Cancer Inst.* 106:dju416. doi: 10.1093/jnci/dju416

Sexton, W. M., Lin, Y., Kryscio, R. J., Dawson, D. R. III, Ebersole, J. L., and Miller, C. S. (2011). Salivary biomarkers of periodontal disease in response to treatment. *J. Clin. Periodontol.* 38, 434–441. doi: 10.1111/j.1600-051X.2011.01706.x

Shaila, M., Pai, G. P., and Shetty, P. (2013). Salivary protein concentration, flow rate, buffer capacity and pH estimation: a comparative study among young and elderly subjects, both normal and with gingivitis and periodontitis. *J. Indian Soc. Periodontol.* 17, 42–46. doi: 10.4103/0972-124X.107473

Silva, T. A., Garlet, G. P., Fukada, S. Y., Silva, J. S., and Cunha, F. Q. (2007). Chemokines in oral inflammatory diseases: apical periodontitis and periodontal disease. *J. Dent. Res.* 86, 306–319. doi: 10.1177/154405910708600403

Song, G. G., Choi, S. J., Ji, J. D., and Lee, Y. H. (2013). Association between tumor necrosis factor-α promoter −308 A/G, −238 A/G, interleukin-6 −174 G/C and −572 G/C polymorphisms and periodontal disease: a meta-analysis. *Mol. Biol. Rep.* 40, 5191–5203. doi: 10.1007/s11033-013-2621-4

Sorsa, T., Mantyla, P., Ronka, H., Kallio, P., Kallis, G. B., Lundqvist, C., et al. (1999). Scientific basis of a matrix metalloproteinase-8 specific chair-side test for monitoring periodontal and peri-implant health and disease. *Ann. N.Y. Acad. Sci.* 878, 130–140. doi: 10.1111/j.1749-6632.1999.tb07679.x

Swift, R. (2003). Direct measurement of alcohol and its metabolites. *Addiction* 98, 73–80. doi: 10.1046/j.1359-6357.2003.00605.x

Syndergaard, B., Al-Sabbagh, M., Kryscio, R. J., Xi, J., Ding, X., Ebersole, J. L., et al. (2014). Salivary biomarkers associated with gingivitis and response to therapy. *J. Periodontol.* 85, e295–e303. doi: 10.1902/jop.2014.130696

Terpos, E., Politou, M., Viniou, N., and Rahemtulla, A. (2005). Significance of macrophage inflammatory protein-1 α (MIP-1α) in multiple myeloma. *Leuk. Lymphoma* 46, 1699–1707. doi: 10.1080/104281905001 75049

Tervahartiala, T., Pirilä, E., Ceponis, A., Maisi, P., Salo, T., Tuter, G., et al. (2000). The *in vivo* expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J. Dent. Res.* 79, 1969–1977. doi: 10.1177/00220345000790120801

Thomas, M. V., Branscum, A., Miller, C. S., Ebersole, J., Al-Sabbagh, M., and Schuster, J. L. (2009). Within-subject variability in repeated measures

of salivary analytes in healthy adults. *J. Periodontol.* 80, 1146–1153. doi: 10.1902/jop.2009.080654

Tobón-Arroyave, S. I., Jaramillo-González, P. E., and Isaza-Guzmán, D. M. (2008). Correlation between salivary IL-1β levels and periodontal clinical status. *Arch. Oral Biol.* 53, 346–352. doi: 10.1016/j.archoralbio.2007.11.005

Trombelli, L. (2004). Susceptibility to gingivitis: a way to predict periodontal disease? *Oral Health Prev. Dent.* 2(Suppl. 1), 265–269.

van Andel, H. W., Jansen, L. M., Grietens, H., Knorth, E. J., and van der Gaag, R. J. (2014). Salivary cortisol: a possible biomarker in evaluating stress and effects of interventions in young foster children? *Eur. Child Adolesc. Psychiatry* 23, 3–12. doi: 10.1007/s00787-013-0439-1

Van Dyke, T. E., and Sheilesh, D. (2005). Risk factors for periodontitis. *J. Int. Acad. Periodontol.* 7, 3–7.

Wu, T., Sajitharan, D., and Mohan, C. (2010). Biomarkers of rheumatoid arthritis: recent progress. *Expert Opin. Med. Diagn.* 4, 293–305. doi: 10.1517/17530059.2010.492828

Yoon, A. J., Cheng, B., Philipone, E., Turner, R., and Lamster, I. B. (2012). Inflammatory biomarkers in saliva: assessing the strength of association of diabetes mellitus and periodontal status with the oral inflammatory burden. *J. Clin. Periodontol.* 39, 434–440. doi: 10.1111/j.1600-051X.2012.01866.x

Yucel-Lindberg, T., and Båge, T. (2013). Inflammatory mediators in the pathogenesis of periodontitis. *Expert Rev. Mol. Med.* 15:e7. doi: 10.1017/erm.2013.8

Zakynthinos, E., and Pappa, N. (2009). Inflammatory biomarkers in coronary artery disease. *J. Cardiol.* 53, 317–333. doi: 10.1016/j.jjcc.2008.12.007

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 01 October 2015
doi: 10.3389/fcimb.2015.00069

Quantitative PCR analysis of salivary pathogen burden in periodontitis

Aino Salminen[1], K. A. Elisa Kopra[1], Kati Hyvärinen[1], Susanna Paju[1], Päivi Mäntylä[1], Kåre Buhlin[1,2], Markku S. Nieminen[3], Juha Sinisalo[3] and Pirkko J. Pussinen[1]*

[1] Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Helsinki, Finland, [2] Division of Periodontology, Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden, [3] Department of Cardiology, Heart and Lung Center, Department of Medicine, Helsinki University Hospital, Helsinki, Finland

Our aim was to investigate the value of salivary concentrations of four major periodontal pathogens and their combination in diagnostics of periodontitis. The Parogene study included 462 dentate subjects (mean age 62.9 ± 9.2 years) with coronary artery disease (CAD) diagnosis who underwent an extensive clinical and radiographic oral examination. Salivary levels of four major periodontal bacteria were measured by quantitative real-time PCR (qPCR). Median salivary concentrations of *Porphyromonas gingivalis, Tannerella forsythia*, and *Prevotella intermedia*, as well as the sum of the concentrations of the four bacteria, were higher in subjects with moderate to severe periodontitis compared to subjects with no to mild periodontitis. Median salivary *Aggregatibacter actinomycetemcomitans* concentrations did not differ significantly between the subjects with no to mild periodontitis and subjects with moderate to severe periodontitis. In logistic regression analysis adjusted for age, gender, diabetes, and the number of teeth and implants, high salivary concentrations of *P. gingivalis*, *T. forsythia*, and *P. intermedia* were significantly associated with moderate to severe periodontitis. When looking at different clinical and radiographic parameters of periodontitis, high concentrations of *P. gingivalis* and *T. forsythia* were significantly associated with the number of 4–5 mm periodontal pockets, ≥6 mm pockets, and alveolar bone loss (ABL). High level of *T. forsythia* was associated also with bleeding on probing (BOP). The combination of the four bacteria, i.e., the bacterial burden index, was associated with moderate to severe periodontitis with an odds ratio (OR) of 2.40 (95% CI 1.39–4.13). When *A. actinomycetemcomitans* was excluded from the combination of the bacteria, the OR was improved to 2.61 (95% CI 1.51–4.52). The highest OR 3.59 (95% CI 1.94–6.63) was achieved when *P. intermedia* was further excluded from the combination and only the levels of *P. gingivalis* and *T. forsythia* were used. Salivary diagnostics of periodontitis has potential especially in large-scale population studies and health promotion. The cumulative strategy appears to be useful in the analysis of salivary bacteria as markers of periodontitis.

Keywords: cumulative approach, oral pathogen, pathogen burden, periodontitis, quantitative PCR, saliva, salivary diagnostics

OPEN ACCESS

Edited by:
Thomas A. Ficht, Texas A&M University, USA

Reviewed by:
Daniel Jupiter, Scott & White Memorial Clinic and Hospital, USA
Nick Stephen Jakubovics, Newcastle University, UK
Gena D. Tribble, University of Texas Health Science Center at Houston, USA

****Correspondence:***
Aino Salminen, Department of Oral and Maxillofacial Diseases, University of Helsinki, P.O. Box 63, 00014 Helsinki, Finland
aino.m.salminen@helsinki.fi

Received: *12 June 2015*
Accepted: *18 September 2015*
Published: *01 October 2015*

Citation:
Salminen A, Kopra KAE, Hyvärinen K, Paju S, Mäntylä P, Buhlin K, Nieminen MS, Sinisalo J and Pussinen PJ (2015) Quantitative PCR analysis of salivary pathogen burden in periodontitis. Front. Cell. Infect. Microbiol. 5:69. doi: 10.3389/fcimb.2015.00069

Introduction

Periodontitis is a multifactorial and multibacterial disease of the supporting tissues of teeth initiated by disturbances in the subgingival biofilm and host homeostasis. The symptoms of the disease involve gingival swelling and bleeding, formation of deepened periodontal pockets, and inflammatory destruction of periodontal ligament and alveolar bone. Finally, untreated periodontitis may lead to the loss of teeth.

The tissue destruction results from the host defense response against bacterial challenge. The progression of periodontitis is characterized by an increase in subgingival bacterial load and by the transformation of the dominance of Gram-positive bacteria to a majority of Gram-negative bacteria. The bacteria associated with periodontitis have been classified into five major microbial color-coded complexes, since these bacteria are repeatedly found together in periodontitis. The "red complex" periodontopathogens, including *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, have a particularly strong association with periodontitis-related clinical parameters such as pocket probing depth (PPD) and bleeding on probing (BOP) (Socransky et al., 1998). The "orange complex" involves typical periodontopathogens related to PPD, e.g., *Prevotella intermedia* (Socransky et al., 1998). In addition, *Aggregatibacter actinomycetemcomitans* is among the bacteria involved in the pathology of periodontitis (Genco, 1996; Henderson et al., 2010; Könönen and Müller, 2014).

There are numerous methods to detect and quantify periodontal bacteria, e.g., microbial cultivation, species-specific DNA probes, or conventional end-point PCR. All these methods have limitations to measure the concentrations of specific bacteria accurately. Most of the previous reports on the number of periodontal bacteria use subgingival plaque, not saliva, as sample material. The presence of salivary bacteria, as well as the number of bacterial species in saliva, have been associated with periodontitis and periodontitis-related variables in several previous studies (Umeda et al., 1998; Könönen et al., 2007; Paju et al., 2009). However, the number of studies investigating salivary bacterial concentrations determined by qPCR and especially the combinations of multiple bacteria is limited (Hyvärinen et al., 2009; Saygun et al., 2011). Moreover, the number of subjects in these studies has been relatively small.

Saliva is a promising diagnostic fluid and it has been widely analyzed for biomarkers of health and disease over the past decade (Giannobile et al., 2011). The benefit of saliva samples over subgingival bacterial samples is that saliva is inexpensively and easily collected (Zhang et al., 2009). Saliva samples can be taken by non-dental healthcare professionals or even by the patients themselves. The relative levels of periodontal pathogens seem to be similar in whole saliva or mouthwash compared to periodontal lesions (Umeda et al., 1998; Boutaga et al., 2007; Haririan et al., 2014). Moreover, saliva reflects the overall conditions in mouth; in addition to tooth surfaces and periodontal pockets, periodontal pathogens can also be found on tongue and mucosa.

In addition to periodontitis, various systemic conditions, and behavioral factors such as smoking may affect salivary biomarkers and bacterial composition (Mager et al., 2003). For example, our previous study showed that high salivary concentrations of *A. actinomycetemcomitans* were associated with increased risk for coronary artery disease (CAD) (Hyvärinen et al., 2012).

In this study, we investigated the salivary levels of four important periodontal pathogens, *P. gingivalis*, *T. forsythia*, *P. intermedia*, and *A. actinomycetemcomitans*, in 462 individuals who underwent a detailed periodontal examination. Overall, the study population represents systemically compromised middle-aged and older subjects who are known to frequently suffer from periodontal disease. Our aim was to study if the salivary bacteria are adequate biomarkers of periodontitis. Moreover, we investigated if the combination of salivary pathogens, i.e., the salivary bacterial burden, provides more diagnostic value than the levels of individual pathogens. Our final aim is to find biomarkers that can be used in developing salivary diagnostic tools. Salivary diagnostics could provide an easy assessment of periodontal risk, e.g., for health care promotion or home testing purposes.

Materials and Methods

Participants

The study population included 462 dentate participants of the Parogene study (Buhlin et al., 2011). The original aim of the study is to identify genetic loci and variations disclosing the association between periodontitis and CAD. The Parogene study was a substudy of the Corogene study that included 5295 native symptomatic Finnish patients who were assigned to coronary angiography in Helsinki University Central Hospital, Finland, between 2006 and 2008 (Vaara et al., 2012). Ten percent of the Corogene population was randomly chosen to be invited to a comprehensive oral examination. Five hundred and eight patients participated in the clinical and radiographic oral examination at the Institute of Dentistry, University of Helsinki. The study was approved by the Helsinki University Central Hospital ethics committee (#106/2007) and all subjects gave a written informed consent.

Oral Examination

The oral examination was performed by two calibrated periodontists (Buhlin et al., 2011). Probing pocket depths (PPD) were measured from six sites of each tooth with a manual periodontal probe and BOP was registered from four sites of each tooth. The amount of alveolar bone loss (ABL) was calculated from digital panoramic radiographs by choosing the tooth with most severe attachment loss from each dentate sextant and graded into four categories by calculating the mean of the sextants: no ABL; mild, ABL in cervical third of the root; moderate, ABL in the middle third of the root; and from severe to total loss, ABL from the apical third of the root to total ABL. The study subjects completed a questionnaire on their smoking habits, recent intake of antibiotics, and previous periodontal treatment.

The dentate subjects were divided into two groups based on their periodontal status: 338 subjects (73.2%) with no

periodontitis or mild periodontitis (no ABL or mild ABL or less than four sites with PPD of ≥4 mm) and 124 subjects (26.8%) with moderate to severe periodontitis (patients with ABL from moderate to severe and at least four sites with PPD of ≥4 mm) (Salminen et al., 2014).

Saliva Samples

Before the oral examination, the subjects chewed a piece of paraffin for 5 min and a minimum of 2 ml of stimulated saliva was collected. The concentrations of bacteria were analyzed from saliva samples of 492 subjects. Thirty edentulous patients were excluded from further statistical analyses.

Quantitative PCR

Quantitative real-time PCR (qPCR) assays for *P. gingivalis*, *T. forsythia*, *P. intermedia*, and *A. actinomycetemcomitans* were performed for the saliva samples in an earlier study (Hyvärinen et al., 2012). Briefly, total bacterial DNA was isolated from the pellets derived from 500 µl of saliva and reference strain cultures using a ZR Fungal/Bacterial DNA Kit™ (Zymo Research) according to the manufacturer's instructions. The DNA concentrations were analyzed using NanoDrop 1000 spectrofotometer (Thermo Fisher Scientific). The target gene for primers (Thermo Fisher Scientific) and TaqMan probes (DNA Technology A/S) was Kdo transferase waaA and amplifications were conducted in duplicate 25 µl reactions by using Brilliant QPCR Master Mix (Agilent Technologies Inc.) and optimized concentrations of primer/probe sets (Hyvärinen et al., 2009). The thermocycling protocol used in Mx3005P Real-Time QPCR System (Agilent Technologies Inc.) was as follows: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The results were analyzed using Mx3005P Real-Time QPCR System software and the bacterial concentrations were calculated from standard curves derived from serial dilutions (1×10^{-5}–1 ng) of reference strains. The masses of bacterial genomes were calculated from genomic size data and the final results were expressed as genomic equivalents (GE)/ml saliva (Hyvärinen et al., 2009).

Statistical Analysis

The statistical analysis was performed with IBM SPSS Statistics software (version 22.0; IBM, New York, USA).

The differences of characteristics, periodontal parameters, presence of the bacteria, and salivary concentrations of the bacteria between subjects with no to mild periodontitis and subjects with moderate to severe periodontitis were analyzed by the Pearson Chi-Squared test (for categorical variables), *t*-test (for continuous, normally distributed variables: age), and the Mann-Whitney test (for continuous, non-normally distributed variables: bacterial concentrations and the number of teeth and implants).

In addition to the main periodontal diagnosis (no to mild periodontitis vs. moderate to severe periodontitis), the subjects were divided into subgroups defined by different clinical and radiographic parameters of periodontitis (the number of periodontal pockets with PPD of 4–5 mm, the number of pockets with PPD ≥ 6 mm, the percentage of BOP, and the degree of ABL). The concentrations of the bacteria were analyzed in these subgroups. Concentrations of salivary bacteria were expressed as medians with interquartile range as they were not normally distributed, and the comparisons between the subgroups of study subjects defined by different periodontal characteristics were made with the Jonckheere-Terpstra test.

The concentrations of each salivary bacteria were divided into three levels/scores as follows: low level, score 1: 0 GE/ml (concentration below the detection limit); medium level, score 2: concentration above the detection limit but below the median detectable concentration; and high level, score 3: concentration above the median detectable concentration.

The number of subjects with low, medium, or high levels of each pathogen were calculated in the groups divided according to periodontal diagnosis and in the subgroups divided according to periodontal parameters. The comparisons between the groups were made with the Chi-Squared test.

To obtain a bacterial burden index reflecting the total bacterial load in saliva for each individual, the scores of the four bacteria were summed up resulting in a number between 4 (if the concentrations of all four bacteria were below the detection limit) and 12 (if the level of each pathogen was high). After calculating the bacterial scores together, the sums were divided into tertiles, resulting in a bacterial burden index value of I (low burden), II (medium burden), or III (high burden) for each individual. The idea of a cumulative approach in salivary diagnostics has been described earlier in Gursoy et al. (2011), where the concentrations of MMP-8, IL-1β, and *P. gingivalis* were used to obtain a cumulative risk score. In the present study, we used the concentrations of the four salivary bacteria instead.

Logistic regression models were used to analyze the association of salivary bacterial concentrations and their combination as three levels and as continuous variables with periodontal diagnosis (no to mild periodontitis vs moderate to severe periodontitis) and with individual parameters of periodontitis. The models were adjusted for the number of teeth and implants (continuous variable), age (continuous variable), gender (male/female), diabetes (no/yes), and smoking (never/former/current). In further models, CAD diagnosis was added as a covariate to ensure that the heart disease status of the patients did not have an effect on the results. For the regression analyses with continuous variables, the concentrations of the bacteria were log-transformed (10-based logarithm). If the concentration was below the detection limit, the value of logarithm was set to 0. For the combinations of bacteria as continuous variables, the log-transformed bacterial concentrations were summed up.

Results

In the whole study population, 55.2, 65.7, 24.1, and 10.8% of the subjects were positive for saliva *P. gingivalis*, *T. forsythia*, *P. intermedia*, and *A. actinomycetemcomitans*, respectively.

TABLE 1 | Characteristics of the study subjects.

			No—mild periodontitis (n = 340)	Moderate—severe periodontitis (n = 124)	
			N (%)		p^d
Positive for saliva	Pg		177 (52.1)	79 (63.7)	**0.03**
	Tf		215 (63.6)	90 (72.6)	0.07
	Pi		75 (22.1)	37 (29.8)	0.08
	Aa		35 (10.3)	15 (12.1)	0.58
	n of species	0	81 (23.8)	24 (19.4)	0.07
		1	90 (26.5)	22 (17.7)	
		2	104 (30.6)	42 (33.9)	
		3	56 (16.5)	29 (23.4)	
		4	9 (2.6)	7 (5.6)	
Gender (men)			210 (61.8)	92 (74.2)	**0.01**
Smokers	Current		27 (7.9)	28 (22.6)	**<0.001**
	Former		127 (37.4)	59 (47.6)	**<0.001**
Diabetes			69 (20.7)	36 (29.0)	0.06
Antibiotic treatment[a]			136 (41.3)	46 (38.7)	0.66
Periodontal treatment[b]			33 (10.3)	22 (20.4)	**0.01**
			MEDIAN (IQR)		p^e
Salivary concentration (GE/ml)[c]	Pg		679 (21,300)	31,500 (311,000)	**<0.001**
	Tf		33,100 (297,000)	265,000 (1,280,000)	**<0.001**
	Pi		4040 (70,900)	55,900 (824,000)	**<0.01**
	Aa		1100 (5780)	539 (3510)	0.43
	Sum of bacteria		38,900 (372,000)	518,000 (1,950,000)	**<0.001**
Number of teeth and implants			25.0 (7)	21.0 (12)	**<0.001**
			MEAN (SD)		p^f
Age			62.1 (9.6)	65.3 (7.5)	**<0.001**

[a]Self-reported intake of antibiotics during past 6 months; [b]Self-reported periodontal treatment in the past; [c]In pathogen-positive subjects; [d]Chi-squared test; [e]Mann-Whitney test; [f]t-test; Significant p-values are presented in bold face.

The study subjects were divided into two groups based on their periodontal diagnosis: no to mild periodontitis and moderate to severe periodontitis. The characteristics, periodontal parameters, the presence of the bacteria in saliva, and the salivary concentrations of the bacteria in these two groups are shown in **Table 1**. The subjects with moderate to severe periodontitis were older, they were more frequently current or former smokers and male, they had received periodontal treatment more often, and they had fewer teeth than those with no or mild periodontitis. They were also more frequently positive for salivary *P. gingivalis*, but not for other bacteria. Median salivary concentrations of *P. gingivalis, T. forsythia*, and *P. intermedia*, as well as the sum of the concentrations of the four bacteria, were higher in subjects with moderate to severe periodontitis compared to subjects with no to mild periodontitis (**Table 1**). Salivary *A. actinomycetemcomitans* concentrations did not differ significantly between the two groups (**Table 1**).

In addition to the main periodontal diagnosis, the subjects were divided into subgroups defined by different clinical and radiographic parameters of periodontitis. The median concentrations of salivary bacteria in these subgroups are presented in **Table 2**, and the number of subjects with low, medium, or high levels of each bacterium in these subgroups are presented in Supplementary Table 1. The median concentrations of *A. actinomycetemcomitans* did not differ between any of the subgroups (**Table 2**). The median *P. intermedia* concentrations differed between subgroups divided according to the number of sites with PPD $\geq$ 6 mm or the degree of ABL. The median concentrations of *T. forsythia* differed additionally between the subgroups divided based on the number of sites with PPD of 4–5 mm. The median concentrations of *P. gingivalis* differed between subgroups defined by all periodontal parameters investigated (**Table 2**).

The combination of the four bacteria, i.e., the bacterial burden index, was calculated for each individual. The number of subjects with the bacterial burden index I, II, and III in the subgroups divided according to periodontal parameters are presented in Supplementary Table 2.

TABLE 2 | Median concentrations of salivary pathogens in subgroups of pathogen-positive subjects divided according to periodontal parameters.

	Concentration, median (IQR)			
	Pg (GE/ml)	**Tf (GE/ml)**	**Pi (GE/ml)**	**Aa (GE/ml)**
NUMBER OF SITES WITH PPD 4–5 mm				
0	425 (1390)	19,600 (198,000)	681 (47,600)	320 (-)
1–6	338 (25,800)	26,700 (196,000)	8115 (57,000)	2330 (41,700)
7–16	3640 (83,500)	100,000 (960,000)	6360 (144,000)	2280 (14,200)
17–	12,000 (222,000)	103,000 (652,000)	42,300 (697,000)	539 (1600)
	***p* < 0.001**	***p* = 0.01**	$p = 0.20$	$p = 0.25$
NUMBER OF SITES WITH PPD ≥ 6 mm				
0	499 (7710)	25,700 (309,000)	4250 (38,000)	939 (8820)
1–3	12,000 (60,100)	107,289 (852,000)	7830 (340,000)	627 (1950)
4–6	48,200 (931,000)	60,745 (329,000)	180,000 (836,000)	3710 (11,100)
7–	32,000 (364,000)	284,000 (1,570,000)	53,500 (833,000)	808 (2580)
	***p* < 0.001**	***p* < 0.001**	***p* < 0.01**	$p = 0.70$
BLEEDING ON PROBING, %, TERTILES				
0–26	639 (16,900)	41,631 (303,000)	4250 (107,000)	664 (28,000)
27–44	1200 (31,000)	33,100 (738,000)	21,500 (288,000)	3170 (7980)
45–100	30,300 (317,000)	88,000 (708,000)	7140 (205,000)	404 (1540)
	***p* < 0.001**	***p* = 0.03**	$p = 0.36$	$p = 0.31$
ALVEOLAR BONE LOSS				
None	464 (2320)	31,400 (246,000)	5420 (21300)	519 (2300)
Mild	1240 (45,700)	39,400 (308,000)	3260 (118,000)	1420 (13,000)
Moderate	21,500 (174,000)	103,000 (952,000)	46,300 (401,000)	843 (8140)
Severe—total	291,000 (884,000)	634,000 (2,390,000)	519,000 (1,790,000)	300 (4040)
	***p* < 0.001**	***p* < 0.001**	***p* = 0.01**	$p = 0.77$
NO. OF TEETH + IMPLANTS				
1–10	2030 (7790)	25,300 (242,000)	55,900 (864,000)	356 (1070)
11–20	32,600 (292,000)	66,100 (1,180,000)	21,500 (533,000)	8470 (47,900)
21–25	3540 (70800)	117,000 (646,000)	5850 (225,000)	808 (8980)
26–	438 (9530)	31,400 (373,000)	4040 (45,800)	797 (1720)
	***p* < 0.001**	$p = 0.34$	***p* < 0.01**	$p = 0.59$

PPD, pocket probing depth; IQR, interquartile range.
P-values were obtained by Jonckheere-Terpstra test.
Significant p-values are presented in bold face.

Table 3 indicates the correlations between the salivary concentrations of the four bacteria. The concentrations of all bacterial species correlated significantly with each other. The strongest correlation was found between *P. gingivalis* and *T. forsythia*.

Logistic regression models adjusted for age, gender, smoking, diabetes, and the number of teeth plus implants were used to evaluate the associations between salivary pathogen levels and periodontal parameters. **Table 4** and **Figure 1** present the odds ratios (OR) and 95% confidence intervals (95% CI) for the associations between periodontal parameters and salivary pathogen levels in the study population. High salivary concentrations of *P. gingivalis*, *T. forsythia*, and *P. intermedia* were significantly associated with moderate to severe periodontitis. When looking at individual parameters of periodontitis, high levels of *P. gingivalis* and *T. forsythia* were

TABLE 3 | Correlations between salivary pathogen concentrations.

		Pg	**Tf**	**Pi**	**Aa**
Pg	Correlation coefficient	1.00	0.50	0.31	0.17
	p-value	.	**<0.001**	**<0.001**	**<0.001**
Tf	Correlation coefficient		1.00	0.27	0.23
	p-value		.	**<0.001**	**<0.001**
Pi	Correlation coefficient			1.00	0.10
	p-value			.	**0.03**
Aa	Correlation coefficient				1.00
	p-value				.

Spearman's correlation.
Significant p-values are presented in bold face.

TABLE 4 | The associations (OR) between periodontal parameters and the four salivary pathogens.

Dependent parameter	Bacterial concentration	Pg OR (95% CI)	*p*	Tf OR (95% CI)	*p*	Pi OR (95% CI)	*p*	Aa OR (95% CI)	*p*
High ABL (moderate-total) reference: none-mild ABL	Low[a]	1		1		1		1	
	Medium[a]	0.99 (0.56–1.77)	0.98	1.00 (0.56–1.78)	0.99	0.90 (0.43–1.85)	0.77	2.30 (0.91–5.83)	0.08
	High[a]	2.19 (1.29–3.73)	**<0.01**	2.01 (1.15–3.49)	**0.01**	1.88 (0.96–3.65)	0.06	1.13 (0.39–3.23)	0.82
	Continuous[b]	1.19 (1.07–1.32)	**<0.001**	1.14 (1.04–1.26)	**<0.01**	1.10 (0.98–1.24)	0.12	1.14 (0.92–1.41)	0.25
High PPD4 (≥17 sites with PPD 4–5 mm) reference: <17 sites with PPD 4–5 mm	Low	1		1		1		1	
	Medium	0.89 (0.52–1.55)	0.69	1.62 (0.93–2.81)	0.09	0.82 (0.42–1.60)	0.55	4.14 (1.69–10.2)	**0.01**
	High	2.03 (1.20–3.43)	**<0.01**	1.89 (1.10–3.27)	**0.02**	1.10 (0.57–2.14)	0.78	1.71 (0.70–4.18)	0.24
	Continuous	1.17 (1.06–1.30)	**<0.01**	1.12 (1.03–1.23)	**0.01**	1.03 (0.92–1.16)	0.62	1.25 (1.02–1.52)	**0.03**
High PPD6 (≥7 sites with PPD ≥ 6 mm) reference: <7 sites with PPD ≥ 6 mm	Low	1		1		1		1	
	Medium	0.59 (0.27–1.28)	0.18	1.11 (0.53–2.34)	0.78	0.94 (0.40–2.25)	0.90	1.64 (0.57–4.67)	0.36
	High	1.86 (1.01–3.45)	**0.05**	2.33 (1.19–4.59)	**0.02**	1.51 (0.71–3.24)	0.29	1.46 (0.47–4.52)	0.51
	Continuous	1.14 (1.01–1.29)	**0.04**	1.19 (1.05–1.34)	**<0.01**	1.07 (0.94–1.23)	0.30	1.12 (0.88–1.43)	0.35
High BOP (highest tertile) reference: tertiles 1–2	Low	1		1		1		1	
	Medium	0.25 (0.14–0.46)	**<0.001**	1.46 (0.86–2.47)	0.16	1.65 (0.89–3.06)	0.11	1.80 (0.75–4.31)	0.19
	High	0.98 (0.60–1.60)	0.94	2.24 (1.34–3.76)	**<0.01**	1.21 (0.64–2.27)	0.56	1.29 (0.52–3.18)	0.58
	Continuous	0.98 (0.89–1.09)	0.74	1.16 (1.06–1.26)	**<0.001**	1.07 (0.96–1.19)	0.24	1.09 (0.89–1.32)	0.41
Moderate—severe periodontitis reference: no—mild periodontitis	Low	1		1		1		1	
	Medium	0.77 (0.42–1.41)	0.39	0.99 (0.55–1.78)	0.98	1.02 (0.50–2.10)	0.96	2.22 (0.89–5.51)	0.09
	High	2.20 (1.30–3.72)	**<0.01**	2.24 (1.30–3.90)	**<0.01**	2.17 (1.13–4.16)	**0.02**	1.01 (0.34–2.98)	0.99
	Continuous	1.19 (1.08–1.32)	**<0.001**	1.16 (1.06–1.28)	**<0.01**	1.14 (1.02–1.28)	**0.02**	1.11 (0.90–1.39)	0.34

[a] *Low, below the detection limit; Medium, above the detection limit but below the median concentration; High, above the median concentration.*

[b] *The continuous analyses are expressed as OR / log-transformed GE/ml.*

Models were adjusted for age, gender, diabetes, smoking, and number of teeth plus implants.

ABL, alveolar bone loss; PPD, pocket probing depth; BOP, bleeding on probing; OR, odds ratio; CI, confidence interval.

Significant p-values are presented in bold face.

significantly associated with the number of 4–5 mm periodontal pockets, ≥6 mm pockets, and ABL (**Table 4** and **Figure 1**). High level of *T. forsythia* was also associated with BOP. When the models were further adjusted for CAD status, the ORs remained similar.

In the adjusted regression models, the bacterial burden index reflecting the total bacterial load was associated with moderate to severe periodontitis with an OR of 2.40 (95% CI 1.39–4.13) (**Table 5** and **Figure 2**). When *A. actinomycetemcomitans* was excluded from the combination of the bacteria, the OR was improved to 2.61 (95% CI 1.51–4.52). When *P. intermedia* was further excluded from the combination, and only the concentrations of *P. gingivalis* and *T. forsythia* were used, the OR was increased to 3.59 (95% CI 1.94–6.63) (**Table 5** and **Figure 2**). The bacteria to be excluded one-by-one from the model were chosen because they had the weakest association with periodontitis.

Since the combination of *P. gingivalis* and *T. forsythia* was most strongly associated with periodontitis, we investigated this combination further using the modified bacterial burden index calculated based on these two species. **Table 6** shows the number of individuals with no to mild periodontitis and with moderate to severe periodontitis having the modified bacterial burden index of I, II, or III. Half of those with no to mild periodontitis had an index value of I. However, also more than one third of individuals with moderate to severe periodontitis had an index value of I. The sensitivity of the bacterial burden index III for detecting periodontitis was 31% and the specificity was 89%.

Discussion

Our results show that salivary concentrations of *P. gingivalis*, *T. forsythia*, and *P. intermedia* are associated with periodontitis. The combination of salivary *P. gingivalis* and *T. forsythia* had the strongest association with periodontitis when compared to the four pathogens analyzed individually and in combinations. The results were independent of age, gender, smoking, presence of diabetes, or CAD, as well as of the number of teeth and implants.

There is a limited number of previous studies that utilize salivary bacterial concentrations measured by qPCR in diagnostics of periodontitis. Saygun et al. showed in a sample of 150 systemically healthy subjects that *P. gingivalis*, *T. forsythia*, and *P. intermedia* occurred with significantly higher copy numbers in the saliva of patients with gingivitis, chronic periodontitis, and aggressive periodontitis when compared to periodontally healthy individuals (Saygun et al., 2011). Similarly, Hyvärinen et al. found that salivary levels of *P. gingivalis*, *T. forsythia*, and *P. intermedia* were higher in subjects with

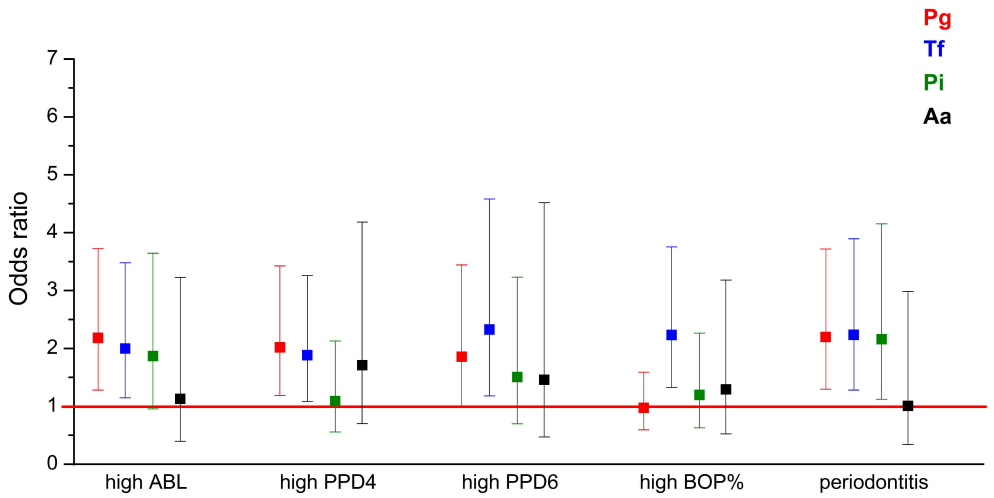


FIGURE 1 | The associations (OR) between periodontal parameters and the four bacteria investigated. ORs are calculated for the association between the periodontal parameters and highest levels of periodontal pathogen concentrations. The regression models were adjusted for the number of teeth and implants, age, gender, smoking, and diabetes. Error bars represent 95% confidence intervals. High ABL: ABL from moderate to severe compared to no to mild ABL; high PPD4: ≥17 sites with PPD 4–5 mm compared to <17 sites; high PPD6: ≥7 sites with PPD ≥ 6 mm compared to <7 sites; high BOP: BOP% ≥ 40 compared to <40%, periodontitis: moderate to severe periodontitis compared to no to mild periodontitis. ABL, alveolar bone loss; PPD, pocket probing depth; BOP, bleeding on probing.

periodontitis ($n = 84$) compared to periodontally healthy controls ($n = 81$) (Hyvärinen et al., 2009). Sawamoto et al. detected higher *P. gingivalis* and *T. forsythia* concentrations in the saliva of periodontitis patients ($n = 29$) compared to healthy subjects ($n = 20$) (Sawamoto et al., 2005), and He et al. found higher concentrations of *P. gingivalis* and *P. intermedia* in patients with chronic periodontitis ($n = 25$) compared to controls ($n = 60$) (He et al., 2012). The results of these previous reports are concordant with our results. However, the sample sizes have been relatively small and the study design has been case-control.

Since the bacterial burden index calculated using *P. gingivalis* and *T. forsythia* was most strongly associated with periodontitis, we investigated this combination further. Half of the patients with no to mild periodontitis had bacterial burden index value of I representing the lowest pathogen burden. However, also more than one third of individuals with moderate to severe periodontitis had index value of I. This suggests that the levels of the salivary pathogens analyzed are likely to be low in periodontally healthy subjects, but they might be low also in individuals with periodontitis. Since periodontitis results from a dysbiotic state of oral microbiota, a large number of pathogenic species are involved. Possibly analyzing a higher number of species among the 1000 found in the oral cavity (Wade, 2013) would increase the sensitivity of the burden index. Furthermore, the salivary bacterial composition might not always directly reflect the bacteria in plaque biofilm. In Yamanaka et al. (2012), bacterial populations of saliva and supragingival plaque were analyzed before and after periodontal treatment. Following periodontal therapy, microbial richness and biodiversity were significantly decreased in the plaque microbiota, but not in saliva. These results suggest that the contribution of plaque microbes to salivary bacterial composition is limited. It is possible that saliva reflects better other oral bacterial communities than dental plaque, such as the mucosal microbiota.

In a previous study, *A. actinomycetemcomitans* showed higher salivary copy numbers only in subjects with aggressive periodontitis, but not in subjects with chronic periodontitis (Saygun et al., 2011). In Hyvärinen et al. (2009), the presence of *A. actinomycetemcomitans* was more common in periodontitis patients, but the salivary concentrations did not differ between periodontitis cases and controls. Also in the present study, high level of *A. actinomycetemcomitans* was not associated with any of the periodontal parameters investigated. This may be due to the serological heterogeneity of *A. actinomycetemcomitans*. The serotype b is considered to be etiologically linked to certain types of periodontal disease (Zambon et al., 1983), while the role of other serotypes is not that clear. The assay used in the present study was carefully designed to recognize all serotypes of the species (Hyvärinen et al., 2009), but it may be useful to combine it with serotype detection. *P. intermedia* was not associated with any of the individual parameters either, but it was associated with moderate—severe periodontitis.

In the study of Saygun et al., the diagnostic sensitivity for periodontitis was 89.2 for *P. gingivalis* and *T. forsythia* and 86.5 for *P. intermedia*, with specificities ranging from 83.8 to 94.6 (Saygun et al., 2011). The sensitivities are considerably higher than in our study, but the specificities are at a comparable level. In addition to methodological differences, this may result from the different study designs. Their study had a case-control setting which included only individuals with teeth without any deepened periodontal pockets or attachment loss, and patients with at least nine posterior teeth with 5–7 mm pocket depth, i.e., patients with moderate to severe periodontitis. No "borderline" cases or cases with mild periodontitis were included, even though they are likely to complicate the diagnostics in reality. In our study, the

TABLE 5 | The associations (OR) between periodontal parameters and bacterial burden index calculated for different bacterial combinations.

Dependent parameter	Bacterial burden index	With Pg, Tf, Pi, and Aa OR (95% CI)	*p*	With Pg, Tf, and Pi OR (95% CI)	*p*	With Pg and Tf OR (95% CI)	*p*
High ABL (moderate-total) reference: none-mild ABL	I	1		1		1	
	II	1.06 (0.61–1.85)	0.84	0.97 (0.56–1.67)	0.90	1.05 (0.63–1.76)	0.85
	III	2.06 (1.19–3.56)	**0.01**	2.27 (1.30–3.97)	**<0.01**	3.32 (1.78–6.21)	**<0.01**
	Continuous[a]	1.08 (1.03–1.12)	**<0.001**	1.08 (1.03–1.13)	**<0.001**	1.11 (1.04–1.17)	**<0.001**
High PPD4 (≥17 sites with PPD 4–5 mm) reference: <17 sites with PPD 4–5 mm	I	1		1		1	
	II	0.76 (0.44–1.33)	0.34	0.73 (0.43–1.26)	0.26	0.96 (0.58–1.57)	0.87
	III	1.95 (1.16–3.28)	**0.01**	1.90 (1.11–3.23)	**0.02**	2.28 (1.25–4.18)	**< 0.01**
	Continuous	1.06 (1.02–1.11)	**<0.01**	1.06 (1.02–1.11)	**<0.01**	1.10 (1.03–1.16)	**<0.01**
High PPD6 (≥7 sites with PPD ≥ 6 mm) reference: <7 sites with PPD ≥ 6 mm	I	1		1		1	
	II	0.85 (0.41–1.76)	0.66	0.70 (0.34–1.44)	0.33	0.76 (0.38–1.49)	0.42
	III	2.07 (1.10–3.89)	**0.02**	2.10 (1.12–3.93)	**0.02**	3.04 (1.55–5.97)	**<0.01**
	Continuous	1.07 (1.02–1.13)	**<0.01**	1.08 (1.02–1.14)	**<0.01**	1.11 (1.03–1.19)	**<0.01**
High BOP (highest tertile) reference: tertiles 1–2	I	1		1		1	
	II	0.93 (0.56–1.55)	0.78	0.98 (0.60–1.61)	0.93	0.78 (0.49–1.26)	0.31
	III	1.65 (1.01–2.71)	**0.05**	1.63 (0.98–2.71)	0.06	2.10 (1.18–3.73)	**0.01**
	Continuous	1.04 (1.00–1.08)	0.05	1.04 (1.00–1.09)	0.06	1.05 (1.00–1.11)	0.06
Moderate—severe periodontitis reference: no—mild periodontitis	I	1		1		1	
	II	0.90 (0.51–1.60)	0.72	0.81 (0.46–1.43)	0.46	0.92 (0.54–1.56)	0.76
	III	2.40 (1.39–4.13)	**<0.01**	2.61 (1.51–4.52)	**<0.01**	3.59 (1.94–6.63)	**<0.001**
	Continuous	1.09 (1.04–1.13)	**<0.001**	1.09 (1.04–1.14)	**<0.001**	1.12 (1.05–1.19)	**<0.001**

[a] *Calculated by summing up the log-transformed concentrations of the bacteria.*

Models were adjusted for age, gender, diabetes, smoking, and number of teeth plus implants.

ABL, alveolar bone loss; PPD, pocket probing depth; BOP, bleeding on probing; OR, odds ratio; CI, confidence interval.

Significant p-values are presented in bold face.

subjects were randomly selected from the Corogene study cohort, which included all patients assigned to coronary angiography in Helsinki University Central Hospital under a certain time period. The selection was not based on the periodontal status of the individuals and they represented all stages of periodontal conditions. Study subjects were middle-aged or older, and thus belonging to the age group that is most often affected by chronic periodontitis.

Systemic diseases may affect the composition of saliva, thereby posing challenges to salivary diagnostics. For example, the concentrations of salivary interleukins and matrix metalloproteinases may be influenced by smoking, diabetes, tumors, or joint diseases (Costa et al., 2010; Rathnayake et al., 2013a,b). In the present study, all study subjects were assigned to coronary angiography because of cardiologic problems. 25% of the patients also had diabetes, which was taken into account in the analyses. These systemic diseases or smoking did not seem to confound the diagnostics of periodontitis based on salivary pathogens.

A good diagnostic marker of periodontitis should be applicable in entire populations regardless of systemic diseases, the number of teeth, or smoking habits of the individuals. Two previous studies indicated that a combination of salivary biomarkers and subgingival plaque pathogens is associated with periodontitis and its progression more strongly than individual markers (Ramseier et al., 2009; Kinney et al., 2011). Moreover, the combination of the levels of three selected salivary biomarkers, namely MMP-8, IL-1β, and *P. gingivalis*, was associated with periodontitis better than any of the markers alone (Gursoy et al., 2011; Salminen et al., 2014). The OR for the association between periodontitis and the combination of *P. gingivalis*, IL-1β, and MMP-8 in the Parogene population was 6.13 (95% CI 3.11–12.09) compared to 3.59 (1.94–6.63) in the present study for the combination of *P. gingivalis* and *T. forsythia*, which suggests that analyzing host-derived salivary biomarkers in addition to periodontal pathogens would be beneficial to periodontal diagnostics (Salminen et al., 2014).

Hyvärinen et al. observed that the combination of salivary *P. gingivalis*, *P. intermedia, and A. actinomycetemcomitans* had the highest area under the ROC curve when different pathogen combinations were analyzed for diagnostics of periodontitis (Hyvärinen et al., 2009). However, the sample size of the study was limited and it was a case-control study including only subjects with healthy periodontium or substantial periodontitis, which might cause discrepancy in the results.

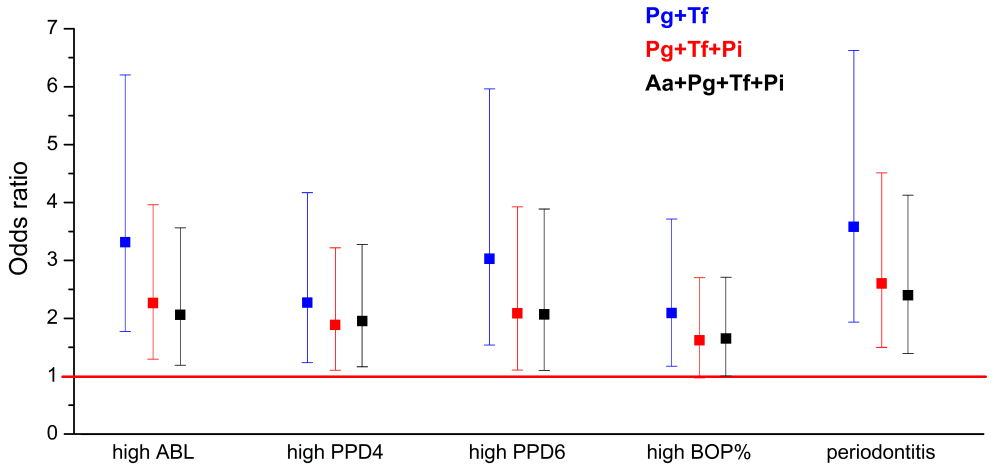


FIGURE 2 | The associations (OR) between periodontal parameters and bacterial burden index. ORs are calculated for the association between the periodontal parameters and bacterial burden index III calculated for different bacterial combinations. The OR was calculated for the following combinations of bacteria: *P. gingivalis*, *T. forsythia*, *P. intermedia*, and *A. actinomycetemcomitans*; for *P. gingivalis*, *T. forsythia*, and *P. intermedia*; and for *P. gingivalis* and *T. forsythia*. ABL, alveolar bone loss; PPD, pocket probing depth; BOP, bleeding on probing.

TABLE 6 | Distribution of patients according to their periodontal diagnosis in the classes of the bacterial burden index calculated using the combination of salivary *P. gingivalis* and *T. forsythia* concentrations.

	No—mild periodontitis N (%)	Moderate—severe periodontitis N (%)
Bacterial burden index I	169 (50%)	47 (38%)
Bacterial burden index II	133 (39%)	38 (31%)
Bacterial burden index III	36 (11%)	39 (31%)
Total count	338 (100%)	124 (100%)

In our study, the highest OR for the presence of moderate—severe periodontitis was achieved by combining salivary *P. gingivalis* and *T. forsythia* concentrations. As these pathogens belong to the "red complex," this finding strengthens their importance in periodontitis. The cumulative strategy appears to be useful in the analysis of salivary bacteria as biomarkers of periodontitis.

Funding

This work was supported by the Finnish Dental Society Apollonia, the Helsinki Doctoral School in Health Sciences, the Sigrid Juselius Foundation (grant to PP), and the Academy of Finland (grant to PP #1266053).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2015.00069

References

Boutaga, K., Savelkoul, P. H. M., Winkel, E. G., and van Winkelhoff, A. J. (2007). Comparison of subgingival bacterial sampling with oral lavage for detection and quantification of periodontal pathogens by real-time polymerase chain reaction. *J. Periodontol.* 78, 79–86. doi: 10.1902/jop.2007.060078

Buhlin, K., Mäntylä, P., Paju, S., Peltola, J. S., Nieminen, M. S., Sinisalo, J., et al. (2011). Periodontitis is associated with angiographically verified coronary artery disease. *J. Clin. Periodontol.* 38, 1007–1014. doi: 10.1111/j.1600-051x.2011.01775.x

Costa, P. P., Trevisan, G. L., Macedo, G. O., Palioto, D. B., Souza, S. L. S., Grisi, M. F. M., et al. (2010). Salivary Interleukin-6, Matrix Metalloproteinase-8, and Osteoprotegerin in patients with periodontitis and diabetes. *J. Periodontol.* 81, 384–391. doi: 10.1902/jop.2009.090510

Genco, R. J. (1996). Consensus report periodontal diseases: pathogenesis and microbial factors. *Ann. Periodontol.* 1, 926–932. doi: 10.1902/annals.1996.1.1.926

Giannobile, W. V., McDevitt, J. T., Niedbala, R. S., and Malamud, D. (2011). Translational and clinical applications of salivary diagnostics. *Adv. Dent. Res.* 23, 375–380. doi: 10.1177/00220345114 20434

Gursoy, U. K., Könönen, E., Pussinen, P. J., Tervahartiala, T., Hyvärinen, K., Suominen, A. L., et al. (2011). Use of host- and bacteria-derived salivary markers in detection of periodontitis: a cumulative approach. *Dis. Markers* 30, 299–305. doi: 10.1155/2011/621484

Haririan, H., Andrukhov, O., Bertl, K., Lettner, S., Kierstein, S., Moritz, A., et al. (2014). Microbial analysis of subgingival plaque samples compared to that of whole saliva in patients with periodontitis. *J. Periodontol.* 85, 819–828. doi: 10.1902/jop.2013.130306

He, J., Huang, W., Pan, Z., Cui, H., Qi, G., Zhou, X., et al. (2012). Quantitative analysis of microbiota in saliva, supragingival, and subgingival plaque of Chinese adults with chronic periodontitis. *Clin. Oral Investig.* 16, 1579–1588. doi: 10.1007/s00784-011-0654-4

Henderson, B., Ward, J. M., and Ready, D. (2010). Aggregatibacter (Actinobacillus) actinomycetemcomitans: a triple A* periodontopathogen? *Periodontol. 2000* 54, 78–105. doi: 10.1111/j.1600-0757.2009.00331.x

Hyvärinen, K., Laitinen, S., Paju, S., Hakala, A., Suominen-Taipale, L., Skurnik, M., et al. (2009). Detection and quantification of five major periodontal pathogens by single copy gene-based real-time PCR. *Innate Immun.* 15, 195–204. doi: 10.1177/1753425908101920

Hyvärinen, K., Mäntylä, P., Buhlin, K., Paju, S., Nieminen, M. S., Sinisalo, J., et al. (2012). A common periodontal pathogen has an adverse association with both acute and stable coronary artery disease. *Atherosclerosis* 223, 478–484. doi: 10.1016/j.atherosclerosis.2012.05.021

Kinney, J. S., Morelli, T., Braun, T., Ramseier, C. A., Herr, A. E., Sugai, J. V., et al. (2011). Saliva/pathogen biomarker signatures and periodontal disease progression. *J. Dent. Res.* 90, 752–758. doi: 10.1177/0022034511399908

Könönen, E., and Müller, H. (2014). Microbiology of aggressive periodontitis. *Periodontol. 2000* 65, 46–78. doi: 10.1111/prd.12016

Könönen, E., Paju, S., Pussinen, P. J., Hyvönen, M., Di Tella, P., Suominen-Taipale, L., et al. (2007). Population-based study of salivary carriage of periodontal pathogens in adults. *J. Clin. Microbiol.* 45, 2446–2451. doi: 10.1128/jcm.02560-06

Mager, D. L., Haffajee, A. D., and Socransky, S. S. (2003). Effects of periodontitis and smoking on the microbiota of oral mucous membranes and saliva in systemically healthy subjects. *J. Clin. Periodontol.* 30, 1031–1037. doi: 10.1046/j.0303-6979.2003.00418.x

Paju, S., Pussinen, P. J., Suominen-Taipale, L., Hyvönen, M., Knuuttila, M., and Könönen, E. (2009). Detection of multiple pathogenic species in saliva is associated with periodontal infection in adults. *J. Clin. Microbiol.* 47, 235–238. doi: 10.1128/jcm.01824-08

Ramseier, C. A., Kinney, J. S., Herr, A. E., Braun, T., Sugai, J. V., Shelburne, C. A., et al. (2009). Identification of pathogen and host-response markers correlated with periodontal disease. *J. Periodontol.* 80, 436–446. doi: 10.1902/jop.2009.080480

Rathnayake, N., Åkerman, S., Klinge, B., Lundegren, N., Jansson, H., Tryselius, Y., et al. (2013a). Salivary biomarkers of oral health - a cross-sectional study. *J. Clin. Periodontol.* 40, 140–147. doi: 10.1111/jcpe.12038

Rathnayake, N., Åkerman, S., Klinge, B., Lundegren, N., Jansson, H., Tryselius, Y., et al. (2013b). Salivary biomarkers for detection of systemic diseases. *PLoS ONE* 8:e61356. doi: 10.1371/journal.pone.0061356

Salminen, A., Gursoy, U. K., Paju, S., Hyvärinen, K., Mäntylä, P., Buhlin, K., et al. (2014). Salivary biomarkers of bacterial burden, inflammatory response, and tissue destruction in periodontitis. *J. Clin. Periodontol.* 41, 442–450. doi: 10.1111/jcpe.12234

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Saygun, I., Nizam, N., Keskiner, I., Bal, V., Kubar, A., Açıkel, C., et al. (2011). Salivary infectious agents and periodontal disease status. *J. Periodont. Res.* 46, 235–239. doi: 10.1111/j.1600-0765.2010.01335.x

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., and Kent, R. L. (1998). Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25, 134–144. doi: 10.1111/j.1600-051x.1998.tb02419.x

Umeda, M., Contreras, A., Chen, C., Bakker, I., and Slots, J. (1998). The utility of whole saliva to detect the oral presence of periodontopathic bacteria. *J. Periodontol.* 69, 828–833. doi: 10.1902/jop.1998.69.7.828

Vaara, S., Nieminen, M. S., Lokki, M., Perola, M., Pussinen, P. J., Allonen, J., et al. (2012). Cohort profile: the corogene study. *Int. J. Epidemiol.* 41, 1265–1271. doi: 10.1093/ije/dyr090

Wade, W. G. (2013). The oral microbiome in health and disease. *Pharmacol. Res.* 69, 137–143. doi: 10.1016/j.phrs.2012.11.006

Yamanaka, W., Takeshita, T., Shibata, Y., Matsuo, K., Eshima, N., Yokoyama, T., et al. (2012). Compositional stability of a salivary bacterial population against supragingival microbiota shift following periodontal therapy. *PLoS ONE* 7:e42806. doi: 10.1371/journal.pone.0042806

Zambon, J. J., Slots, J., and Genco, R. J. (1983). Serology of oral Actinobacillus actinomycetemcomitans and serotype distribution in human periodontal disease. *Infect. Immun.* 41, 19–27.

Zhang, L., Henson, B. S., Camargo, P. M., and Wong, D. T. (2009). The clinical value of salivary biomarkers for periodontal disease. *Periodontol. 2000* 51, 25–37. doi: 10.1111/j.1600-0757.2009.00315.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 05 January 2016
doi: 10.3389/fcimb.2015.00092

Salivary Cytoprotective Proteins in Inflammation and Resolution during Experimental Gingivitis—A Pilot Study

Guy M. Aboodi[1†], Corneliu Sima[1†], Eduardo B. Moffa[2,3], Karla T. B. Crosara[2], Yizhi Xiao[2], Walter L. Siqueira[2] and Michael Glogauer[2*]*

[1] Department of Periodontology and Matrix Dynamics Group, Faculty of Dentistry, University of Toronto, Toronto, ON, Canada, [2] Department of Biochemistry and Schulich Dentistry, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, ON, Canada, [3] Department of Prosthodontics, CEUMA University, Sao Luis, Brazil

OPEN ACCESS

Edited by:
Ulvi Kahraman Gürsoy, University of Turku, Finland

Reviewed by:
John T. Belisle, Colorado State University, USA
Rui Vitorino, University of Aveiro, Portugal

***Correspondence:**
Michael Glogauer
michael.glogauer@utoronto.ca;
Walter L. Siqueira
walter.siqueira@uwo.ca

[†] *These authors have contributed equally to this work.*

Received: *17 September 2015*
Accepted: *30 November 2015*
Published: *05 January 2016*

Citation:
Aboodi GM, Sima C, Moffa EB, Crosara KTB, Xiao Y, Siqueira WL and Glogauer M (2016) Salivary Cytoprotective Proteins in Inflammation and Resolution during Experimental Gingivitis—A Pilot Study. Front. Cell. Infect. Microbiol. 5:92. doi: 10.3389/fcimb.2015.00092

Objective: The protective mechanisms that maintain periodontal homeostasis in gingivitis and prevent periodontal tissue destruction are poorly understood. The aim of this study was to identify changes in the salivary proteome during experimental gingivitis.

Study design: We used oral neutrophil quantification and whole saliva (WS) proteomics to assess changes that occur in the inflammatory and resolution phases of gingivitis in healthy individuals. Oral neutrophils and WS samples were collected and clinical parameters measured on days 0, 7, 14, 21, 28, and 35.

Results: Increased oral neutrophil recruitment and salivary cytoprotective proteins increased progressively during inflammation and decreased in resolution. Oral neutrophil numbers in gingival inflammation and resolution correlated moderately with salivary β-globin, thioredoxin, and albumin and strongly with collagen alpha-1 and G-protein coupled receptor 98.

Conclusions: Our results indicate that changes in salivary cytoprotective proteins in gingivitis are associated with a similar trend in oral neutrophil recruitment and clinical parameters.

Clinical relevance: We found moderate to strong correlations between oral neutrophil numbers and levels of several salivary cytoprotective proteins both in the development of the inflammation and in the resolution of gingivitis. Our proteomics approach identified and relatively quantified specific cytoprotective proteins in this pilot study of experimental gingivitis; however, future and more comprehensive studies are needed to clearly identify and validate those protein biomarkers when gingivitis is active.

Keywords: gingivitis, proteomics, saliva, oral neutrophils, mass spectrometry, inflammation

INTRODUCTION

Periodontal diseases are a diverse group of inherited or acquired conditions that affect the tooth-supporting tissues in more than half of world population. Different pathogenic mechanisms including inflammatory, traumatic, genetic, and neoplastic contribute to the onset and progression of periodontal diseases (Madianos et al., 2005). The main etiologic factor for these conditions is the bacterial biofilm while the most common forms of periodontal diseases are plaque-induced gingivitis (GI) and chronic periodontitis (CP).

Plaque-induced gingivitis (GI) is defined as an inflammation of the gingiva induced by bacteria located at the gingival margin. The causative relationship between bacterial plaque (biofilm) and gingival inflammation was well demonstrated in experimental gingivitis (Loe et al., 1965). Characteristic GI clinical signs include erythema, edema, loss of gingival stippling, and bleeding upon probing (Mariotti, 1999). Interestingly, the host response to similar plaque levels varies significantly among patients (Trombelli et al., 2004). Histologic changes in the tissue include proliferation of junctional epithelium, vasculitis of blood vessels adjacent to the junctional epithelium, collagen degradation, cytopathologic alteration of fibroblasts, and inflammatory infiltrate (Page and Schroeder, 1976). GI is reversible upon removal of the etiologic biofilm (Loe et al., 1965). CP is characterized by extension of gingival inflammation to the alveolar bone, connective tissue degradation, and net loss of tooth attachment to periodontium (American Academy of Periodontology, 2000). The transition from GI to CP is incompletely understood. On the one hand, GI is an established risk factor for CP (Lang et al., 2009). On the other hand, clinical studies have demonstrated that in some individuals GI never progresses to CP, regardless of periodontal care (Pihlstrom et al., 2005).

Although certain pathogenic bacteria in subgingival biofilms produce specific virulence factors that could cause direct damage to periodontal tissues, current evidence suggest that it is the host factors that drive periodontal tissue degradation at sites with CP. These factors include an increase abundance of inflammatory cytokines, host proteolytic enzymes, and increased oxidative stress (Chapple and Matthews, 2007; Bartold et al., 2010). The rate limiting steps in onset and progression of clinical attachment loss are incompletely understood. Increasing evidence that emerged in recent years indicates that failure to resolve biofilm-induced periodontal inflammation results in chronicity and pro-osteolytic environments (Bartold and Van Dyke, 2013; Freire and Van Dyke, 2013; Van Dyke, 2014). Well-functioning resolution programs in periodontal tissues may be more critical that in other tissues because as a result of continuous challenge by subgingival bacteria, neutrophils traffic between the vasculature, gingival tissues, and gingival crevicular fluid (GCF) to maintain the host–biofilm balance, prevent tissue invasion by pathogens and ultimately bone loss.

The development of proteomics techniques allows us to identify, characterize and quantitate large numbers of proteins in a single study (Neilson et al., 2011). Utilizing proteomics in periodontitis research may reveal changes in the protein profile (proteome) during disease progression and the identification of disease-specific biomarkers (Baliban et al., 2012). WS and GCF samples were used for proteome analysis during GI (Ozdemir et al., 2009; Grant et al., 2010; Gonçalves Lda et al., 2011) and CP (Gonçalves Lda et al., 2010; Baliban et al., 2012; Rangé et al., 2012; Salazar et al., 2013; Silva-Boghossian et al., 2013). Blood proteins levels were reported to increase in both CP and GI patients (Gonçalves Lda et al., 2010, 2011), confirming the inflammatory nature of both diseases. While GCF and tissue samples provide site-specific information, salivary proteome analysis provides a comprehensive approach to oral health status, as WS proteins are originated from numerous sources (salivary glands, mucosal cells, immune cells, serum, and bacteria). The purpose of the current investigation was to identify changes in proteome profile during the development, progression and resolution of GI in an EG (experimental gingivitis) model. Findings of specific cytoprotective proteins and their correlation with clinical parameters and oral neutrophil numbers may suggest different protective mechanisms during GI that prevent tissue destruction and attachment loss that can be used for disease activity biomarker characterization and new preventive therapies for CP.

MATERIALS AND METHODS

Study Design

Twenty-one day experimental gingivitis (EG) model was used to investigate protein dynamics at health (day 0 of EG) and GI (day 21 of EG). Study design and goals were presented to all participants, and consent was obtained in writing. The study was approved by the Scientific and Ethics Review Boards at the University of Toronto (protocols #24295/#24567) and conducted in the Graduate Periodontics Clinic at the Faculty of Dentistry from September–December 2012. Five periodontally healthy participants (2 males, 3 females, age range 20–36) completed EG trial. All study participants were systemically healthy and non-smokers. Participants completed baseline periodontal exam and professional scaling by a registered dental hygienist, followed by 7 days of enhanced oral hygiene (pre-study hygiene phase). On day 0 of EG, full periodontal exam was completed, and participants were asked to refrain from any oral hygiene procedures (including brushing, flossing, use of mouthwash, and gum chewing) for the remainder of the trial. EG inflammatory phase was concluded after 21 days. Oral neutrophils and WS samples were collected and clinical parameters measured on days 0, 7, 14, 21, 28, and 35. Participants received scaling and oral hygiene instructions. Participants were followed for 2 weeks during healing phase (**Figure 1**). During the trial, participants were followed weekly. Full periodontal exam and sample collection was completed at each visit. EG participants were used both as healthy control group (EG0–day 0 samples) and GI group (EG21–day 21 samples).

Oral Neutrophil Quantification

Oral neutrophil quantification was completed utilizing hemocytometer technique, as previously described (Landzberg

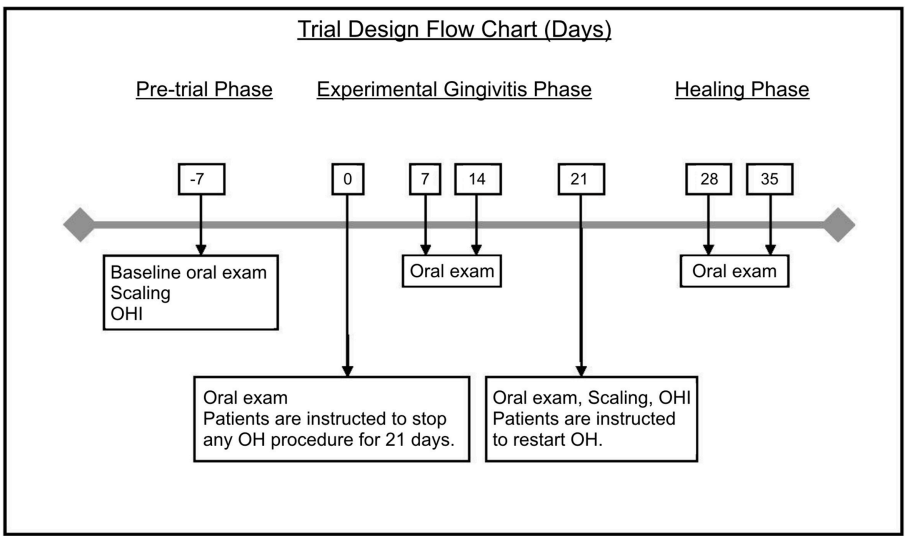


FIGURE 1 | Experimental gingivitis trial design flow chart (days). Stimulated whole saliva samples were collected at each time point.

et al., 2015). Rinse samples for oral neutrophil cell counts were collected prior to any periodontal instrumentation to avoid sample contamination with blood. Subjects were asked to rinse with 10 mL of 0.9% isotonic sodium chloride solution (Baxter, Toronto, ON) for 30 s, and then expectorate into a 50 mL polypropylene tube (Sigma-Aldrich, St. Louis, MO). A 500 μl sample was separated into an 1.5 ml polypropylene tube and fixed with 50 μl with 37% formaldehyde (Sigma-Aldrich, St. Louis, MO). Samples were kept in 4°C until analysis. All cell counts were completed by the same examiner (GMA). Samples were centrifuged at 1139 × g for 5 min (Hettich Zentrifugen, Tuttlingen, Germany). The supernatant was removed and the pellet was resuspended in 100 μL of phosphate buffered saline (PBS. Sigma-Aldrich, St. Louis, MO). One microliter acridine orange (Sigma Chemical, Burlington, ON, Canada) was added to the cell suspension. Acridine orange is a fluorescent nucleic acid marker. Its interaction with DNA and RNA allows the identification of neutrophils under fluorescence microscope. Following acridine orange staining, samples were incubated, light protected, for 15 min at room temperature. A 10 μL aliquot of this suspension was loaded on to a hemocytometer (Bright-Line; Hausser Scientific, Horsham, PA, USA), and the neutrophils were visually counted using fluorescence microscopy (Leitz Orthoplan Microscope; Leitz, Wetzlar, Germany). Neutrophils were counted and quantified based on the standard protocol for hemocytometer use.

Whole Saliva (WS) Sample Collection and Preparation

Stimulated WS samples were collected prior to any periodontal instrumentation to avoid sample contamination with blood. Chewing has been demonstrated to increase GCF flow, therefore subjects were asked to chew on a 5 × 5 cm parafilm (around 1.4 g, Parafilm M, Brand, Wertheim, Germany). This allowed for an increased GCF component in the analyzed WS samples (Griffiths, 2003). All EG sample collection took place in the morning, at the same time for each subject. Stimulated saliva in the first 30 s was discarded (swallowed). Patients were then asked to expectorate stimulated saliva into a 50 ml polypropylene tube (Sigma-Aldrich, St. Louis, MO) until 15 ml were collected. Salivary flow rate (ml/min) was acquired for each saliva collection. Saliva samples were placed on ice until aliquoting, which was completed within 3 h of sample collection. Samples were aliquoted into 1 ml portions. Three 1 ml samples were kept at −80°C until analysis and analyzed as whole saliva samples. Six 1 ml samples were centrifuged at 14,000 g over 20 min (Eppendorf Centrifuge 5415R, Eppendorf, Hauppauge, NY). Supernatant was separated from pellet, and both were kept at −80°C until analysis. The remaining 6 ml were stored at −80°C and were used as a reservoir for additional testing (Siqueira et al., 2004). Our goal was to establish a disease-specific proteome profile and a trend that characterizes the inflammatory and healing phases of gingival inflammation rather than individual investigations. Therefore, samples for each of the study groups were pooled together (Siqueira et al., 2012). As previously described (Silva-Boghossian et al., 2013), pooled samples were denatured and reduced for 2 h by buffer containing 4M urea, 10 mM dithiothreitol (DTT), and 50 mM ammonium bicarbonate (NH_4HCO_3), pH 7.8. Following dilution with 50 mM ammonium bicarbonate and the addition of 2% w/w sequencing-grade trypsin (Promega, Madison, WI), tryptic digestion was carried out for 18 h at 37°C.

Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS)

Mass spectrometric analyses were carried out with a LTQ-Velos (Thermo Scientific, San Jose, CA, USA) which allows in-line liquid chromatography with the capillary fused silica C18 column

(column length 10 mm, column ID 75 μm, 3 μm spherical beads and 100 Å pores size) linked to mass spectrometer using an electrospray ionization in a survey scan in the range of m/z values 390–2000 tandem MS/MS. All samples were dried by rotary evaporator and re-suspended in 20 μL of 97.5% H_2O/2.4% acetonitrile/0.1% formic acid and then subjected to reversed-phase LC-ESI-MS/MS. The nano-flow reversed-phase HPLC was developed with linear 80-min gradient ranging from 5 to 55% of solvent B (97.5% acetonitrile, 0.1% formic acid) at a flow rate of 300 nL/min with a maximum pressure of 280 bar. Electrospray voltage and the temperature of the ion transfer capillary were 1.8 kV and 250°C, respectively. Each survey scan (MS) was followed by automated sequential selection of seven peptides for CID, with dynamic exclusion of the previously selected ions. The obtained MS/MS spectra were searched against human protein databases (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, http://ca.expasy.org/sprot/) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA). Search results were filtered for a false discovery rate of 1% employing a decoy search strategy utilizing a reverse database. An additional inclusion criterion for positive identification of proteins was the same protein passing the filter score at least in two different MS analyses from a total of three MS analyses per condition.

Relative Proteome Quantitation

For quantitative proteome analysis, three MS raw files from each pooled group were analyzed using SIEVE technology (Version 2.0 Thermo Scientific, San Jose, CA, USA) as previously described (Siqueira et al., 2012). Relative Proteome quantitation for day 0 vs. day 21 of EG, were carried out. Baseline samples were compared to each of the study groups. Initial alignment step was carried out using a single MS raw file belonging to the baseline group. This file was selected as the reference file and all of other files were adjusted accordingly. Following the alignment, the feature detection and integration (or framing) process was performed through the "Frames from MS2 Scans" feature, using the MS level data. This framing process employs only MS mass-to-charge ratio (m/z) values that were associated with MS2 scan only. The parameters used consisted of a frame m/z width of 1500 ppm and a retention time width of 1.75 min. Peak integration was performed for each frame and these values were used for statistic analysis. Next, peptide sequences obtained from the database search using SEQUEST algorithm were imported into SIEVE. Peptides were grouped into proteins and a protein ratio and *p*-value were calculated. SIEVE uses a weighted average of the peptide intensities for the protein calculation. By using the weighted average, peptides with lower variance in their intensity measurements have a higher weight on the overall protein ratio. This is done to decrease variance in protein level quantities based on variance of the peptides that compose the proteins (Siqueira et al., 2012).

Correlation Assessment Between Salivary Proteins Abundance and Oral Neutrophils Quantification

Pearson correlation was calculated for each of the whole saliva cytoprotective proteins and the oral neutrophil using JMP software (SAS, Cary, NC). The association between oral neutrophil levels and the level of cytoprotective proteins was tested through correlation analysis during the experimental gingivitis phase (days 0–21), and the healing period which followed (days 28–35).

RESULTS

Worsening of clinical inflammatory parameters and biofilm accumulation were evident in all EG participants by day 21, including Bleeding on Probing (BOP), and Gingival Index (Löe, 1967). Changes in clinical parameters were detected by day 7, and increased significantly by day 14 reaching a peak on day 21 and returned to baseline levels by day 35, after subjects returning to regular OH on day 21. BOP was significantly higher on days 14 and 21 compared to day 0 and 35 (day 0, 12.8 ± 1.6 %; day 7, 22.2 ± 4.4 %; day 14, 36.5 ± 4.6 %; day 21, 38.1 ± 5.1 %; day 28, 30.9 ± 5.2 %; day 35, 19.8 ± 1.4 %; **Figure 2A**). Similarly, GI increased progressively during the inflammatory phase and decreased in the healing phase (day 0, 4.4 ± 1.3; day 7, 8.8 ± 0.7; day 14, 11.2 ± 0.4; day 21, 11.6 ± 0.24; day 28, 10 ± 0.7; day 35, 8.8 ± 0.4; **Figure 2B**). A significant increase in oral neutrophil numbers was found on day 21 of the inflammatory phase of GI compared to baseline (**Figure 2C**). Oral neutrophil counts decreased in the resolution phase by day 35, reaching levels close to baseline (day 0, 198 ± 40 × 10^4; day 7, 322 ± 32 × 10^4; day 14, 363 ± 27 × 10^4; day 21, 722 ± 44 × 10^4; day 28, 242 ± 73 × 10^4; day 35, 220 ± 79 × 10^4).

Salivary flow rate (ml/min) measured during all time-points of the study demonstrated no statistical significance difference among the groups (day 7, 1.23 ± 0.08 day 1.23 ± 0.21; day 7, 1.21 ± 0.07; day 14, 1.11 ± 0.09; day 21, 1.11 ± 0.18; day 28, 1.16 ± 0.19; day 35, 1.21 ±0.24).

Proteome data analysis revealed that 89 proteins showed significant level changes (at $p < 0.05$; **Table 1**) during EG. Twenty-one percent of these proteins (25 proteins) demonstrated a significant increase of at least 2 fold by day 21 compared to baseline (**Table 2**). Serum albumin levels identified in WS samples significantly increased during the inflammatory EG phase (2.3 fold increase). Furthermore, collagen fragment abundance increased significantly (7.35 fold increase), in line with the histologic findings previously described (Page and Schroeder, 1976).

Significant increases in several cytoprotective proteins were observed during the inflammatory phase, including proteins involved in inflammatory regulation (Annexin A1 and Vitamin D binding protein–4.15 and 2.78 fold increase, respectively), antibacterial (Lactotransferrin–3.5 fold increase), antioxidants (β-globin and Thioredoxin–3.75 and 2.29 fold increase, respectively), and protease inhibitor proteins (Cystatin SN, Cystatin S–2.33 and 2.14 fold increase, respectively; **Figure 3A**). Abundance of these cytoprotective proteins went back to baseline levels in the resolution phase of EG. Eighteen of the 25 salivary proteins that increased by >2 fold by day 21, followed a trend of reduction in the resolution phase. Aryl hydrocarbon receptor repressor, cDNA

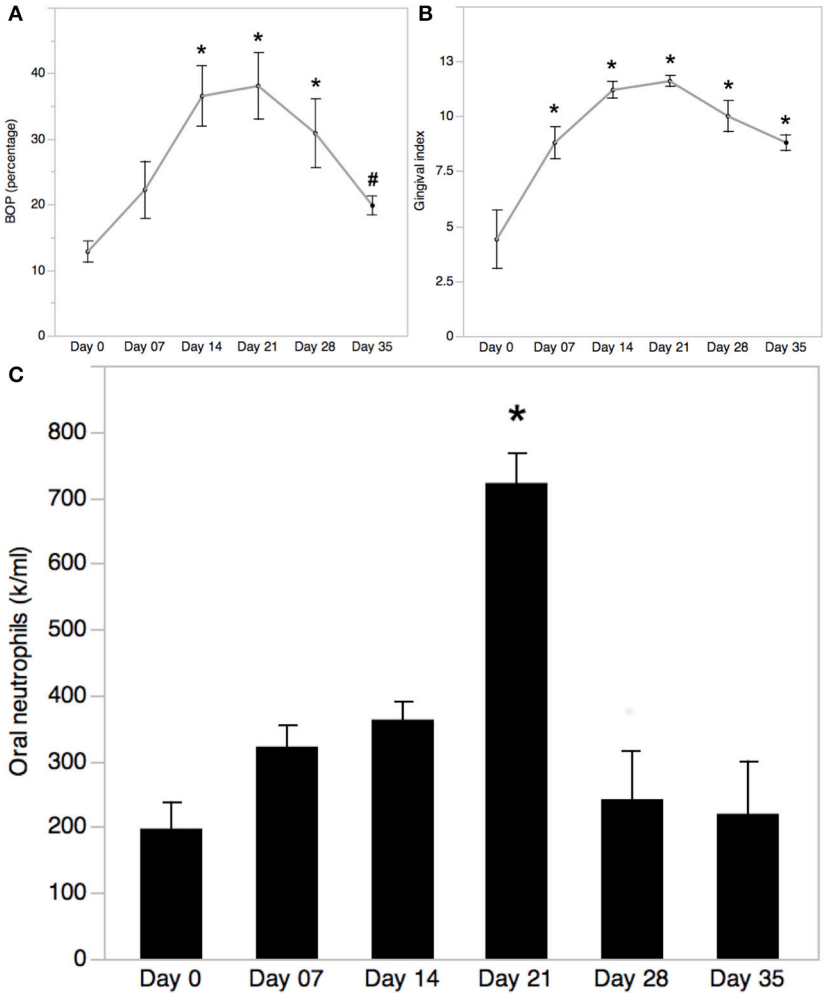


FIGURE 2 | Bleeding on Probing (BOP) trend in experimental gingivitis. (A) Bleeding on probing (BOP) was measured at baseline (day 0) and on days 7, 14, 21, 28, and 35 during EG. Mean ± SEM; $n = 5$; One-way ANOVA with Turkey-Kramer HSD, $^{*}p < 0.05$ vs. day 0, $^{\#}p < 0.05$ vs. day 21. **(B)** Gingival index was measured at baseline (day 0) and on days 7, 14, 21, 28, and 35 during EG. Mean ± SEM; $n = 5$; One-way ANOVA with Turkey-Kramer HSD, $^{*}p < 0.05$. **(C)** Oral neutrophils isolated from oral rinses collected on days 0, 7, 14, 21, 28, and 35 of EG were quantified using a hemocytometer. Cell numbers were normalized to the rinse volume collected. Mean ± SEM; $n = 5$; One-way ANOVA with Turkey-Kramer HSD; $^{*}p < 0.05$.

FLJ3590 fis and lactotransferrin increased further by day 35 (**Figure 3B**).

The inflammatory response observed during gingivitis is mediated by neutrophils migrating from the blood stream to the gingival tissues and in to the oral cavity through the GCF. We speculate that the increase in oral neutrophil levels during the experimental gingivitis phase is responsible in part for the observed increase in whole saliva cytoprotective proteins. Pearson correlation assessment between salivary proteins abundance and oral neutrophils quantification demonstrated that oral neutrophil numbers correlated moderately with salivary β-globin and thioredoxin ($R^2 = 0.649$ and 0.564, respectively), and strongly with collagen alpha-1(XXVII) chain and G-protein coupled receptor 98 ($R^2 = 0.883$ and 0.884, respectively), in the inflammatory and resolution phases of GI (**Figure 4**).

DISCUSSION

The comparison of proteome profile at baseline and day 21 of EG demonstrated that several salivary proteins increased by at least 2 fold during the inflammatory phase of GI. Among these proteins, we identified 10 cytoprotective proteins. The overall increase in these proteins, which are mostly involved in tissue protection and inflammatory control, suggests that activation of protective pathways, including antibacterial activity, regulation of the inflammatory process, antioxidants, and protease inhibitors characterizes host responses of healthy individuals to increasing bacterial burden on the gingiva. The moderate correlation between oral neutrophil numbers and salivary β-globin and thioredoxin in the inflammation and resolution phases of GI indicates that salivary antioxidants may not be the primary source of protection against neutrophil-mediated oxidative damage in

TABLE 1 | Proteins with significant change in WS levels during the inflammatory phase of EG (Day 0 vs. Day 21).

Accession	Protein name	Fold change	*p*-Value
A7E2D6	NAV2 protein	1.52	0.000
A8K6R0	cDNA FLJ75726, highly similar to Homo sapiens basic leucine zipper nuclear factor 1 (JEM-1) (BLZF1), mRNA	1.92	0.004
B0I1T1	MYO1F variant protein	1.71	0.013
B0ZBF6	Mineralocorticoid receptor	1.07	0.007
B1AN48	Small proline-rich protein 3	1.03	0.000
B3KTH9	cDNA FLJ38275 fis, highly similar to GAS2-like protein 3	2.31	0.004
B3KVS4	cDNA FLJ41312 fis, clone BRAMY2042804, highly similar to Homo sapiens zinc finger RNA binding protein (ZFR), mRNA	1.81	0.000
B3KXQ8	cDNA FLJ45892 fis, clone OCBBF3023175, highly similar to Protein neurobeachin	1.85	0.005
B4DG67	cDNA FLJ58842, highly similar to Homo sapiens zinc and ring finger 1 (ZNRF1), mRNA	1.72	0.008
B4DH81	cDNA FLJ61250, highly similar to Homo sapiens GTPase activating Rap/RanGAP domain-like 3 (GARNL3), mRNA	1.59	0.006
B4DSR5	Kinesin-like protein KIF3B	2.23	0.013
B4DSW4	cDNA FLJ51541, moderately similar to Transcription factor Sp8	1.64	0.048
B4DT16	B-cell lymphoma/leukemia 11A	1.71	0.000
B4DVQ0	cDNA FLJ58286, highly similar to Actin, cytoplasmic 2	1.75	0.005
B4DYH2	cDNA FLJ53243	1.72	0.012
B4DYQ3	cDNA FLJ60974, highly similar to Mediator of RNA polymerase II transcription subunit 12	1.69	0.009
B7ZAX4	cDNA, FLJ79338, highly similar to Krueppel-like factor 11	1.20	0.000
B7ZMD7	Amylase, alpha 1A	0.79	0.001
C3PTT6	Pancreatic adenocarcinoma unregulated factor	2.25	0.012
C8C504	Beta-globin	1.77	0.018
C9J185	Eukaryotic translation initiation factor 2-alpha kinase 3 (Fragment)	1.64	0.006
C9JAJ5	Putative uncharacterized protein LOC349136	2.18	0.036
C9JC68	Fetuin-B (Fragment)	1.09	0.009
D3DPB9	Nitric oxide synthase trafficker, isoform CRA_a	1.09	0.031
D3DRN4	KIAA1539, isoform CRA_a	4.72	0.003
D6RGV6	Serine/threonine-protein kinase Nek11	0.72	0.005
E5KRP6	Spastin	1.88	0.036
E7EMQ1	Carbonic anhydrase 6	2.23	0.039
E7ETI5	G-protein-coupled receptor 98	3.41	0.018
E9PAV3	Nascent polypeptide-associated complex subunit alpha	1.50	0.010
E9PIJ5	Transmembrane protease serine 13	2.21	0.000
F2Z2U9	Myosin-14	1.89	0.009
F4MH44	Ubiquitously transcribed tetratricopeptide repeat protein Y-linked transcript variant 22	1.68	0.010
F5H2N0	Zinc finger protein 574	1.37	0.028
F8W696	Apolipoprotein A-I	2.32	0.027
G3ClG0	MUC19 variant 12	1.21	0.000
H0Y3D5	Fibroblast growth factor receptor 4	1.72	0.022
H0YBS7	Gamma-aminobutyric acid receptor subunit alpha-6 (Fragment)	1.72	0.002
H0YD40	Collagen alpha-1 (XXVII) chain (Fragment)	7.35	0.001
H0YL38	Zinc finger protein 280D	1.82	0.032
H6VRF8	Keratin 1	1.00	0.001
O14513	Nck-associated protein 5	1.72	0.027
O15047	Histone-lysine N-methyltransferase SETD1A	1.61	0.012
O75443	Alpha-tectorin	1.62	0.009
O75592	Probable E3 ubiquitin-protein ligase MYCBP2	1.95	0.000
O95447	Lebercilin-like protein	1.66	0.001
O95613	Pericentrin	1.55	0.026

(Continued)

TABLE 1 | Continued

Accession	Protein name	Fold change	*p*-Value
P01036	Cystatin-S	2.14	0.006
P01037	Cystatin-SN	2.33	0.046
P01833	Polymeric immunoglobulin receptor	1.10	0.007
P01834	Ig kappa chain C region	2.87	0.005
P01857	Ig gamma-1 chain C region	0.62	0.001
P01876	Ig alpha-1 chain C region	1.56	0.039
P02768	Albumin	2.33	0.022
P02774	Vitamin D-binding protein	2.78	0.001
P02788	Lactotransferrin	3.50	0.002
P02808	Statherin	1.23	0.001
P02814	Submaxillary gland androgen-regulated protein 3B	1.59	0.006
P04083	Annexin A1	4.15	0.002
P06733	Alpha-enolase	1.88	0.001
P10599	Thioredoxin	2.29	0.000
P15515	Histatin-1	1.29	0.000
P19961	Alpha-amylase 2B	0.87	0.003
P20930	Filaggrin	1.87	0.017
P22748	Carbonic anhydrase 4	0.64	0.002
P27482	Calmodulin-like protein 3	2.11	0.000
P61626	Lysozyme C	1.67	0.003
Q05BP9	OLIG2 protein	2.47	0.042
Q12912	Lymphoid-restricted membrane protein	1.21	0.001
Q12955	Ankyrin-3	0.07	0.001
Q14118	Dystroglycan	0.68	0.005
Q14484	Beta-globin	3.75	0.001
Q14515	SPARC-like protein 1	2.10	0.005
Q14C71	GLRA1 protein	1.41	0.032
Q16378	Proline-rich protein 4	0.93	0.008
Q502W4	IGKC protein	1.57	0.013
Q569J1	IGHA1 protein	1.33	0.026
Q59FG1	Calcium channel, voltage-dependent, alpha 1E subunit variant (Fragment)	1.49	0.012
Q5H9S0	Putative uncharacterized protein DKFZp781N1974	1.57	0.000
Q5JPC9	ABI gene family, member 3 (NESH) binding protein, isoform CRA_d	1.82	0.009
Q6N092	Putative uncharacterized protein DKFZp686K18196 (Fragment)	1.27	0.008
Q6PL43	Uncharacterized protein	1.96	0.014
Q6W4X9	Mucin-6	2.13	0.045
Q86UD4	Zinc finger protein 329	1.60	0.002
Q8TDL5	Long palate, lung and nasal epithelium carcinoma-associated protein 1	0.38	0.001
Q8WXI7	Mucin-16	1.99	0.000
Q96AY4	Tetratricopeptide repeat protein 28	2.20	0.020
Q96FS4	Signal-induced proliferation-associated protein 1	1.40	0.030
Q9BZG5	Androgen receptor	2.21	0.002

periodontal tissue inflammation. The inflammatory response observed during gingivitis is mediated by neutrophils migrating from the blood stream to the gingival tissues and in to the oral cavity through the GCF. We speculate that the increase in oral neutrophil levels during the experimental gingivitis phase is responsible in part for the observed increase in whole saliva cytoprotective proteins. The association between oral neutrophil levels and levels of proteins of interest was tested through correlation analysis during the experimental gingivitis phase (days 0–21), and the healing period which followed (days 28–35). In general, we found moderate to strong correlations between oral neutrophil numbers and levels of several salivary cytoprotective proteins in inflammation and resolution of gingivitis.

Lactotransferrin (also known as lactoferrin) is an iron-binding protein (Brock, 2002; Ammons and Copié, 2013), found in

TABLE 2 | Proteins with significant increase of >2 fold during the inflammatory phase of EG.

Accession	Protein name	Fold change	*p*-Value
B3KTH9	cDNA FLJ38275 fis, highly similar to GAS2-like protein 3	2.31	0.004
B4DSR5	Kinesin-like protein KIF3B	2.23	0.013
C3PTT6	Pancreatic adenocarcinoma unregulated factor	2.25	0.012
C9JAJ5	Putative uncharacterized protein LOC349136	2.18	0.036
D3DRN4	KIAA1539, isoform CRA_a	4.72	0.003
E7EMQ1	Carbonic anhydrase 6	2.23	0.039
E7ETI5	G-protein-coupled receptor 98	3.41	0.018
E9PIJ5	Transmembrane protease serine 13	2.21	0.000
F8W696	Apolipoprotein A-I	2.32	0.027
H0YD40	Collagen alpha-1 (XXVII) chain (Fragment)	7.35	0.001
P01036	Cystatin-S	2.14	0.006
P01037	Cystatin-SN	2.33	0.046
P01834	Ig kappa chain C region	2.87	0.005
P02768	Albumin	2.33	0.022
P02774	Vitamin D-binding protein	2.78	0.001
P02788	Lactotransferrin	3.50	0.002
P04083	Annexin A1	4.15	0.002
P10599	Thioredoxin	2.29	0.000
P27482	Calmodulin-like protein 3	2.11	0.000
Q05BP9	OLIG2 protein	2.47	0.042
Q14484	Beta-globin	3.75	0.001
Q14515	SPARC-like protein 1	2.10	0.005
Q6W4X9	Mucin-6	2.13	0.045
Q96AY4	Tetratricopeptide repeat protein 28	2.20	0.020
Q9BZG5	Androgen receptor	2.21	0.002

Highlighted proteins are proteins considered cytoprotective proteins.

serum and secreted fluids, and also present in specific neutrophil granules (Masson et al., 1969). Its antibacterial properties result from bacteriostatic activity, directly related to its iron-chelating capacity; and bactericidal activity, as a result of a direct interaction between the protein and bacteria where the N-terminal region of LTF, known as lactoferricin, can disrupt or possibly even penetrate bacterial cell membranes (Brock, 2002). Anti-biofilm activity of LTF was also demonstrated, as it inhibits biofilm formation and reduces the established biofilm of oral bacteria at physiological concentrations (Wakabayashi et al., 2010; Ammons and Copié, 2013). It has been speculated that LTF inhibits biofilm formation and disrupts existing biofilm by preventing bacterial adhesion or stimulating bacterial motility. Similar to our current findings, significant increase in LTF abundance was previously demonstrated in GCF samples during EG (15). The reported antibacterial and anti-biofilm properties of LTF can explain its increased abundance during EG, as an innate protective response to bacterial accumulation. Due to these protective properties, LTF was suggested as a potential treatment for CP (Wakabayashi et al., 2010).

In addition to biofilm control-associated factors, several anti-inflammatory proteins were changed during the course of inflammation-resolution in our EG study. Two inflammation regulating proteins were increased in the inflammatory phase of EG: Annexin A1 and Vitamin D binding protein. Annexins are a family of calcium and phospholipid binding proteins (Gerke and Moss, 2002), which dampen the inflammatory response via inhibition of neutrophil activation (Chatterjee et al., 2005). Annexin A1 levels in GCF were previously demonstrated to be stable during 21 days EG, opposing our current findings (Grant et al., 2010). One possible explanation is that we used WS samples and not GCF samples. Therefore, it is possible that salivary sources contribute to annexin A1 production and its involvement in regulating gingival inflammation.

Vitamin D binding protein (DBP) is a multifunctional protein found in plasma. Its ability to bind to vitamin D (calcitriol) and its metabolites and to transfer them to target cells has a major role in the involvement of vitamin D in inflammatory regulation. Vitamin D was shown to be involved in both the innate and adaptive immune systems, with vitamin D insufficiency being linked to many inflammatory disorders, including periodontal diseases. It has been suggested that vitamin D may act similarly to cytokines, and regulate the inflammatory process by several mechanisms: stimulating phagocytosis and antibody-presenting actions to enhance the initial immune response. As the inflammatory process progresses, vitamin D plays a role in inhibition of T-cell proliferation and thus inflammatory resolution (Stein et al., 2014). DBP itself

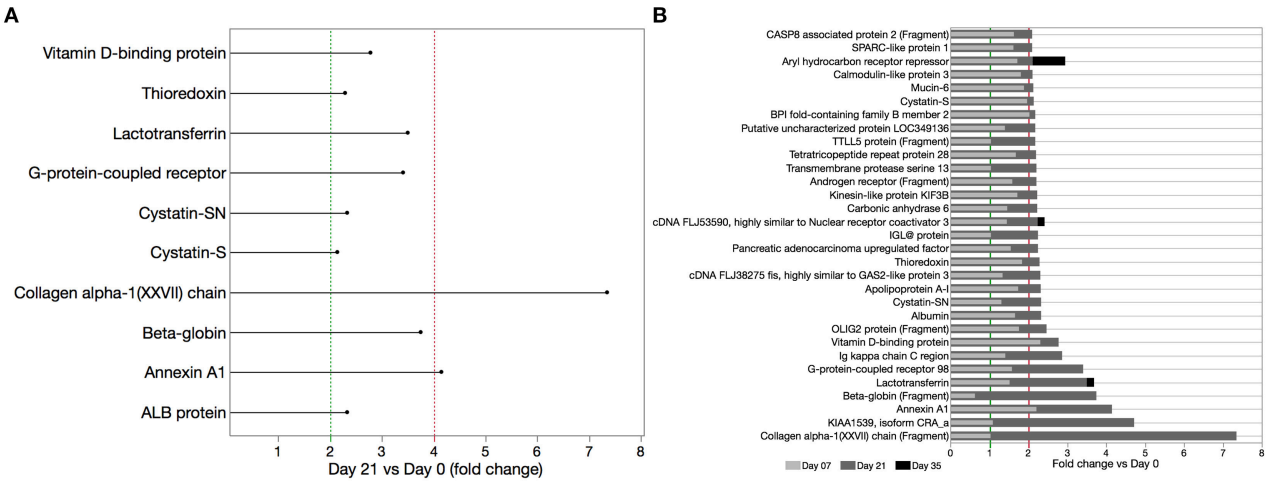


FIGURE 3 | Cytoprotective proteins significantly increased during EG (day 21 vs. day 0). (A) WS samples were analyzed by LC-ESI-MS/MS and relative proteome analysis performed based on peptide sequences obtained from the database search using SEQUEST algorithm after peak integration from acquired frames and SIEVE-assisted recognition of proteins form peptide sequences. **(B)** Time course of salivary protein levels for select proteins that increased by >2 fold on day 21 vs. baseline. With exception of aryl hydrocarbon receptor repressor, cDNA FLJ3590 fis and lactotransferrin all proteins with >2 fold increase in the inflammatory phase were reduced by day 35, 2 weeks after oral hygiene was resumed.

was demonstrated to have significant neutrophil chemotactic activity *in vivo* where DBP knock-out mice demonstrated significant decreases in neutrophil recruitment to the site of infection when compared to the wild type group. Exogenous addition of DBP was shown to restore neutrophil response (Trujillo et al., 2013). Our observation of increased DBP levels during EG may suggest an increased abundance of vitamin D during this phase. High vitamin D levels were suggested to reduce inflammation during gingivitis (Dietrich et al., 2005).

Increases in oxidative stress, where reactive oxygen species (ROS) levels exceed antioxidant levels, have been shown to directly contribute to periodontal inflammation and connective tissue breakdown during periodontal disease. The antioxidant defense systems have an important role in balancing physiological oxidative stress (Chapple and Matthews, 2007). Total antioxidant capacity (TAC) levels were found to be significantly lower in periodontitis patients when compared to healthy controls (Chapple et al., 2007). Our current findings demonstrate an increase in antioxidant proteins–β-globin and Thioredoxin during the development of EG, suggesting an additional protective mechanism during this reversible phase of periodontal disease.

The globin superfamilies are hemeproteins which can be found in all known life forms. Its common role in O2 transport in vertebrate erythrocytes is recognized as a relatively recent adaptation from its more primative functions in non-erythroid cells, including iron metabolism regulation, intracellular oxygen transport, oxygen sensing, NO scavenging, and hydrogen peroxide scavenging (Vinogradov and Moens, 2008). Several human studies demonstrated that hemoglobin overexpression reduces oxidative stress, suggesting its cytoprotective role as an antioxidant (Liu et al., 2011; Li et al., 2013). Increased β-globin levels were demonstrated in mice macrophages treated with lipopolysaccharide and interferon-γ (Liu et al., 1999). In-line with our current findings, salivary proteome analysis of samples collected from gingivitis patients with gingivitis revealed increase in both α- and β-globin compared to healthy controls (Gonçalves Lda et al., 2011).

Thioredoxin (Trx) is an intra-cellular protein that together with thioredoxin reductase and NADPH, comprises the thioredoxin system. This system has been shown to play a key role in many intra-cellular pathways including H2O2 as a means to reduce oxidative stress (Holmgren and Lu, 2010). The cytoprotective effects of Trx were demonstrated in transgenic mice overexpressing human Trx1 that are resistant to oxidative stress conditions, and are more resistant to inflammation (Yoshida et al., 2005). In humans, increased extracellular Trx levels were reported for various systemic conditions, including rheumatoid arthritis (RA; Maurice et al., 1999). Our current results demonstrate an increase in Trx during EG, supporting its protective role during this reversible inflammatory phase.

Several proteolytic enzymes have been demonstrated to play a key role in periodontal tissue destruction. The main sources for these enzymes are the neutrophils that populate the periodontal pocket and produce proteolytic enzymes as part of the non-oxidative killing function (Meyer-Hoffert and Wiedow, 2011). We identified a significant increase in the protease inhibitor proteins Cystatin SN and Cystatin S during EG. Cystatins SN and S were first identified in saliva, and have been identified in other secreted fluids. Their production in secretory glands suggests their role as cytoprotective inhibitors of exogenous cysteine peptidases (Abrahamson et al., 2003). Cystatin S levels in GCF have been demonstrated to be stable during the 21 days EG model, which once again is in contrast

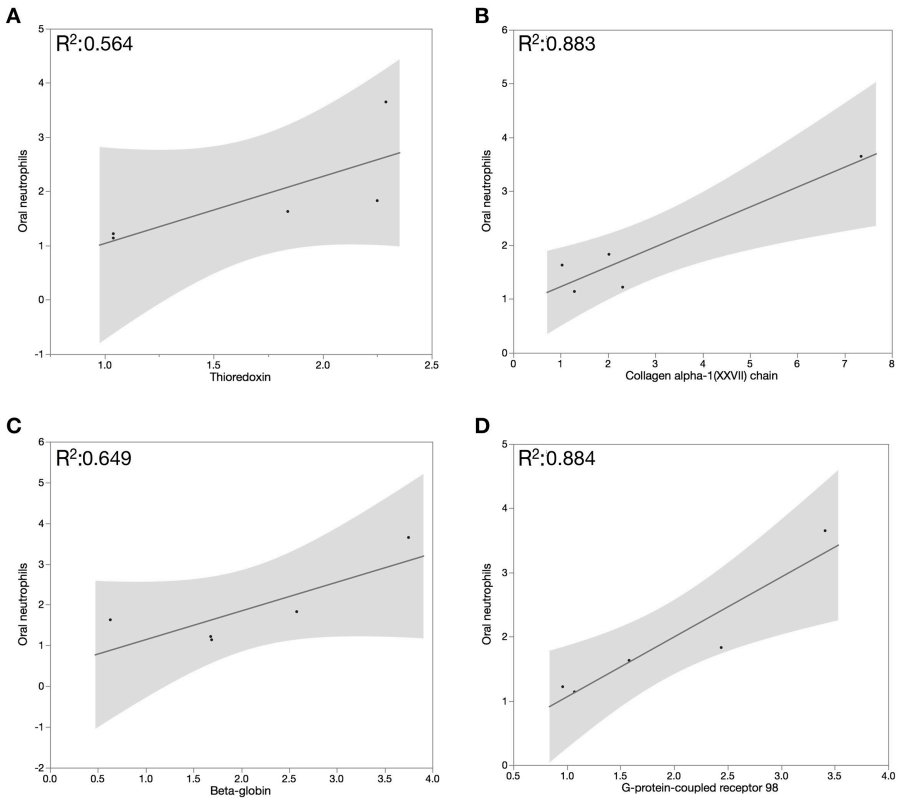


FIGURE 4 | Pearson correlation assessment between salivary proteins abundance and oral neutrophils quantification demonstrated that oral neutrophil numbers correlated moderately with salivary β-globin (A) and thioredoxin (B) and strongly with (C) collagen alpha-1(XXVII) chain and (D) G-protein coupled receptor 98 in the inflammatory and resolution phases of GI.

to our current findings (Grant et al., 2010). In a different study, GCF proteome analysis reported decrease in cystatin S abundance in GCF samples obtained from GI patients (Huynh et al., 2014). Salivary glands contribute to cystatin S production, which can explain the differences in our analysis of WS samples, as opposed to GCF samples. In support of our findings, cystatin activity was demonstrated to increase in WS samples obtained from GI patients (Henskens et al., 1993), as well as in inflamed gingival tissues (Babnik et al., 1988).

The increase in cytoprotective proteins during the development of EG, suggests that production of proteins that can dampen the effector proteins involved in the inflammatory process is important during inflammatory diseases including GI (**Figures 3**, **4**). Similar findings were reported in a human tears study, which demonstrated increase in cystatin S and lactotransferrin levels in tears collected from patients with autoimmune conditions (Katunuma et al., 2003). Interestingly, reduced levels of several of these cytoprotective proteins were previously reported in patients diagnosed with CP. Reduced cystatins (S, SN, SA) levels were previously reported in WS samples obtained from CP patients when compared to healthy controls (Henskens et al., 1996; Ito et al., 2008; Gonçalves Lda et al., 2010). Reports of LTF levels during CP were inconsistent: WS proteome analysis demonstrated a 2-fold increase in LTF levels when compared to healthy controls (Salazar et al., 2013). Contrary to this report, low levels of LTF were demonstrated in WS samples obtained from patients diagnosed with *Aggregatibacter actinomycetemcomitans*-associated periodontitis (Groenink et al., 1999), and in WS samples obtained from patients diagnosed with generalized aggressive periodontitis (GAgP) when compared to healthy controls (Wu et al., 2009). The same study also showed increased vitamin D binding protein levels in GAgP patients when compared to healthy controls (Wu et al., 2009).

Several theories may explain the reduced levels of protective proteins during CP: as the inflammatory process progresses, increased levels of endogenous and exogenous ROS and proteases may lead to damage of the cytoprotective proteins. A second theory suggests that once bacterial and inflammatory overload reach a specific threshold, the cytoprotective mechanisms are reduced. This threshold can vary between subjects, and may be responsible for the observed variability in the development of CP among subjects. The findings of the present study demonstrate that in the initial inflammatory phase of GI levels of salivary cytoprotective proteins are increased and that they decrease as inflammation resolves.

CONCLUSIONS

We were able to demonstrate in the current pilot study a significant increase in several cytoprotective proteins during the inflammatory phase of GI. Further, we found that levels of these proteins decreased in the healing phase of GI indicating a dose-response relationship with gingival inflammation-resolution. The increase in cytoprotective proteins in onset of GI may prevent periodontal tissue destruction and clinical attachment loss in patients at risk of developing CP due to other host and environmental factors. Future studies to investigate whether these proteins can serve as biomarkers for GI progression to CP are necessary.

AUTHOR CONTRIBUTIONS

GA, CS, WS, MG contributed to conception or design. GA, CS, WS, MG, YX, EM, KC contributed to acquisition, analysis, or interpretation. GA, CS, WS, MG drafted the manuscript. GA, CS, WS, MG, YX, EM, KC critically revised the manuscript. GA, CS, WS, MG, YX, EM, KC gave final approval. GA, CS, WS, MG, YX, EM, KC agrees to be accountable for all aspects of work ensuring integrity and accuracy.

ACKNOWLEDGMENTS

The authors would like to give special thanks to Ricky Livay-Shakked (Matrix Dynamics Group, Faculty of Dentistry, University of Toronto). Research funds provided by Canadian Institute for Health Research (CIHR) grants to MG (grant number #122068) and to WLS grant number #97577; Canadian Foundation for Innovation (CFI-LOF grant to WLS); Harron scholarship, Faculty of Dentistry, University of Toronto; Department of Periodontology, Faculty of Dentistry, University of Toronto; and the Alpha Omega Foundation of Canada. WLS is recipient of a CIHR New Investigator Award grant#113166.

REFERENCES

American Academy of Periodontology. (2000). Parameter on chronic periodontitis with advanced loss of periodontal support. *J. Periodontol.* 71, 856–858. doi: 10.1902/jop.2000.71.5-S.856

Abrahamson, M., Alvarez-Fernandez, M., and Nathanson, C. M. (2003). Cystatins. *Biochem. Soc. Symp.* 70, 179–199. doi: 10.1042/bss0700179

Ammons, M. C., and Copié, V. (2013). Mini-review: lactoferrin: a bioinspired, anti-biofilm therapeutic. *Biofouling* 29, 443–455. doi: 10.1080/08927014.2013.773317

Babnik, J., Curin, V., Lah, T., Turk, V., and Skaleric, U. (1988). Cysteine proteinase inhibitors in inflamed human gingiva. *Biol. Chem. Hoppe Seyler* 369(Suppl.), 271–276.

Baliban, R. C., Sakellari, D., Li, Z., DiMaggio, P. A., Garcia, B. A., and Floudas, C. A. (2012). Novel protein identification methods for biomarker discovery via a proteomic analysis of periodontally healthy and diseased gingival crevicular fluid samples. *J. Clin. Periodontol.* 39, 203–212. doi: 10.1111/j.1600-051X.2011.01805.x

Bartold, P. M., Cantley, M. D., and Haynes, D. R. (2010). Mechanisms and control of pathologic bone loss in periodontitis. *Periodontol. 2000* 53, 55–69. doi: 10.1111/j.1600-0757.2010.00347.x

Bartold, P. M., and Van Dyke, T. E. (2013). Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. *Periodontol. 2000* 62, 203–217. doi: 10.1111/j.1600-0757.2012.00450.x

Brock, J. H. (2002). The physiology of lactoferrin. *Biochem. Cell Biol* 80, 1–6. doi: 10.1139/o01-212

Chapple, I. L., Brock, G. R., Milward, M. R., Ling, N., and Matthews, J. B. (2007). Compromised GCF total antioxidant capacity in periodontitis: cause or effect? *J. Clin. Periodontol.* 34, 103–110. doi: 10.1111/j.1600-051X.2006.01029.x

Chapple, I. L., and Matthews, J. B. (2007). The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol. 2000* 43, 160–232. doi: 10.1111/j.1600-0757.2006.00178.x

Chatterjee, B. E., Yona, S., Rosignoli, G., Young, R. E., Nourshargh, S., Flower, R. J., et al. (2005). Annexin 1-deficient neutrophils exhibit enhanced transmigration *in vivo* and increased responsiveness *in vitro*. *J. Leukocyte Biol.* 78, 639–646. doi: 10.1189/jlb.0405206

Dietrich, T., Nunn, M., Dawson-Hughes, B., and Bischoff-Ferrari, H. A. (2005). Association between serum concentrations of 25-hydroxyvitamin D and gingival inflammation. *Am. J. Clin. Nutr.* 82, 575–580.

Freire, M. O., and Van Dyke, T. E. (2013). Natural resolution of inflammation. *Periodontol. 2000* 63, 149–164. doi: 10.1111/prd.12034

Gerke, V., and Moss, S. E. (2002). Annexins: from structure to function. *Physiol. Rev.* 82, 331–371. doi: 10.1152/physrev.00030.2001

Gonçalves Lda, R., Soares, M. R., Nogueira, F. C., Garcia, C., Camisasca, D. R., Domont, G., et al. (2010). Comparative proteomic analysis of whole saliva from chronic periodontitis patients. *J. Proteomics* 73, 1334–1341. doi: 10.1016/j.jprot.2010.02.018

Gonçalves Lda, R., Soares, M. R., Nogueira, F. C., Garcia, C. H., Camisasca, D. R., Domont, G., et al. (2011). Analysis of the salivary proteome in gingivitis patients. *J. Periodontal Res.* 46, 599–606. doi: 10.1111/j.1600-0765.2011.01378.x

Grant, M. M., Creese, A. J., Barr, G., Ling, M. R., Scott, A. E., Matthews, J. B., et al. (2010). Proteomic analysis of a noninvasive human model of acute inflammation and its resolution: the twenty-one day gingivitis model. *J. Proteome Res.* 9, 4732–4744. doi: 10.1021/pr100446f

Griffiths, G. S. (2003). Formation, collection and significance of gingival crevice fluid. *Periodontol. 2000* 31, 32–42. doi: 10.1034/j.1600-0757.2003.03103.x

Groenink, J., Walgreen-Weterings, E., Nazmi, K., Bolscher, J. G., Veerman, E. C., van Winkelhoff, A. J., et al. (1999). Salivary lactoferrin and low-Mr mucin MG2 in Actinobacillus actinomycetemcomitans-associated periodontitis. *J. Clin. Periodont.* 26, 269–275. doi: 10.1034/j.1600-051X.1999.260501.x

Henskens, Y. M., van den Keijbus, P. A., Veerman, E. C., Van der Weijden, G. A., Timmerman, M. F., Snoek, C. M., et al. (1996). Protein composition of whole and parotid saliva in healthy and periodontitis subjects. Determination of cystatins, albumin, amylase and IgA. *J. Periodontal Res.* 31, 57–65. doi: 10.1111/j.1600-0765.1996.tb00464.x

Henskens, Y. M., van der Velden, U., Veerman, E. C., and Nieuw Amerongen, A. V. (1993). Protein, albumin and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis or periodontitis. *J.Periodontal Res.* 28, 43–48. doi: 10.1111/j.1600-0765.1993.tb01049.x

Holmgren, A., and Lu, J. (2010). Thioredoxin and thioredoxin reductase: current research with special reference to human disease. *Biochem. Biophys. Res. Commun.* 396, 120–124. doi: 10.1016/j.bbrc.2010.03.083

Huynh, A. H., Veith, P. D., McGregor, N. R., Adams, G. G., Chen, D., Reynolds, E. C., et al. (2014). Gingival crevicular fluid proteomes in health, gingivitis and chronic periodontitis. *J. Periodontal Res.* 50, 637–649. doi: 10.1111/jre.12244

Ito, T., Komiya-Ito, A., Arataki, T., Furuya, Y., Yajima, Y., Yamada, S., et al. (2008). Relationship between antimicrobial protein levels in whole saliva and periodontitis. *J. Periodontol.* 79, 316–322. doi: 10.1902/jop.2008.070348

Katunuma, N., Shiota, H., and Le, Q. T. (2003). Medical significance of cysteine protease inhibitors in mammalian secretory fluids. *J. Med. Invest.* 50, 154–161.

Landzberg, M., Doering, H., Aboodi, G. M., Tenenbaum, H. C., and Glogauer, M. (2015). Quantifying oral inflammatory load: oral neutrophil counts

in periodontal health and disease. *J. Periodontal Res.* 50, 330–336. doi: 10.1111/jre.12211

Lang, N. P., Schätzle, M. A., and Löe, H. (2009). Gingivitis as a risk factor in periodontal disease. *J. Clin. Periodontol.* 36(Suppl. 10), 3–8. doi: 10.1111/j.1600-051X.2009.01415.x

Li, X., Wu, Z., Wang, Y., Mei, Q., Fu, X., and Han, W. (2013). Characterization of adult alpha- and beta-globin elevated by hydrogen peroxide in cervical cancer cells that play a cytoprotective role against oxidative insults. *PLoS ONE* 8:e54342. doi: 10.1371/journal.pone.0054342

Liu, L., Zeng, M., and Stamler, J. S. (1999). Hemoglobin induction in mouse macrophages. *Proc. Natl. Acad. Sci.U.S.A.* 96, 6643–6647. doi: 10.1073/pnas.96.12.6643

Liu, W., Baker, S. S., Baker, R. D., Nowak, N. J., and Zhu, L. (2011). Upregulation of hemoglobin expression by oxidative stress in hepatocytes and its implication in nonalcoholic steatohepatitis. *PLoS ONE* 6:e24363. doi: 10.1371/journal.pone.0024363

Löe, H. (1967). The gingival index, the plaque index and the retention index systems. *J. Periodontol.* 38(Suppl.), 610–616. doi: 10.1902/jop.1967.38.6_part2.610

Loe, H., Theilade, E., and Jensen, S. B. (1965). Experimental Gingivitis in Man. *J. Periodontol.* 36, 177–187. doi: 10.1902/jop.1965.36.3.177

Madianos, P. N., Bobetsis, Y. A., and Kinane, D. F. (2005). Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. *J. Clin. Periodontol.* 32(Suppl. 6), 57–71. doi: 10.1111/j.1600-051X.2005.00821.x

Mariotti, A. (1999). Dental plaque-induced gingival diseases. *Ann. Periodontol.* 4, 7–19. doi: 10.1902/annals.1999.4.1.7

Masson, P. L., Heremans, J. F., and Schonne, E. (1969). Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* 130, 643–658. doi: 10.1084/jem.130.3.643

Maurice, M. M., Nakamura, H., Gringhuis, S., Okamoto, T., Yoshida, S., Kullmann, F., et al. (1999). Expression of the thioredoxin-thioredoxin reductase system in the inflamed joints of patients with rheumatoid arthritis. *Arthritis Rheum.* 42, 2430–2439.

Meyer-Hoffert, U., and Wiedow, O. (2011). Neutrophil serine proteases: mediators of innate immune responses. *Curr. Opin. Hematol.* 18, 19–24. doi: 10.1097/MOH.0b013e32834115d1

Neilson, K. A., Ali, N. A., Muralidharan, S., Mirzaei, M., Mariani, M., Assadourian, G., et al. (2011). Less label, more free: approaches in label-free quantitative mass spectrometry. *Proteomics* 11, 535–553. doi: 10.1002/pmic.201000553

Ozdemir, B., Ozcan, G., Karaduman, B., Teoman, A. I., Ayhan, E., Ozer, N., et al. (2009). Lactoferrin in gingival crevicular fluid and peripheral blood during experimental gingivitis. *Eur. J. Dent.* 3, 16–23.

Page, R. C., and Schroeder, H. E. (1976). Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab. Invest.* 34, 235–249.

Pihlstrom, B. L., Michalowicz, B. S., and Johnson, N. W. (2005). Periodontal diseases. *Lancet* 366, 1809–1820. doi: 10.1016/S0140-6736(05)67728-8

Rangé, H., Léger, T., Huchon, C., Ciangura, C., Diallo, D., Poitou, C., et al. (2012). Salivary proteome modifications associated with periodontitis in obese patients. *J. Clin. Periodontol.* 39, 799–806. doi: 10.1111/j.1600-051X.2012.01913.x

Salazar, M. G., Jehmlich, N., Murr, A., Dhople, V. M., Holtfreter, B., Hammer, E., et al. (2013). Identification of periodontitis associated changes in the proteome of whole human saliva by mass spectrometric analysis. *J. Clin. Periodontol.* 40, 825–832. doi: 10.1111/jcpe.12130

Silva-Boghossian, C. M., Colombo, A. P., Tanaka, M., Rayo, C., Xiao, Y., and Siqueira, W. L. (2013). Quantitative proteomic analysis of gingival crevicular fluid in different periodontal conditions. *PLoS ONE* 8:e75898. doi: 10.1371/journal.pone.0075898

Siqueira, W. L., Bakkal, M., Xiao, Y., Sutton, J. N., and Mendes, F. M. (2012). Quantitative proteomic analysis of the effect of fluoride on the acquired enamel pellicle. *PLoS ONE* 7:e42204. doi: 10.1371/journal.pone.0042204

Siqueira, W. L., de Oliveira, E., Mustacchi, Z., and Nicolau, J. (2004). Electrolyte concentrations in saliva of children aged 6-10 years with Down syndrome. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 98, 76–79. doi: 10.1016/j.tripleo.2004.04.007

Stein, S. H., Livada, R., and Tipton, D. A. (2014). Re-evaluating the role of vitamin D in the periodontium. *J. Periodontal Res.* 49, 545–553. doi: 10.1111/jre.12149

Trombelli, L., Tatakis, D. N., Scapoli, C., Bottega, S., Orlandini, E., and Tosi, M. (2004). Modulation of clinical expression of plaque-induced gingivitis. II. Identification of "high-responder" and "low-responder" subjects. *J. Clin. Periodontol.* 31, 239–252. doi: 10.1111/j.1600-051x.2004.00478.x

Trujillo, G., Habiel, D. M., Ge, L., Ramadass, M., Cooke, N. E., and Kew, R. R. (2013). Neutrophil recruitment to the lung in both C5a- and CXCL1-induced alveolitis is impaired in vitamin D-binding protein-deficient mice. *J. Immunol.* 191, 848–856. doi: 10.4049/jimmunol.1202941

Van Dyke, T. E. (2014). Commentary: periodontitis is characterized by an immuno-inflammatory host-mediated destruction of bone and connective tissues that support the teeth. *J. Periodontol.* 85, 509–511. doi: 10.1902/jop.2014.130701

Vinogradov, S. N., and Moens, L. (2008). Diversity of globin function: enzymatic, transport, storage, and sensing. *J. Biol. Chem.* 283, 8773–8777. doi: 10.1074/jbc.R700029200

Wakabayashi, H., Kondo, I., Kobayashi, T., Yamauchi, K., Toida, T., Iwatsuki, K., et al. (2010). Periodontitis, periodontopathic bacteria and lactoferrin. *Biometals* 23, 419–424. doi: 10.1007/s10534-010-9304-6

Wu, Y., Shu, R., Luo, L. J., Ge, L. H., and Xie, Y. F. (2009). Initial comparison of proteomic profiles of whole unstimulated saliva obtained from generalized aggressive periodontitis patients and healthy control subjects. *J. Periodontal Res.* 44, 636–644. doi: 10.1111/j.1600-0765.2008.01172.x

Yoshida, T., Nakamura, H., Masutani, H., and Yodoi, J. (2005). The involvement of thioredoxin and thioredoxin binding protein-2 on cellular proliferation and aging process. *Ann. N.Y. Acad. Sci.* 1055, 1–12. doi: 10.1196/annals.1323.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

REVIEW
published: 20 October 2015
doi: 10.3389/fcimb.2015.00073

Salivary markers of oxidative stress in oral diseases

L'ubomíra Tóthová[1,2], *Natália Kamodyová*[1], *Tomáš Červenka*[1] and *Peter Celec*[1,2,3,4]*

[1] Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia, [2] Center for Molecular Medicine, Slovak Academy of Sciences, Bratislava, Slovakia, [3] Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia, [4] Institute of Pathophysiology, Faculty of Medicine, Comenius University, Bratislava, Slovakia

Saliva is an interesting alternative diagnostic body fluid with several specific advantages over blood. These include non-invasive and easy collection and related possibility to do repeated sampling. One of the obstacles that hinders the wider use of saliva for diagnosis and monitoring of systemic diseases is its composition, which is affected by local oral status. However, this issue makes saliva very interesting for clinical biochemistry of oral diseases. Periodontitis, caries, oral precancerosis, and other local oral pathologies are associated with oxidative stress. Several markers of lipid peroxidation, protein oxidation and DNA damage induced by reactive oxygen species can be measured in saliva. Clinical studies have shown an association with oral pathologies at least for some of the established salivary markers of oxidative stress. This association is currently limited to the population level and none of the widely used markers can be applied for individual diagnostics. Oxidative stress seems to be of local oral origin, but it is currently unclear whether it is caused by an overproduction of reactive oxygen species due to inflammation or by the lack of antioxidants. Interventional studies, both, in experimental animals as well as humans indicate that antioxidant treatment could prevent or slow-down the progress of periodontitis. This makes the potential clinical use of salivary markers of oxidative stress even more attractive. This review summarizes basic information on the most commonly used salivary markers of oxidative damage, antioxidant status, and carbonyl stress and the studies analyzing these markers in patients with caries or periodontitis.

Keywords: saliva, biomarkers, oxidative stress, antioxidant status, oral diseases

OPEN ACCESS

Edited by:
Ulvi Kahraman Gürsoy, University of Turku, Finland

Reviewed by:
Fares Zeidán-Chuliá, Universidade Federal do Rio Grande do Sul, Brazil
Daisuke Ekuni, Okayama University, Japan

***Correspondence:**
Peter Celec
petercelec@gmail.com

Received: *01 September 2015*
Accepted: *02 October 2015*
Published: *20 October 2015*

Citation:
Tóthová L', Kamodyová N, Červenka T and Celec P (2015) Salivary markers of oxidative stress in oral diseases. Front. Cell. Infect. Microbiol. 5:73. doi: 10.3389/fcimb.2015.00073

INTRODUCTION

Saliva has become a popular diagnostic fluid for research and clinics in recent years. Its availability, easy collection and possibility of repeated non-invasive sampling makes it ideal for screening, diagnosis, or monitoring of many diseases. However, many technical issues have to be overcome and sensitivity as well as specificity have to be increased before routine clinical use. Several articles have been published dealing with saliva and its diagnostic potential in the past (Kaufman and Lamster, 2002; Chiappin et al., 2007; Lee and Wong, 2009). One review article has summarized the literature on oxidative stress in oral cavity-related pathologies. However, the review was not specifically oriented on oxidative stress in saliva (Iannitti et al., 2012). One critical review on this topic has been published recently, but was mainly focused on the methodology and statistical analysis used in the published studies (Wang et al., 2015a).

Periodontitis and dental caries represent the most common oral diseases. Very often, low adherence to oral hygiene and follow-up treatment lead to worsening of the disease and subsequently to loss of teeth (Renz et al., 2007). A non-invasive approach to diagnose and to monitor the progress of periodontitis and dental caries is needed that would improve the adherence and the overall therapeutic outcome. This review therefore aims to summarize the current findings in the research of oxidative stress analyzed in saliva in relation to oral diseases.

SALIVA AS A DIAGNOSTIC FLUID

Saliva is produced by secretion from the three major salivary glands (the parotid, submandibular, and sublingual glands) and numerous minor salivary glands. The collected whole saliva is a more complex mixture of fluids including gingival cevicular fluid, as well as oral, nasal, and mucosal transudate (Humphrey and Williamson, 2001). Additionally, oral bacteria and their metabolites, desquamated epithelial and blood cells, food debris and various chemical products are present in the saliva (de Almeida Pdel et al., 2008). Daily saliva production is estimated to be between 0.75 and 1.5 L in healthy adults. Salivary secretion is under both neural and hormonal control (Proctor and Carpenter, 2007). Nevertheless, salivary flow rate can be affected by various factors, such as circadian cycle, age, hydratation, chewing, oral hygiene, physical exercise, and others (Dawes, 1972; Chicharro et al., 1998; Chiappin et al., 2007; de Almeida Pdel et al., 2008).

Physiologial pH of saliva is between 6.2 and 7.4 (Schipper et al., 2007). From biochemical point of view, saliva is an aqueous solution (more than 99% is water) containing numerous organic and anorganic molecules (Greabu et al., 2009; Lima et al., 2010). Saliva may reflect the current physiological condition of the body and therefore is often called "the mirror of health of the organism" (Farnaud et al., 2010; Yoshizawa et al., 2013). The exchange between plasma and saliva takes place in the salivary ducts, which are separated from the circulation system as a thin layer of epithelial cells. The exchange includes active transport, diffusion across the cell membrane by passive diffusion directed by the concentration gradient (Lee and Wong, 2009). In patients suffering from oral diseases such as periodontitis there is a higher probability of blood leakage into saliva. This leads to the occurrence of blood components in saliva (Schwartz and Granger, 2004). This could interfere with various analytical methods and, thus, hinder the diagnostic use of saliva. However, at least for some of the most commonly used markers of oxidative stress we have found that blood contamination up to 1% does not affect even spectrophotometric methods and samples with higher blood concentrations can easily be excluded (Kamodyová et al., 2015). Since saliva represents a rapidly changing dynamic environment, it can potentially be used for long-term monitoring of oral diseases. In addition, new high-throughput approaches are being introduced for fast, reliable, and reproducible salivary diagnostic tests. Research on saliva cannot escape the current technological revolution. The whole salivary metabolome has already been described using several methodological approaches (Dame et al., 2015).

Similarly to plasma and tissues, free radicals and reactive oxygen/nitrogen species (ROS/RNS) in saliva play an important role in redox-dependent signaling and are necessary for physiological functions (Valko et al., 2007). On the other hand, excessive production of free radicals can lead to oxidative stress (Sies, 1997). Redox balance is than shifted in favor of oxidants. ROS/RNS can induce oxidative damage to cellular components with serious pathophysiological consequences (Devasagayam et al., 2004). On contrary, various antioxidant mechanisms are present in saliva including low molecular antioxidants—glutathione, ascorbic, and uric acid as well as melatonin (Moore et al., 1994; Balaji et al., 2015). Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are present in saliva (Battino et al., 2002). Their function is to protect oral cavity against the negative effects of endogenous and exogenous ROS/RNS. Additionally, membrane and DNA repair enzymes as well as proteases that degrade oxidatively modified proteins reduce the consequences of oxidative damage in saliva. Saliva is intended to be the first line defense against free radicals (Amerongen and Veerman, 2002; Battino et al., 2002). The dysbalance between the production of free radicals and antioxidant status in favor of oxidants is called oxidative stress. Oxidative stress has been implicated in the etiology and pathogenesis of several oral diseases including dental caries and periodontitis (Iannitti et al., 2012). This has already been shown by expression analysis of antioxidant genes in periodontitis patients (Zeidan-Chulia et al., 2013). Although it is not clear whether it is a cause or consequence of the disease process.

SALIVARY BIOMARKERS OF OXIDATIVE, CARBONYL STRESS, AND ANTIOXIDANT STATUS

Biomarkers are any characteristics which can be objectively measured and allow to predict the diagnosis, onset, or progression of a disease (Maiese et al., 2010). Optimal biomarkers for diagnostics of oxidative stress related diseases should be stable, accumulated in detectable concentrations, reflect specific oxidation pathways, and correlate with disease severity (Dalle-Donne et al., 2006). Considering free radicals are highly reactive and have a short half-life, the products formed from the reaction of ROS/RNS with cellular macromolecules are used preferentially as biomarkers of oxidative damage (Palmieri and Sblendorio, 2007a,b). Lipid peroxidation products, oxidized proteins, and products of DNA oxidation and fragmentation—are used for the assessment of oxidative stress. Measurement of various antioxidants or total antioxidant status represent another option of analyzing the redox status. Nevertheless, the use of a panel of biomarkers instead of a single parameter provides more informative results, reduces false positive and false negative results and enables a better understanding of the underlying pathomechanisms. The recent technological advances enabled the progress in systems biology and saliva research is moving into the omics world. Also using the metabolomic approach markers of oxidative stress were found to be among the discriminative between patients with periodontitis and healthy controls (Barnes

et al., 2014). This confirms that the research of salivary markers of oxidative stress is worth the effort.

Lipid Peroxidation

Lipid peroxidation is a reaction of lipids such as polyunsaturated fatty acids with ROS/RNS leading to formation of lipid hydroperoxides. This is accompanied by a complex process of degradation and decomposition reactions of hydroperoxides, whereby a wide range of products is formed (Dotan et al., 2004; Palmieri and Sblendorio, 2007a). The endproducts of lipid peroxidation are more stable than free radicals. Additionally, they can further react with other macromolecules, including DNA, proteins and phospholipids, far from the site of their production (Dalle-Donne et al., 2006). The most studied marker of lipid peroxidation is malondialdehyde (MDA). MDA is produced from fatty acids with two or more methylene-interrupted double bonds (Ayala et al., 2014). Standard method used for MDA detection is the spectrophotometric assay developed by Yagi, which is based on the reaction with thiobarbituric acid in acidic environment. It is also referred to as thiobarbituric acid reacting substances (TBARS) assay (Yagi, 1976). This assay is not specific for MDA and other aldehydes may react with thiobarbituric acid to produce a compound that absorbs wavelengths in the same range as MDA. Liquid chromatography or mass spectroscopy methods were reported as more reliable and specific for the measurement of MDA (Akalin et al., 2007), but the TBARS assay still represents a commonly used, cheap and high throughput method for the quantitative analysis of lipid peroxidation.

Another marker produced during lipid peroxidation is 4-hydroxy-2-nonenal that is generated by free radical attack on *ω*—6 polyunsaturated fatty acids (arachidonic, linoleic, and linolenic acids) (Sayre et al., 2006). Isoprostanes are unique products of lipid peroxidation of arachidonic acid and are considered to be reliable biomarkers of free radical mediated lipid peroxidation *in vivo* (Devasagayam et al., 2004; Spickett, 2013). Conjugated dienes are another option for analysis of lipid peroxidation. Unfortunately, few studies dealt with several of these markers, so the data about their usefulness for oral disease monitoring are limited.

Protein Oxidation

Proteins are major targets for ROS/RNS because they are highly abundant and are responsible for most functional processes in the cell (Dalle-Donne et al., 2003). The oxidation of proteins can take place at the level of single amino acid residues, it can lead to fragmentation of polypeptide chains or to covalent cross-linking of two amino acids either of the same or of two different proteins (Shacter, 2000). Oxidized proteins are either catabolized in proteosomal and lysosomal pathways or aggregated and accumulated in cellular compartments (Stadtman and Berlett, 1998).

Widespread methods for assessment of protein oxidation are the measurement of carbonyl groups by specific antibodies in ELISA or Western blot and by spectrophotometric assay based on dinitrophenylhydrazine derivitization (Dalle-Donne et al., 2003; Cabiscol et al., 2014). The advantages of protein carbonyls as a marker in comparison to lipid peroxidation products include early production and greater stability of oxidized proteins. Nonetheless, the production of protein carbonyl groups can be induced by almost all types of ROS and so, the protein carbonyl assay does not provide information about the source of oxidative stress. A major factors influencing most biomarkers of oxidative stress and protein carbonyl especially is aging. The relatively high correlation coefficients between salivary carbonyls and age led to a suggestion of protein carbonyls as an alternative biomarker of aging (Wang et al., 2015b).

Advanced oxidation protein products (AOPP) represent a sensitive biomarker of protein oxidation, especially due to neutrophil activation and the enzymatic activity of myeloperoxidase (Witko-Sarsat et al., 1996). AOPP were formerly thought to be a novel uremic toxin, reflecting highly oxidized protein status, but oxidized fibrinogen was found to be the major molecule responsible for the increase of the AOPP concentration (Selmeci, 2011). This should be taken into account, when interpreting the results.

Oxidative DNA Damage

The ROS/RNS react with DNA inducing damage to purine and pyrimidine bases and also the deoxyribose backbone (Halliwell, 2000). Pyrimidine damage products include thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoin, and others. Purine damage products are 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines, and other less characterized purine oxidative products (Cadet et al., 2003).

Measurement of 8-OHdG has been used to assess "whole-body" oxidative DNA damage using various analytical methods (Henderson et al., 2010). The issue with measuring 8-OHdG concentration is that 8-OHdG may not truly reflect oxidative damage to DNA. 8-OHdG can arise not just from removal of oxidized guanine residues from DNA by repair processes but also from degradation of oxidized dGTP in the DNA precursor pool. Recently published data, however, show that there is a clear strong association between the salivary concentrations of 8-OHdG and the clinical indices of periodontal status (Villa-Correa et al., 2015).

Carbonyl Stress

Carbohydrates such as glucose react non-enzymatically with the free amino groups of proteins and proceeds from reversible Schiff base adducts to more stable Amadori products. Some Amadori products are further converted to advanced glycation end products (AGEs) through a series of chemical rearrangements, dehydration, and fragmentation reactions (Ott et al., 2014). In addition to endogenously formed, AGEs can also be derived from exogenous sources such as smoking and food (Singh et al., 2001; Nass et al., 2007). Carbonyl precursors can form oxidative AGEs such as N^{ε}-(carboxymethyl)lysine and pentosidine or non-oxidative AGEs such as 3-deoxyglucosone or methylglyoxal (Singh et al., 2001). Pentosidine is derived exclusively from carbohydrate-derived carbonyl group and protein amino group while N^{ε}-(carboxymethyl)lysine originates not only from carbohydrates but also from autooxidation of lipids and amino acids (Zoccali et al., 2000). N^{ε}-(carboxymethyl)lysine

therefore serves as a general biomarker of oxidative stress, mostly assessed by ELISA or Western blotting.

AGEs are a heterogeneous group of protein-bound moieties and are characterized by browning, fluorescence, and crosslinking. Their determination is based on detection of specific fluorescence of AGEs at 370 nm excitation and 440 nm emission (Münch et al., 1997). Other methods such as ELISA, polyclonal antibodies, HPLC, or immunohistochemistry have been also applied to determine specific AGEs (Ahmed et al., 2002; Lapolla et al., 2005; Schmitt et al., 2005). Measurement of non-enzymatically glycated total proteins is referred to as fructosamine assay (Armbruster, 1987). Glucose bound to protein by aldimine linkage undergoes Amadori rearrangement through nonenzymatic glycation to the ketoamine (generically termed fructosamine). Fructosamine spectrophotometric assay is a simple test based upon the property of fructosamines to act as reducing agents in alkaline solution (San-Gil et al., 1985).

Nitrosative Stress

Similarly to oxidative stress, nitrosative stress is characterized as the imbalance between reactive nitrogen species and the antioxidants in favor of pro-oxidant RNS. Immoderate/excessive production of nitric oxide (NO) have detrimental effects on biomacromolecules. Due to its highly reactive nature, NO reacts with other reactive species (i.e., superoxide) leading to formation of more reactive compounds resulting in cytotoxic effects (Eiserich et al., 1998). On the other hand, NO can be oxidized to nitrite (NO_2^-) and subsequently oxidized to nitrate (NO_3^-). Therefore, the total concentrations of nitrite and nitrate are summed to adequately determine complex NO production. Salivary nitrates/nitrites are measured by Gries colorimetric assay (Andrukhov et al., 2013). Salivary NO and its derivates have been studied in relation to plasma NO, endothelial function, dietary intake of NO donors etc. However, most of the analyzed correlations were not significant (Clodfelter et al., 2015). Although the test was originally developed to help cardiologists, it is clear that the salivary NO concentrations are most affected by local oral processes and could, thus, be more usable for dentists and their patients.

Antioxidant Status

The measurement of individual antioxidants in biological samples is time consuming, labor-intensive, costly and requires complicated chemical techniques. In addition, the antioxidant effects are additive and therefore total antioxidant capacity of samples is preferentially measured (Erel, 2004). Antioxidant capacity assays can be divided into assays involving oxidants that are not necessarily pro-oxidants and assays involving oxidants that are pro-oxidants (Prior and Cao, 1999). To the first group of assays belong for example the ferric reducing/antioxidant power (FRAP) assay and the Trolox equivalent antioxidant capacity. The second group of assays includes total radical trapping parameter assay, luminol-based assays, dichlorofluorescin-diacetate based assay, crocin based assays, phycoerythrin based assays, and oxygen radical absorbance capacity assay (Prior and Cao, 1999). The increased antioxidant capacity can be a consequence of an adaptive response to a long term increased oxidative stress. On the other hand, the decrease in antioxidant capacity is not necessarily an undesirable condition when the production of reactive species decreases. The results of antioxidant assays should therefore be interpreted with caution.

SALIVARY OXIDATIVE STRESS IN ORAL DISEASES

Dental Caries

Dental caries is the most prevalent oral disease worldwide affecting both, children and adults and leading to pain and tooth loss. Systematic review dealing with the pathogenesis, epidemiology, diagnosis, and treatment of dental caries was published previously (Selwitz et al., 2007). Dental caries is a multifactorial inflammatory disease. However, the primary trigger is usually acidic by-products formed during bacterial fermentation of carbohydrates (Selwitz et al., 2007). In a recent review, the concept of inflammatory response in dentin, tightly connected with oxidative stress leading to the destruction of dental hard tissues is discussed (Southward, 2011). It was shown that dentinal fluid movement is inhibited by high sucrose levels causing down-regulation of parotid hormone from hypothalamic signaling. The consequence is that teeth become susceptible to bacterial acids (Leonora et al., 1993). The important role of ROS and antioxidants on the regulation of parotid hormone was hypothesized in a review dealing with the systemic theory of dental caries (Southward, 2011).

The importance of saliva in terms of salivary flow and antibacterial protection is generally accepted for the pathogenesis of dental caries (Lenander-Lumikari and Loimaranta, 2000; Stookey, 2008). The role of oxidative stress in dental caries is less clear, but it is the focus of intense research. Nine studies performed on children and four studies performed on adult subjects analyzing salivary oxidative stress in relation to dental caries are summarized in **Table 1**. Most research dealing with salivary oxidative stress in relation to dental caries is analyzing the antioxidant properties of saliva. Tulunoglu et al. have measured the antioxidant capacity of saliva in 80 children with and without caries activity (Tulunoglu et al., 2006). Children were divided into 8 groups according to age, gender, and caries activity. Non-significantly higher TAC was observed in caries active children and this observation was attributed to higher protein concentrations in caries active children. The exception was the group of 11–15 years old girls, where total protein and antioxidant capacity were lower in caries active group (Tulunoglu et al., 2006). In a very similar study TAC was analyzed in 120 children with and without caries activity (Preethi et al., 2010). Similarly to the previous study, children were divided into 8 groups. TAC was significantly higher in caries active girls and also boys in comparison to age matched control subjects. Total proteins were significantly higher in caries active in comparison to caries free children (Preethi et al., 2010; Dodwad et al., 2011). In the next study, relationship between the total antioxidant capacity of saliva and dental caries was assessed in deciduous and permanent teeth of Saharan children (Uberos et al., 2008).

TABLE 1 | Association studies investigating salivary markers of oxidative stress and dental caries.

Patients	Analyzed markers and methods	Detected concentrations	Results	Ref.
82 consecutive pediatric dental patients	AOPP (SPH), TBARS (SFL), AGES (SFL), FRAP (SPH), TAC (SPH)	Children with CI: 0, AOPP (160 μmol/L$^{\#}$), TBARS (0.029 μmol/L$^{\#}$), AGES (1 g/L$^{\#}$), FRAP (80 μmol/L$^{\#}$), TAC (1.6 mmol/L$^{\#}$) Children with CI: 1, AOPP (125 μmol/L$^{\#}$), TBARS (0.035 μmol/L$^{\#}$), AGES (0.95 g/L$^{\#}$), FRAP (68 μmol/L$^{\#}$), TAC (1.55 mmol/L$^{\#}$); Children with CI: 2, AOPP (130 μmol/L$^{\#}$), TBARS (0.034 μmol/L$^{\#}$), AGES (0.85 g/L$^{\#}$), FRAP (68 μmol/L$^{\#}$), TAC (1.58 mmol/L$^{\#}$)	AOPP were related to CI (eta 8.6%, $p < 0.03$) in general linear model	Tóthová et al., 2013a
50 children with severe early childhood caries (S-ECC), 50 healthy control children	TAC (SPH)	Control children, TAC (0.568 ± 0.169 mmol/L); Children with caries, TAC (1.729 ± 0.297 mmol/L)	↑ TAC in children with S-ECC ($p < 0.001$), linear regression between TAC and DMFT score ($p < 0.001$)	Kumar et al., 2011
80 children, 8 groups—according to age, gender and caries activity ($n = 10$/per group)	TAC (SPH)	Girls, age 7–10, caries free, TAC (0.39 ± 0.08 mmol/L); Boys, age 7–10, caries free, TAC (0.48 ± 0.20 mmol/L); Girls, age 11–15, caries free, TAC (0.65 ± 0.16 mmol/L); Boys, age 11–15, caries free, TAC (0.56 ± 0.30 mmol/L); Girls, age 7–10, caries active, TAC (0.45 ± 0.13 mmol/L); Boys, age7–10, caries active, TAC (0.60 ± 0.15 mmol/L); Girls, age 11–15, caries active, TAC (0.57 ± 0.18 mmol/L); Boys, age 11–15, caries active, TAC (0.69 ± 0.25 mmol/L)	↑ TAC in caries active groups (p = ns) except the 11 to 15-year-old girls group (p = ns)	Tulunoglu et al., 2006
120 children, 8 groups—according to age, gender and caries activity ($n = 15$/per group)	TAC (SPH)	Girls, age 7–10, caries free, TAC (0.16 ± 0.03 μmol/L); Boys, age 7–10, caries free, TAC (0.16 ± 0.05 μmol/L); Girls, age 11–14, caries free, TAC (0.19 ± 0.04 μmol/L); Boys, age 11–14, caries free, TAC (0.19 ± 0.04 μmol/L); Girls, age 7–10, caries active, TAC (0.23 ± 0.05 μmol/L); Boys, age7–10, caries active, TAC (0.20 ± 0.04 μmol/L); Girls, age 11–14, caries active, TAC (0.23 ± 0.04 μmol/L); Boys, age 11–14, caries active, TAC (0.22 ± 0.04 μmol/L)	↑ TAC in children with caries in comparison to children without caries ($p < 0.05$)	Preethi et al., 2010
126 children (4.5–14.5 years), caries in deciduous teeth (78.6% of children), caries in permanent teeth (77.8% of children)	TAC (SPH)	Caries free in deciduous teeth, TAC (7.8 ± 4.0 1/IC50); caries free in permanent teeth, TAC (7.8 ± 5.0 1/IC50); caries active in deciduous teeth, TAC (10.6 ± 11.1 1/IC50); caries active in permanent teeth, TAC (9.0 ± 8.0 1/IC50)	↑ TAC in patients with caries in deciduous teeth than among those without caries (p=0.06); linear regression between the number of deciduous teeth affected by caries and TAC ($p = 0.004$)	Uberos et al., 2008

(Continued)

TABLE 1 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	Ref.
		Caries free group, TAC (7.3 ± 4.3 1/IC50); Group with caries activity 1–2, TAC (7.2 ± 4.2 1/IC50); Group with caries activity 3–4, TAC (8.7 ± 4.7 1/IC50); Group with caries activity 5+, TAC (11.5 ± 12.4 1/IC50)		
100 children, 4 groups—with early childhood caries and controls (below 71 months), with rampant caries and controls (6–12 years)	TAC (SPH)	Early childhood caries TAC (25.58 ± 12.12 mmol/l); Early childhood caries free controls, TAC (14.15 ± 4.24 mmol/l) Rampant caries, TAC (46.12 ± 0.99 mmol/l); Rampant caries free controls, TAC (22.96 ± 4.76 mmol/l)	↑ TAC in children with caries ($p < 0.001$), TAC was increased with the age of the children ($p < 0.001$)	Hegde et al., 2009
80 children, (aged 3–5 years), 2 groups—with severe early childhood caries and controls ($n = 40$/per group)	FRAP (SPH)	N/A	↑ FRAP in children with severe early childhood caries ($p = 0.025$)	Mahjoub et al., 2014
50 children, (aged 3–5 years), 2 groups—with severe early childhood caries and controls ($n = 25$/per group)	TAC (SPH)	Severe early caries, TAC (1.82 ± 0.19 mmol/l saliva); Control group TAC (1.08 ± 0.17 mmol/l saliva)	↑ TAC in children with severe early childhood caries ($p < 0.0001$)	Muchandi et al., 2015
100 healthy high school students (age 15–17 years), 4 groups according to gender and caries activity. (Caries active was confirmed when volunteer had at least 5 caries surfaces)	TAC (SPH)	Boys, caries active TAC (59.72 ± 12.15); Boys, caries free TAC (41.32 ± 9.92); Girls, caries active TAC(40.95 ± 15.48); Girls, caries free TAC (40.16 ± 11.88)	↑ TAC in caries active males when compared to caries free males ($p < 0.001$). In females, no difference in TAC ($p < 0.84$). ↑ TAC in males when compared to females ($p < 0.001$)	Ahmadi-Motamayel et al., 2013
21 adult patients with caries, 16 controls	GSH (SPH), LPO (SPH)	Caries group, GSH (1.6 ± 0.75 mg/g protein), LPO (0.3 ± 0.15 μmol MDA/g protein); Control group, GSH (2.2 ± 0.8 mg/g protein), LPO (0.33 ± 0.28 μmol MDA/g protein)	↓ GSH in caries group ($p < 0.05$), negative correlation between DMFT and GSH ($p < 0.05$)	Oztürk et al., 2008
67 adult patients with dental caries, 50 controls	TBARS (SPH)	Females, caries free, MDA (3.24 ± 0.54 ng/mL); Males, caries free, MDA (3.38 ± 0.33 ng/mL); Females, caries active, MDA (3.36 ± 1.42 ng/mL); Males, caries active, MDA (3.42 ± 0.22 ng/mL)	↑ MDA in caries active patients (p = ns)	Rai et al., 2006
100 adult patients, 4 groups ($n = 25$/per group)—control group, group I (DMFT < 3), group II (DMFT < 10), group III (DMFT > 10)	TAC (SPH)	Control group, TAC (0.34 ± 0.13 μmol/L); Group I, TAC (0.44 ± 0.13 μmol/L); Group II, TAC (0.56 ± 0.26 μmol/L); Group III, TAC (0.59 ± 0.18 μmol/L)	↑ TAC was observed in groups with higher DMFT score ($p < 0.001$)	Hegde et al., 2013

TAC, total antioxidant capacity; AOPP, advanced oxidation protein products; TBARS, thiobarbituric acid reacting substances; AGES, advanced glycation end products; FRAP, ferric reducing antioxidant power; GSH, reduced glutathione; LPO, lipid peroxidation; SPH, spectrophotometry; SFL, spectrofluorometry; # denotes levels estimated from graph.

This study is one of the largest focusing on caries and salivary markers of antioxidant status. The authors have found a higher TAC in deciduous teeth of caries active patients than of caries free patients. Linear association was observed between the number of deciduous, but not permanent teeth affected by caries and TAC (Uberos et al., 2008). Hegde et al. and Kumar et al. have found that TAC in children with early childhood caries is higher than in controls with age being a significant confounding factor (Hegde et al., 2009; Kumar et al., 2011). The results were confirmed in another study in very young children (Muchandi et al., 2015). In a case-control study performed on 80 children FRAP as a measure of antioxidant status higher in children with caries (Mahjoub et al., 2014). Similarly, TAC was higher in adolescent males with active caries (Ahmadi-Motamayel et al., 2013). The reason for the higher antioxidant status in all studies remains unclear. A likely mechanism could include the response of the host to an infection/inflammation. All above mentioned studies had a case-control design. The study conducted by Tóthová et al. had a cross-sectional design with 82 children (4–18 years old) (Tóthová et al., 2013a). In this study beyond salivary antioxidant status (TAC and FRAP) also oxidative stress markers were analyzed in relation to caries index in children. These included TBARS, AOPP, and AGEs. Multivariate analysis showed that salivary AOPP are related to caries index (eta 8.6%), however ANOVA revealed no significant association between CI and AOPP (Tóthová et al., 2013a).

The study conducted in adults by Öztürk et al. was the first study analyzing the role of salivary glutathione (GSH) as an antioxidant in relation to dental caries (Oztürk et al., 2008). Significantly lower GSH concentrations were detected in adults with caries compared to subjects without caries and a negative correlation was observed between clinical indices and GSH. In this study no difference in lipid peroxidation was observed between subjects with and without caries (Oztürk et al., 2008). Rai et al. have analyzed TBARS in the saliva of caries active patients. Higher lipid peroxidation was detected in caries active patients than in control patients (Rai et al., 2006). Ahmadi-Motamayel et al. have analyzed TAC in caries active and caries free students (Ahmadi-Motamayel et al., 2013). In this study higher TAC was found in the group with caries, especially in males. Subtle gender differences were indicated by lower TAC in caries active females than in caries active males (Ahmadi-Motamayel et al., 2013). The association between TAC and caries severity was confirmed later (Hegde et al., 2013).

Most of the studies found that the antioxidant status is higher in caries active probands. As emphasized by Prior and Cao, an increased antioxidant capacity may be an adaptive response to increased oxidative stress (Prior and Cao, 1999). On the other hand, decreased GSH levels could suggest a tendency to decreased antioxidant status (Oztürk et al., 2008). The contradictory findings could be partially explained by temporal differences. As an acute effect the antioxidant status could be increased, but long term effects could contribute to consumption of antioxidants. It is clear that a panel of oxidative stress and antioxidant status markers is needed to interpret the role of oxidative stress in disease pathogenesis and progression (Prior and Cao, 1999). The only study in which a panel of salivary oxidative stress and antioxidant status markers was analyzed was the study conducted in children by Tóthová et al. (2013a). Although association studies have a clear outcome, there is a lack of mechanistic studies on the role of ROS or oxidative damage in saliva in relation to dental caries. Based on previous studies, it appears that there is no association between salivary lipid peroxidation and dental caries (Rai et al., 2006; Oztürk et al., 2008; Tóthová et al., 2013a). However, to interpret the relationship between dental caries and oxidative stress in saliva it is necessary to perform more carefully designed studies incorporating whole palette of oxidative stress and antioxidant status markers and, more importantly, experimental animal studies focusing on the underlying mechanism associated with ROS/RNS.

Periodontal Diseases

Periodontitis is affecting 11% of the global population (Marcenes et al., 2013). Its incidence varies in different populations and depends on oral hygiene and socio-economic status (Rylev and Kilian, 2008). Periodontitis is a chronic infectious disease of the supporting tissues of teeth and can lead to loss of connective tissue attachment, alveolar bone resorption, increased mobility of teeth and subsequent teeth loss. The etiology and pathogenesis of periodontitis is multifactorial and includes periodontal pathogens, biofilm formation, host immune response, and genetic risk factors (Laine et al., 2012).

The production of proteolytic enzymes and the respiratory burst of neutrophils mediated by enzymes such as NADPH oxidase and myeloperoxidase lead to generation of ROS/RNS and induce oxidative stress (Nizam et al., 2014; Syndergaard et al., 2014). These mechanisms play a key role in the pathogenesis of periodontitis as summarized by Chapple and Matthews in their comprehensive review (Chapple and Matthews, 2007). Salivary myeloperoxidase was found to be higher in periodontitis patients (Meschiari et al., 2013). The most common parameters of oxidative damage in association to periodontal diseases are markers of lipid peroxidation. Tsai et al. found higher lipid peroxidation in saliva of chronic periodontitis patients in a case-control study. An important secondary finding was that lipid peroxidation in saliva and gingival crevicular fluid correlated positively (Tsai et al., 2005). The association between periodontal status and salivary TBARS was confirmed in our large cross-sectional study with 217 patients (Celec et al., 2005). In a preliminary part of this study we found no correlation between salivary and plasma TBARS and, thus, local intraoral production of TBARS was hypothesized (Celec et al., 2005). Other studies confirmed higher lipid peroxidation in patients with severe but not moderate periodontitis (Mashayekhi et al., 2005; Khalili and Biloklytska, 2008) suggesting that the association between periodontitis and salivary TBARS might not be linear (Dalai et al., 2013). More recently, male patients, but not women with chronic periodontitis showed higher concentrations of salivary TBARS compared to healthy controls (Banasová et al., 2015). This could be due to changes in the changes in salivary cytokines during the menstrual cycle (Becerik et al., 2010). However, menstrual cycle related changes in salivary TAC concentrations have also been described with lower TAC during the ovulation period

(Kawamoto et al., 2012). In line with these findings are the results of unique study on pregnant women. It has been shown that pregnancy has an effect on several markers of oxidative stress and, more importantly, that the association between TBARS and periodontal status found in the general population could not be proved for pregnant women or women who gave birth recently (Gümüs et al., 2015). This is very likely related to the hormonal changes in pregnancy that have major impact on oral health and local immune status (Gürsoy et al., 2014).

MDA is the most studied product of lipid peroxidation. However, MDA is only one of many products formed during lipid peroxidation. TOS assay developed by Erel provides a possibility to measure additive effects of oxidants (Erel, 2005). Akalin et al. utilized TOS assay to measure oxidants in saliva of chronic periodontitis patients. Higher MDA and TOS levels were observed in saliva and also gingival crevicular fluid of chronic periodontitis patients (Akalin et al., 2007). The importance of lipid peroxidation in saliva was confirmed in another showing that the lipid peroxidation in saliva of patients who smoke and suffer from periodontitis is higher when compared to healthy probands (Guentsch et al., 2008). Increased oxidative damage of DNA, lipids and proteins were observed in periodontitis patients in a cross-sectional study (Su et al., 2009). In our cross-sectional study with more than 200 participants salivary TBARS, AOPP, AGEs, and TAC were assessed in relation to dental health, gender, and age (Celecová et al., 2013). We have found a strong association between TBARS and papillary bleeding index and confirmed the results from a previous study (Celec et al., 2005). Moreover, it was shown that this association is age independent. Only TBARS and no other analyzed markers of oxidative stress were associated with periodontitis (Celecová et al., 2013). When MDA was assessed using HPLC periodontitis patients had higher MDA in gingival crevicular fluid but not in saliva (Wei et al., 2010). These results are in accordance with our results showing that salivary TBARS other than MDA are associated with periodontitis (Celec et al., 2005). On the other hand, superoxide dismutase and TOS levels were higher in periodontitis patients in both, gingival crevicular fluid and saliva. In addition, 16 weeks of non-surgical treatment decreased lipid peroxidation. Significant positive correlations were observed between clinical indices and MDA, TOS, and superoxide dismutase levels in both oral fluids (Wei et al., 2010). Scaling and root planing resulted in an increase in TAC, uric acid, and glutathione peroxidase, but also in a decrease in superoxide dismutase activity (Novakovic et al., 2014). This finding has been further strengthened by mass spectrometry analysis of ions in patients with periodontitis where Cu, Zn, and Mn ions—all important for superoxide dismutase isoforms were lower in patients vs. controls. In line are the found higher concentrations of isoprostanes as a consequence of lipid peroxidation (Huang et al., 2014). Several authors have assumed that higher lipid peroxidation in saliva is associated with increased percentage of gingival crevicular fluid in the saliva of periodontitis patients (Akalin et al., 2007; Su et al., 2009; Wei et al., 2010). Lipid peroxidation products in saliva might arise from ROS/RNS production by neutrophils activated by periodontal pathogens (Chapple and Matthews, 2007) or from direct microbial production of ROS/RNS (Vlková and Celec, 2009). Especially DNA damage markers seem to be associated with the presence and abundance of specific periodontal pathogens (Almerich-Silla et al., 2015).

Salivary 8-OHdG as a marker of DNA damage was higher in periodontitis patients in comparison to controls (Novakovic et al., 2013) but not in gingivitis patients (Sezer et al., 2012). Higher concentrations of salivary 8-OHdG in periodontal diseases were found also in several other studies (Takane et al., 2002; Sawamoto et al., 2005; Canakçi et al., 2009; Su et al., 2009; Novakovic et al., 2013; Miricescu et al., 2014). Initial periodontal therapy led to a decrease in salivary 8-OHdG. Thus, 8-OHdG could become a useful biomarker for evaluating the efficacy of periodontal treatment and individual prognosis (Takane et al., 2002, 2005). In another study, however, these findings could not be confirmed (Dede et al., 2013). Some studies have found a correlation between salivary 8-OHdG and the presence of *Porphyromonas gingivalis* or the large mitochondrial DNA deletion in gingival tissues (Canakçi et al., 2009).

Oxidative damage to proteins may have dangerous consequences in a cell due to their important catalytic functions (Dalle-Donne et al., 2003). Protein carbonyl content is the most widely used marker of protein oxidation (Sezer et al., 2012). It is associated with clinical indices of oral health, especially if gender of the patients is taken into account (Sculley and Langley-Evans, 2003). This has been confirmed in another study focusing on the prognostic potential of specifically oxidized salivary proteins such as transferrin, human IgG1 heavy chain fragment and amylase (Su et al., 2009). AOPP was originally suggested as a marker of oxidative damage in plasma of chronic uremic patients (Witko-Sarsat et al., 1996). Only two studies coming from our group have analyzed AOPP in saliva in relation to periodontal status. However, we have found an association between salivary AOPP and papillary bleeding index neither in adults (Celecová et al., 2013) nor in children (Tóthová et al., 2013a). Oxidative and carbonyl stress are closely linked. Reactive carbonyl groups can non-enzymatically react with amino groups of proteins leading to the production of AGEs. Lipid peroxidation products including MDA possess reactive carbonyl groups and can substitute carbohydrates in the Maillard reaction as the basis of AGEs production (Miyata et al., 2000). Similarly to AOPP, salivary AGEs were not associated with periodontal status in any of the studied group of patients (Celecová et al., 2013; Tóthová et al., 2013a).

Relatively few studies examined the concentrations of salivary NO in patients with gingivitis or periodontitis. Most of them observed higher NO concentrations depending on the periodontitis severity when compared to healthy controls (Reher et al., 2007; Parwani et al., 2012; Sundar et al., 2013; Wadhwa et al., 2013; Poorsattar Bejeh-Mir et al., 2014), but NO was decreased by the treatment (Parwani et al., 2012). Interestingly, in another study nitrite concentrations were lowered by treatment only in erythrocytes and not in plasma or saliva (Meschiari et al., 2015). Salivary concentrations of NO can be altered by other factors, especially smoking. Some studies, however, found lower salivary NO in patients with periodontitis compared to the healthy controls (Aurer et al., 2001; Andrukhov et al., 2013). The reasons for these discrepancies might include

differences in the analytical methods, but also in the pre-analytic phase.

Similar reasons could explain some of the contradictory findings on TAC (Chapple and Matthews, 2007). In most of the studies TAC was found to be lower in patients with periodontitis (Chapple, 1997; Diab-Ladki et al., 2003; Mashayekhi et al., 2005). This has been confirmed by another study, while the three main specific salivary antioxidants assessed—uric acid, ascorbic acid, and albumin were not significantly different from controls (Diab-Ladki et al., 2003). A study focusing on the antioxidant melatonin found that neither plasma, nor salivary concentrations were different between patients and controls. The difference with lower concentrations for patients was found in the gingival tissue showing that saliva is not representative for all analyzed markers in the oral cavity (Balaji et al., 2015). Mashayekhi et al. have shown that TAC depends on the clinical severity of periodontitis. In addition, the role of cyclic nucleotides cAMP and cGMP, which were lower in patients vs. controls was highlighted in this study (Mashayekhi et al., 2005). Two other studies, however, found no difference between patients and controls in salivary TAC (Brock et al., 2004; Tóthová et al., 2013b). In contrast, higher salivary TAC was observed in periodontitis patients in a cross-sectional study (Su et al., 2009). Patients with periodontitis in that study were, however, older and consumed higher amounts of antioxidants than controls. Nevertheless, after adjusting for age and antioxidant intake, the multivariate analysis showed positive correlation between salivary TAC and periodontitis (Su et al., 2009). The crucial difference might, thus, lie in the dynamics of the disease that could differ between studies and is rarely reported. There might be also an often overlooked sex difference in salivary TAC being lower in women (Sculley and Langley-Evans, 2003). Miricescu et al. have reported lower salivary TAC and also uric acid as well as glutathione peroxidase in whole saliva of patients with periodontitis (Novakovic et al., 2013). In other studies lower salivary glutathione—the main intracellular antioxidant and ceruloplasmin—an important extracellular antioxidant were reported in periodontitis patients in comparison to healthy probands (Tsai et al., 2005; Dalai et al., 2013). This could be explained by a depletion of the antioxidants due to increased ROS/RNS generation. It is unclear whether periodontal pathogens could be directly involved (Tsai et al., 2005). The studies on salivary oxidative stress and antioxidant status markers in relation to periodontal disease are summarized in **Table 2**.

ISSUES AND LIMITATIONS

Numerous studies summarized in this review show the potential of salivary markers of oxidative, carbonyl stress and antioxidant status as they seem to be associated with oral diseases, their severity and respond to treatment (**Figure 1**). However, there are several limitations that explain why these markers are not in routine clinical use yet. From the summary tables it is clear that the reported concentrations of the salivary markers differ by orders of magnitude between studies. With such a variability, contradictory results from the studies are not surprising. The reasons for such a variability are both, technical and biological. Various different methods and protocols for each particular assay are used as comprehensively reviewed by others (Palmieri and Sblendorio, 2007a; Wang et al., 2015a). Our research uncovered several pre-analytical factors that affect the salivary markers of oxidative stress including saliva collection, daytime, intake of antioxidants, tooth-brushing and others (Kamodyová and Celec, 2011; Kamodyová et al., 2013). Importantly, salivation should not be induced as such samples are biased in comparison to spontaneous salivation. And although collection devices such as Salivette make saliva collection even more effective, their use should be limited to biomarkers that are resistant to such a collection method. This is not the case for most of the studied markers of oxidative stress. Our unpublished preliminary data show that a major impact on the subsequent analyses can come from such a small step as centrifugation after saliva collection. Although our data do not support this assumption, it can be expected that prolonged storage at different temperatures can lead to variable results in the analysis. The stability of the biomarkers very likely differs from marker to marker.

Beyond periodontitis and caries there are other oral and systemic diseases that were studied in relation to salivary markers of oxidative stress. Oral precanceroses such as lichen planus and leukoplakia but also patients with oral squamous cell carcinoma were found to be associated with higher MDA (Lopez-Jornet et al., 2014; Metgud and Bajaj, 2014). In one study autistic children had lower salivary TAC in comparison to their healthy siblings, although this difference can be attributed to worse oral hygiene (Rai et al., 2012). Markers of carbonyl stress such as salivary AGEs are modified by the treatment in sleep apnea syndrome (Celec et al., 2012). Even patients with the Down syndrome have higher salivary MDA and superoxide dismutase when compared with controls (de Sousa et al., 2015). It is very likely that other physiological and pathological factors including renal diseases, diabetes mellitus, and systemic inflammatory disorders influence salivary concentrations of biomarkers and a lot of research is yet to be conducted. A summary of the current research on salivary oxidative stress markers including studies on extra-oral or systemic diseases was published recently (Buczko et al., 2015).

The level of evidence varies between the studies. Only few of them report results from more than 100 patients. With such high technical and biological variability it is clear that the number of patients/samples has to be high to extract meaningful information from the gathered data. The most important limitations are, however, the unclear causality of the observed associations. Even tight correlation do not have to be due to a causal relationship. The causality can only be tested in interventional experiments in animal models or human patients.

FUTURE OUTLOOK

Especially, the easy and non-invasive collection of saliva makes it a very interesting diagnostic fluid. Although numerous pitfalls have to be taken into account, analysis of salivary biomarkers could make it into routine clinic when it comes to salivary steroids, DNA, RNA, or the oral microflora. It is possible that in the future also salivary markers of oxidative stress will be

TABLE 2 | Association studies investigating salivary markers of oxidative stress and periodontal diseases.

Patients	Analyzed markers and methods	Detected concentrations	Results	References
36 CP patients, 28 controls	MDA (HPLC), TOS (SPH)	Control group, MDA (median 0.06 μM), TOS (4.16 ± 0.63 mM H_2O_2 Equivalent); CP group, MDA (median 0.1 μM), TOS (6.03 ± 1.37 mM H_2O_2 Equivalent)	↑ MDA and ↑ TOS in CP patients ($p < 0.05$); positive correlations was observed between periodontal parameters and MDA and TOS ($p < 0.05$)	Akalin et al., 2007
30 CP patients (15 smokers), 30 controls (15 smokers)	MDA (SFL), GSHPx (SPH), TAC (PCL) (baseline and 6 moth after non-surgical treatment)	Control group, non-smokers, MDA (0.065 ± 0.05 μmol/L), GSHPx (5.78 ± 3.77 U/L), TAC flow rate (0.52 ± 0.20 μmol/mL); Control group, smokers, MDA (0.085 ± 0.08 μmol/L), GSHPx (7.72 ± 2.70 U/L), TAC flow rate (0.75 ± 0.24 μmol/mL) CP group, non-smokers, MDA (0.095 ± 0.05 μmol/L), GSHPx (16.08 ± 13.34 U/L), TAC flow rate (0.37 ± 0.24 μmol/mL) CP group, smokers, MDA (0.123 ± 0.08 μmol/L), GSHPx (21.10 ± 18.65 U/L), TAC flow rate (0.29 ± 0.21 μmol/mL) Post-treatment, CP group, non-smokers, MDA (0.060 ± 0.09 μmol/L), GSHPx (6.54 ± 2.84 U/L), TAC flow rate (0.44 ± 0.22 μmol/mL) Post-treatment, CP group, smokers, MDA (0.060 ± 0.05 μmol/l), GSHPx (6.74 ± 4.33 U/l), TAC flow rate (0.42 ± 0.17 μmol/ml)	↑ MDA in smoking CP patients compared to non-smoking controls ($p < 0.05$);↑ GSHPx and ↓ TAC flow rate in CP patients ($p < 0.05$); non-surgical periodontal treatment lead to ↓ MDA and ↓ GSHPx ($p < 0.05$)	Guentsch et al., 2008
48 CP patients, 35 controls	MDA (HPLC), TOS (SPH), SOD (SPH) (baseline and 16 weeks after non-surgical treatment)	Control 1, MDA (0.10 ± 0.02 mM), TOS (6.75 ± 1.02 mM), SOD (174.9 ± 21.07 U/mg protein); Control 2, MDA (0.11 ± 0.03 mM), TOS (5.69 ± 1.03), SOD (177.6 ± 24.61 U/mg protein); CP 1 (before therapy), MDA (0.11 ± 0.05 mM), TOS (9.12 ± 1.77 mM), SOD (216.4 ± 36.78 U/mg protein); CP 2 (after therapy), MDA (0.09 ± 0.01 mM), TOS (5.61 ± 0.95 mM), SOD (169.8 ± 23.65 U/mg protein)	↑ TOS and ↑ SOD in CP group ($p < 0.05$); ↓ SOD and ↓ TOS after therapy ($p < 0.05$); positive correlations between clinical parameters and MDA, TOS and SOD levels	Wei et al., 2010
74 patients with periodontitis, 3 groups -early ($n = 30$), moderate ($n = 30$), severe ($n = 14$), 30 controls	MDA (SPH)	Control group, MDA (5.16 ± 0.03 μmol/mL) Early periodontitis group, MDA (28.08 ± 1.56 μmol/mL); Moderate periodontitis group, MDA (39.01 ± 1.59 μmol/mL); Severe periodontitis group, MDA (65.20 ± 2.00 μmol/mL)	Significant differences in MDA levels of patients with early, moderate and severe periodontitis in comparison to control patients ($p < 0.05$)	Khalili and Biloklytska, 2008

(Continued)

TABLE 2 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	References
Preliminary study: 13 CP patients, 9 controls	GSH (SPH), GPx (SPH), LPO (SPH)	Preliminary stud Control group, GSH (606.67 ± 191.02 μmol/L), GPx (92.90 ± 58.58 mU/mL), LPO (0.13 ± 0.08 μmol/L);	↓ GSH ($p < 0.05$), ↑ lipid peroxidation ($p < 0.0005$) and no difference in GPx activity in patients than in controls; ↓ lipid peroxidation ($p < 0.05$), ↑ GSH ($p < 0.001$), and no change in GPx activity in patients after periodontal treatment	Tsai et al., 2005
Subsequent study: 22 CP patients	Subsequent study: (baseline and 1 month after initial periodontal treatment)	Periodontitis group, GSH (373.04 ± 287.42 μmol/L), GPx (92.99 ± 74.40 mU/mL), LPO (0.66 ± 0.36 μmol/L) Subsequent study: Periodontitis group before treatment, GSH (353.59 ± 141.93 μmol/L), GPx (96.50 ± 35.14 mU/mL), LPO (0.63 ± 0.49 μmol/L); Periodontitis group after treatment, GSH (602.92 ± 170.15 μmol/L), GPx (99.34 ± 45.72 mU/mL), LPO (0.41 ± 0.26 μmol/L)		
217 consecutive stomatologic patients	TBARS (SFL), MDA (HPLC)	TBARS (0.05–2.2 μmol/L$^{\#}$), MDA (0.3 μmol/mL$^{\#}$)	↑ TBARS tightly associated with ↑ PBI (adjusted for age and sex, $p < 0.001$)	Celec et al., 2005
204 consecutive stomatologic patients	TBARS (SPH), AOPP (SPH), TAC (SPH), AGEs (SFL)	Group with PBI = 0, TBARS (0.038 μmol/L$^{\#}$); Group with PBI = 1, TBARS (0.045 μmol/L$^{\#}$); Group with PBI = 2, TBARS (0.055 μmol/L$^{\#}$)	TBARS associated with PBI ($p = 0.004$); ↑ TBARS ($r = 0.235$, $P = 0.004$), ↑ AGEs ($r = 0.141$, $P = 0.043$) and ↑ TAC ($r = 0.225$, $P = 0.002$) with age	Celecová et al., 2013
82 consecutive pediatric dental patients	AOPP (SPH), TBARS (SFL), AGES (SFL), FRAP (SPH), TAC (SPH)	Children with PBI: 0, AOPP (140 μmol/L$^{\#}$), TBARS (0.029 μmol/L$^{\#}$), AGES (1 g/L$^{\#}$), FRAP (75 μmol/L$^{\#}$), TAC (1.6 mmol/L$^{\#}$); Children with PBI: 1, AOPP (125 μmol/L$^{\#}$), TBARS (0.038 μmol/L$^{\#}$), AGES (0.95 g/L$^{\#}$), FRAP (65 μmol/L$^{\#}$), TAC (1.65 mmol/L$^{\#}$); Children with PBI: 2, AOPP (150 μmol/L$^{\#}$), TBARS (0.056 μmol/L$^{\#}$), AGES (0.85 g/L$^{\#}$), FRAP (70 μmol/L$^{\#}$), TAC (1.5 mmol/L$^{\#}$)	variability of PBI explains 10.9% of the variance of TBARS ($p = 0.02$); TAC and FRAP were partially determined by PBI (16.9% and 7.9%, $p < 0.05$)	Tóthová et al., 2013a
29 periodontitis patients, 20 controls	8-HOdG (C-ELISA kit) (baseline and 2–4 month after initial periodontal treatment)	Control group, 8-HOdG (1.48 ± 0.08 ng/mL) Periodontitis group, 8-HOdG (4.36 ± 0.18 ng/mL) Before therapy, 8-HOdG (4.05 ± 0.17 ng/mL) After therapy, 8-HOdG (1.75 ± 0.09 ng/mL)	↑ 8-HOdG in periodontitis patients ($p < 0.01$); 8-OHdG correlated with P. gingivalis ($p < 0.01$); ↓ 8-OHdG after initial periodontal treatment ($p < 0.01$)	Sawamoto et al., 2005

(Continued)

TABLE 2 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	References
24 patients with periodontitis, 3 groups—early ($n = 8$), moderate ($n = 8$), advanced ($n = 8$), 8 controls	TBARS (SPH), TAP (SPH), cAMP (ELISA), cGMP (ELISA)	Control group, TBARS (1.2 μmol/mL$^{\#}$), TAP (2 μmol/mL$^{\#}$), cAMP (32 pmol/mL$^{\#}$), cGMP (3.6 μmol/mL$^{\#}$) Early periodontitis group, TBARS (1.22 μmol/mL$^{\#}$), TAP (1.8 μmol/mL$^{\#}$), cAMP (27 pmol/mL$^{\#}$), cGMP (3.3 μmol/mL$^{\#}$); Moderate periodontitis group, TBARS (1.22 μmol/mL$^{\#}$), TAP (1.75 μmol/mL$^{\#}$), cAMP (23 pmol/mL$^{\#}$), cGMP (3.1 μmol/mL$^{\#}$); Advanced periodontitis group, TBARS (1.35 μmol/mL$^{\#}$), TAP (1.5 μmol/mL$^{\#}$), cAMP (25 pmol/mL$^{\#}$), cGMP (3 μmol/mL$^{\#}$)	↓ cAMP and ↓ cGMP in patients with moderate and advanced periodontitis ($p < 0.01$); ↑ TBARS ($p < 0.01$) and ↓ TAP ($p < 0.01$) in patients with advanced periodontitis	Mashayekhi et al., 2005
58 periodontitis patients, 234 controls	8-OHdG (ELISA), 8-epi-PGF2α (ELISA), total protein carbonyls (ELISA), TAC (SPH)	Control group, 8-OHdG (42.65 ng/ml), 8-epi-PGF2α (43.57 pg/ml), protein carbonyls (0.96 nmol/mg protein), TAC (0.46 mM); Periodontitis group, 8-OHdG(66.84 ng/ml), 8-epi-PGF2α (62.72 pg/ml), protein carbonyls (1.79 nmol/mg protein), TAC (0.71 mM)	↑ 8-OHdG ($p = 0.0003$), ↑ 8-epi-PGF2α ($p = 0.0001$), and ↑ carbonylated proteins ($p < 0.0001$) in periodontal patients; 8-OHdG, 8-epi-PGF2α, and carbonylated proteins independently negatively associated with CPITN ($P = 0.004$, 0.02, and 0.0001); a positive correlation between TAC and periodontal disease status ($p < 0.0001$)	Su et al., 2009
16 patients with stage I periodontitis, 16 patients with stage II periodontitis, 15 controls	MDA, ceruloplasmin (detailed methods not provided)	Control group, MDA (0.58 ± 0.14 nmol/mL), ceruloplasmin (3.46 ± 1.25 mg%); Stage I periodontitis, MDA (2.17 ± 0.55 nmol/mL), ceruloplasmin (2.14 ± 1.18 mg%); Stage II periodontitis, MDA (2.05 ± 0.48 nmol/mL), ceruloplasmin (1.11 ± 0.66 mg%)	↑ MDA in patients with stage I and stage II periodontitis in comparison to control ($p < 0.001$); ↓ MDA in patients with stage II periodontitis compared to the patients with stage I periodontitis ($p > 0.1$); ↓ ceruloplasmin in stage II periodontitis patients compared to control patients ($p < 0.001$)	Dalai et al., 2013
20 CP patients, 20 controls	8-HOdG (ELISA), MDA (SPH), uric acid (SPH), TAC (SPH), GPx (SPH)	Control group, 8-HOdG (6.46 ± 0.93 ng/mg albumin), MDA (0.25 ± 0.4 nmol/mg albumin), uric acid (3.12 ± 0.85 mg/mg albumin), TAC (1.24 ± 0.16 nmol/mg albumin), GPx (28.16 ± 11.95 U/mg albumin); CP group, 8-HOdG (6.78 ± 1.80 ng/mg albumin), MDA (0.296 ± 0.10 nmol/mg albumin), uric acid (2.41 ± 0.265 mg/mg albumin), TAC (0.75 ± 0.16 nmol/mg albumin), GPx (15.81 ± 7.22 U/mg albumin)	8-OHdG, MDA higher in CP group ($p < 0.05$); uric acid, TAC and GPx decreased in CP patients vs. controls ($p < 0.05$), oxidative stress markers associated with alveolar bone loss biomarkers	Miricescu et al., 2014

(Continued)

TABLE 2 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	References
32 patients with periodontits, 32 control patients	8-OHdG (C-ELISA kit)	Control group, 8-OHdG (1.41 ± 0.22 ng/ml) CP group, 8-OHdG (3.76 ± 0.30 ng/ml)	↑ 8-OHdG in patients with periodontitis ($p < 0.01$); positive correlation between the occurrence of the 5-kbp mtDNA deletion and salivary 8-OHdG concentrations	Canakçi et al., 2009
78 patients with periodontitis, 17 controls	8-HOdG (ELISA)	Control group, 8-OHdG (1.56 ± 0.10 ng/mL); Periodontitis group, 8-OHdG (4.28 ± 0.10 ng/mL)	↑ 8-HOdG in patients with periodontitis ($p < 0.01$)	Takane et al., 2002
20 CP patients, 20 CG patients, 20 controls	8-HOdG (C-ELISA)	Control group, 8-OHdG (1.56 ± 0.12 ng/mL) CG group, 8-OHdG (1.58 ± 0.13 ng/mL) CP group, 8-OHdG (3.13 ± 0.22 ng/mL)	↑ 8-HOdG in CP than in CG and H groups ($p < 0.001$); correlation between the salivary 8-OHdG and CAL in patients with CAL > 3 mm ($p < 0.001$)	Sezer et al., 2012
34 CP patients, two groups- with periodontally involved teeth of hopeless prognosis ($n = 16$), without teeth of hopeless prognosis ($n = 18$), 17 controls	8-OHdG (C-ELISA) (baseline and 2–6 months after initial periodontal treatment)	Control group, 8-OHdG (1.56 ± 0.1 ng/mL) Before treatment: Group without periodontally hopeless teeth, 8-OHdG (2.35 ± 0.18 ng/mL); Group with periodontally hopeless teeth, 8-OHdG (4.78 ± 0.14 ng/mL); After treatment: Group without periodontally hopeless teeth, 8-OHdG (1.73 ± 0.16 ng/mL); Group with periodontally hopeless teeth, 8-OHdG (2.02 ± 0.31 ng/mL)	↑ 8-OHdG in those with than in subjects without periodontally-involved teeth of hopeless prognosis ($p < 0.05$) and healthy controls ($p < 0.01$); ↓ 8-OHdG levels after treatment of subjects with ($p < 0.01$) but not those without periodontally involved teeth of hopeless prognosis	Takane et al., 2005
24 patients with CP, 24 controls	8-OHdG (baseline and 10 days, 1 month, 3 months after initial periodontal therapy)	CP baseline 8-OHdG (605.5 ± 139.1) controls 8-OHdG (550.52 ± 150.28) CP 10 days 8-OHdG (543.1 ± 154.8) CP 1 month 8-OHdG (542.0 ± 154.6) CP 3 months 8-OHdG (534.3 ± 151.2)	salivary 8-OHdG did not differ between groups or during initial periodontal therapy ($p > 0.05$)	Dede et al., 2013
129 patients, cohort study	TAC (SPH), ascorbate (SPH), urate (SPH), albumin (SPH), protein carbonyl (SPH)	Men, protein carbonyl (8.46 ± 1.71 fmol/g of protein), TAC flow rate (0.31 ± 0.02 μmol/mL/min), ascorbate flow rate (4.27 ± 0.44 nmol/mL/min), albumin flow rate (5.44 ± 0.74 nmol/mL/min), urate flow rate (108.8 ± 12.0 nmol/mL/min);	↓ TAC in women than in men ($p < 0.01$), protein carbonyls 2.3 times ↑ in women than in men (p = ns); TAC corrected for sex ↑ in severe disease group ($p < 0.05$), protein carbonyls corrected for sex ↓ in severe disease group ($p < 0.05$);association between protein carbonyl concentration and low CPITN score ($p = 0.018$) and female sex ($p = 0.020$); antioxidant status and risk of disease (classified as CPITN < 18) was significantly related to TAC flow rate ($p = 0.043$)	Sculley and Langley-Evans, 2003

(Continued)

TABLE 2 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	References
		Women, protein carbonyl (19.26 ± 7.09 fmol/g of protein), TAC flow rate (0.19 ± 0.09 μmol/mL/min), ascorbate flow rate (2.88 ± 0.30 nmol/mL/min), albumin flow rate (4.03 ± 0.74 nmol/mL/min), urate flow rate (58.7 ± 6.6 nmol/mL/min);		
17 patients with severe periodontitis, 20 controls	Uric acid (SPH), albumin (SPH), ascorbic acid (SPH), TAC (SPH)	Control group, uric acid (160 μmol/L$^{\#}$), albumin (14 μmol/L$^{\#}$), ascorbic acid (9 μmol/L$^{\#}$), TAC (340 μmol/L$^{\#}$); Periodontitis group, uric acid (140 μmol/L$^{\#}$), albumin (11 μmol/L$^{\#}$), ascorbic acid (7 μmol/L$^{\#}$), TAC (220 μmol/L$^{\#}$)	↓ TAC in patients with periodntitis ($p < 0.05$); uric acid, ascorbic acid, and albumin are not significantly affected (p = ns)	Diab-Ladki et al., 2003
20 CP patients, 20 controls	TAC (ECL)	Control group, TAC (0.14 ± 0.06 nmoles/30 s sample) CP group, TAC (0.18 ± 0.08 nmoles/30 s sample)	↓TAC in CP group than in control group (p = ns)	Brock et al., 2004
18 patients with periodontitis, 16 controls	TAC (ECL)	Control group, TAC (254 ± 110 mumol/L); Peridontitis group, TAC (175 ± 53 mumol/L)	↓ TAC in the peridontitis patients than in control patients ($p < 0.01$)	Chapple et al., 1997
24 patients with gingivitis, 23 with periodontitis, and 23 controls	SOD (SPH), thiol antioxidant concentrations (SPH) (baseline and 15 days after non-surgical treatment)	Before treatment: Control group, SOD (129.93 ± 3.98 U/0.5 mL), thiol (36.99 ± 2.14 μmol/L) Gingivitis group, SOD (88.28 ± 2.56 U/0.5 mL), thiol (30.15 ± 3.13 μmol/L) Periodontitis group, SOD (39.99 ± 3.52 U/0.5 mL), thiol (15.09 ± 2.26 μmol/L) After treatment: Control group, SOD (158.69 ± 3.61 U/0.5 mL), thiol (53.96 ± 1.72 μmol/L) Gingivitis group, SOD (133.56 ± 2.16 U/0.5 mL), thiol (45.83 ± 2.09 μmol/L) Periodontitis group, SOD (61.44 ± 2.67 U/0.5 mL), thiol (27.41 ± 1.88 μmol/L)	↑ SOD and thiol concentrations post-treatment in all the three groups, comparison between the three groups post-treatment did not show any significant difference in improvement of superoxide dismutase or thiol concentrations	Karim et al., 2012
21 CP patients	GPx, SOD, albumin, uric acid, total antioxidative status (TAS) (before and after non-surgical treatment)	N/A	↑ uric acid, albumin, GPx, TAS; ↓ SOD activity after treatment; correlation between GPx and plaque index, SOD and gingival index before therapy; correlation between SOD and bleeding on probing, and TAS and bleeding on probing after therapy	Novakovic et al., 2013
23 CP patients (14 females, 9 males)	TBARS, AGEs (SFL), TAC FRAP (SPH)	N/A	↑ TBARS in male CP ($p < 0.01$); ↓ TAC in female CP ($p < 0.001$)	Banasová et al., 2015

(Continued)

TABLE 2 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	References
19 controls (8 females, 11 males)				
30 CP patients 30 controls	MDA, SOD, GR, CAT (all markers SPH)	CP group MDA (9.34 ± 8.15 nmol/ml), SOD (19.76 ± 11.53 U/ml), CAT (0.08 ± 0.13 U/min/mg prot), GR (12.51 ± 6.39 U/min/mg prot); Control group MDA (1.39 ± 1.28 nmol/ml), SOD (30.22 ± 7.03 U/ml), CAT (0.37 ± 0.28 U/min/mg prot), GR (25.50 ± 9.11 U/min/mg prot)	↑ MDA in CP; ↓ SOD, CAT and GR in CP compared to control group Positive correlation between MDA and periodontal status; Negative correlation between SOD, CAT and GR and periodontal status	Trivedi et al., 2015
30 CP patients with diabetes mellitus type 2	SOD GR	CP group MDA (9.09 ± 8.16 nmol/ml) SOD (19.93 ± 12.05 U/ml)	↑ MDA in CP irrespective of diabetes;	Trivedi et al., 2014
30 CP patients without systemic disease	CAT MDA (all markers SPH)	GR (13.63 ± 6.46 U/min/mg prot) CAT (0.08 ± 0.14 U/min/mg prot)	SOD and GR differed in CP patients with diabetes mellitus type 2 compared to CP without systemic disease	
30 controls with diabetes mellitus type 2 30 healthy controls		CP group with DM type 2 MDA (10.79 ± 8.07 nmol/ml) SOD (14.08 nmol/ml 4.28 U/ml) GR (18.33 ± 7.47 U/min/mg prot) CAT (0.04 ± 0.03 U/min/mg prot) controls without systemic disease MDA (1.53 ± 1.30 nmol/ml) SOD (29.64 ± 6.98 U/ml) GR (24.57 ± 7.97 U/min/mg prot) CAT (0.38 ± 0.29 U/min/mg prot) Controls with DM type 2 MDA (1.91 ± 1.72 nmol/ml) SOD (13.45 ± 2.80 U/ml) GR (13.73 ± 2.79 U/min/mg prot) CAT (0.04 ± 0.04 U/min/mg prot)		
33 CP patients 35 patients with generalized aggressive periodontitis (GAP) 30 healthy controls	MDA (HPLC) TOS TAC (SPH)	CP group MDA 0.15 μmol/L (0.1 to 0.18), TOS 6.32 μmol/L (5.51 to 7), TAC (0.53 ± 0.11 mmol/L); GAP group MDA 0.15 μmol/L (0.14 to 0.18), TOS 7.80 μmol/L (7.5 to 8.5), TAC (7.80 ± 0.08 mmol/L); Control group MDA 0.08 μmol/L (0.06 to 0.12), TOS 4.22 μmol/L (3.75 to 4.53), TAC (4.22 ± 0.08 mmol/L)	↓ TAC ↑ MDA, TOS and oxidation stress index in CP and GAP in comparison with controls	Baltacioglu et al., 2014
47 CP patients (24 smokers, 23 non-smokers)	8-OHdG, 4-HNE, GPx (ELISA)	Smokers with CP 8-OHdG (8.02 ng/ml ± 1.46), 4-HNE (144.28 pg/ml ± 59.18), GPx (36.81 U/ml ± 9.16)	↑ 8-OHdG in patients with CP (irrespective of smoking) compared to controls;	Hendek et al., 2015

(Continued)

TABLE 2 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	References
46 controls (23 smokers, 23 non-smokers)		Non-smokers with CP 8-OHdG (7.85 ng/ml ± 1.74), 4-HNE (133.09 pg/ml ± 61.92), GPx (30.59 U/ml ± 15.06) Smokers controls 8-OHdG (7.50 ng/ml ± 1.46), 4-HNE (131.40 pg/ml ± 23.03), GPx (29.22 U/ml ± 16.45) Non-smokers controls 8-OHdG (6.74 ng/ml ± 1.96), 4-HNE (130.36 pg/ml ± 21.09), GPx (24.36 U/ml ± 13.28)	↓ 8-OHdG after treatment	
30 CP (baseline, 1 month after periodontal treatment) 30 controls	8-OHdG (ELISA)	8-OHdG CP group (645.18 pg/ml ± 84.91), control group (527.23 pg/ml ± 62.19) after treatment 8-OHdG CP group (532.18 pg/ml ± 91.37)	↑ 8-OHdG in patients with CP; ↓ 8-OHdG after treatment	Arunachalam et al., 2015
23 CP (8 females, 15 males) 25 controls (15 females, 10 males); (baseline, 6 weeks after periodontal treatment)	8-OHdG (ELISA and LC-MS/MS)	N/A	↓ 8-OHdG after treatment; correlation between plaque index, gingival index, probing pocke depth, clinical attachment level, bleeding on probing	Kurgan et al., 2015
31 CP patients baseline and after periodontal treatment	TAC (SPH)	TAC before treatment (0.655 μmol/L ± 0.281) TAC after treatment (0.962 μmol/L ± 0.287)	↑ TAC after periodontal treatment	Shirzaiy et al., 2014
CP patient aggressive periodontitis patients controls (n = 20/per group) aged 25–55; both genders	NO (SPH)	CP NO (16.53 ± 1.51) Aggressive periodontitis NO (16.39 ± 2.38) Control NO (5.69 ± 0.93)	↑ NO in both periodontal groups compared to controls positive correlation of NO and periodontal parameters in both groups of patients	Sundar et al., 2013
89 generalized severe CP patients, 56 healthy controls (non-smokers); both gender	NO metabolites (SPH)	Only graphs available	↓ NO in CP compared to healthy controls	Andrukhov et al., 2013
CP patients divided into 2 groups according smoking status and healthy controls (n = 20/per group)	NO (SPH)	Non-smoking CP NO (79.52 μmol/L ± 24.88) Smoking CP NO (153.84 μmol/L ± 44.04) Controls NO (27.70 μmol/L ± 8.04)	↑ NO in both periodontal groups compared to controls; smoking significantly effects NO levels	Wadhwa et al., 2013
Patients with gingivitis periodontits and controls (n = 14/per group); age (30–50 years); both gender	Nitrite Nitrate Total NO (ELISA)	Gingivitis group nitrite (79.64 ± 4.62) Nitrate (79.36 ± 4.62) Total NO (159 ± 9.25) CP group nitrite (173.26 ± 9.26) Nitrate (172.99 ± 9.26)	↑ NO in gingivitis and periodontitis patients compared to controls	Poorsattar Bejeh-Mir et al., 2014

(Continued)

TABLE 2 | Continued

Patients	Analyzed markers and methods	Detected concentrations	Results	References
		Total NO (346.25 ± 18.52) Control group nitrite (33.19 ± 4.69) Nitrate (50.39 ± 17.79) Total NO (100.95 ± 35.60)		
Patients with gingivitis periodontits and controls (n = 30/per group)	NO (SPH)	Gingivitis group NO pretreatment (430.60 ± 67.97) NO postreatment (269.07 ± 53.08) CP group NO pretreatment (537.67 ± 80.06) NO postreatment (326.73 ± 41.03) Control group NO pretreatment (241.10 ± 83.72)	↑ NO in gingivitis and periodontitis patients compared to controls	Parwani et al., 2012
Rapidly progressive periodontitis, adult periodontitis and healthy controls (n = 25/per group)	NO_2(SPH)	Progressive periodontitis group NO_2(2.5 ± 3.27 μmol/l) Adult periodontitis group NO_2(11.1 ± 8.23 μmol/l) Control group NO_2(22.4 ± 17.04 μmol/l)	↓ NO_2 in CP patients compared to healthy controls; patiemts with rapidly progresive perriodontitis had ↓ NO_2compared to patients with adult form of periodontitis	Aurer et al., 2001

CP, chronic periodontitis; CG, chronic gingivitis; TAC, total antioxidant capacity; TAP, total antioxidant power; AOPP, advanced oxidation protein products; TBARS, thiobarbituric acid reacting substances; AGES, advanced glycation end products; FRAP, ferric reducing antioxidant power; *8-HOdG, 8-hydroxydeoxyguanosine*; *8-epi PGF2α, 8-epi prostaglandin F2α*; *GPx, glutathione peroxidase*; *GSH, reduced glutathione*; *GR, glutathione reductase*; *MDA, malodialdehyde*; *LPO, lipid peroxidation*; *TOS, total oxidant status*; *SOD, superoxid dismutase; SPH, spectrophotometry; SFL, spectrofluorometry; PCL, photochemiluminescence; ECL, enhanced chemiluminescent assay; HPLC, high performance liquid chromatography; C-ELISA, competitive ELISA; # denotes levels estimated from graph.*

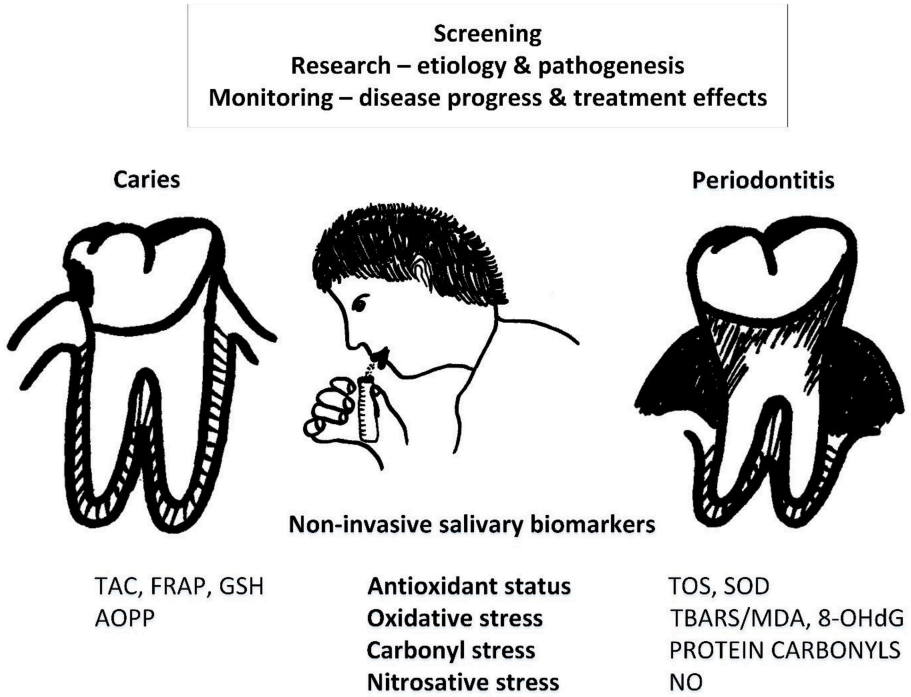


FIGURE 1 | Potential for non-invasive use of salivary biomarkers associated with caries and periodontitis. Saliva can be used for disease screening, monitoring of progress, and treatment as well as in basic research on etiology or pathogenesis. 8-OHdG, 8-hydroxyguanosine; AOPP, advanced oxidation protein products; FRAP, ferric reducing antioxidant power; NO, nitric oxide; TAC, total antioxidant capacity; TBARS/MDA, thiobarbituric acid reacting substances/malondialdehyde; TOS, total oxidant status; SOD, superoxide dismutase.

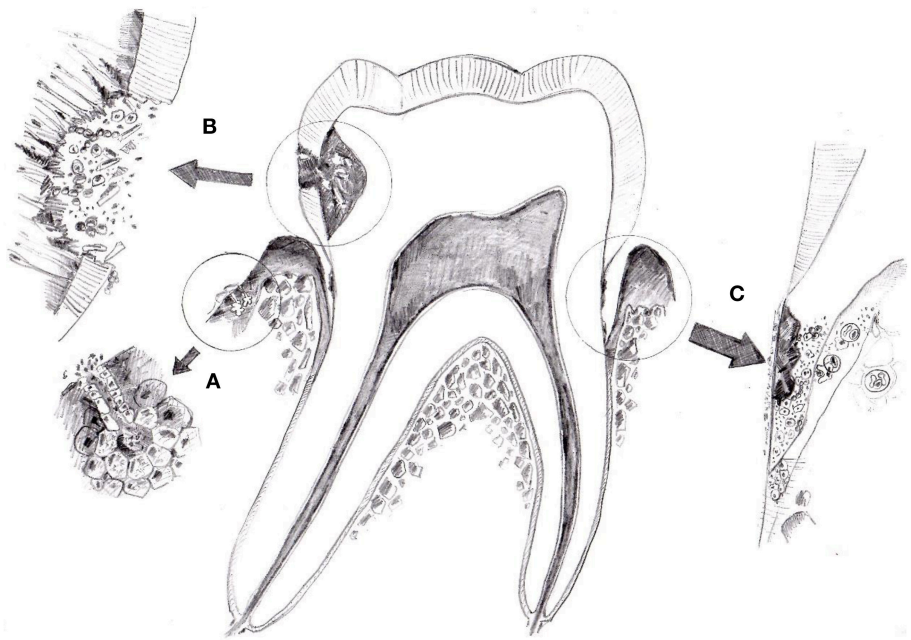


FIGURE 2 | The origin of salivary markers of oxidative stress. Three possible sources are shown—blood plasma **(A)**, oral bacteria **(B)**, and immune cells **(C)**. Although direct evidence is lacking, salivary markers of oxidative stress seem to be of local oral origin. At least in periodontitis the production of reactive oxygen species by oral bacteria or activated neutrophils seems to be of importance. In systemic diseases diffusion from plasma could be the main source of salivary markers of oxidative stress.

used for screening and monitoring of oral diseases such as periodontitis or caries as these markers seem to be mostly of local oral origin (**Figure 2**). It should not be seen as a substitute for proper clinical examination but rather an adjuvant tool for various applications such monitoring of the patients with poor adherence to dental visits. Especially, if the biochemical detection methods will be developed into simple strips that change color based on the concentration of the particular marker similarly to urine strips. Such first example already exist with a strip detecting thiol compounds known to be higher in periodontitis patients (Khocht et al., 2013). However, before that numerous mentioned obstacles have to be solved. Future studies should concentrate on identification of the sources of the observed variability, widening the palette of the available markers and experiments proving the role of oxidative stress in the pathogenesis of oral diseases. Inflammatory markers measured in saliva are very informative regarding the periodontal status of patients (Rathnayake et al., 2013; Salminen et al., 2014). Cooperative efforts focused on large multi-centric studies with sufficient power and standardized analysis are needed. Promising targeted antioxidant treatments could be a way how to approach cases of therapy-resistant periodontitis as partially shown for melatonin (Kara et al., 2013; Arabaci et al., 2015). The potential of non-invasive diagnosis and treatment of oral diseases makes salivary markers of oxidative stress very attractive for further research. And for this future a paradigm shift will be needed as the research will very likely by dominated by high-throughput approaches such as salivary metabolome analysis (Dame et al., 2015).

ACKNOWLEDGMENTS

This work was supported by grants VEGA 1/0674/12, 1/0222/14, and 1/0172/14.

REFERENCES

Ahmadi-Motamayel, F., Goodarzi, M. T., Hendi, S. S., Kasraei, S., and Moghimbeigi, A. (2013). Total antioxidant capacity of saliva and dental caries. *Med. Oral. Patol. Oral. Cir. Bucal.* 18, e553–e556. doi: 10.4317/medoral.18762

Ahmed, N., Argirov, O. K., Minhas, H. S., Cordeiro, C. A., and Thornalley, P. J. (2002). Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatization by 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate and application to Nepsilon-carboxymethyl-lysine- and Nepsilon-(1-carboxyethyl)lysine-modified albumin. *Biochem. J.* 364, 1–14. doi: 10.1042/bj3640001

Akalin, F. A., Baltacioglu, E., Alver, A., and Karabulut, E. (2007). Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. *J. Clin. Periodontol.* 34, 558–565. doi: 10.1111/j.1600-051X.2007.01091.x

Almerich-Silla, J. M., Montiel-Company, J. M., Pastor, S., Serrano, F., Puig-Silla, M., and Das, F. (2015). Oxidative stress parameters in saliva and its association with periodontal disease and types of bacteria. *Dis. Markers* 2015:653537. doi: 10.1155/2015/653537

Amerongen, A. V., and Veerman, E. C. (2002). Saliva–the defender of the oral cavity. *Oral Dis.* 8, 12–22. doi: 10.1034/j.1601-0825.2002.1o816.x

Andrukhov, O., Haririan, H., Bertl, K., Rausch, W. D., Bantleon, H. P., Moritz, A., et al. (2013). Nitric oxide production, systemic inflammation and lipid metabolism in periodontitis patients: possible gender aspect. *J. Clin. Periodontol.* 40, 916–923. doi: 10.1111/jcpe.12145

Arabaci, T., Kermen, E., Özkanlar, S., Köse, O., Kara, A., Kizildag, A., et al. (2015). Therapeutic effects of melatonin on alveolar bone resorption after experimental periodontitis in rats: a biochemical and immunohistochemical study. *J. Periodontol.* 86, 874–881. doi: 10.1902/jop.2015.140599

Armbruster, D. A. (1987). Fructosamine: structure, analysis, and clinical usefulness. *Clin. Chem.* 33, 2153–2163.

Arunachalam, R., Reshma, A. P., Rajeev, V., Kurra, S. B., Prince, M. R. J., and Syam, N. (2015). Salivary 8-hydroxydeoxyguanosine – a valuable indicator for oxidative DNA damage in periodontal disease. *Saudi J. Dent. Res.* 6, 15–20. doi: 10.1016/j.sjdr.2014.04.002

Aurer, A., Aleksic, J., Ivic-Kardum, M., Aurer, J., and Culo, F. (2001). Nitric oxide synthesis is decreased in periodontitis. *J. Clin. Periodontol.* 28, 565–568. doi: 10.1034/j.1600-051x.2001.028006565.x

Ayala, A., Muñoz, M. F., and Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxid. Med. Cell. Longev.* 2014:360438. doi: 10.1155/2014/360438

Balaji, T. M., Vasanthi, H. R., and Rao, S. R. (2015). Gingival, plasma and salivary levels of melatonin in periodontally healthy individuals and chronic periodontitis patients: a pilot study. *J. Clin. Diagn. Res.* 9, Zc23–Zc25. doi: 10.7860/JCDR/2015/11311.5652

Baltacioglu, E., Yuva, P., Aydin, G., Alver, A., Kahraman, C., Karabulut, E., et al. (2014). Lipid peroxidation levels and total oxidant/antioxidant status in serum and saliva from patients with chronic and aggressive periodontitis. Oxidative stress index: a new biomarker for periodontal disease? *J. Periodontol.* 85, 1432–1441. doi: 10.1902/jop.2014.130654

Banasová, L., Kamodyová, N., Janšóková, K., Tóthová, Ľ., Stanko, P., Turna, J., et al. (2015). Salivary DNA and markers of oxidative stress in patients with chronic periodontitis. *Clin. Oral Investig.* 19, 201–207. doi: 10.1007/s00784-014-1236-z

Barnes, V. M., Kennedy, A. D., Panagakos, F., Devizio, W., Trivedi, H. M., Jönsson, T., et al. (2014). Global metabolomic analysis of human saliva and plasma from healthy and diabetic subjects, with and without periodontal disease. *PLoS ONE* 9:e105181. doi: 10.1371/journal.pone.0105181

Battino, M., Ferreiro, M. S., Gallardo, I., Newman, H. N., and Bullon, P. (2002). The antioxidant capacity of saliva. *J. Clin. Periodontol.* 29, 189–194. doi: 10.1034/j.1600-051X.2002.290301x.x

Becerik, S., Ozçaka, O., Nalbantsoy, A., Atilla, G., Celec, P., Behuliak, M., et al. (2010). Effects of menstrual cycle on periodontal health and gingival crevicular fluid markers. *J. Periodontol.* 81, 673–681. doi: 10.1902/jop.2010.090590

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. (2004). Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.* 31, 515–521. doi: 10.1111/j.1600-051X.2004.00509.x

Buczko, P., Zalewska, A., and Szarmach, I. (2015). Saliva and oxidative stress in oral cavity and in some systemic disorders. *J. Physiol. Pharmacol.* 66, 3–9.

Cabiscol, E., Tamarit, J., and Ros, J. (2014). Protein carbonylation: proteomics, specificity and relevance to aging. *Mass Spectrom. Rev.* 33, 21–48. doi: 10.1002/mas.21375

Cadet, J., Douki, T., Gasparutto, D., and Ravanat, J. L. (2003). Oxidative damage to DNA: formation, measurement and biochemical features. *Mutat. Res.* 531, 5–23. doi: 10.1016/j.mrfmmm.2003.09.001

Canakçi, C. F., Canakçi, V., Tatar, A., Eltas, A., Sezer, U., Ciçek, Y., et al. (2009). Increased salivary level of 8-hydroxydeoxyguanosine is a marker of premature oxidative mitochondrial DNA damage in gingival tissue of patients with periodontitis. *Arch. Immunol. Ther. Exp. (Warsz).* 57, 205–211. doi: 10.1007/s00005-009-0026-9

Celec, P., Hodosy, J., Behuliak, M., Pálffy, R., Gardlík, R., Halcák, L., et al. (2012). Oxidative and carbonyl stress in patients with obstructive sleep apnea treated with continuous positive airway pressure. *Sleep Breath.* 16, 393–398. doi: 10.1007/s11325-011-0510-4

Celec, P., Hodosy, J., Celecová, V., Vodrázka, J., Cervenka, T., Halcák, L., et al. (2005). Salivary thiobarbituric acid reacting substances and malondialdehyde–their relationship to reported smoking and to parodontal

status described by the papillary bleeding index. *Dis. Markers* 21, 133–137. doi: 10.1155/2005/693437

Celecová, V., Kamodyová, N., Tóthová, Ľ., Kúdela, M., and Celec, P. (2013). Salivary markers of oxidative stress are related to age and oral health in adult non-smokers. *J. Oral Pathol. Med.* 42, 263–266. doi: 10.1111/jop.12008

Chapple, I. L. (1997). Reactive oxygen species and antioxidants in inflammatory diseases. *J. Clin. Periodontol.* 24, 287–296. doi: 10.1111/j.1600-051X.1997.tb00760.x

Chapple, I. L., Mason, G. I., Garner, I., Matthews, J. B., Thorpe, G. H., Maxwell, S. R. J., et al. (1997). Enhanced chemiluminescent assay for measuring the total antioxidant capacity of serum, saliva and crevicular fluid. *Ann. Clin. Biochem.* 34, 412–421. doi: 10.1177/000456329703400413

Chapple, I. L., and Matthews, J. B. (2007). The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol. 2000* 43, 160–232. doi: 10.1111/j.1600-0757.2006.00178.x

Chiappin, S., Antonelli, G., Gatti, R., and De Palo, E. F. (2007). Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin. Chim. Acta.* 383, 30–40. doi: 10.1016/j.cca.2007.04.011

Chicharro, J. L., Lucía, A., Pérez, M., Vaquero, A. F., and Ureña, R. (1998). Saliva composition and exercise. *Sports Med.* 26, 17–27. doi: 10.2165/00007256-199826010-00002

Clodfelter, W. H., Basu, S., Bolden, C., Dos Santos, P. C., King, S. B., and Kim-Shapiro, D. B. (2015). The relationship between plasma and salivary NOx. *Nitric Oxide* 47, 85–90. doi: 10.1016/j.niox.2015.04.003

Dalai, C., Ignat-Romanul, I., Rosca, E., Muresan, M., Micle, O., and Bodog, F. (2013). Correlation between histopathological aspects of periodontitis and biochemical changes of oxidative stress. *Rom. J. Morphol. Embryol.* 54, 817–822.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., and Milzani, A. (2006). Biomarkers of oxidative damage in human disease. *Clin. Chem.* 52, 601–623. doi: 10.1373/clinchem.2005.061408

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., and Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* 329, 23–38. doi: 10.1016/S0009-8981(03)00003-2

Dame, Z. T., Aziat, F., Mandal, R., Krishnamurthy, R., Bouatra, S., Borzouie, S., et al. (2015). The human saliva metabolome. *Metabolomics* 11, 1864–1883. doi: 10.1007/s11306-015-0840-5

Dawes, C. (1972). Circadian rhythms in human salivary flow rate and composition. *J. Physiol.* 220, 529–545. doi: 10.1113/jphysiol.1972.sp009721

de Almeida Pdel, V., Grégio, A. M., Machado, M. A., de Lima, A. A., and Azevedo, L. R. (2008). Saliva composition and functions: a comprehensive review. *J. Contemp. Dent. Pract.* 9, 72–80.

Dede, F. Ö., Ozden, F. O., and Avci, B. (2013). 8-Hydroxy-deoxyguanosine levels in gingival crevicular fluid and saliva in patients with chronic periodontitis after initial periodontal treatment. *J. Periodontol.* 84, 821–828. doi: 10.1902/jop.2012.120195

de Sousa, M. C., Vieira, R. B., Dos Santos, D. S., Carvalho, C. A., Camargo, S. E., Mancini, M. N., et al. (2015). Antioxidants and biomarkers of oxidative damage in the saliva of patients with down's syndrome. *Arch. Oral Biol.* 60, 600–605. doi: 10.1016/j.archoralbio.2014.09.013

Devasagayam, T. P., Tilak, J. C., Boloor, K. K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. D. (2004). Free radicals and antioxidants in human health: current status and future prospects. *J. Assoc. Phys. India* 52, 794–804.

Diab-Ladki, R., Pellat, B., and Chahine, R. (2003). Decrease in the total antioxidant activity of saliva in patients with periodontal diseases. *Clin. Oral Investig.* 7, 103–107. doi: 10.1007/s00784-003-0208-5

Dodwad, R., Betigeri, A. V., and Preeti, B. P. (2011). Estimation of total antioxidant capacity levels in saliva of caries-free and caries-active children. *Contemp. Clin. Dent.* 2, 17–20. doi: 10.4103/0976-237X.79296

Dotan, Y., Lichtenberg, D., and Pinchuk, I. (2004). Lipid peroxidation cannot be used as a universal criterion of oxidative stress. *Prog. Lipid Res.* 43, 200–227. doi: 10.1016/j.plipres.2003.10.001

Eiserich, J. P., Patel, R. P., and O'Donnell, V. B. (1998). Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. *Mol. Aspects Med.* 19, 221–357. doi: 10.1016/S0098-2997(99)00002-3

Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* 37, 277–285. doi: 10.1016/j.clinbiochem.2003.11.015

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* 38, 1103–1111. doi: 10.1016/j.clinbiochem.2005.08.008

Farnaud, S. J., Kosti, O., Getting, S. J., and Renshaw, D. (2010). Saliva: physiology and diagnostic potential in health and disease. *ScientificWorldJournal* 10, 434–456. doi: 10.1100/tsw.2010.38

Greabu, M., Battino, M., Mohora, M., Totan, A., Didilescu, A., Spinu, T., et al. (2009). Saliva–a diagnostic window to the body, both in health and in disease. *J. Med. Life* 2, 124–132.

Guentsch, A., Preshaw, P. M., Bremer-Streck, S., Klinger, G., Glockmann, E., and Sigusch, B. W. (2008). Lipid peroxidation and antioxidant activity in saliva of periodontitis patients: effect of smoking and periodontal treatment. *Clin. Oral Investig.* 12, 345–352. doi: 10.1007/s00784-008-0202-z

Gümüs, P., Emingil, G., Öztürk, V. Ö., Belibasakis, G. N., and Bostanci, N. (2015). Oxidative stress markers in saliva and periodontal disease status: modulation during pregnancy and postpartum. *BMC Infect. Dis.* 15, 261. doi: 10.1186/s12879-015-1003-z

Gürsoy, M., Zeidán-Chuliá, F., Könönen, E., Moreira, J. C., Liukkonen, J., Sorsa, T., et al. (2014). Pregnancy-induced gingivitis and OMICS in dentistry: *in silico* modeling and *in vivo* prospective validation of estradiol-modulated inflammatory biomarkers. *OMICS* 18, 582–590. doi: 10.1089/omi.2014.0020

Halliwell, B. (2000). Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come? *Am. J. Clin. Nutr.* 72, 1082–1087.

Hegde, A. M., Rai, K., and Padmanabhan, V. (2009). Total antioxidant capacity of saliva and its relation with early childhood caries and rampant caries. *J. Clin. Pediatr. Dent.* 33, 231–234. doi: 10.17796/jcpd.33.3.c730518021m56077

Hegde, M. N., Hegde, N. D., Ashok, A., and Shetty, S. (2013). Evaluation of total antioxidant capacity of saliva and serum in caries-free and caries-active adults: an *in-vivo* study. *Indian J. Dent. Res.* 24, 164–167. doi: 10.4103/0970-9290.116670

Hendek, M. K., Erdemir, E. O., Kisa, U., and Ozcan, G. (2015). Effect of initial periodontal therapy on oxidative stress markers in gingival crevicular fluid, saliva, and serum in smokers and non-smokers with chronic periodontitis. *J. Periodontol.* 86, 273–282. doi: 10.1902/jop.2014.140338

Henderson, P. T., Evans, M. D., and Cooke, M. S. (2010). Salvage of oxidized guanine derivatives in the (2'-deoxy)ribonucleotide pool as source of mutations in DNA. *Mutat. Res.* 703, 11–17. doi: 10.1016/j.mrgentox.2010.08.021

Huang, Y., Zhu, M., Li, Z., Sa, R., Chu, Q., Zhang, Q., et al. (2014). Mass spectrometry-based metabolomic profiling identifies alterations in salivary redox status and fatty acid metabolism in response to inflammation and oxidative stress in periodontal disease. *Free Radic. Biol. Med.* 70, 223–232. doi: 10.1016/j.freeradbiomed.2014.02.024

Humphrey, S. P., and Williamson, R. T. (2001). A review of saliva: normal composition, flow, and function. *J. Prosthet. Dent.* 85, 162–169. doi: 10.1067/mpr.2001.113778

Iannitti, T., Rottigni, V., and Palmieri, B. (2012). Role of free radicals and antioxidant defences in oral cavity-related pathologies. *J. Oral Pathol. Med.* 41, 649–661. doi: 10.1111/j.1600-0714.2012.01143.x

Kamodyová, N., Banasová, L., Janšáková, K., Koborová, I., Tóthová, Ľ., Stanko, P., et al. (2015). Blood contamination in saliva: impact on the measurement of salivary oxidative stress markers. *Dis. Markers* 2015:479251. doi: 10.1155/2015/479251

Kamodyová, N., and Celec, P. (2011). Salivary markers of oxidative stress and salivette collection systems. *Clin. Chem. Lab. Med.* 49, 1887–1890. doi: 10.1515/cclm.2011.677

Kamodyová, N., Táthová, L., and Celec, P. (2013). Salivary markers of oxidative stress and antioxidant status: influence of external factors. *Dis. Markers* 34, 313–321. doi: 10.1155/2013/341302

Kara, A., Akman, S., Ozkanlar, S., Tozoglu, U., Kalkan, Y., Canakci, C. F., et al. (2013). Immune modulatory and antioxidant effects of melatonin in experimental periodontitis in rats. *Free Radic. Biol. Med.* 55, 21–26. doi: 10.1016/j.freeradbiomed.2012.11.002

Karim, S., Pratibha, P. K., Kamath, S., Bhat, G. S., Kamath, U., Dutta, B., et al. (2012). Superoxide dismutase enzyme and thiol antioxidants in gingival crevicular fluid and saliva. *Dent. Res. J. (Isfahan)* 9, 266–272.

Kaufman, E., and Lamster, I. B. (2002). The diagnostic applications of saliva–a review. *Crit. Rev. Oral Biol. Med.* 13, 197–212. doi: 10.1177/154411130201300209

Kawamoto, A., Sugano, N., Motohashi, M., Matsumoto, S., and Ito, K. (2012). Relationship between salivary antioxidant capacity and phases of the menstrual cycle. *J. Periodont. Res.* 47, 593–598. doi: 10.1111/j.1600-0765.2012.01471.x

Khalili, J., and Biloklytska, H. F. (2008). Salivary malondialdehyde levels in clinically healthy and periodontal diseased individuals. *Oral Dis.* 14, 754–760. doi: 10.1111/j.1601-0825.2008.01464.x

Khocht, A., Seyedain, M., Hardan, S., Gaughan, J., and Suzuki, J. B. (2013). Salivary thiol levels and periodontal parameters assessed with a chromogenic strip. *Gen. Dent.* 61, 50–54.

Kumar, D., Pandey, R. K., and Agrawal, D. (2011). An estimation and evaluation of total antioxidant capacity of saliva in children with severe early childhood caries. *Int. J. Paediatr. Dent.* 21, 459–464. doi: 10.1111/j.1365-263X.2011.01154.x

Kurgan, S., Önder, C., Altingöz, S. M., Bagis, N., Uyanik, M., Serdar, M. A., et al. (2015). High sensitivity detection of salivary 8-hydroxy deoxyguanosine levels in patients with chronic periodontitis. *J. Periodont. Res.* doi: 10.1111/jre.12263

Laine, M. L., Crielaard, W., and Loos, B. G. (2012). Genetic susceptibility to periodontitis. *Periodontology 2000* 58, 37–68. doi: 10.1111/j.1600-0757.2011.00415.x

Lapolla, A., Traldi, P., and Fedele, D. (2005). Importance of measuring products of non-enzymatic glycation of proteins. *Clin. Biochem.* 38, 103–115. doi: 10.1016/j.clinbiochem.2004.09.007

Lee, Y. H., and Wong, D. T. (2009). Saliva: an emerging biofluid for early detection of diseases. *Am. J. Dent.* 22, 241–248.

Lenander-Lumikari, M., and Loimaranta, V. (2000). Saliva and dental caries. *Adv. Dent. Res.* 14, 40–47. doi: 10.1177/08959374000140010601

Leonora, J., Tieche, J. M., and Steinman, R. R. (1993). Further evidence for a hypothalamus-parotid gland endocrine axis in the rat. *Arch. Oral Biol.* 38, 911–916. doi: 10.1016/0003-9969(93)90101-Q

Lima, D. P., Diniz, D. G., Moimaz, S. A., Sumida, D. H., and Okamoto, A. C. (2010). Saliva: reflection of the body. *Int. J. Infect. Dis.* 14, e184–e188. doi: 10.1016/j.ijid.2009.04.022

Lopez-Jornet, P., Martinez-Canovas, A., and Pons-Fuster, A. (2014). Salivary biomarkers of oxidative stress and quality of life in patients with oral lichen planus. *Geriatr. Gerontol. Int.* 14, 654–659. doi: 10.1111/ggi.12153

Mahjoub, S., Ghasempour, M., Gharage, A., Bijani, A., and Masrourroudsari, J. (2014). Comparison of total antioxidant capacity in saliva of children with severe early childhood caries and caries-free children. *Caries Res.* 48, 271–275. doi: 10.1159/000355581

Maiese, K., Chong, Z. Z., Hou, J., and Shang, Y. C. (2010). Oxidative stress: biomarkers and novel therapeutic pathways. *Exp. Gerontol.* 45, 217–234. doi: 10.1016/j.exger.2010.01.004

Marcenes, W., Kassebaum, N. J., Bernabé, E., Flaxman, A., Naghavi, M., Lopez, A., et al. (2013). Global burden of oral conditions in 1990-2010: a systematic analysis. *J. Dent. Res.* 92, 592–597. doi: 10.1177/0022034513490168

Mashayekhi, F., Aghahoseini, F., Rezaie, A., Zamani, M. J., Khorasani, R., and Abdollahi, M. (2005). Alteration of cyclic nucleotides levels and oxidative stress in saliva of human subjects with periodontitis. *J. Contemp. Dent. Pract.* 6, 46–53.

Meschiari, C. A., Marcaccini, A. M., Santos Moura, B. C., Zuardi, L. R., Tanus-Santos, J. E., and Gerlach, R. F. (2013). Salivary MMPs, TIMPs, and MPO levels in periodontal disease patients and controls. *Clin. Chim. Acta* 421, 140–146. doi: 10.1016/j.cca.2013.03.008

Meschiari, C. A., Zuardi, L. R., Gomes, V. A., Costa de Almeida, G. R., Novaes, A. B. Jr., Gerlach, R. F., et al. (2015). Salivary, blood and plasma nitrite concentrations in periodontal patients and healthy individuals before and after periodontal treatment. *Clin. Chim. Acta* 444, 293–296. doi: 10.1016/j.cca.2015.02.045

Metgud, R., and Bajaj, S. (2014). Evaluation of salivary and serum lipid peroxidation, and glutathione in oral leukoplakia and oral squamous cell carcinoma. *J. Oral Sci.* 56, 135–142. doi: 10.2334/josnusd.56.135

Miricescu, D., Totan, A., Calenic, B., Mocanu, B., Didilescu, A., Mohora, M., et al. (2014). Salivary biomarkers: relationship between oxidative stress and alveolar bone loss in chronic periodontitis. *Acta Odontol. Scand.* 72, 42–47. doi: 10.3109/00016357.2013.795659

Miyata, T., Kurokawa, K., and Van Ypersele De Strihou, C. (2000). Advanced glycation and lipoxidation end products: role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. *J. Am. Soc. Nephrol.* 11, 1744–1752.

Moore, S., Calder, K. A., Miller, N. J., and Rice-Evans, C. A. (1994). Antioxidant activity of saliva and periodontal disease. *Free Radic. Res.* 21, 417–425. doi: 10.3109/10715769409056594

Muchandi, S., Walimbe, H., Bijle, M. N., Nankar, M., Chaturvedi, S., and Karekar, P. (2015). Comparative evaluation and correlation of salivary total antioxidant capacity and salivary ph in caries-free and severe early childhood caries children. *J. Contemp. Dent. Pract.* 16, 234–237. doi: 10.5005/jp-journals-10024-1667

Münch, G., Keis, R., Wessels, A., Riederer, P., Bahner, U., Heidland, A., et al. (1997). Determination of advanced glycation end products in serum by fluorescence spectroscopy and competitive ELISA. *Eur. J. Clin. Chem. Clin. Biochem.* 35, 669–677. doi: 10.1515/cclm.1997.35.9.669

Nass, N., Bartling, B., Navarrete Santos, A., Scheubel, R. J., Börgermann, J., Silber, R. E., et al. (2007). Advanced glycation end products, diabetes and ageing. *Z. Gerontol. Geriatr.* 40, 349–356. doi: 10.1007/s00391-007-0484-9

Nizam, N., Gumus, P., Pitkanen, J., Tervahartiala, T., Sorsa, T., and Buduneli, N. (2014). Serum and salivary matrix metalloproteinases, neutrophil elastase, myeloperoxidase in patients with chronic or aggressive periodontitis. *Inflammation* 37, 1771–1778. doi: 10.1007/s10753-014-9907-0

Novakovic, N., Cakic, S., Todorovic, T., Raicevic, B. A., Dozic, I., Petrovic, V., et al. (2013). Antioxidative status of saliva before and after non-surgical periodontal treatment. *Srp. Arh. Celok. Lek.* 141, 163–168. doi: 10.2298/SARH1304163N

Novakovic, N., Todorovic, T., Rakic, M., Milinkovic, I., Dozic, I., Jankovic, S., et al. (2014). Salivary antioxidants as periodontal biomarkers in evaluation of tissue status and treatment outcome. *J. Periodont. Res.* 49, 129–136. doi: 10.1111/jre.12088

Ott, C., Jacobs, K., Haucke, E., Navarrete Santos, A., Grune, T., and Simm, A. (2014). Role of advanced glycation end products in cellular signaling. *Redox Biol.* 2, 411–429. doi: 10.1016/j.redox.2013.12.016

Oztürk, L. K., Furuncuoglu, H., Atala, M. H., Uluköylü, O., Akyüz, S., and Yarat, A. (2008). Association between dental-oral health in young adults and salivary glutathione, lipid peroxidation and sialic acid levels and carbonic anhydrase activity. *Braz. J. Med. Biol. Res.* 41, 956–959.

Palmieri, B., and Sblendorio, V. (2007a). Oxidative stress tests: overview on reliability and use. Part I. *Eur. Rev. Med. Pharmacol. Sci.* 11, 309–342.

Palmieri, B., and Sblendorio, V. (2007b). Oxidative stress tests: overview on reliability and use. Part II. *Eur. Rev. Med. Pharmacol. Sci.* 11, 383–399.

Parwani, S. R., Chitnis, P. J., and Parwani, R. N. (2012). Salivary nitric oxide levels in inflammatory periodontal disease - a case-control and interventional study. *Int. J. Dent. Hyg.* 10, 67–73. doi: 10.1111/j.1601-5037.2011.00508.x

Poorsattar Bejeh-Mir, A., Parsian, H., Akbari Khoram, M., Ghasemi, N., Bijani, A., and Khosravi-Samani, M. (2014). Diagnostic role of salivary and gcf nitrite, nitrate and nitric oxide to distinguish healthy periodontium from gingivitis and periodontitis. *Int. J. Mol. Cell. Med.* 3, 138–145.

Preethi, B. P., Reshma, D., and Anand, P. (2010). evaluation of flow rate, ph, buffering capacity, calcium, total proteins and total antioxidant capacity levels of saliva in caries free and caries active children: an *in vivo* study. *Indian J. Clin. Biochem.* 25, 425–428. doi: 10.1007/s12291-010-0062-6

Prior, R. L., and Cao, G. (1999). *In vivo* total antioxidant capacity: comparison of different analytical methods1. *Free Radic. Biol. Med.* 27, 1173–1181. doi: 10.1016/S0891-5849(99)00203-8

Proctor, G. B., and Carpenter, G. H. (2007). Regulation of salivary gland function by autonomic nerves. *Auton. Neurosci.* 133, 3–18. doi: 10.1016/j.autneu.2006.10.006

Rai, B. K. S., Jain, R., and Anand, S. C. (2006). Salivary LPO product malonaldehyde in various dental diseases. *World J. Med. Sci.* 1, 100–101.

Rai, K., Hegde, A. M., and Jose, N. (2012). Salivary antioxidants and oral health in children with autism. *Arch. Oral Biol.* 57, 1116–1120. doi: 10.1016/j.archoralbio.2012.03.006

Rathnayake, N., Akerman, S., Klinge, B., Lundegren, N., Jansson, H., Tryselius, Y., et al. (2013). Salivary biomarkers of oral health: a cross-sectional study. *J. Clin. Periodontol.* 40, 140–147. doi: 10.1111/jcpe.12038

Reher, V. G., Zenábio, E. G., Costa, F. O., Reher, P., and Soares, R. V. (2007). Nitric oxide levels in saliva increase with severity of chronic periodontitis. *J. Oral Sci.* 49, 271–276. doi: 10.2334/josnusd.49.271

Renz, A., Ide, M., Newton, T., Robinson, P. G., and Smith, D. (2007). Psychological interventions to improve adherence to oral hygiene instructions in adults with periodontal diseases. *Cochrane Database Syst. Rev.* CD005097. doi: 10.1002/14651858.CD005097.pub2

Rylev, M., and Kilian, M. (2008). Prevalence and distribution of principal periodontal pathogens worldwide. *J. Clin. Periodontol.* 35, 346–361. doi: 10.1111/j.1600-051X.2008.01280.x

Salminen, A., Gursoy, U. K., Paju, S., Hyvärinen, K., Mäntylä, P., Buhlin, K., et al. (2014). Salivary biomarkers of bacterial burden, inflammatory response, and tissue destruction in periodontitis. *J. Clin. Periodontol.* 41, 442–450. doi: 10.1111/jcpe.12234

San-Gil, F., Schier, G. M., Moses, R. G., and Gan, I. E. (1985). Improved estimation of fructosamine, as a measure of glycated serum protein, with the Technicon RA-1000 analyzer. *Clin. Chem.* 31, 2005–2006.

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Sayre, L. M., Lin, D., Yuan, Q., Zhu, X., and Tang, X. (2006). Protein adducts generated from products of lipid oxidation: focus on HNE and one. *Drug Metab. Rev.* 38, 651–675. doi: 10.1080/03602530600959508

Schipper, R. G., Silletti, E., and Vinyerhoeds, M. H. (2007). Saliva as research material: biochemical, physicochemlical and practical aspects. *Arch. Oral Biol.* 52, 1114–1135. doi: 10.1016/j.archoralbio.2007.06.009

Schmitt, A., Schmitt, J., Münch, G., and Gasic-Milencovic, J. (2005). Characterization of advanced glycation end products for biochemical studies: side chain modifications and fluorescence characteristics. *Anal. Biochem.* 338, 201–215. doi: 10.1016/j.ab.2004.12.003

Schwartz, E. B., and Granger, D. A. (2004). Transferrin enzyme immunoassay for quantitative monitoring of blood contamination in saliva. *Clin. Chem.* 50, 654–656. doi: 10.1373/clinchem.2003.028266

Sculley, D. V., and Langley-Evans, S. C. (2003). Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. *Clin. Sci.* 105, 167–172. doi: 10.1042/CS20030031

Selmeci, L. (2011). Advanced oxidation protein products (AOPP): novel uremic toxins, or components of the non-enzymatic antioxidant system of the plasma proteome? *Free Radic. Res.* 45, 1115–1123. doi: 10.3109/10715762.2011.602074

Selwitz, R. H., Ismail, A. I., and Pitts, N. B. (2007). Dental caries. *Lancet* 369, 51–59. doi: 10.1016/S0140-6736(07)60031-2

Sezer, U., Ciçek, Y., and Canakçi, C. F. (2012). Increased salivary levels of 8-hydroxydeoxyguanosine may be a marker for disease activity for periodontitis. *Dis. Markers* 32, 165–172. doi: 10.1155/2012/215430

Shacter, E. (2000). Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* 32, 307–326. doi: 10.1081/DMR-100102336

Shirzaiy, M., Ansari, S. M., Dehghan, J. H., and Ghaeni, S. H. (2014). Total antioxidant capacity of saliva in chronic periodontitis patients before and after periodontal treatment. *J. Nepal Health Res. Counc.* 12, 172–176.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* 82, 291–295. doi: 10.1113/expphysiol.1997.sp004024

Singh, R., Barden, A., Mori, T., and Beilin, L. (2001). Advanced glycation end-products: a review. *Diabetologia* 44, 129–146. doi: 10.1007/s001250051591

Southward, K. (2011). The systemic theory of dental caries. *Gen. Dent. 59,* 367–373; quiz 374–375.

Spickett, C. M. (2013). The lipid peroxidation product 4-hydroxy-2-nonenal: advances in chemistry and analysis. *Redox Biol.* 1, 145–152. doi: 10.1016/j.redox.2013.01.007

Stadtman, E. R., and Berlett, B. S. (1998). Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab. Rev.* 30, 225–243. doi: 10.3109/03602539808996310

Stookey, G. K. (2008). The effect of saliva on dental caries. *J. Am. Dent. Assoc.* 139(Suppl.), 11S–17S. doi: 10.14219/jada.archive.2008.0347

Su, H., Gornitsky, M., Velly, A. M., Yu, H., Benarroch, M., and Schipper, H. M. (2009). Salivary DNA, lipid, and protein oxidation in nonsmokers with periodontal disease. *Free Radic. Biol. Med.* 46, 914–921. doi: 10.1016/j.freeradbiomed.2009.01.008

Sundar, N. M., Krishnan, V., Krishnaraj, S., Hemalatha, V. T., and Alam, M. N. (2013). Comparison of the salivary and the serum nitric oxide levels in chronic and aggressive periodontitis: a biochemical study. *J. Clin. Diagn. Res.* 7, 1223–1227. doi: 10.7860/jcdr/2013/5386.3068

Syndergaard, B., Al-Sabbagh, M., Kryscio, R. J., Xi, J., Ding, X., Ebersole, J. L., et al. (2014). Salivary biomarkers associated with gingivitis and response to therapy. *J. Periodontol.* 85, e295–e303. doi: 10.1902/jop.2014.130696

Takane, M., Sugano, N., Ezawa, T., Uchiyama, T., and Ito, K. (2005). A marker of oxidative stress in saliva: association with periodontally-involved teeth of a hopeless prognosis. *J. Oral Sci.* 47, 53–57. doi: 10.2334/josnusd.47.53

Takane, M., Sugano, N., Iwasaki, H., Iwano, Y., Shimizu, N., and Ito, K. (2002). New biomarker evidence of oxidative DNA damage in whole saliva from clinically healthy and periodontally diseased individuals. *J. Periodontol.* 73, 551–554. doi: 10.1902/jop.2002.73.5.551

Tóthová, Ľ., Celecová, V., and Celec, P. (2013a). Salivary markers of oxidative stress and their relation to periodontal and dental status in children. *Dis. Markers* 34, 9–15. doi: 10.1155/2013/591765

Tóthová, Ľ., Hodosy, J., Mettenburg, K., Fábryová, H., Wagneroví, A., Bábícková, J., et al. (2013b). No harmful effect of different coca-cola beverages after 6 months of intake on rat testes. *Food Chem. Toxicol.* 62, 343–348. doi: 10.1016/j.fct.2013.08.073

Trivedi, S., Lal, N., Mahdi, A. A., Mittal, M., Singh, B., and Pandey, S. (2014). Evaluation of antioxidant enzymes activity and malondialdehyde levels in patients with chronic periodontitis and diabetes mellitus. *J. Periodontol.* 85, 713–720. doi: 10.1902/jop.2013.130066

Trivedi, S., Lal, N., Mahdi, A. A., Singh, B., and Pandey, S. (2015). Association of salivary lipid peroxidation levels, antioxidant enzymes, and chronic periodontitis. *Int. J. Periodontics Restorative Dent.* 35, e14–e19. doi: 10.11607/prd.2079

Tsai, C. C., Chen, H. S., Chen, S. L., Ho, Y. P., Ho, K. Y., Wu, Y. M., et al. (2005). Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. *J. Periodont. Res.* 40, 378–384. doi: 10.1111/j.1600-0765.2005.00818.x

Tulunoglu, O., Demirtas, S., and Tulunoglu, I. (2006). Total antioxidant levels of saliva in children related to caries, age, and gender. *Int. J. Paediatr. Dent.* 16, 186–191. doi: 10.1111/j.1365-263X.2006.00733.x

Uberos, J., Alarcon, J. A., Penalver, M. A., Molina-Carballo, A., Ruiz, M., Gonzalez, E., et al. (2008). Influence of the antioxidant content of saliva on dental caries in an at-risk community. *Br. Dent. J.* 205, E5. doi: 10.1038/sj.bdj.2008.520

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39, 44–84. doi: 10.1016/j.biocel.2006.07.001

Villa-Correa, Y. A., Isaza-Guzmán, D. M., and Tobón-Arroyave, S. I. (2015). Prognostic value of 8-hydroxy-2′-deoxyguanosine and human neutrophil elastase/α1-proteinase inhibitor complex as salivary biomarkers of oxidative stress in chronic periodontitis. *J. Periodontol.* 1–11. doi: 10.1902/jop.2015.150293. [Epub ahead of print].

Vlková, B., and Celec, P. (2009). Does Enterococcus faecalis contribute to salivary thiobarbituric acid-reacting substances? *In Vivo* 23, 343–345.

Wadhwa, D., Bey, A., Hasija, M., Moin, S., Kumar, A., Aman, S., et al. (2013). Determination of levels of nitric oxide in smoker and nonsmoker patients with chronic periodontitis. *J. Periodontal. Implant Sci.* 43, 215–220. doi: 10.5051/jpis.2013.43.5.215

Wang, J., Schipper, H. M., Velly, A. M., Mohit, S., and Gornitsky, M. (2015a). Salivary biomarkers of oxidative stress: a critical review. *Free Radic. Biol. Med.* 85, 95–104. doi: 10.1016/j.freeradbiomed.2015.04.005

Wang, Z., Wang, Y., Liu, H., Che, Y., Xu, Y., and E, L. (2015b). Age-related variations of protein carbonyls in human saliva and plasma: is saliva protein carbonyls an alternative biomarker of aging? *Age (Dordr).* 37:9781. doi: 10.1007/s11357-015-9781-1

Wei, D., Zhang, X. L., Wang, Y. Z., Yang, C. X., and Chen, G. (2010). Lipid peroxidation levels, total oxidant status and superoxide dismutase in serum, saliva and gingival crevicular fluid in chronic periodontitis patients before and after periodontal therapy. *Aust. Dent. J.* 55, 70–78. doi: 10.1111/j.1834-7819.2009.01123.x

Witko-Sarsat, V., Friedlander, M., Capeillère-Blandin, C., Nguyen-Khoa, T., Nguyen, A. T., Zingraff, J., et al. (1996). Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int.* 49, 1304–1313. doi: 10.1038/ki.1996.186

Yagi, K. (1976). A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* 15, 212–216. doi: 10.1016/0006-2944(76)90049-1

Yoshizawa, J. M., Schafer, C. A., Schafer, J. J., Farrell, J. J., Paster, B. J., and Wong, D. T. (2013). Salivary biomarkers: toward future clinical and diagnostic utilities. *Clin. Microbiol. Rev.* 26, 781–791. doi: 10.1128/CMR.00021-13

Zeidan-Chulia, F., Neves de Oliveira, B. H., Gursoy, M., Kononen, E., Fonseca Moreira, J. C., Gursoy, U. K., et al. (2013). MMPREDOX/NO interplay in periodontitis and its inhibition with Satureja hortensis L. *Chem. Biodivers.* 10, 507–523. doi: 10.1002/cbdv.201200375

Zoccali, C., Mallamaci, F., and Tripepi, G. (2000). AGEs and carbonyl stress: potential pathogenetic factors of long-term uraemic complications. *Nephrol. Dial. Transplant*. 15(Suppl. 2), 7–11. doi: 10.1093/ndt/15.suppl_1.7

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 06 January 2016
doi: 10.3389/fcimb.2015.00097

Total Antioxidant Capacity and Total Oxidant Status in Saliva of Periodontitis Patients in Relation to Bacterial Load

Taowen Zhang[1,2†], Oleh Andrukhov[2†], Hady Haririan[2], Michael Müller-Kern[2], Shutai Liu[1], Zhonghao Liu[1] and Xiaohui Rausch-Fan[2]*

[1] Department of Science and Education, Yantai Stomatological Hospital, Binzhou Medical University, Yantai, China, [2] Division of Conservative Dentistry and Periodontology, University Clinic of Dentistry, Medical University of Vienna, Vienna, Austria

The detection of salivary biomarkers has a potential application in early diagnosis and monitoring of periodontal inflammation. However, searching sensitive salivary biomarkers for periodontitis is still ongoing. Oxidative stress is supposed to play an important role in periodontitis progression and tissue destruction. In this cross-sectional study, we investigated total antioxidant capacity (TAC) and total oxidant status (TOS) in saliva of periodontitis patients compared to healthy controls and their relationship with periodontopathic bacteria and periodontal disease severity. Unstimulated saliva was collected from 45 patients with generalized severe periodontitis and 37 healthy individuals and the TAC/TOS were measured. In addition, salivary levels of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Fusobacterium nucleatum* in saliva were measured. Salivary TAC was lower in periodontitis patients compared to healthy controls. Moreover, a significant negative correlation of salivary TAC with clinical attachment loss was observed in periodontitis patients. No significant difference in the salivary TOS was observed between periodontitis patients and healthy controls. Bacterial load was enhanced in periodontitis patients and exhibited correlation with periodontal disease severity but not with salivary TAC/TOS. Our data suggest that changes in antioxidant capacity in periodontitis patients are not associated with increased bacterial load and are probably due to a dysregulated immune response.

Keywords: oxidative stress, periodontopathic bacteria, saliva, total oxidant status, total antioxidant capacity

OPEN ACCESS

Edited by:
Ulvi Kahraman Gürsoy, University of Turku, Finland

Reviewed by:
Peter Celec, Comenius University, Slovakia
Sivge Kurgan, Ankara University, Turkey

***Correspondence:**
Xiaohui Rausch-Fan
xiaohui.rausch-fan@meduniwien.ac.at

† These authors have contributed equally to this work.

Received: *25 September 2015*
Accepted: *09 December 2015*
Published: *06 January 2016*

Citation:
Zhang T, Andrukhov O, Haririan H, Müller-Kern M, Liu S, Liu Z and Rausch-Fan X (2016) Total Antioxidant Capacity and Total Oxidant Status in Saliva of Periodontitis Patients in Relation to Bacterial Load. Front. Cell. Infect. Microbiol. 5:97. doi: 10.3389/fcimb.2015.00097

INTRODUCTION

Periodontitis is an inflammatory disease caused by specific bacteria and is characterized by gingival bleeding, periodontal pocket formation, destruction of connective tissue attachment, and alveolar bone resorption (Armitage, 1995). Periodontitis is initiated by the sub-gingival biofilm, but the progression of this destructive disease appears to depend upon an abnormal host response to those organisms (Cekici et al., 2014; Hasturk and Kantarci, 2015; Meyle and Chapple, 2015). Some specific bacteria such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Fusobacterium nucleatum* are strongly related to periodontitis (Socransky et al., 1998; Signat et al., 2011).

The classical parameters for diagnosis of periodontitis are clinical parameters such as probing depths of the gingival crevice, bleeding on probing, clinical attachment levels, and radiographic analysis (Goodson, 1986). These parameters are reliable to assess the severity of periodontitis under the condition of significant tissue destruction. However, measuring these parameters has poor prognostic impact (Lindhe et al., 1983) and therefore oral fluids have been investigated as an alternative diagnostic and prognostic approach (Kinney et al., 2011). In particular, salivary analysis has the potential to reflect current disease activity and severity, which can be advantageous for providing information used in risk assessment and monitoring of disease progress (Miller et al., 2010; Kinney et al., 2011).

Recently the advanced researches in molecular mechanisms in the pathogenesis of periodontitis have provided information about the specific biological pathways and biomolecules that could be used as biomarkers for risk assessment, diagnosis, and prognosis (Kinney et al., 2011). Obviously, most studies focused on markers that hold potential diagnostic significance relevant to three important biological phases of periodontal disease: inflammatory phase, connective-tissue degradation phase, and bone-turnover phase (Miller et al., 2010). Moreover, many factors associated with host-immune reaction to periodontal pathogens have been detected in saliva of periodontitis patients, for instance cytokines, chemokines, enzymes and immunoglobulins (Lamster and Grbic, 1995; Kaufman and Lamster, 2000; Seymour and Gemmell, 2001; Lamster et al., 2003). Nevertheless, studies about salivary biomarkers for the assessment of periodontitis are still ongoing (Giannobile et al., 2009).

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of organism. As most inflammatory diseases, periodontitis is characterized by oxidative stress, which might contribute to the host tissue destruction (for review, see Chapple and Matthews, 2007). Salivary markers of oxidative stress are extensively discussed as a possible tool for periodontal diagnostic (Tóthová et al., 2015). Since most oxidants have a very short half-life time, the measuring of oxidation products is widely used as an indicator of oxidative stress (Palmieri and Sblendorio, 2007). Previous studies show that periodontitis patients have the increased salivary levels of lipid peroxidation products, protein oxidation markers, and DNA damage marker (reviewed in Tóthová et al., 2015). Alternatively to measurements of oxidation products, a test measuring total oxidative status (TOS) was recently developed (Erel, 2005). Previous studies show that salivary TOS is increased in periodontitis patients and can be restored by periodontal therapy (Akalin et al., 2007; Wei et al., 2010). Oxidants produced during inflammatory response either react with target proteins or are neutralized by different antioxidants system. Therefore, measuring salivary total antioxidant capacity (TAC) can be also considered as an important tool for periodontal diagnostic. Some previous studies show that salivary TAC is decreased in periodontitis patients (Diab-Ladki et al., 2003; Mashayekhi et al., 2005; Guentsch et al., 2008), some studies report no significant difference in the salivary TAC between periodontitis patients and healthy controls (Brock et al., 2004; Tóthová et al., 2013), and one recent study shows an increase in salivary TAC in periodontitis patients (Almerich-Silla et al., 2015).

Oxidative stress in periodontitis arises from the immune response of the host to periodontal bacteria. Therefore, it would be interesting to investigate if there is some connection between salivary oxidative stress markers and the bacterial load. However, to date this relationship has not been investigated extensively (Almerich-Silla et al., 2015). Therefore, in the present study we investigated salivary TAC and TOS in periodontitis patients in relation to the levels of periodontal pathogens as well as in relation to clinical parameters of periodontitis.

MATERIALS AND METHODS

Study Population and Clinical Periodontal Examination

This cross-sectional study included 45 periodontitis patients and 37 periodontally healthy volunteers recruited from February 2010 until July 2012. The group of periodontitis patients included 15 patients with aggressive periodontitis and 30 patients with chronic periodontitis. All study participants gave written consent for participation in the study. The study protocol was approved by the ethics committee of the Medical University of Vienna (EK 623/2007).

Periodontal disease was diagnosed by experienced periodontologists with a postgraduate specialization in periodontology with clinical assessments using a periodontal probe and radiographs. Probing pocket depth (PD), clinical attachment loss (CAL), and bleeding on probing (BOP) were recorded at six sites per tooth using a periodontal probe (Hu-Friedy, Chicago, US). Bone loss was evaluated with intra-oral and panoramic radiographs. Periodontitis was classified according to the classification of the World Workshop 1999 (Armitage, 1999). Inclusion criteria for periodontitis patients were: $\geq$20 teeth, generalized aggressive periodontitis with a clinical attachment-loss $\geq$5 mm at minimum six different teeth, age of onset $<$35 years; and for those with generalized chronic periodontitis a clinical attachment-loss $\geq$5 mm at minimum six different teeth. In the control group, no radiographic bone loss and no probing pocket depth $\geq$4 mm was recorded. The control group exhibited no clinical signs of gingivitis. Exclusion criteria for all study participants were: systemic diseases, medication, periodontal or antibiotic treatment $>$6months prior to the investigation, pregnancy. Smoking status was recorded based on a questionnaire. Only current smoking status was considered, the amount of cigarette consumption was not recorded.

Saliva Collection

Unstimulated whole saliva samples were used in this study. Salivary collection was carried out between 8:00 and 11:00 a.m. Participants were asked to refrain from eating, drinking, smoking or brushing their teeth after midnight on the day of sampling. Unstimulated saliva was collected for 5 min according to the protocol of Navazesh et al. (2008), aliquoted and stored at -80°C until analysis.

Total Antioxidant Capacity and Total Oxidant Status Measurements

TAC and total oxidant status (TOS) were measured by commercially available TAC kit and TOS kit, respectively (both Rel Assay Diagnostics, Turkey). Measurements were performed according to the manufacturer's instruction.

TAC assay was based on the measurements of the reduction of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; ABTS) radical. For the measurements of salivary TAC 225 μl of assay Reagent 1 (acetate buffer, pH 5.8) was mixed with 5 μl of saliva and the absorbance was measured at 420 nm after 30 s incubation. Afterwards, 20 μl of Reagent 2 (ABTS, 30 mM in acetate buffer, pH 3.6) were added into each sample and the absorbance at 420 nm was measured after 5 min incubation. TAC was calculated based on the differences in the absorbance at 420 nm before and after adding the Reagent 2. The assay was calibrated with trolox and the results were expressed in terms of mM trolox equivalent per liter (mmol trolox Equiv/L).

TOS of saliva samples were measured using a method developed by Erel (Erel, 2005). Briefly, 225 μL Reagent 1 (xylenol orange 150 μM, NaCl 140 mM and glycerol 1.35M in 25 mM H_2SO_4 solution, pH 1.75) was mixed with 35 μL of samples (saliva) and the absorbance of each sample was read spectrophotometrically at 560 nm as a sample blank. After that, 11 μL Reagent 2 (ferrous ion 5 mM and o-dianisidine10 mM in 25 mM H_2SO_4 solution) was added to the mixture, mixed for about 3–4 min and the last absorbance was read at 560 nm. TOS was calculated based on the differences in the absorbance at 560 nm before and after adding the Reagent 2. The assay was calibrated with H_2O_2 and the results were expressed in terms of μM H_2O_2 equivalent per liter (μmol H_2O_2 Equiv/L).

Microbiological Analysis

Salivary bacteria were measured using the method described previously (Haririan et al., 2014). *A. actinomycetemcomitans, P. gingivalis, T. forsythia, T. denticola,* and *F. nucleatum* in saliva were measured. Saliva in the tubes was centrifuged, and the supernatant was transferred into 2 mL tubes. DNA was extracted by means of a DNA Extraction Kit (ParoCheck®, Greiner Bio One, Kremsmuenster, Austria). Protein kinase K, buffer solution, and EtOH were added, and tubes were centrifuged, vortexed, and heated at 95°C. Part of the 16S rRNA gene was amplified by a highly conserved specific primer pair flanking the diversity box of each 16S rRNA gene. A chip (ParoCheckChip®, Greiner Bio One, Kremsmuenster, Austria) was used for hybridization, followed by washes at 50°C and drying by centrifugation. Finally, the DNA-Chip was analyzed by a scanner (CheckScanner) semi quantitatively using specific analytic software (Check Report Software Version 4.0.2, Greiner Bio One, Kremsmuenster, Austria). The signals describing the bacterial load in the samples were scored as −, (+), +, ++, or +++.

Statistical Analysis

Differences in TAC/TOS values between periodontitis and control groups were analyzed using Students *t*-test. The effect of diagnosis, age, gender, smoking status, and bacterial load on the salivary TAC and TOS was tested using multivariate generalized linear model. The differences in the detection frequency of particular bacteria, the proportion of female/male, and the proportion of smokers between groups were examined by χ^2 test. Correlations between different parameters were checked by Spearmans's rank correlation tests. Differences were considered to be statistically significant at $p < 0.05$. Statistical power was calculated using freely available online tool (www.powerandsamplesize.com). All other statistical analysis was performed using statistical program SPSS 21.0.

RESULTS

Demographic and Clinical Characterization of the Study Groups

Demographical characteristics and clinical parameters of healthy controls and periodontitis patients are listed in the **Table 1**. No significant difference in the proportion of female and male participants was found between the groups. The proportion of smokers was significantly higher in the periodontitis group compared to the control group ($p < 0.01$). Clinical parameters were significantly higher in the periodontitis group than in the control group ($p < 0.01$). The results of the semiquantitative analysis of *A. actinomycetemcomitans, P. gingivalis, T. denticola, T. forsythia, and F. nucleatum* in saliva of healthy individuals and

TABLE 1 | Demographic characteristics and clinical parameters of the study population.

	Control	Periodontitis overall	Aggressive periodontitis	Chronic periodontitis
Number of participants	37	45	15	30
Age, years	36.1 ± 10.3	45.2 ± 9.8	34.7 ± 5.8	50.5 ± 6.6
Female/male (n)	21/16	17/28	5/10	12/18
Non-Smoker/Smoker (n)	25/12	15/30[#]	6/9	9/21
PPD, mm	1.71 ± 0.21[*]	3.88 ± 0.86	3.91 ± 1.09[*]	3.86 ± 0.75[*]
CAL, mm	1.74 ± 0.22[*]	4.43 ± 1.12	4.59 ± 1.17[*]	4.41 ± 0.97[*]
BOP, %	2.6 ± 3.1[*]	41.0 ± 25.1	45.7 ± 27.3[*]	38.7 ± 24.0[*]
Number of teeth with PPD ≥ 5 mm	0	19.2 ± 6.2[*]	20.3 ± 6.9[*]	18.6 ± 5.8[*]

Data are presented as mean ± SD. BOP, bleeding on probing; PPD, probing pocket depth; CAL, clinical attachment loss. [*]Significantly different vs. control group, $p < 0.05$, t-test. [#]significantly different vs. control group, $p < 0.05$, χ^2 test.

periodontitis patients are shown in the **Figure 1**. Significantly higher detection rates of *P. gingivalis, T. denticola,* and *T. forsythia,* were observed in periodontitis patients compared to healthy subjects ($p < 0.01$). No significant difference in the detection rate of *A. actinomycetemcomitans* ($p = 0.051$) and *F. nucleatum* ($p = 0.642$) was found between periodontitis and control groups.

Salivary TAC and TOS

The results of a multivariate generalized linear model analysis of the effect of overall diagnosis, smoking status, gender, age, and bacterial load on the salivary TAC and TOS are presented in the **Table 2**. Multivariate test showed a significant dependency of study parameters on the overall diagnosis. Between subject tests showed that only overall diagnosis has a significant effect on the salivary TAC, whereas age, gender, smoking status, and bacterial load had no significant effect on this parameter. None of the factors had a significant effect on salivary TOS. The salivary TAC and TOS in control and periodontitis groups are shown in the **Figure 2**. The salivary TAC values in periodontitis group were significantly lower compared to the control group ($p < 0.05$, power = 0.82). No significant difference in the salivary TOS between control and periodontitis groups was found. No significant difference in the salivary TAC and TOS was found between patients with aggressive and chronic periodontitis (data not shown).

Correlation of TAC and TOS with Clinical Parameters of Periodontitis

In periodontitis patients, the salivary TAC exhibited a significantly negative correlation with CAL parameter ($r = -0.312$, $p < 0.05$, see **Figure 3A**). A negative but non-significant correlation was observed between salivary TAC and BOP ($r = -0.258$, $p = 0.087$, see **Figure 3B**). No significant correlation of TAC with clinical parameters BOP, PPD, and the number of teeth with PPD $\geq$ 5 mm was found. No significant correlation of salivary TOS with any clinical parameter was found.

Clinical Parameters and Salivary TAC/TOS in Relation to Bacterial Load

The dependency of PPD, number of teeth with PPD $\geq$ 5 mm, and BOP on the bacterial load measured in the saliva of periodontitis patients is shown in **Figure 4**. Increased salivary content of *A. actinomycetemcomitans, P. gingivalis, and T. denticola* was associated with significantly higher PPD and the number of teeth with PPD $\geq$ 5 mm in periodontitis patients. No significant effect of the salivary presence of bacteria on BOP (**Figure 4C**) and CAL (data not shown) was observed.

Figure 5 shows salivary TAC and TOS depending on the presence of different bacteria in the saliva of periodontitis patients. No significant effect of any periodontal bacteria on the salivary TAC (**Figure 5A**) and salivary TOS (**Figure 5B**) was observed.

DISCUSSION

In the present study we investigated the differences in TAC and TOS in saliva in individuals with periodontitis and healthy controls and investigated their relationship with clinical parameters of periodontitis and the presence of periodontopathic bacteria *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *T. denticola*, and *F. nucleatum* in saliva. Activation of the host response by periodontal pathogens results in an activation and infiltration of neutrophils, which are the primary source of ROS in periodontitis (Ryder, 2010; Scott and Krauss, 2012). ROS production by neutrophils is considered as an important mechanism of bacteria killing (Nauseef, 2007). However, ROS production by neutrophils might also cause damages of host tissues when ROS are not neutralized by the antioxidant system or in case of an impaired neutrophils clearance (Hajishengallis and Hajishengallis, 2014). A proper balance between ROS production and TAC of the host tissue plays an important role in the homeostasis of periodontal tissue and prevents tissue

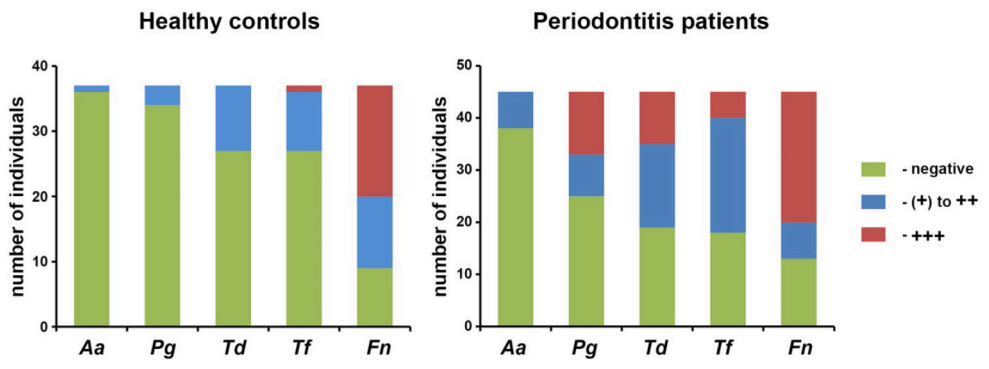


FIGURE 1 | Detection of different periodontal pathogens in saliva of healthy individuals and periodontitis patients. The results of semiquantitative analysis of the presence of periodontal pathogens in the saliva are presented. The detection rate of *P. gingivalis (Pg)*, *T. denticola (Td)*, and *T. forsythia (Tf)* was significantly higehr in periodontits patients than in control group. No significant difference in the detection rate of *A. actinomycetemcomitans* and *F. nucleatum* was detected.

TABLE 2 | Effect of overall diagnosis, gender, smoking status, age, and bacterial load on the salivary TAC and TOS analyzed by multivariate generalized linear model.

	Multivariate test Wilks-Lambda		Salivary TAC			Salivary TOS		
	***F*-value**	***P*-value**	***F*-value**	***P*-value**	***β***	***F*-value**	***P*-value**	***β***
Overall diagnosis "0"–control "1"–periodontitis	5.104	0.014	5.289	0.028	−0.386	0.138	0.713	0.139
Gender "0"–male "1"–female	1.042	0.368	2.990	0.093	0.160	0.037	0.848	−0.088
Smoking "0"–non-smokers "1"–smokers	1.498	0.244	2.272	0.141	0.045	0.141	0.731	0.124
Age	1.381	0.118	1.611	0.085	0.021	1.047	0.449	0.013
A. actinomycetemcomitans "0"–negative; "1"– ≤ ++ "2"– + ++	0.554	0.765	0.001	0.981	0.063	0.161	0.691	−0.107
P. gingivalis "0"–negative; "1"– ≤ ++ "2"– + ++	1.543	0.184	0.120	0.888	−0.204	0.491	0.616	0.263
T. denticola "0"–negative; "1"– ≤ ++ "2"– + ++	1.322	0.256	3.384	0.064	0.355	2.707	0.082	−0.401
T. forsythia "0"–negative; "1"– ≤ ++ "2"– + ++	1.418	0.214	0.639	0.639	−0.098	0.952	0.396	−0.107
F. nucleatum "0"–negative; "1"– ≤ ++ "2"– + ++	1.113	0.369	1.529	0.232	−0.176	0.250	0.780	0.282

Parameters are calculated by multivariate generalized linear models with TAC and TOS as dependent variable and overall diagnosis, gender, smoking status, age, or bacterial load as independent variables. β, standardized regression coefficient.

destruction upon activation of the immune system by periodontal pathogens.

Analysis using multivariate generalized linear model showed that salivary TAC was significantly dependent on the overall diagnosis but not on other parameters such as age, gender, smoking status, and bacterial load. None of the parameters, such as overall diagnosis, age, gender, smoking status, and bacterial load exhibited a significant effect on salivary TOS. The antioxidant capacity of saliva is due to low molecular antioxidants such as uric acid, ascorbic acid, and albumin (Moore et al., 1994) as well as due to antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (Battino et al., 2002). Salivary TOS might be accounted by different reactive oxygen and nitrogen species, such as hydroxyl radical, hydrogen peroxide, or peroxynitrite (Takahama et al., 2005; Chitra et al., 2012). Our data suggest that changes in the oxidative stress parameters in periodontitis patients are presumably associated with the inflammatory processes in periodontitis and not directly with a bacterial load. The contribution of other factors such as age, gender, and smoking status on the oxidative stress parameters must be considered in the future studies with higher number of patients.

Salivary TAC in periodontitis patients was significantly lower than those in healthy subjects. There was a significant negative relationship between salivary TAC and CAL. Moreover, a tendency of a negative relationship of salivary TAC with BOP was also observed. Our results are supported by some previous studies showing that salivary TAC is lower in periodontitis patients and negatively correlates with periodontitis severity (Diab-Ladki et al., 2003; Mashayekhi et al., 2005; Guentsch et al., 2008). Immune response against periodontal pathogens is associated with an enhanced production of ROS by neutrophils

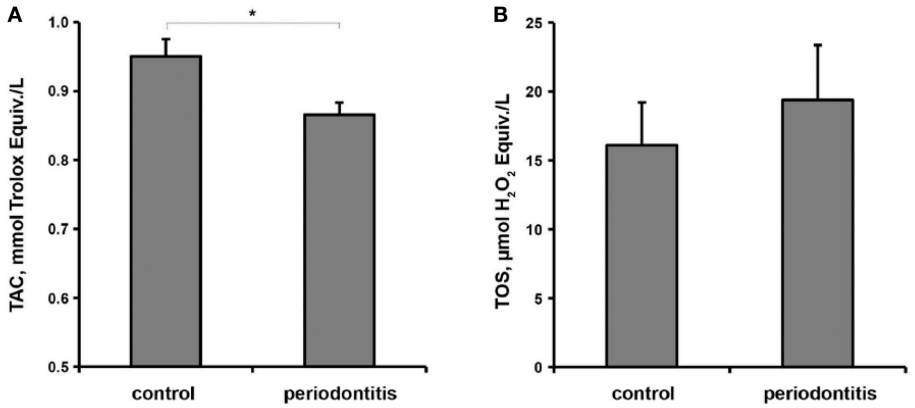


FIGURE 2 | Oxidative stress parameter in the control and periodontitis groups. Salivary total antioxidant capacity **(A)** and total oxidant status **(B)** are presented as mean ± s.e.m. *Significantly different vs. control group, $p < 0.05$, *t*-test.

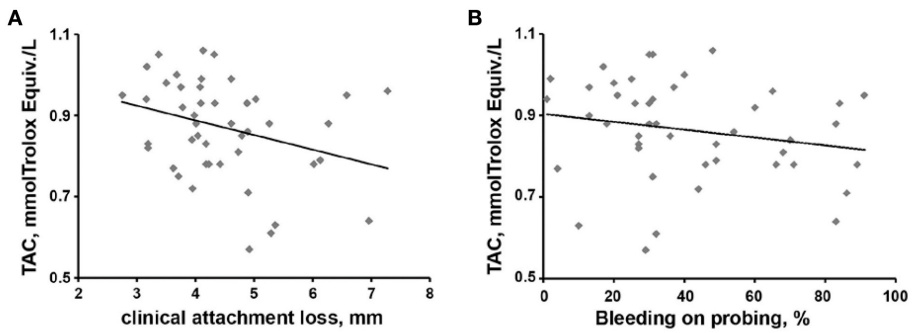


FIGURE 3 | Relationship between salivary TAC and clinical parameters in periodontitis patients. Relationships salivary TAC vs. CAL **(A)** and salivary TAC vs. BOP **(B)** are presented. Each point represents one periodontitis patient. Spearman rank correlation test revealed a significant negative correlation between salivary TAC and CAL ($r = -0.312$, $p < 0.05$) and a non-significant negative correlation between salivary TAC and BOP ($r = -0.258$, $p = 0.087$).

and macrophages (for review, see Chapple and Matthews, 2007). To avoid host tissue destruction these ROS are neutralized by antioxidants, which might result in decreased TAC. Systemic and local TAC in periodontitis patients might reflect increased oxygen radical activity during periodontal inflammation and can be restored to control subject levels by non-surgical therapy (Chapple and Matthews, 2007). However, there are also some studies showing that salivary TAC is either increased or remains on the same level in periodontitis patients compared to healthy controls (Brock et al., 2004; Tóthová et al., 2013; Almerich-Silla et al., 2015). The differences in the results of TAC between different studies could be explained by different analytical methods used by TAC assessment (reviewed in Wang et al., 2015). Particularly, in our study, TAC was measured based on the ability of saliva sample to reduce ABTS radical, whereas in another study TAC measurements were based on the saliva ability to prevent ABTS oxidation (Almerich-Silla et al., 2015). It should be also noted that TAC is a complex parameter which includes the integrated activity of different antioxidants and often depends on their interaction and synergistic effects (Ghiselli et al., 2000).

We did not find any significant difference in the salivary TOS between periodontitis patients and healthy controls. Salivary TOS exhibited no correlation with clinical parameters and the bacterial load. This observation is in contrast to recent studies reporting salivary TOS in periodontitis patients of about 1.5 times higher compared to healthy individuals as well as a strong correlation of TOS with clinical parameter of periodontitis (Akalin et al., 2007; Wei et al., 2010). However, in these two studies, patients' selection was more restrictive: they include only never smokers, all studies participants have similar age, socio-economic status and nutrition habits. Therefore, patient selection could be a crucial parameter influencing salivary levels of TOS and this fact is rather limiting the use of salivary TOS as a marker of periodontal disease.

An assumption that an increased salivary TOS reflects an increased production of ROS in periodontitis is rather questionable. Most ROS produced by neutrophils and macrophages either reacts with target proteins or are neutralized by antioxidants. Furthermore, the production of ROS as a result of an inflammatory reaction occurs either before or at the same time of tissue damages (Halliwell, 2000). Therefore, lipid peroxidation products malondialdehyde (MDA) and thiobarbituric acid-reacting substances (TBARS), advanced

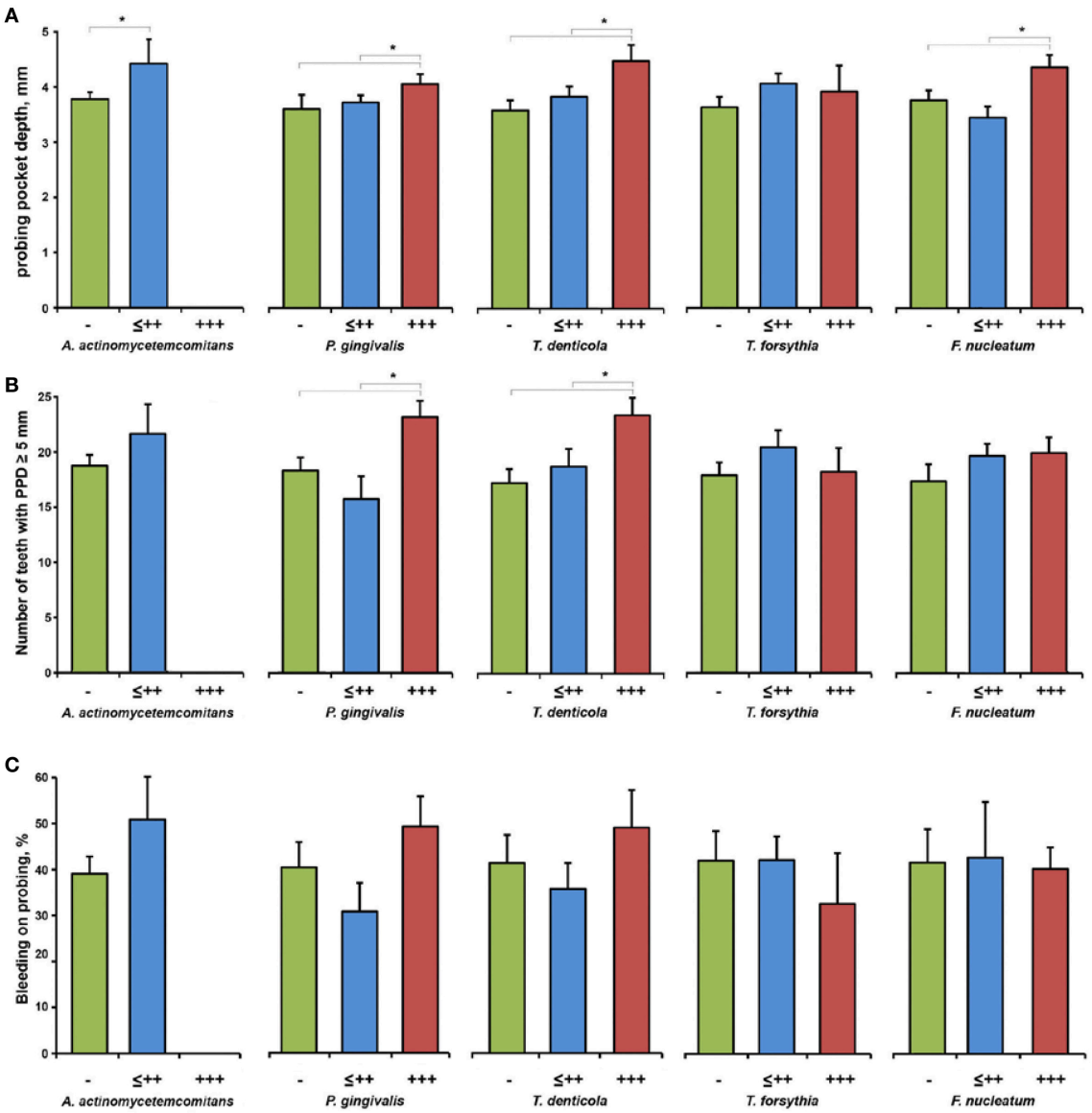


FIGURE 4 | Clinical parameters of periodontitis patients depending on the bacterial load. PPD **(A)**, number of teeth with PPD ≥ 5 mm **(B)**, and BOP **(C)** in subgroups of periodontitis patients based on the results of the semiquantitative bacterial analysis in the saliva: negative (group "−"), from (+) to ++ (group "≤ ++"), and + + + (group "+ + +"). Data are presented as mean ± s.e.m. *Significantly different with $p < 0.05$.

oxidation protein products (AOPP), and oxidative DNA damage product 8-hydroxydeoxyguanosine (8-OHdG) are often used as an indicator of oxidative stress (Chapple and Matthews, 2007; Tóthová et al., 2015; Wang et al., 2015). Previous studies suggest that salivary TBARS and 8-OHdG levels are enhanced in periodontitis patients compared to controls (for review, see Tóthová et al., 2015). Two previous studies show a significant positive correlation between the levels of periodontal pathogens and oxidative stress markers in saliva from periodontitis patients (Sawamoto et al., 2005; Almerich-Silla et al., 2015). Moreover, analysis with predictive model shows that salivary markers of lipid peroxidation and DNA damage might have good diagnostic potential for periodontitis (Almerich-Silla et al., 2015).

We have determined the levels of different bacteria in saliva. As shown by our previous study, salivary bacteria levels exhibit a good correlation with the subgingival bacterial load in the dental plaque of periodontitis patients (Haririan et al., 2014). We observed that the salivary levels of *P. gingivalis*, *T. forsythia*, and *T. denticola* were significantly enhanced in periodontitis patients compared to healthy subjects. This confirms the previous finding suggesting the importance of red complex bacteria as true pathogens in adult periodontitis patients (Socransky et al., 1998). We further found that a high bacterial load was associated with increased clinical parameters of periodontitis such as PPD and BOP but exhibited no correlation with salivary TAC and TOS. However, a negative correlation of salivary TAC with CAL

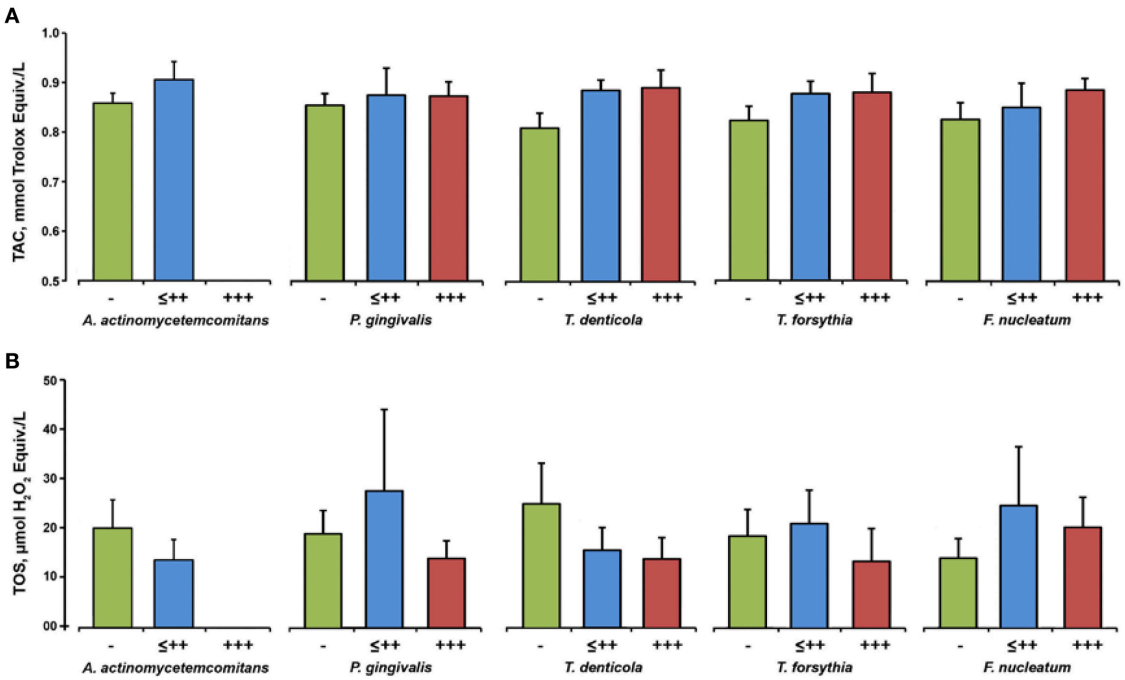


FIGURE 5 | Oxidative stress parameters depending on the bacterial load. Salivary TAC **(A)** and TOS **(B)** in subgroups of periodontitis patients based on the results of the semiquantitative bacterial analysis in the saliva: negative (group "−"), from (+) to ++ (group "≤ ++"), and + + + (group "+ + +"). Data are presented as mean ± s.e.m.

and BOP was observed. Our observations suggest that changes in salivary antioxidant capacity are associated rather with the inflammatory response than with an enhanced bacterial load. This conclusion is generally in line with the current opinion that periodontal tissue destruction is mainly due to a dysregulated unresolved immune response than due to an enhanced bacterial load (Hasturk and Kantarci, 2015; Meyle and Chapple, 2015).

As well-known, periodontitis is a local inflammatory disease with multifactorial character, with fluctuations in bacterial burden, systemic host immune response, and tissue destruction. Up to date, various host-derived factors in periodontitis can be detected in saliva, which provide important information regarding the status of periodontal tissues. Most of the studies focused on biomarkers, which hold a potential diagnostic significance relevant to three important biological phases of periodontal disease, i.e., well-known markers C-reactive protein, IL-1β, IL-6, TNF-α involved in inflammatory phase; matrix metalloproteinases (MMP-8 and MMP-9) in connective-tissue degradation phase and alkaline phosphatase, receptor activator of NF-κB ligand, and osteoprotegerin in bone-turnover phase (for review, see Miller et al., 2010). Moreover, our previous studies demonstrate other biomolecules such as histamine, chromogranin A (CgA), melatonin, and nitric oxide derivatives to be related to the periodontal bacterial load and severity of periodontitis, as well as some of them were restored by a non-surgical therapy and influenced by smoking and gender (Bertl et al., 2012, 2013; Haririan et al., 2012; Andrukhov et al., 2013).

Before considering oxidative stress parameters as potential markers of periodontitis, it is important to take into account their dependency on different factors such as age, smoking, gender, and nutrition. We did not find any effect of gender on both TAC and TOS. Gender is a well-known risk factor of periodontitis and other oxidative stress related disease (Genco, 1996). Male individuals usually have a higher prevalence and severity of periodontal disease than females (Shiau and Reynolds, 2010). In our previous study we also indicated male gender should be considered an important factor by assessing the risk of CVDs in periodontitis patients (Andrukhov et al., 2013). A recent study reports that changes of TAC and oxidative stress marker TBARS in saliva of periodontitis patients might be gender dependent (Baňasová et al., 2015). Antioxidant protection in females is usually higher due to an increased activity of antioxidant enzymes in pre-menopausal females when compared to men (Pepe et al., 2009; Bloomer and Fisher-Wellman, 2010). Various markers of oxidative stress such as MDA, AOPP, nitric oxide derivatives, as well as Trolox-Equivalent Antioxidant Capacity (TEAC) are reported to differ between men and women (Andrukhov et al., 2013; Bloomer and Lee, 2013). However, the exact effect of gender on salivary TAC and TOS must be investigated in further studies with higher patients' numbers.

Smoking is another important factor influencing the salivary levels of oxidative stress parameters. Smoking stimulates the oxidative burst of neutrophils and therefore influences the oxidative balance in the whole body (Ryder et al., 1998). In our study we did not observe any significant effect of smoking

on salivary TAC and TOS. Similarly to our observation, some previous studies also do not observe any significant effect of smoking on salivary TAC (Charalabopoulos et al., 2005; Guentsch et al., 2008). In contrast, another study reports that the activity of antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase is increased in smoking periodontitis patients compared to non-smokers (Tonguç et al., 2011). Salivary levels of lipid peroxidation marker MDA are reported to be higher in smoking patients than in non-smokers (Guentsch et al., 2008; Tonguç et al., 2011). The dependency of oxidative stress markers on current smoking status, smoking history, and smoking intensity (cigarette per day or pack per years) must be taken into account before considering these parameters as candidate for periodontal disease markers.

Age might also influence the salivary levels of oxidative stress parameters. Previous studies suggest that the levels of salivary antioxidants as well as protein oxidation products positively correlate with the age (Hershkovich et al., 2007; Celecová et al., 2013). However, these changes seem to be pronounced mainly in the elderly individuals. In our study, we did not observe any significant effect of age on the salivary TAC and TOS. Periodontitis patients included in our study were about 9 years older than healthy controls. The observed differences in salivary TAC observed in our study cannot be contributed to the different age on the salivary because older individual have higher TAC (Hershkovich et al., 2007; Celecová et al., 2013) but we observed the lower TAC in periodontitis patients.

In conclusion, the present study showed that salivary TAC is decreased in periodontitis patients compared to healthy controls. The change of TAC correlates with the severity of periodontal disease but not with the bacterial load, which implies that changes in the oxidative status in periodontitis patients are rather due to dysregulated immune response than an increased bacterial load.

AUTHOR CONTRIBUTIONS

Designed research: TZ, OA, HH, XR. Performed research and analyzed data: TZ, OA, HH, MM, SL. Edited the manuscript: HH, MM, SL, ZL. Wrote the manuscript: TZ, OA, XR.

ACKNOWLEDGMENTS

The authors would like to thank Mrs. Nguyen Phuong Quynh and Mrs. Hedwig Rutschek (both Medical University of Vienna) for excellent technical assistances. This study was supported by a grant from the Austrian National Bank (ONB 14152) and by Greiner Bio-One, Kremsmuenster, Austria (reagents and saliva collection system).

REFERENCES

Akalin, F. A., Baltacioglu, E., Alver, A., and Karabulut, E. (2007). Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. *J. Clin. Periodontol.* 34, 558–565. doi: 10.1111/j.1600-051X.2007.01091.x

Almerich-Silla, J. M., Montiel-Company, J. M., Pastor, S., Serrano, F., Puig-Silla, M., and Dasí, F. (2015). Oxidative stress parameters in saliva and its association with periodontal disease and types of bacteria. *Dis. Markers* 2015:653537. doi: 10.1155/2015/653537

Andrukhov, O., Haririan, H., Bertl, K., Rausch, W. D., Bantleon, H. P., Moritz, A., et al. (2013). Nitric oxide production, systemic inflammation and lipid metabolism in periodontitis patients: possible gender aspect. *J. Clin. Periodontol.* 40, 916–923. doi: 10.1111/jcpe.12145

Armitage, G. C. (1995). Clinical evaluation of periodontal diseases. *Periodontol.* 7, 39–53. doi: 10.1111/j.1600-0757.1995.tb00035.x

Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. *Ann. Periodontol.* 4, 1–6. doi: 10.1902/annals.1999.4.1.1

Baňasová, L., Kamodyová, N., Janšáková, K., Tóthová, L., Stanko, P., Turňa, J., et al. (2015). Salivary DNA and markers of oxidative stress in patients with chronic periodontitis. *Clin. Oral Investig.* 19, 201–207. doi: 10.1007/s00784-014-1236-z

Battino, M., Ferreiro, M. S., Gallardo, I., Newman, H. N., and Bullon, P. (2002). The antioxidant capacity of saliva. *J. Clin. Periodontol.* 29, 189–194. doi: 10.1034/j.1600-051X.2002.290301x.x

Bertl, K., Haririan, H., Laky, M., Matejka, M., Andrukhov, O., and Rausch-Fan, X. (2012). Smoking influences salivary histamine levels in periodontal disease. *Oral Dis.* 18, 410–416. doi: 10.1111/j.1601-0825.2011.01891.x

Bertl, K., Schoiber, A., Haririan, H., Laky, M., Steiner, I., Rausch, W. D., et al. (2013). Non-surgical periodontal therapy influences salivary melatonin levels. *Clin. Oral Investig.* 17, 1219–1225. doi: 10.1007/s00784-012-0801-6

Bloomer, R. J., and Fisher-Wellman, K. H. (2010). Lower postprandial oxidative stress in women compared with men. *Gend. Med.* 7, 340–349. doi: 10.1016/j.genm.2010.07.001

Bloomer, R. J., and Lee, S. R. (2013). Women experience lower postprandial oxidative stress compared to men. *Springerplus* 2, 553. doi: 10.1186/2193-1801-2-553

Brock, G. R., Butterworth, C. J., Matthews, J. B., and Chapple, I. L. (2004). Local and systemic total antioxidant capacity in periodontitis and health. *J. Clin. Periodontol.* 31, 515–521. doi: 10.1111/j.1600-051X.2004.00509.x

Cekici, A., Kantarci, A., Hasturk, H., and Van Dyke, T. E. (2014). Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontol. 2000* 64, 57–80. doi: 10.1111/prd.12002

Celecová, V., Kamodyová, N., Tóthová, L., Kúdela, M., and Celec, P. (2013). Salivary markers of oxidative stress are related to age and oral health in adult non-smokers. *J. Oral Pathol. Med.* 42, 263–266. doi: 10.1111/jop.12008

Chapple, I. L., and Matthews, J. B. (2007). The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol. 2000* 43, 160–232. doi: 10.1111/j.1600-0757.2006.00178.x

Charalabopoulos, K., Assimakopoulos, D., Karkabounas, S., Danielidis, V., Kiortsis, D., and Evangelou, A. (2005). Effects of cigarette smoking on the antioxidant defence in young healthy male volunteers. *Int. J. Clin. Pract.* 59, 25–30. doi: 10.1111/j.1742-1241.2004.00340.x

Chitra, S., Balasubramaniam, M., and Hazra, J. (2012). Effect of alpha-tocopherol on salivary reactive oxygen species and trace elements in oral submucous fibrosis. *Ann. Clin. Biochem.* 49, 262–265. doi: 10.1258/acb.2011.011050

Diab-Ladki, R., Pellat, B., and Chahine, R. (2003). Decrease in the total antioxidant activity of saliva in patients with periodontal diseases. *Clin. Oral Investig.* 7, 103–107. doi: 10.1007/s00784-003-0208-5

Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clin. Biochem.* 38, 1103–1111. doi: 10.1016/j.clinbiochem.2005.08.008

Genco, R. J. (1996). Current view of risk factors for periodontal diseases. *J Periodontol.* 67, 1041–1049. doi: 10.1902/jop.1996.67.10s.1041

Ghiselli, A., Serafini, M., Natella, F., and Scaccini, C. (2000). Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Radic. Biol. Med.* 29, 1106–1114. doi: 10.1016/S0891-5849(00)00394-4

Giannobile, W. V., Beikler, T., Kinney, J. S., Ramseier, C. A., Morelli, T., and Wong, D. T. (2009). Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol. 2000* 50, 52–64. doi: 10.1111/j.1600-0757.2008.00288.x

Goodson, J. M. (1986). Clinical measurements of periodontitis. *J. Clin. Periodontol.* 13, 446–460. doi: 10.1111/j.1600-051X.1986.tb01489.x

Guentsch, A., Preshaw, P. M., Bremer-Streck, S., Klinger, G., Glockmann, E., and Sigusch, B. W. (2008). Lipid peroxidation and antioxidant activity in saliva of

periodontitis patients: effect of smoking and periodontal treatment. *Clin. Oral Investig.* 12, 345–352. doi: 10.1007/s00784-008-0202-z

Hajishengallis, E., and Hajishengallis, G. (2014). Neutrophil homeostasis and periodontal health in children and adults. *J. Dent. Res.* 93, 231–237. doi: 10.1177/0022034513507956

Halliwell, B. (2000). Oral inflammation and reactive species: a missed opportunity? *Oral Dis.* 6, 136–137. doi: 10.1111/j.1601-0825.2000.tb00324.x

Haririan, H., Andrukhov, O., Bertl, K., Lettner, S., Kierstein, S., Moritz, A., et al. (2014). Microbial analysis of subgingival plaque samples compared to that of whole saliva in patients with periodontitis. *J. Periodontol.* 85, 819–828. doi: 10.1902/jop.2013.130306

Haririan, H., Bertl, K., Laky, M., Rausch, W. D., Böttcher, M., Matejka, M., et al. (2012). Salivary and serum chromogranin A and alpha-amylase in periodontal health and disease. *J. Periodontol.* 83, 1314–1321. doi: 10.1902/jop.2012.110604

Hasturk, H., and Kantarci, A. (2015). Activation and resolution of periodontal inflammation and its systemic impact. *Periodontol. 2000* 69, 255–273. doi: 10.1111/prd.12105

Hershkovich, O., Shafat, I., and Nagler, R. M. (2007). Age-related changes in salivary antioxidant profile: possible implications for oral cancer. *J. Gerontol. A Biol. Sci. Med. Sci.* 62, 361–366. doi: 10.1093/gerona/62.4.361

Kaufman, E., and Lamster, I. B. (2000). Analysis of saliva for periodontal diagnosis–a review. *J. Clin. Periodontol.* 27, 453–465. doi: 10.1034/j.1600-051x.2000.027007453.x

Kinney, J. S., Morelli, T., Braun, T., Ramseier, C. A., Herr, A. E., Sugai, J. V., et al. (2011). Saliva/Pathogen biomarker signatures and periodontal disease progression. *J. Dent. Res.* 90, 752–758. doi: 10.1177/0022034511399908

Lamster, I. B., and Grbic, J. T. (1995). Diagnosis of periodontal disease based on analysis of the host response. *Periodontol. 2000* 7, 83–99. doi: 10.1111/j.1600-0757.1995.tb00038.x

Lamster, I. B., Kaufman, E., Grbic, J. T., Winston, L. J., and Singer, R. E. (2003). Beta-glucuronidase activity in saliva: relationship to clinical periodontal parameters. *J. Periodontol.* 74, 353–359. doi: 10.1902/jop.2003.74.3.353

Lindhe, J., Haffajee, A. D., and Socransky, S. S. (1983). Progression of periodontal disease in adult subjects in the absence of periodontal therapy. *J. Clin. Periodontol.* 10, 433–442. doi: 10.1111/j.1600-051X.1983.tb01292.x

Mashayekhi, F., Aghahoseini, F., Rezaie, A., Zamani, M. J., Khorasani, R., and Abdollahi, M. (2005). Alteration of cyclic nucleotides levels and oxidative stress in saliva of human subjects with periodontitis. *J. Contemp. Dent. Pract.* 6, 46–53.

Meyle, J., and Chapple, I. (2015). Molecular aspects of the pathogenesis of periodontitis. *Periodontol. 2000* 69, 7–17. doi: 10.1111/prd.12104

Miller, C. S., Foley, J. D., Bailey, A. L., Campell, C. L., Humphries, R. L., Christodoulides, N., et al. (2010). Current developments in salivary diagnostics. *Biomark. Med.* 4, 171–189. doi: 10.2217/bmm.09.68

Moore, S., Calder, K. A., Miller, N. J., and Rice-Evans, C. A. (1994). Antioxidant activity of saliva and periodontal disease. *Free Radic. Res.* 21, 417–425. doi: 10.3109/10715769409056594

Nauseef, W. M. (2007). How human neutrophils kill and degrade microbes: an integrated view. *Immunol. Rev.* 219, 88–102. doi: 10.1111/j.1600-065X.2007.00550.x

Navazesh, M., Kumar, S. K., and University Of Southern California School of Dentistry. (2008). Measuring salivary flow: challenges and opportunities. *J. Am. Dent. Assoc.* 139(Suppl.), 35S–40S. doi: 10.14219/jada.archive.2008.0353

Palmieri, B., and Sblendorio, V. (2007). Oxidative stress tests: overview on reliability and use. Part I. *Eur. Rev. Med. Pharmacol. Sci.* 11, 309–342.

Pepe, H., Balci, S. S., Revan, S., Akalin, P. P., and Kurtoglu, F. (2009). Comparison of oxidative stress and antioxidant capacity before and after running exercises in both sexes. *Gend. Med.* 6, 587–595. doi: 10.1016/j.genm.2009.10.001

Ryder, M. I. (2010). Comparison of neutrophil functions in aggressive and chronic periodontitis. *Periodontol. 2000* 53, 124–137. doi: 10.1111/j.1600-0757.2009.00327.x

Ryder, M. I., Fujitaki, R., Johnson, G., and Hyun, W. (1998). Alterations of neutrophil oxidative burst by *in vitro* smoke exposure: implications for oral and systemic diseases. *Ann. Periodontol.* 3, 76–87. doi: 10.1902/annals.1998.3.1.76

Sawamoto, Y., Sugano, N., Tanaka, H., and Ito, K. (2005). Detection of periodontopathic bacteria and an oxidative stress marker in saliva from periodontitis patients. *Oral Microbiol. Immunol.* 20, 216–220. doi: 10.1111/j.1399-302X.2005.00215.x

Scott, D. A., and Krauss, J. (2012). Neutrophils in periodontal inflammation. *Front. Oral Biol.* 15, 56–83. doi: 10.1159/000329672

Seymour, G. J., and Gemmell, E. (2001). Cytokines in periodontal disease: where to from here? *Acta Odontol. Scand.* 59, 167–173. doi: 10.1080/000163501750266765

Shiau, H. J., and Reynolds, M. A. (2010). Sex differences in destructive periodontal disease: a systematic review. *J. Periodontol.* 81, 1379–1389. doi: 10.1902/jop.2010.100044

Signat, B., Roques, C., Poulet, P., and Duffaut, D. (2011). *Fusobacterium nucleatum* in periodontal health and disease. *Curr. Issues Mol. Biol.* 13, 25–36.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., and Kent, R. L. Jr. (1998). Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25, 134–144. doi: 10.1111/j.1600-051X.1998.tb02419.x

Takahama, U., Hirota, S., and Oniki, T. (2005). Production of nitric oxide-derived reactive nitrogen species in human oral cavity and their scavenging by salivary redox components. *Free Radic. Res.* 39, 737–745. doi: 10.1080/10715760500043561

Tonguç, M. Ö., Öztürk, O., Sütçü, R., Ceyhan, B. M., Kilinç, G., Sönmez, Y., et al. (2011). The impact of smoking status on antioxidant enzyme activity and malondialdehyde levels in chronic periodontitis. *J. Periodontol.* 82, 1320–1328. doi: 10.1902/jop.2011.100618

Tóthová, L., Hodosy, J., Mettenburg, K., Fábryová, H., Wagnerová, A., Baḃícková, J., et al. (2013). No harmful effect of different Coca-cola beverages after 6 months of intake on rat testes. *Food Chem. Toxicol.* 62, 343–348. doi: 10.1016/j.fct.2013.08.073

Tóthová, L., Kamodyová, N., Cervenka, T., and Celec, P. (2015). Salivary markers of oxidative stress in oral diseases. *Front. Cell. Infect. Microbiol.* 5:73. doi: 10.3389/fcimb.2015.00073

Wang, J., Schipper, H. M., Velly, A. M., Mohit, S., and Gornitsky, M. (2015). Salivary biomarkers of oxidative stress: a critical review. *Free Radic. Biol. Med.* 85, 95–104. doi: 10.1016/j.freeradbiomed.2015.04.005

Wei, D., Zhang, X. L., Wang, Y. Z., Yang, C. X., and Chen, G. (2010). Lipid peroxidation levels, total oxidant status and superoxide dismutase in serum, saliva and gingival crevicular fluid in chronic periodontitis patients before and after periodontal therapy. *Aust. Dent. J.* 55, 70–78. doi: 10.1111/j.1834-7819.2009.01123.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 01 December 2015
doi: 10.3389/fcimb.2015.00087

Using Salivary Nitrite and Nitrate Levels as a Biomarker for Drug-Induced Gingival Overgrowth

Erkan Sukuroglu[1], Güliz N. Güncü[2], Kamer Kilinc[3] and Feriha Caglayan[2]*

[1] Department of Periodontology, Faculty of Dentistry, Karadeniz Technical University, Trabzon, Turkey, [2] Department of Periodontology, Faculty of Dentistry, Hacettepe University, Ankara, Turkey, [3] Department of Biochemistry, TOBB University of Economics and Technology, Ankara, Turkey

Aim: Drug-induced gingival overgrowth has a multifactorial nature and the pathogenesis is still uncertain. It has been suggested that Nitric Oxide (NO) might play a role in the pathogenesis of drug-induced gingival overgrowth due to the contribution of NO to immune response and matrix degradation. NO levels in biological fluids have been used as a diagnostic biomarker in many diseases. The aim of this study is to determine whether NO levels in plasma, saliva, and gingival crevicular fluid (GCF) can serve as a potential biomarker for the evaluation of drug-induced gingival overgrowth risk.

Materials and Methods: A total of 104 patients, receiving cyclosporine A ($n = 35$), phenytoin ($n = 25$), nifedipine ($n = 26$), or diltiazem ($n = 18$) participated in the study. The amount of gingival overgrowth was evaluated with two indices and was given as percentage. Periodontal clinical parameters including plaque index (PI), gingival index (GI), gingival bleeding time index (GBTI), and probing depth (PD) were also assessed. Saliva, GCF, and plasma samples were obtained from each participants. Nitrite and nitrate levels in saliva, GCF, and plasma were analyzed by Griess reagent.

Results: Salivary nitrite and nitrate levels in responders were significantly higher than those in non-responders in only phenytoin group ($p < 0.05$). Nitrite and nitrate levels of gingival crevicular fluid and plasma did not significantly differ between responders and non-responders in all study groups ($p > 0.05$). Salivary nitrite levels exhibited a significant correlation with PD, GBTI, severity of gingival overgrowth (%GO), and GCF volume ($p < 0.05$). Additionally, a strong positive correlation was detected between saliva and plasma nitrate levels ($p < 0.005$). However, both nitrite and nitrate levels in GCF and plasma demonstrated no significant correlation with clinical parameters, GO severity, and GCF volume ($p > 0.05$).

Conclusion: Salivary nitrite and nitrate levels could be used as periodontal disease biomarkers in phenytoin induced gingival overgrowth, and that saliva seems to have a better diagnostic potential than GCF and plasma for the evaluation of drug-induced gingival overgrowth risk. However, when all drug groups were considered, saliva nitrite and nitrate levels could not be used as a biomarker for drug-induced gingival overgrowth.

Keywords: nitric oxide, nitrite, nitrate, saliva, drug induced gingival overgrowth, biomarker

OPEN ACCESS

Edited by:
Ulvi Kahraman Gürsoy, University of Turku, Finland

Reviewed by:
Gena D. Tribble, University of Texas Health Science Center at Houston, USA
Christian Damgaard, University of Copenhagen, Denmark
Telma Blanca Lombardo Bedran, Centro Universitario Nove de Julho (UNINOVE), Brazil

****Correspondence:***
Erkan Sukuroglu
esukuroglu@gmail.com

Received: *30 August 2015*
Accepted: *16 November 2015*
Published: *01 December 2015*

Citation:
Sukuroglu E, Güncü GN, Kilinc K and Caglayan F (2015) Using Salivary Nitrite and Nitrate Levels as a Biomarker for Drug-Induced Gingival Overgrowth. Front. Cell. Infect. Microbiol. 5:87. doi: 10.3389/fcimb.2015.00087

INTRODUCTION

Drug-induced gingival overgrowth is a well-known side effect of anticonvulsant phenytoin, immunosupressant cyclosporine A (CsA), and antihypertensive calcium channel blockers. Although the structural characteristics and pharmacologic effects of each of these drugs are different, all of them can cause gingival overgrowth that generally has a similar clinical and histological appearance. The overgrowth normally begins at the interdental papillae and is usually confined to the attached gingiva, but it may extend coronally and interfere with the occlusion, mastication and speech, and may also compromise proper plaque control (Marshall and Bartold, 1999). The prevalence rate of gingival overgrowth is about 50% for phenytoin (Güncü et al., 2006) and 25–30% for CsA (Boltchi et al., 1999). This prevalence rate in patients treated with calcium channel blockers varies widely from 10 to 50% (Ellis et al., 1999; Güncü et al., 2007).

Not all patients receiving these drugs develop gingival overgrowth and the severity of overgrowth varies between patients. Although, many clinical and laboratory studies have been performed to clarify the underlying pathogenic mechanisms responsible for drug-induced gingival overgrowth, the etiology remains still undisclosed. Many risk factors including poor plaque control, gender, age of the patient, drug dosage, pharmacokinetic variables, and genetic predisposition have been identified in a number of studies, but it is not possible to determine the patients at high risk for developing gingival overgrowth (Seymour et al., 2000).

The essential feature of drug-induced gingival overgrowth is an increase in the connective tissue matrix and it results from disturbance of the intricate balance between synthesis and degradation of the extracellular matrix of the gingival tissues. Some of the suggested factors responsible for this imbalance are cytokines, inflammatory mediators, and growth factors (Trackman and Kantarci, 2004; Chae et al., 2006; Kantarci et al., 2006).

Nitric oxide (NO) is a gaseous free radical generated from the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) (Jenkins et al., 1995). Three distinct isoforms of NOS are known and identified as endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). eNOS and nNOS release small amounts of NO for a short period of time following receptor stimulation and they play an important role in regulation of physiological processes including platelet aggregation, vascular relaxation, and immune modulation. iNOS produces large amount of NO, and is expressed in response to bacterial endotoxins and cytokines, such as interleukin-1β and TNF-α (Forstermann et al., 1994; Lyons, 1995). Distinct from eNOS and nNOS, iNOS is considered to act in pathological conditions and plays a detrimental role in the regulation of inflammatory reactions. These NO-mediated detrimental effects probably occur in combination with the action of metalloproteinases and collagenases, produced by activated macrophages, polymorphonuclear cells, and fibroblasts (Kröncke et al., 1997; Kendall et al., 2001).

It has been shown that NO takes part in the etiopathogenesis of many diseases, including periodontal disease (Ohashi et al., 1999; Batista et al., 2002). Increased production of NO can cause destruction of periodontal tissues (Batista et al., 2002). Based on the role of NO on both immune response and matrix degradation in the periodontal environment, it can be speculated that NO may play an important role in the pathogenesis of drug-induced gingival overgrowth. However, only few animal and clinical studies have investigated the relationship between NO and drug-induced gingival overgrowth (Fu et al., 2000; Gau et al., 2005; Rezaie et al., 2005; Gürkan et al., 2009). Fu et al. (2000) reported a decrease in gingival dimensions of CsA-fed rats after NO substrate (L-arginine) treatment compared to controls. In another animal study, Gau et al. (2005) demonstrated that CsA-treated rats have increased plasma nitrite/nitrate levels compared to control rats. In a recent study, Gürkan et al. (2009) showed higher iNOS and eNOS immunostaining in connective tissues from patients in the gingivitis and CsA induced gingival overgrowth groups compared to healthy and subjects without gingival overgrowth. However, no intergroup differences were reported regarding nitrite/nitrate levels in gingival crevicular fluid (GCF). There is no clinical human study evaluating the relation of salivary nitrite and nitrate levels to drug-induced gingival overgrowth in the literature. There is only one study conducted on rats, in which the relation of nitric oxide to nifedipine-induced gingival overgrowth and salivary gland function was assessed. It was suggested that nitric oxide mediated positive signal-transduction mechanisms in salivary glands may play an important role in nifedipine-induced gingival overgrowth (Rezaie et al., 2005).

Due to its reactivity and short half-life, direct measurements of NO from tissues and biological fluids are hard to perform. Although NO metabolites have a very short life, nitrite and nitrate are the relatively stable end products of NO oxidation (Lappin et al., 2000; Ugar-Cankal and Ozmeric, 2006). The total levels of nitrite and nitrate in biological fluids are generally used for adequate monitoring of the NO synthesis (Moshage et al., 1995). Based on the findings that showed a marked increase in NO synthesis in overgrown gingiva, measuring nitrite and nitrate levels in saliva, GCF, and plasma might be useful to understand the etiopathogenesis of drug-induced gingival overgrowth. To the best of our knowledge, there is no study evaluating the association of nitrite and nitrate levels in saliva, plasma, and GCF with drug-induced gingival overgrowth. Drug-induced gingival overgrowth may constitute speech, mastication, hygienic, and aesthetic problems. Thus, early diagnosis of drug-induced gingival overgrowth is very important and it needs to have a diagnostic biological marker to determine the risk of developing drug-induced gingival overgrowth in patients receiving these drugs, before the occurrence of gingival overgrowth. Therefore, the present study was conducted to evaluate nitrite and nitrate levels in plasma, saliva, and GCF from responders and non-responders treated with either CsA, phenytoin, nifedipine, or diltiazem, and to determine whether NO levels in plasma, saliva, and GCF can serve as a potential biomarker for the evaluation of drug-induced gingival overgrowth risk.

MATERIALS AND METHODS

Study Population and Design

This research study was conducted at the Department of Periodontology, Hacettepe University between February 2010 and September 2013. A total of 104 Turkish patients (50 male, 54 female) were included in the study. The study groups consisted of 35 renal transplant patients receiving CsA, 25 patients receiving phenytoin, 26 patients receiving nifedipine, and 18 patients receiving diltiazem therapy. Patients participated in this study were obtained from outpatients attending the Department of Nephrology, Neurology, and Cardiology, Faculty of Medicine, Hacettepe University. Only patients receiving one of these drugs (including CsA, phenytoin, nifedipine, and diltiazem) in regular doses for at least 6 months were included in the study. The presence of all maxillary and mandibular anterior teeth was also required. Pregnant women, subjects with systemic diseases such as diabetes mellitus or any other form of systemic inflammatory involvement, patients who have mouth breath and restorative crowns at anterior regions, and used any antibiotics or received periodontal treatment within the last 6 months and who also received one more drug leading to gingival overgrowth were excluded from the study. Due to the fact that NO levels could be affected by smoking, smokers were not included in the study. The protocol of the study was approved by the ethics committee of the Hacettepe University. (#FON 07/15-1) Written informed consent in accordance with the Declaration of Helsinki was obtained from each patient prior to enrollment in the study.

Clinical Parameters

Periodontal examinations were carried out on the upper and lower six anterior teeth by one calibrated periodontal specialist (ES) during the study period. Periodontal clinical parameters including plaque index (PI; Löe, 1967), gingival index (GI; Löe, 1967), probing depth (PD), and gingival bleeding time index (GBTI; Nowicki et al., 1981) were recorded. Demographic variables including age and gender were also recorded.

In order to assess the overgrowth of gingival tissues accurately, alginate impressions were obtained from each patient and gypsum individual models were created. Gingival overgrowth was assessed on these individual models by performing measurements in vertical and horizontal dimensions. Vertical measurements were performed according to the Miller and Damm index (1992). Briefly, the height of the gingival tissue was measured from the cemento-enamel junction (CEJ) to the free gingival margin. The following grades were scored in six points around each tooth on maxillary and mandibular anterior region: 0 = normal gingiva; 1 = minimal enlargement ($\leq$2 mm in size); 2 = moderate enlargement (2–4 mm in size); and 3 = severe enlargement (nodular growth >4 mm). Horizontal gingival overgrowth was measured in the buccal-lingual direction of all interdental papillae of maxillary and mandibular anterior teeth according to Miranda et al. (2001). The increase in the size of buccal and lingual papillae was measured from the enamel surface to the outer papillary surface at the interdental contact point. Horizontal measurements were assigned a score of between 0 and 2, as follows: 0 = normal thickness (1 < mm); 1 = thickness between 1 and 2 mm; and 2 = thickness more than 2 mm.

The vertical and horizontal scores were calculated to determine an overgrowth score for each gingival papillae. The maximum overgrowth score obtained for each interdental unit was five. Twenty interdental units were identified, giving a maximum overgrowth score of 100 for a patient. Therefore, this index could be expressed as a percentage (Thomason et al., 2005). A score of 30% was determined as the critical value for gingival overgrowth index. Patients were classified into two subgroups (Seymour and Smith, 1991) as followed: patients with a gingival overgrowth index of <30% were identified as non-responders (GO–) and patients with a value of $\geq$30% were defined as responders (GO+). The severity of GO was also determined with this percentage.

GCF, Saliva, and Plasma Sampling

The participants were told not to eat any high nitrate foods (e.g., spinach, beets, and other green leafy vegetables) at least 12 h before sampling due to the fact that it may affect the study results. GCF samples were obtained from maxillary anterior teeth. To avoid blood contamination and possible stimulation of GCF flow during clinical measurements, samples were collected before any other clinical recordings except the PI. GCF samples were obtained by the technique described by Rüdin et al. (1970). Prior to sampling, the respective region was dried by isolation with cotton rolls and a 5-s gentle air stream with a 90° angle to the tooth axis, and supragingival plaque was eliminated with a curette. Standardized strips were inserted 1 mm into the sulcus and left for 30 s. Papers with visible blood contaminations were discarded and sampling was replicated from another site of the natural tooth that was not sampled. To eliminate the risk of evaporation, paper strips with GCF were immediately transported to a previously calibrated electronic GCF measuring device for volume determination. The GCF was measured electronically in Periotron units, which were converted to micro liters (μl) by MCCONVRT software (Software version 2.52, Oraflow Inc., Amityville, NY, USA). GCF samples were then placed in sterile Eppendorf tubes and stored at −30°C until the day of laboratory analysis. A total of 10 paper strips with GCF were obtained with this standardize technique from maxillary anterior six teeth of each patient for detecting GCF nitrite and nitrate levels.

After GCF collection, stimulated parotid saliva samples were obtained from each patient using Lashley cup (Lashley, 1916) during stimulation with 1% citric acid. Citric acid was applied to the dorsum of the tongue, held there by the subject for 10 s and 1 ml of saliva was collected from each patient as described in detail before (Francis et al., 2001). Saliva samples were then placed in sterile Eppendorf tubes and stored at −80°C until the laboratory analysis.

After saliva collection, in order to determine the plasma nitrite and nitrate levels, venous blood samples were collected into lithium-heparin tubes. Blood samples were placed within 3 min into a refrigerated (4°C) centrifuge and centrifuged at 4000 rpm

for 10 min according to standard procedures (Uzar et al., 2011). After getting plasma, samples were frozen at −80°C for later analysis of plasma nitrite and nitrate concentrations.

Determination of Nitrite and Nitrate Levels in GCF, Saliva, and Plasma

For the determination of nitrite and nitrate levels in GCF samples, 300 ml of phosphate buffer (50 mM, pH: 7.4) was added onto each sample and the samples were mixed vigorously for the extraction of nitrite and nitrate into buffer. Nitrite and nitrate content of the samples extracts was determined using Griess reagent (Grisham et al., 1996). The sum of nitrate and nitrite was measured by the same assay after reduction of nitrate to nitrite with nitrate reductase from *Aspergillus niger* (Roche Diagnostics, Mannheim, Germany). Nitrite and nitrate levels in both saliva and plasma were determined by the same method without any pretreatment. The nitrate concentrations were calculated by subtracting the nitrite level from the total nitrite level (nitrite + nitrate). This assay is in the routine research analyze protocol of the laboratories of Hacettepe University Faculty of Medicine Department of Biochemistry for more than a decade and its sensitivity and specificity is being tested routinely by the university staff, together with other routine tests. However, commercial standards were implemented to confirm its sensitivity and specificity in the present study.

STATISTICAL ANALYSIS

Statistical analysis was performed with (SPSS Inc.,Chicago, IL; Serial number: 2Z7ZHN12FA70P1JXWYNCMWY5). The normality of data was tested with Kolmogorov Smirnov test. Mann Whitney U test was used to compare each clinical or laboratory findings for individual drug group between responders and non-responders. Spearman correlation test was obtained to determine the interactions between laboratory and clinical parameters for all participants. The differences between the age groups were tested with Independent sample *t*-test, and the distribution differences of gender was analyzed with chi-square test interms of between responders and non-responders. The confidence interval was set as 95%.

All patients participated in this study ($n = 104$) revealed a power of 87.74% with a normal approximation method. A difference in salivary nitrate levels between responders and non-responders in phenytoin group can be detected at an alpha level of 0.05, with a statistical power of 87.74%.

RESULTS

A total of 104 patients consisting of CsA group ($n = 35$), phenytoin group ($n = 25$), nifedipine group ($n = 26$), and diltiazem group ($n = 18$) were included in the study. The demographic variables of groups including mean age and gender ratio are presented in **Table 1.**

Patients in each drug group were divided into two subgroups as responders (overgrowth index ≥30%) and non-responders (overgrowth index <30%) according to gingival overgrowth index. Out of 35 patients in CsA group, 13 (37.1%) were identified as responders, those values in phenytoin, nifedipine, and diltiazem groups were 11 (44%), 11 (42.3%), and 2 (11.1%), respectively. The gingival overgrowth index was significantly higher in responders in all drug groups, as expected (**Table 2**). There were statistically no significant differences between responders and non-responders in mean age and gender ratio in all drug groups except for nifedipine. While the mean age was similar between subgroups in nifedipine group, there was a significant difference in gender ratio ($p < 0.05$).

Clinical Periodontal Parameters

All clinical periodontal parameters including PI, GI, PD, and GBTI were statistically higher in responders compared to non-responders in all drug groups ($p < 0.05$), except for diltiazem and nifedipine group (**Table 3**). In diltiazem group, these clinical parameters were higher in responders but only the differences in GBTI and GI reached statistically significance ($p < 0.05$). In nifedipine group, all clinical periodontal parameters except PI were statistically higher in responders ($p < 0.05$).

Nitrite and Nitrate Levels in Saliva, GCF, and Plasma

Nitrite and nitrate levels of saliva, GCF, and plasma for the subgroups of each study group are shown on **Table 3**. Salivary nitrite and nitrate levels (μM) did not differ significantly between responders and non-responders in all study groups, except for phenytoin group. In phenytoin group, salivary nitrite and nitrate levels in responders were significantly higher than those in non-responders ($p < 0.05$). Nitrite and nitrate levels of GCF (nmol)

TABLE 1 | Demographic variables of study population.

	Phenytoin ($n = 25$)		CsA ($n = 35$)		Nifedipine ($n = 26$)		Diltiazem ($n = 18$)	
	GO+ ($n = 11$, 44%)	GO− ($n = 14$, 56%)	GO+ ($n = 13$, 37.1%)	GO− ($n = 22$, 62.9%)	GO+ ($n = 11$, 42.3%)	GO− ($n = 15$, 57.7%)	GO+ ($n = 2$, 11.2%)	GO− ($n = 16$, 88.8%)
Age	40.1 ± 12.2	47.7 ± 16.2	35.3 ± 9.6	41.0 ± 9.7	51.1 ± 8.9	50.3 ± 9.1	53.5 ± 4.9	57.5 ± 9.6
Gender								
Male	6	8	5	13	7	3	1	7
Female	5	6	8	9	4	12	1	9

TABLE 2 | Clinical periodontal parameters in all groups.

Clinical Periodontal Parameters	Phenytoin ($n = 25$)		CsA ($n = 35$)		Nifedipine ($n = 26$)		Diltiazem ($n = 18$)	
	GO+	GO−	GO+	GO−	GO+	GO−	GO+	GO−
PD	2.26 ± 0.57*	1.45 ± 0.51	2.52 ± 0.49*	1.45 ± 0.32	2.28 ± 1.02*	1.39 ± 0.38	2.46 ± 0.83	1.49 ± 0.33
PI	1.05 ± 0.58*	0.41 ± 0.36	1.52 ± 0.54*	0.62 ± 0.48	0.90 ± 0.63	0.56 ± 0.46	1.22 ± 0.74	0.59 ± 0.44
GI	1.62 ± 0.62*	0.88 ± 0.52	2.04 ± 0.38*	0.83 ± 0.61	1.64 ± 0.41*	0.79 ± 0.57	1.82 ± 0.25*	0.87 ± 0.36
GBTI	2.24 ± 0.91*	1.28 ± 0.75	2.80 ± 0.25*	1.17 ± 0.98	2.29 ± 0.55*	1.08 ± 1.07	2.75 ± 0.35*	1.10 ± 0.73
%GO	53.0 ± 11.93*	17.42 ± 7.41	53.84 ± 17.19*	18.36 ± 5.99	44.81 ± 12.2*	20.13 ± 6.05	33.00 ± 2.82*	18.81 ± 5.77
GCF V(μl)	0.94 ± 0.56	0.78 ± 0.69	1.70 ± 0.99*	0.74 ± 0.44	1.24 ± 0.71	1.00 ± 0.69	1.61 ± 0.26*	0.92 ± 0.39

*PD, probing depth; PI, plaque index; GI, gingival index; GBTI, gingival bleeding time index; %GO, percentage of gingival overgrowth; GCF V, Volume of gingival crevicular fluid. *$p < 0.05$.*

TABLE 3 | Nitrite and nitrate levels in saliva, plasma, and in GCF.

	Phenytoin ($n = 25$)		CsA ($n = 35$)		Nifedipine ($n = 26$)		Diltiazem ($n = 18$)	
	GO+	GO−	GO+	GO−	GO+	GO−	GO+	GO−
Saliva nitrite level (μM)	7.08 ± 6.82*	2.90 ± 2.36	7.44 ± 6.16	4.75 ± 3.42	4.87 ± 4.13	6.18 ± 5.84	3.60 ± 1.81	4.96 ± 4.07
Saliva nitrate level (μM)	474 ± 37.17*	421.30 ± 47.23	456.72 ± 54.05	478.69 ± 52.85	475.65 ± 55.96	430.63 ± 65.02	372.23 ± 79.26	424.09 ± 47.78
Plasma nitrite level (μM)	4.33 ± 4.69	3.13 ± 1.76	4.30 ± 3.15	2.60 ± 2.17	3.74 ± 2.82	3.00 ± 2.54	6.16 ± 4.89	4.84 ± 3.76
Plasma nitrate level (μM)	27.89 ± 9.49	26.77 ± 10.58	32.24 ± 18.60	42.89 ± 20.43	32.45 ± 7.18	26.21 ± 7.90	25.65 ± 12.11	27.22 ± 12.97
GCF nitrite level (nmol)	6.39 ± 3.64	5.12 ± 4.72	7.51 ± 4.38	9.17 ± 5.07	7.27 ± 5.39	6.21 ± 4.94	7.94 ± 3.11	4.28 ± 2.91
GCF nitrate level (nmol)	226.22 ± 47.57	253.96 ± 75.61	215.91 ± 64.58	221.01 ± 49.20	289.09 ± 47.78	288.83 ± 64.40	231.93 ± 34.02	253.70 ± 53.65

*$p < 0.05$.

and plasma (μM) did not significantly differ between responders and non-responders in all study groups ($p > 0.05$).

Correlations between saliva, GCF, and plasma nitrite/nitrate levels, periodontal clinical parameters, severity of GO, and GCF volume were presented in **Table 4**. Salivary nitrite level exhibited a significant correlation with PD, GBTI, severity of GO (%GO), and GCF volume ($p < 0.05$). There was no significant correlation between salivary nitrate level and periodontal clinical parameters, except PD. A strong positive correlation was detected between salivary and plasma nitrate levels ($p < 0.005$). There was a negative significant correlation between nitrite and nitrate levels in GCF ($p < 0.05$). Both nitrite and nitrate levels in GCF and plasma demonstrated no significant correlation with clinical parameters, GO severity, and GCF volume ($p > 0.05$).

DISCUSSION

To examine the role of NO in the pathogenesis of drug-induced gingival overgrowth and also to determine whether NO levels in plasma, saliva, and GCF can serve as a potential biomarker for the evaluation of drug-induced gingival overgrowth risk, nitrite and nitrate levels in saliva, GCF, and plasma were evaluated in patients receiving either CsA, phenytoin, nifedipine, or diltiazem therapy. To the best of our knowledge, this is the first study that has evaluated the association of plasma, saliva, and GCF nitrite/nitrate levels with drug-induced gingival overgrowth in patients receiving either CsA, phenytoin, nifedipine, or diltiazem therapy.

Although the pathogenesis of drug-induced gingival overgrowth is still uncertain, it is characterized by excessive deposition of extracellular matrix reflecting both elevated synthesis and reduced degradation of matrix components (Seymour et al., 1996). Fibroblasts are well-known to play a critical role in collagen synthesis and connective tissue turnover. The expression of iNOS has been demonstrated in human dermal fibroblasts (Wang et al., 1996). And, it has been reported that iNOS expression and NO production were elevated in keloid scar tissue which is characterized by excess collagen deposition (Hsu et al., 2006). Furthermore, exposure of fibroblasts to exogenous NO resulted in increased expression of collagen in a dose dependent manner (Hsu et al., 2006). Similar to these findings, increased iNOS enzyme activities were detected in overgrown gingiva from patients taking CsA (Gürkan et al., 2009). Additionally, iNOS is

TABLE 4 | Correlations between nitrite/nitrate levels and clinical periodontal parameters in whole population.

	PD	PI	GBTI	GI	%GO	GCF volume	Salivary nitrite	Salivary nitrate	Plasma nitrite	Plasma nitrate	GCF nitrite	GCF nitrate
Salivary	$r = 0.270$	$r = 0.181$	$r = 0.202$	$r = 0.190$	$r = 0.256$	$r = 0.199$	–	$r = -0.010$	$r = -0.002$	$r = -0.063$	$r = 0.000$	$r = -0.120$
Nitrite	$p = 0.006^{*}$	$p = 0.067$	$p = 0.040^{*}$	$p = 0.053$	$p = 0.009^{*}$	$p = 0.043^{*}$		$p = 0.923$	$p = 0.987$	$p = 0.527$	$p = 0.996$	$p = 0.226$
Salivary	$r = 0.231$	$r = 0.121$	$r = 0.176$	$r = 0.181$	$r = 0.088$	$r = -0.037$	$r = -0.010$	–	$r = -0.168$	$r = 0.365$	$r = 0.033$	$r = -0.161$
Nitrate	$p = 0.018^{*}$	$p = 0.222$	$p = 0.074$	$p = 0.066$	$p = 0.374$	$p = 0.711$	$p = 0.923$		$p = 0.089$	$p = 0.001^{**}$	$p = 0.740$	$p = 0.101$
Plasma	$r = 0.081$	$r = 0.023$	$r = 0.027$	$r = 0.053$	$r = 0.060$	$r = 0.030$	$r = -0.002$	$r = -0.168$	–	$r = -0.133$	$r = -0.085$	$r = -0.012$
Nitrite	$p = 0.414$	$p = 0.818$	$p = 0.787$	$p = 0.591$	$p = 0.548$	$p = 0.763$	$p = 0.987$	$p = 0.089$		$p = 0.178$	$p = 0.392$	$p = 0.901$
Plasma	$r = 0.113$	$r = 0.022$	$r = -0.008$	$r = 0.029$	$r = -0.060$	$r = 0.071$	$r = -0.063$	$r = 0.365$	$r = -0.133$	–	$r = -0.025$	$r = -0.054$
Nitrate	$p = 0.253$	$p = 0.826$	$p = 0.933$	$p = 0.772$	$p = 0.543$	$p = 0.474$	$p = 0.527$	$p = 0.001^{**}$	$p = 0.178$		$p = 0.802$	$p = 0.589$
GCF	$r = 0.163$	$r = 0.158$	$r = 0.148$	$r = 0.158$	$r = 0.003$	$r = -0.097$	$r = 0.000$	$r = 0.033$	$r = -0.085$	$r = -0.025$	–	$r = -0.267$
Nitrite	$p = 0.099$	$p = 0.109$	$p = 0.134$	$p = 0.109$	$p = 0.973$	$p = 0.327$	$p = 0.996$	$p = 0.740$	$p = 0.392$	$p = 0.802$		$p = 0.006^{*}$
GCF	$r = -0.141$	$r = -0.123$	$r = -0.127$	$r = -0.096$	$r = -0.025$	$r = 0.084$	$r = -0.120$	$r = -0.161$	$r = -0.012$	$r = -0.054$	$r = -0.267$	–
Nitrate	$p = 0.154$	$p = 0.213$	$p = 0.199$	$p = 0.332$	$p = 0.803$	$p = 0.398$	$p = 0.226$	$p = 0.101$	$p = 0.901$	$p = 0.589$	$p = 0.006^{*}$	

PD, probing depth; PI, plaque index; GI, gingival index; GBTI, gingival bleeding time index; %GO, percentage of gingival overgrowth; GCF, gingival crevicular fluid. $^{*}p < 0.05$; $^{**}p < 0.005$.

largely expressed for sustained periods as a consequence of induction by bacterial lipopolysaccharide and pro-inflammatory mediators such as interleukin 1, tumor necrosis factor-α, and interferon-γ, which are present in inflamed periodontal tissues (Kendall et al., 2001). In drug-induced gingival overgrowth patients due to the abundant gingiva, plaque elimination becomes harder. When plaque accumulates and could not be eliminated, gingival inflammation takes place. Thus, prolonged production of NO by inflammatory cells in gingiva may result in excess collagen deposition via fibroblasts.

In the present study we did not collect whole saliva samples from participants due to the fact that nitrite and nitrate concentrations in whole saliva originate from sources other than the glandular cells, such as GCF and intraoral bacteria. Salivary nitrate is converted to salivary nitrite by oral bacteria (Kleinbongard et al., 2003). Thus, we used pure stimulated parotid saliva for analyzing saliva nitrite and nitrate levels.

In the present study, no significant differences were detected according to age between responders and non-responders in each drug groups. Although age has been suggested as a predisposing factor associated with drug-induced gingival overgrowth (Hefti et al., 1994; Somacarrera et al., 1994), in the present study no significant differences were detected according to age among the study groups for all the drug groups investigated. Similar to our results, Ellis et al. (1999) have reported no relations between age and drug-induced gingival overgrowth.

There were no significant differences in gender ratios between responders and non-responders in all drug groups, except for nifedipine group. In nifedipine group, males were found to be more dominant in patients developing gingival overgrowth, as previously shown before (Hattori et al., 1995; Tavassoli et al., 1998). This difference can be explained with the alteration in androgen metabolism in males.

All periodontal clinical parameters including PI, GI, GBTI, and PD were significantly higher in responders in all drug groups except for diltiazem group. These differences in periodontal clinical parameters between responders and non-responders can be explained by poor plaque control in responders due to the gingival overgrowth. In diltiazem group, although all periodontal clinical parameters were higher in responders, only the GI, GBTI, and %GO scores were significantly different. The reason for the lack of significant differences in PD and PI scores may be the limited number of responders in this drug group.

A total of 104 patients were analyzed separately in four groups according to drugs that patients were taking due to the fact that these drugs may affect directly or indirectly the NO metabolism. Patients receiving nifedipine or diltiazem were not evaluated within the same group although both of the drugs have been known as calcium channel blockers. Because it has been reported that nifedipine had an inhibitory effect on induction of NO synthesis, and also inhibited nitrite production in vascular smooth muscle cells, mesangial cells, and cardiac myocytes. However, diltiazem had no effect on nitrite formation in these three cell types (Hattori et al., 1995). Like diltiazem, it has not been demonstrated such an inhibitory effect of phenytoin on NO production (Nagatomo et al., 2000). On the other hand, it was

suggested that CsA could increase gingival iNOS expression (Gau et al., 2005).

The results of the present study demonstrated a significant difference in salivary nitrite and nitrate levels between responders and non-responders in phenytoin group. However, nitrite and nitrate levels in saliva did not differ significantly between responders and non-responders in other drug groups. Similarly, nitrite and nitrate levels in GCF and plasma were not significantly different between subgroups in all study groups.

Although, there have been many studies evaluating the nitrite and nitrate levels of biological fluids as a diagnostic biomarker in oral diseases and periodontitis, only few of them have focused on NO contribution to drug-induced gingival overgrowth. Fu et al. (2000) reported that significantly decreased dimensions of gingival tissues from CsA fed rats receiving L-arginine (NO substrate) or L-NAME (NO blocker) compared to control rats. They also found that plasma nitrite/nitrate concentrations were higher in L-arginine supplement. In another study conducted on rats, it was reported that significantly greater iNOS enzyme activities in gingival tissues obtained from CsA-treated rats than from control rats (Gau et al., 2005). Similarly, the plasma nitrite/nitrate levels were higher in CsA-treated rats than those in control group. In these two studies conducted on rats, nitrite and nitrate were reduced to NO and depicted as total nitrite and nitrate concentration, however nitrite and nitrate concentrations were not reported separately in studied groups. In contrast to the results of these studies, we did not found any significant differences in plasma nitrite and nitrate concentrations between responders and non-responders in all drug groups. In a clinical study, iNOS production significantly increased in connective tissue from gingivitis and CsA-induced gingival overgrowth groups (Gürkan et al., 2009). However, no intergroup differences were found regarding nitrite/nitrate levels in GCF. It was suggested that the reason of this situation might be related to the higher dilution of nitrite and nitrate by GCF volume, which was clearly elevated in responders than non-responders. Another reason might be environmental differences in oral cavity such as composition of oral flora. In the present study, similar to these results, we did not observe any significant difference in nitrite and nitrate levels of GCF between responders and non-responders in all groups.

In the present study, both nitrite and nitrate levels in saliva were found to be significantly increased in responders in phenytoin group. However, no significant differences in salivary nitrite and nitrate levels were observed between responders and non-responders in other drug groups. To the results of a clinical study comparing the NO levels in saliva with the severity of chronic periodontitis, NO levels in saliva increased with severity of chronic periodontitis (Reher et al., 2007). On contrast to these results, Topcu et al. (2014) suggested that nitrite in GCF is a better periodontal disease marker than nitrate in GCF and those in saliva. In another study of the same research group, a tendency to increase in nitrite levels in GCF and peri-implant sulcular fluid with the presence of gingival/peri-implant inflammation was reported (Tözüm et al., 2007).

When the correlations between nitrite and nitrate levels in GCF, saliva, and plasma, periodontal clinical parameters, GCF volume and severity of GO are taken into account, the nitrite level in saliva was found to be significantly correlated to PD and GBTI scores, severity of GO and GCF volume. However, nitrate levels in saliva significantly correlated only to PD score and plasma nitrate level. On the contrary, no significant correlation was found between periodontal clinical parameters, severity of GO and nitrite/nitrate levels in plasma and GCF. Based on these results, it can be suggested that nitrite and nitrate levels in saliva, especially nitrite levels, are more powerful biomarkers than those in plasma and GCF for evaluating drug-induced gingival overgrowth risk. Similar to our results, in a clinical study evaluating the diagnostic role of salivary and GCF nitrite, nitrate and nitric oxide to distinguish healthy periodontium from gingivitis and periodontitis, it was reported that detecting NO biomarker and its end metabolites in saliva is of more value to assess the periodontal health comparing to GCF (Poorsattar Bejeh-Mir et al., 2014). We also found a significant positive correlation between nitrate levels in saliva and plasma, as previously shown before (Clodfelter et al., 2015). The positive correlation between salivary nitrate and plasma nitrate might be explained by the nitrate–nitrite–NO cycle (Lundberg et al., 2008). Ingested nitrate enters the plasma and is taken back up from the circulation by the salivary glands (Lundberg and Govoni, 2004; Lundberg et al., 2008, 2009). Thus, nitrate levels in plasma and saliva are interrelated.

Nitrite and nitrate levels in biological fluids such as plasma, GCF, and saliva might be useful for diagnosis and monitoring of many diseases, including periodontal disease. However, nitrite and nitrate levels in these biological fluids do not always demonstrate the activity and expression of NOS in tissues. It is well-known that NO is produced from L-arginine by NOS in cells (Jenkins et al., 1995). On the other hand, cigarette smoking, oral medicines and supplements that contain nitro-substances, and foods such as spinach, beets, and other green leafy vegetables are exogenous sources of systemic NO (Jobgen et al., 2007). Thus, it is not appropriate to measure nitrite and nitrate levels in biological fluids as an indicator of NO synthesis when these factors are not taken into account.

The most effective treatment of drug-induced gingival overgrowth is substitution of these medications by the patient's physician. The resolution of gingival lesions may take from 1 to 8 weeks depending on the severity of overgrowth (Khocht and Schneider, 1997). However, not all patients respond to this treatment modality, especially those with long-standing gingival overgrowth (Harel-Raviv et al., 1995). Thus, surgical intervention is commonly required to solve the aesthetic and functional problems caused by gingival overgrowth.

CONCLUSION

Within the limitations of the present study, nitrite and nitrate levels in saliva could be used as periodontal disease biomarkers in phenytoin-induced gingival overgrowth. And also, saliva has a better diagnostic potential than GCF and plasma for the evaluation of drug-induced gingival overgrowth risk. However, when all drug groups were considered, salivary nitrite and nitrate levels could not be used as a biomarker for drug-induced gingival

overgrowth. Additionally, present interpretations require caution due to the limited number of subjects evaluated in drug groups. Further studies including higher subjects will be needed to clarify the use of salivary nitrite/nitrate levels as a biomarker for drug-induced gingival overgrowth.

ACKNOWLEDGMENTS

This study was supported by Hacettepe University, Scientific Research Projects Coordination Unit, Ankara, Turkey (Grant number: 07 01 201 001).

REFERENCES

Batista, A. C., Silva, T. A., Chun, J. H., and Lara, V. S. (2002). Nitric oxide synthesis and severity of human periodontal disease. *Oral Dis.* 8, 254–260. doi: 10.1034/j.1601-0825.2002.02852.x

Boltchi, F. E., Rees, T. D., and Iacopino, A. M. (1999). Cyclosporine A-induced gingival overgrowth: a comprehensive review. *Quintessence Int.* 30, 775–783.

Chae, H. J., Ha, M. S., Yun, D. H., Pae, H. O., Chung, H. T., Chae, S. W., et al. (2006). Mechanism of cyclosporine-induced overgrowth in gingiva. *J. Dent. Res.* 85, 515–519. doi: 10.1177/154405910608500607

Clodfelter, W. H., Basu, S., Bolden, C., Dos Santos, P. C., King, S. B., and Kim-Shapiro, D. B. (2015). The relationship between plasma and salivary NOx. *Nitric Oxide* 47, 85–90. doi: 10.1016/j.niox.2015.04.003

Ellis, J. S., Seymour, R. A., Steele, J. G., Robertson, P., Butler, T. J., and Thomason, J. M. (1999). Prevalence of gingival overgrowth induced by calcium channel blockers: a community-based study. *J. Periodontol.* 70, 63–67. doi: 10.1902/jop.1999.70.1.63

Forstermann, U., Closs, E. I., Pollock, J. S., Nakane, M., Schwarz, P., Gath, I., et al. (1994). Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* 23, 1121–1131. doi: 10.1161/01.HYP.23.6.1121

Francis, C. A., Hector, M. P., and Proctor, G. B. (2001). Levels of pre-kallikrein in resting and stimulated human parotid and submandibular saliva. *Eur. J. Oral Sci.* 109, 365–368. doi: 10.1034/j.1600-0722.2001.00091.x

Fu, E., Tz-Chong, C., Liu, D., and Chiu, S. C. (2000). Ameliorated effect of L-arginine supplementation on gingival morphology in cyclosporin-treated rats. *J. Periodontol.* 71, 1737–1742. doi: 10.1902/jop.2000.71.11.1737

Gau, C. H., Chou, T. C., Chiu, H. C., Shen, E. C., Nieh, S., Chiang, C. Y., et al. (2005). Effect of cyclosporin A on the expression of inducible nitric oxide synthase in the gingiva of rats. *J. Periodontol.* 76, 2260–2266. doi: 10.1902/jop.2005.76.12.2260

Grisham, M. B., Johnson, G. G., and Lancaster, J. R. Jr. (1996). Quantitation of nitrate and nitrite in extracellular fluids. *Meth. Enzymol.* 268, 237–246. doi: 10.1016/S0076-6879(96)68026-4

Güncü, G. N., Caglayan, F., Dinçel, A., Bozkurt, A., Ozmen, S., and Karabulut, E. (2007). Clinical and pharmacological variables as a risk factor for nifedipine-induced gingival overgrowth. *Aust. Dent. J.* 52, 295–299. doi: 10.1111/j.1834-7819.2007.tb00505.x

Güncü, G. N., Caglayan, F., Dinçel, A., Bozkurt, A., Saygi, S., and Karabulut, E. (2006). Plasma and gingival crevicular fluid phenytoin concentrations as risk factors for gingival overgrowth. *J. Periodontol.* 77, 2005–2010. doi: 10.1902/jop.2006.060103

Gürkan, A., Emingil, G., Oktem, G., Selvi, N., Afacan, B., Tunç, I., et al. (2009). Immunohistochemical analysis of inducible and endothelial forms of nitric oxide synthase in cyclosporin A-induced gingival overgrowth. *J. Periodontol.* 80, 1638–1647. doi: 10.1902/jop.2009.090138

Harel-Raviv, M., Eckler, M., Lalani, K., Raviv, E., and Gornitsky, M. (1995). Nifedipine-induced gingival hyperplasia. A comprehensive review and analysis. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 79, 715–722. doi: 10.1016/S1079-2104(05)80306-3

Hattori, Y., Kasai, K., So, S., Hattori, S., Banba, N., and Shimoda, S. (1995). Effects of calcium channel antagonists on the induction of nitric oxide synthase in cultured cells by immunostimulants. *Life Sci.* 57, 1833–1840. doi: 10.1016/0024-3205(95)02163-D

Hefti, A. F., Eshenaur, A. E., Hassell, T. M., and Stone, C. (1994). Gingival overgrowth in cyclosporine A treated multiple sclerosis patients. *J. Periodontol.* 65, 744–749. doi: 10.1902/jop.1994.65.8.744

Hsu, Y. C., Hsiao, M., Wang, L. F., Chien, Y. W., and Lee, W. R. (2006). Nitric oxide produced by iNOS is associated with collagen synthesis in keloid scar formation. *Nitric Oxide* 14, 327–334. doi: 10.1016/j.niox.2006.01.006

Jenkins, D. C., Charles, I. G., Thomsen, L. L., Moss, D. W., Holmes, L. S., Baylis, S. A., et al. (1995). Roles of nitric oxide in tumor growth. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4392–4396. doi: 10.1073/pnas.92.10.4392

Jobgen, W. S., Jobgen, S. C., Li, H., Meininger, C. J., and Wu, G. (2007). Analysis of nitrite and nitrate in biological samples using high-performance liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 851, 71–82. doi: 10.1016/j.jchromb.2006.07.018

Kantarci, A., Black, S. A., Xydas, C. E., Murawel, P., Uchida, Y., Yucekal-Tuncer, B., et al. (2006). Epithelial and connective tissue cell CTGF/CCN2 expression in gingival fibrosis. *J. Pathol.* 210, 59–66. doi: 10.1002/path.2000

Kendall, H. K., Marshall, R. I., and Bartold, P. M. (2001). Nitric oxide and tissue destruction. *Oral Dis.* 7, 2–10. doi: 10.1034/j.1601-0825.2001.70102.x

Khocht, A., and Schneider, L. C. (1997). Periodontal management of gingival overgrowth in the heart transplant patient: a case report. *J. Periodontol.* 68, 1140–1146. doi: 10.1902/jop.1997.68.11.1140

Kleinbongard, P., Dejam, A., Lauer, T., Rassaf, T., Schindler, A., Picker, O., et al. (2003). Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic. Biol. Med.* 35, 790–796. doi: 10.1016/S0891-5849(03)00406-4

Kröncke, K. D., Fehsel, K., and Kolb-Bachofen, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection–how, why, when, and where? *Nitric Oxide* 1, 107–120. doi: 10.1006/niox.1997.0118

Lappin, D. F., Kjeldsen, M., Sander, L., and Kinane, D. F. (2000). Inducible nitric oxide synthase expression in periodontitis. *J. Periodont. Res.* 35, 369–373. doi: 10.1034/j.1600-0765.2000.035006369.x

Lashley, K. S. (1916). Reflex secretion of the human parotid gland. *Exp. Psych.* 1, 461–493. doi: 10.1037/h0073282

Löe, H. (1967). The Gingival Index, the Plaque Index and the Retention Index Systems. *J. Periodontol.* 38(Suppl.), 610–616. doi: 10.1902/jop.1967.38.6_part2.610

Lundberg, J. O., Gladwin, M. T., Ahluwalia, A., Benjamin, N., Bryan, N. S., Butler, A., et al. (2009). Nitrate and nitrite in biology, nutrition and therapeutics. *Nat. Chem. Biol.* 5, 865–869. doi: 10.1038/nchembio.260

Lundberg, J. O., and Govoni, M. (2004). Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic. Biol. Med.* 37, 395–400. doi: 10.1016/j.freeradbiomed.2004.04.027

Lundberg, J. O., Weitzberg, E., and Gladwin, M. T. (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* 7, 156–167. doi: 10.1038/nrd2466

Lyons, C. R. (1995). The role of nitric oxide in inflammation. *Adv. Immunol.* 60, 323–371. doi: 10.1016/S0065-2776(08)60589-1

Marshall, R. I., and Bartold, P. M. (1999). A clinical review of drug-induced gingival overgrowths. *Aust. Dent. J.* 44, 219–232. doi: 10.1111/j.1834-7819.1999.tb00224.x

Miller, C. S., and Damm, D. D. (1992). Incidence of verapamil-induced gingival hyperplasia in a dental population. *J. Periodontol.* 63, 453–456. doi: 10.1902/jop.1992.63.5.453

Miranda, J., Brunet, L., Roset, P., Berini, L., Farré, M., and Mendieta, C. (2001). Prevalence and risk of gingival enlargement in patients treated with nifedipine. *J. Periodontol.* 72, 605–611. doi: 10.1902/jop.2001.72.5.605

Moshage, H., Kok, B., Huizenga, J. R., and Jansen, P. L. (1995). Nitrite and nitrate determinations in plasma: a critical evaluation. *Clin. Chem.* 41, 892–896.

Nagatomo, I., Akasaki, Y., Uchida, M., Tominaga, M., Hashiguchi, W., and Takigawa, M. (2000). Effects of combined administration of zonisamide and valproic acid or phenytoin to nitric oxide production, monoamines, and

zonisamide concentrations in the brain of seizure-susceptible EL mice. *Brain Res. Bull.* 53, 211–218. doi: 10.1016/S0361-9230(00)00333-6

Nowicki, D., Vogel, R. I., Melcer, S., and Deasy, M. J. (1981). The gingival bleeding time index. *J. Periodontol.* 52, 260–262. doi: 10.1902/jop.1981.52.5.260

Ohashi, M., Iwase, M., and Nagumo, M. (1999). Elevated production of salivary nitric oxide in oral mucosal diseases. *J. Oral Pathol. Med.* 28, 355–359. doi: 10.1111/j.1600-0714.1999.tb02053.x

Poorsattar Bejeh-Mir, A., Parsian, H., Akbari Khoram, M., Ghasemi, N., Bijani, A., and Khosravi-Samani, M. (2014). Diagnostic role of salivary and GCF nitrite, nitrate and nitric oxide to distinguish healthy periodontium from gingivitis and periodontitis. *Int. J. Mol. Cell. Med.* 3, 138–145.

Reher, V. G., Zenóbio, E. G., Costa, F. O., Reher, P., and Soares, R. V. (2007). Nitric oxide levels in saliva increase with severity of chronic periodontitis. *J. Oral Sci.* 49, 271–276. doi: 10.2334/josnusd.49.271

Rezaie, S., Rezaie, A., Minaiee, B., Khorasani, R., and Abdollahi, M. (2005). On the relation of nitric oxide to nifedipine-induced gingival hyperplasia and impaired submandibular glands function in rats *in vivo*. *Fundam. Clin. Pharmacol.* 19, 65–71. doi: 10.1111/j.1472-8206.2004.00313.x

Rüdin, H. J., Overdiek, H. F., and Rateitschak, K. H. (1970). Correlation between sulcus fluid rate and clinical and histological inflammation of the marginal gingiva. *Helv. Odontol. Acta* 14, 21–26.

Seymour, R. A., Ellis, J. S., and Thomason, J. M. (2000). Risk factors for drug-induced gingival overgrowth. *J. Clin. Periodontol.* 27, 217–223. doi: 10.1034/j.1600-051x.2000.027004217.x

Seymour, R. A., and Smith, D. G. (1991). The effect of a plaque control programme on the incidence and severity of cyclosporin-induced gingival changes. *J. Clin. Periodontol.* 18, 107–110. doi: 10.1111/j.1600-051X.1991.tb01698.x

Seymour, R. A., Thomason, J. M., and Ellis, J. S. (1996). The pathogenesis of drug-induced gingival overgrowth. *J. Clin. Periodontol.* 23, 165–175. doi: 10.1111/j.1600-051X.1996.tb02072.x

Somacarrera, M. L., Hernandez, G., Acero, J., and Moskow, B. S. (1994). Factors related to the incidence and severity of cyclosporin-induced gingival overgrowth in transplant patients. A longitudinal study. *J. Periodontol.* 65, 671–675. doi: 10.1902/jop.1994.65.7.671

Tavassoli, S., Yamalik, N., Caglayan, F., Caglayan, G., and Eratalay, K. (1998). The clinical effects of nifedipine on periodontal status. *J. Periodontol.* 69, 108–112. doi: 10.1902/jop.1998.69.2.108

Thomason, J. M., Ellis, J. S., Jovanovski, V., Corson, M., Lynch, E., and Seymour, R. A. (2005). Analysis of changes in gingival contour from three-dimensional co-ordinate data in subjects with drug-induced gingival overgrowth. *J. Clin. Periodontol.* 32, 1069–1075. doi: 10.1111/j.1600-051X.2005.00769.x

Topcu Al. O., Akalin, F. A., Sahbazoglu, K. B., Yamalik, N., Kilinc, K., Karabulut, E., et al. (2014). Nitrite and nitrate levels of gingival crevicular fluid and saliva in subjects with gingivitis and chronic periodontitis. *J. Oral Maxillofac. Res.* 5:e5. doi: 10.5037/jomr.2014.5205

Tözüm, T. F., Akman, A. C., Yamalik, N., Tulunoglu, I., Turkyilmaz, I., Karabulut, E., et al. (2007). Analysis of the inflammatory process around endosseous dental implants and natural teeth: myeloperoxidase level and nitric oxide metabolism. *Int. J. Oral Maxillofac. Implants* 22, 969–979.

Trackman, P. C., and Kantarci, A. (2004). Connective tissue metabolism and gingival overgrowth. *Crit. Rev. Oral Biol. Med.* 15, 165–175. doi: 10.1177/154411130401500305

Ugar-Cankal, D., and Ozmeric, N. (2006). A multifaceted molecule, nitric oxide in oral and periodontal diseases. *Clin. Chim. Acta* 366, 90–100. doi: 10.1016/j.cca.2005.10.018

Uzar, E., Evliyaoglu, O., Toprak, G., Acar, A., Yucel, Y., Calisir, T., et al. (2011). Increased asymmetric dimethylarginine and nitric oxide levels in patients with migraine. *J. Headache Pain* 12, 239–243. doi: 10.1007/s10194-011-0323-7

Wang, R., Ghahary, A., Shen, Y. J., Scott, P. G., and Tredget, E. E. (1996). Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms. *J. Invest. Dermatol.* 106, 419–427. doi: 10.1111/1523-1747.ep12343428

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 24 December 2015
doi: 10.3389/fcimb.2015.00099

Salivary Antimicrobial Peptides in Early Detection of Periodontitis

Güliz N. Güncü[1], Dogukan Yilmaz[2], Eija Könönen[3,4] and Ulvi K. Gürsoy[3]*

[1] Faculty of Dentistry, University of Hacettepe, Ankara, Turkey, [2] Faculty of Dentistry, University of Istanbul Medipol, Istanbul, Turkey, [3] Periodontology, Institute of Dentistry, University of Turku, Turku, Finland, [4] Welfare Division, Oral Health Care, Turku, Finland

In the pathogenesis of periodontitis, an infection-induced inflammatory disease of the tooth-supporting tissues, there is a complex interaction between the subgingival microbiota and host tissues. A periodontal diagnostic tool for detecting the initiation and progression of the disease, monitoring the response to therapy, or measuring the degree of susceptibility to future disease progression has been of interest for a long time. The value of various enzymes, proteins, and immunoglobulins, which are abundant constituents of saliva, as potential biomarkers has been recognized and extensively investigated for periodontal diseases. Gingival defensins and cathelicidins are small cationic antimicrobial peptides that play an important role in innate immune response. However, their applicability as salivary biomarkers is still under debate. The present review focuses on proteomic biomarkers and antimicrobial peptides, in particular, to be used at early phases of periodontitis.

Keywords: saliva, biomarker, periodontitis, antimicrobial peptides

OPEN ACCESS

Edited by:
Richard Lamont,
University of Louisville, USA

Reviewed by:
Gill Diamond,
University of Florida, USA
Jill L. Maron,
Tufts Medical Center, USA

****Correspondence:***
Güliz N. Güncü
guliz@hacettepe.edu.tr

Received: *16 September 2015*
Accepted: *11 December 2015*
Published: *24 December 2015*

Citation:
Güncü GN, Yilmaz D, Könönen E and Gürsoy UK (2015) Salivary Antimicrobial Peptides in Early Detection of Periodontitis. Front. Cell. Infect. Microbiol. 5:99. doi: 10.3389/fcimb.2015.00099

PERIODONTITIS: AN ORCHESTRAL MASTERPIECE

Periodontitis, an infection-induced inflammatory disease of the tooth-supporting tissues, is initiated by the formation of pathogenic biofilms at and under the gingival margin. Out of more than 700 resident bacterial species of the oral cavity, about a half can be found in subgingival biofilms in both healthy and diseased sites (Teles et al., 2006). Periodontitis-associated pathogens and their toxins in bacterial biofilms perturb gingival epithelial cells triggering a sequence of inflammatory and immune responses. The initial inflammatory response aims to limit bacterial invasion by promoting the infiltration of neutrophils and macrophages to the site of bacterial challenge. This infiltration is achieved by the secretion of proinflammatory cytokines and chemokines from gingival epithelial cells and fibroblasts (Preshaw and Taylor, 2011). After their migration to inflamed tissues, leukocytes suppress bacterial invasion by their oxygen dependent or independent mechanisms. Subsequently, T- and B-cells emerge to the site of infection and secrete immunoglobulins as an antigen-specific response. If the host defense fails to suppress the level of infection by eliminating pathogens, continuing inflammation finally ends up in alveolar bone destruction (Bartold and Narayanan, 2006; Preshaw and Taylor, 2011). In the course of the disease, a number of matrix metalloproteases (MMPs), MMP-8, MMP-9, and MMP-13, in particular, are produced and activated by host cells in a cascade leading to degradation of gingival tissues and alveolar bone (Sorsa et al., 2004). Osteoclastogenesis is connected to an increased expression of Receptor Activator for Nuclear Factor κB Ligand (RANKL) and a decreased expression of osteoprotegerin in osteoblast cells. A consensus report of the 7th European Workshop on Periodontology recently highlighted interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α,

and RANKL as important players in the periodontitis network (Kinane et al., 2011). Therefore, it does not seem an exaggeration to argue that rather than the bacterial infection, the magnitude of the inflammatory response raised against pathogens seems to be the determinant for developing a destructive periodontal disease (Page and Kornman, 1997; Van Dyke, 2007; Silva et al., 2008). Without an adequate therapy, chronic inflammation may result in the destruction in the attachment between the tooth and the gingival tissue, formation of periodontal pockets, alveolar bone loss and, eventually, tooth loss (Darveau, 2010). Yet, this complex interplay between the subgingival microbiota and host tissues is not the same for everyone; several factors, such as smoking, age, systemic disease, and genetic susceptibility, modify the formation and progression of periodontal diseases (Marsh et al., 2011).

SALIVA AS A DIAGNOSTIC FLUID

In general, dental clinicians are looking for a diagnostic tool, preferably a non-invasive one, to determine the current status of periodontal disease, to monitor the response to therapy, and to measure the degree of susceptibility to future disease progression (Giannobile et al., 2009). Conventional diagnostic tools, such as probing pocket depths, bleeding on probing, and clinical attachment level, are inadequate to identify patients who are at risk for disease progression (Goodson, 1992). Saliva has a major importance in the maintenance of oral health, and, during the past two decades, it has been considered a potential specimen to detect oral and systemic diseases (Ji and Choi, 2015). Saliva as a diagnostic fluid has been evaluated for detecting caries (Bratthall and Hänsel Petersson, 2005), periodontitis (Christodoulides et al., 2007; Gursoy et al., 2011), oral and breast cancer (Li et al., 2004; Streckfus and Bigler, 2005), and hepatitis (Ohnishi and Daikuhara, 2003).

With the development of different—omics technologies, analysis of saliva has become particularly fascinating, not only in dentistry but also in general medicine (Zhang et al., 2009; Cuevas-Córdoba and Santiago-García, 2014). By analyzing an array of constituents present in saliva, it is possible to estimate the risk of disease onset, to monitor disease progression, and to evaluate therapeutic efficacy of oral infections as well as oropharyngeal lesions (Zhang et al., 2009).

SALIVARY BIOMARKERS OF PERIODONTITIS: WHAT HAVE BEEN FOUND SO FAR?

Specific biomarkers have been identified from saliva, reflecting the three key features of pathogenic processes in periodontal disease, i.e., infection-induced inflammation, collagen degradation, and bone turnover (Zhang et al., 2009). Host- and bacteria-derived enzymes, proteins, and other inflammatory mediators appear to hold great promise as salivary biomarkers for the diagnosis of periodontal disease (Giannobile et al., 2009). In infected periodontal tissues, numerous cytokines are secreted as part of the innate response by resident cells (epithelial cells, fibroblasts) and neutrophils. There is strong evidence to suggest that salivary IL-1β is a relevant biomarker of periodontitis, while no significant association has been found between salivary TNF-α or IL-6 levels and the presence of periodontitis (Gursoy et al., 2009; Ebersole et al., 2013; Taylor, 2014). Host-derived MMPs are considered initiators of the extracellular matrix degradation associated with periodontitis (Sorsa et al., 1990; Uitto et al., 1990). Especially MMP-8, a neutrophil collagenase, has the potential to be used as a biomarker of periodontal destruction. In a number of studies, it has been shown that MMP-8 activity is elevated in saliva of periodontitis patients as compared to their periodontally healthy controls, regardless of study subjects' smoking status (Sorsa et al., 2004; Miller et al., 2006; Gursoy et al., 2010).

ANTIMICROBIAL PEPTIDES: FUNCTIONS AND INTERACTIONS IN PERIODONTAL TISSUES

The oral cavity with its various surfaces offers an open environment, which allows a constant exposure of microorganisms to be colonized and, in favorable circumstances, this can lead to disease (Diamond et al., 2008). Bacterial infection in the mouth is resisted by the stratified squamous epithelium, which acts as a mechanical barrier, and saliva provides a mechanical rinsing action (Gorr, 2012). The oral epithelium and saliva are the most central defense systems in the mouth. These two defense systems do not act as passive protection mechanisms, but both of them contain several types of antimicrobial peptides, including histatins, defensins, and hCAP18/LL-37 (De Smet and Contreras, 2005). Antimicrobial peptides are small cationic peptides with a broad spectrum of antimicrobial activity. A complex mixture of over 45 antimicrobial proteins and peptides are found in oral fluids (Denny et al., 2008); of these, 13 are up-regulated in periodontal disease, while 11 are down-regulated. Defensins and hCAP18/LL-37 belong to key components of the mucosal antimicrobial defense (Hosokawa et al., 2006; Gorr, 2012).

Defensins are divided into subfamilies of α- and β-defensins (**Table 1**). These defensins differ in their cysteine motifs, but share a similar secondary structure, and both of them are rich in cationic residues (Beckloff and Diamond, 2008). Four types of α-defensins [Human Neutrophilic Peptide (HNP) 1-4] are found predominantly in neutrophils (Ganz et al., 1985), whereas human β-defensins (hBDs) are mainly produced by epithelial cells (Gursoy and Könönen, 2012). The number of hBDs has been suggested to be over 20 based on genomic targeting (Lehrer, 2011). Of the four hBDs known so far, hBD 1-3 are expressed and secreted in the human oral cavity (Dale and Krisanaprakornkit, 2001; Vardar-Sengul et al., 2007). The sole human cathelicidin, hCAP18/LL-37, was initially identified in infiltrating neutrophils in the oral cavity (Dale and Krisanaprakornkit, 2001). However, it has been observed in the salivary glands and gingival epithelium as well (Woo et al., 2003; Gursoy et al., 2012).

Antimicrobial peptides play a major role in the innate host defense. HNPs, hCAP18/LL-37, and hBDs exhibit a broad-spectrum antimicrobial activity against Gram-positive

TABLE 1 | Comparisons between human β-defensins (hBDs) and human α-defensins (human neutrophil peptides, HNPs).

	Expressed and secreted by	Associations with common oral diseases	Activation in periodontal tissues	Regulatory effects on other host-cells and tissues
Human β-defensins (hBDs)	Epithelial cells	Caries and periodontitis, oral cancers	hBD-1 is secreted constitutively, while infection and inflammation influence the secretions of hBD-2 and hBD-3	Chemoattraction of dendritic and T cells, macrophages; wound healing in epithelium
Human α-defensins (HNPs)	Neutrophils	Periodontitis, oral cancers	Synthesized in promyelocytes and myelocytes as proHNPs and stored as mature HNPs in azurophil granules before they reach to periodontal tissues	Epithelial cell viability, adhesion, spread

and -negative bacteria, fungi, and enveloped viruses (Gomes Pde and Fernandes, 2010). Like HNPs, hBDs are considered to exert their antibacterial effect by permeabilizing the bacterial cellular membrane. In addition to their direct antimicrobial activity, both HNPs and hBDs exhibit numerous other biological activities (Dommisch and Jepsen, 2015). HNP 1-3 and hBD 1-3 have a selective chemotactic activity for a variety of host defense cells like immature dendritic cells and mast cells (Soruri et al., 2007). They function as both proinflammatory and anti-inflammatory agents in the periodontal disease pathogenesis (Bowdish et al., 2006). Besides their antimicrobial and immune regulatory functions, hBDs contribute to the healing process of wounds (Niyonsaba et al., 2007) and HNP-1 regulates epithelial cell adhesion and spread (Gursoy et al., 2013). While initially isolated as an antimicrobial peptide, hCAP18/LL-37 has been proposed to play additional roles in inflammation. hCAP18/LL-37 demonstrates a chemotactic activity for neutrophils, monocytes, and some T-cells. Furthermore, hCAP18/LL-37 affects dendritic cell maturation (Kai-Larsen and Agerberth, 2008). Taken together, these multiple activities of antimicrobial peptides suggest that they play an important, multifunctional role in host defense (Gorr and Abdolhosseini, 2011).

It is considered that HNPs, hBDs, and hCAP18/LL-37 have the same function in health and disease, but in a coordinated manner. Periodontal infection and inflammation is thought to affect the expression of each antimicrobial peptide (Gursoy and Könönen, 2012). Up-regulated expression of hBDs has been demonstrated in infections, inflammatory stimulations, and keratinocyte differentiation. In non-inflamed gingival tissues, both hBD-1 and hBD-2 are expressed; their levels are highest at gingival margin close to dental plaque (Yilmaz et al., 2015). During the inflammatory state, these peptides are expressed also in the sulcular epithelium (Dale and Krisanaprakornkit, 2001). The expression of hBD-3 is primarily located in the basal layer of healthy gingival tissues, but it is extended toward superficial layers of the gingival epithelium in periodontitis (Lu et al., 2005; Yilmaz et al., 2015). Although a number of studies have described expression levels and localizations of hCAP18/LL-37, hBDs, and HNPs in healthy and inflamed gingival tissues (Krisanaprakornkit et al., 2000; Dale and Krisanaprakornkit, 2001; Dommisch et al., 2005; Hosokawa et al., 2006; Kuula et al., 2008; Brancatisano et al., 2011; Yilmaz et al., 2015), their relation to the initiation and progression of periodontal disease is still poorly understood.

INTERACTIONS BETWEEN SALIVA AND ANTIMICROBIAL PEPTIDES: ROAD TO BIOMARKERS

The main sources of antimicrobial peptides in the oral cavity are the gingival epithelium and neutrophils, although salivary glands also secrete some amounts of defensins and hCAP18/LL-37 (Mathews et al., 1999; Mizukawa et al., 1999). By using high performance liquid chromatography, it has been demonstrated that healthy adults have a mean value of 0.5–0.9 mg/ml HNP-1 in whole saliva (Goebel et al., 2000). As a large number of neutrophils continuously enter the oral cavity through the junctional epithelium, it is possible that HNPs are mainly derived from these neutrophils. Immunohistochemical staining for HNP 1-3 showed the presence of peptides in parts of the ductal cells in submandibular glands and in minor salivary glands, while no HNP was detected in an individual major salivary gland (Mizukawa et al., 1999). This indicates that ductal cells can be a source of HNPs in saliva (Abiko and Saitoh, 2007). The sources of hBDs in saliva are assumed to be the oral epithelium and salivary glands. hBD-1, -2, and -3 mRNA have been detected in salivary glands, including the parotid, submandibular, and minor glands, as well as the oral epithelium (Bonass et al., 1999; Dunsche et al., 2001). The mean concentrations of hBD-1 and -2 in whole saliva of healthy subjects are around 150 ng/ml (Mathews et al., 1999), while that of hBD-3 is about 730 ng/ml (Abiko and Saitoh, 2007). In humans, hCAP18/LL-37 is mainly secreted by neutrophils and it is present in saliva at concentrations of 0.14–3 μg/ml (Gorr, 2009).

Saliva carries a significant amount of antimicrobial peptides as part of its defense mechanism but also impairs their antimicrobial functions. As an example, saliva can reduce the antibacterial activities of hBD 1-3 and hCAP18/LL-37 by 20–50% in *in vitro* conditions (Mineshiba et al., 2003). This *in vitro* effect is generally explained by the salt concentration of saliva. However, this is probably unlikely because of the low salt concentrations in saliva. Moreover, hBD activity in saliva may get affected by proteases and redox enzymes. On the one hand, proteases, at least in *in vitro* conditions, affect the activity and concentration of

antimicrobial peptides (Kuula et al., 2008), thereby may reduce their value to be used as salivary biomarkers of periodontal disease. On the other hand, defensins are reduced by thioredoxin reductases to their active forms. For instance, glutaredoxin can reduce hBD-1 to its antibacterial form (Jaeger et al., 2013). The activation or inactivation by other proteins in saliva can have a significant effect on the use of antimicrobial peptides as biomarkers, since a selected method for analysis may detect only one form of the peptide, depending on the antibody chosen. Therefore, interactions of antimicrobial peptides with other proteins in saliva should be thoroughly analyzed (Wilson et al., 1999).

ANTIMICROBIAL PEPTIDES AS SALIVARY BIOMARKERS: HOW MUCH EVIDENCE DO WE HAVE?

Although, the levels of single markers in saliva can be statistically distinguished between subjects with and without periodontitis, the large variation in their values between individuals make a prospective assignment difficult (Miller et al., 2010). Antimicrobial peptides are typically expressed in response to oral bacteria or bacterial toxins, which makes them suitable biomarkers for the diagnosis of periodontal disease (Gorr, 2009; Gorr and Abdolhosseini, 2011). Information on the association between salivary antimicrobial peptide concentrations and periodontal disease status is limited. Pereira et al. (2013) studied salivary levels of hBD-2 in 31 chronic periodontitis and 27 gingivitis patients, compared to 31 periodontally healthy controls, and detected elevated hBD-2 levels in chronic periodontitis patients. No relationship between the frequency of examined periodontopathogens and hBD-2 protein concentrations was found. Salazar et al. (2013) examined 20 periodontally healthy and 20 diseased subjects to identify periodontitis-associated changes in the proteome of the whole saliva. Twenty proteins, including HNP-1, were elevated in periodontitis patients in comparison to their controls (Salazar et al., 2013). It is important to note that peptide concentrations can be significantly diluted in saliva and, therefore, much lower than those in periodontal pockets and gingival tissues (Gorr, 2012). Salivary LL-37 concentrations have been demonstrated to correlate to periodontal tissue destruction in subjects with chronic periodontitis (Takeuchi et al., 2012).

Advances in genomic technologies offer hitherto unprecedented observations on complex human diseases. To date, however, there is only one study by Jaradat et al. (2013) where associations between the genomic copy number of hBD-2 and periodontitis are evaluated. According to their results, there is an association between decreased hBD-2 genomic copy numbers and severity periodontitis. With increasing information, it may be possible to avoid some of the limitations that currently exist in the use of gingival defensins as biomarkers of periodontitis. Moreover, the outcomes of genomic research would help in understanding clinically distinct diseases, for example Crohn's disease, and periodontitis, with a view on their shared molecular targets, such as hBD-2 (Keskin et al., 2015).

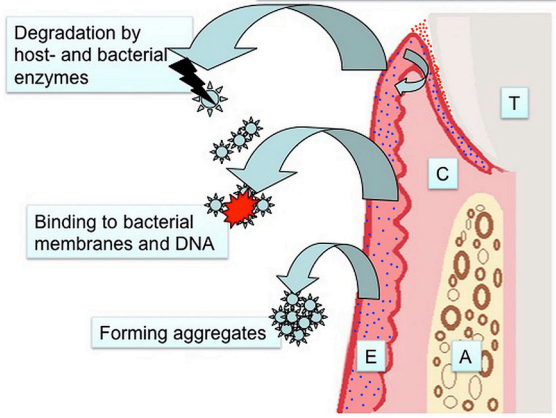


FIGURE 1 | Limitations in the use of antimicrobial defensins as salivary biomarkers of periodontitis (T, Tooth; C, Connective Tissue; A, Alveolar Bone; E, Epithelium; red dots, α-defensins; blue dots, β-defensins).

THINGS TO CONSIDER

In this review, we evaluated the evidence on salivary antimicrobial peptides as biomarkers of periodontitis. These small peptides form the initial tissue response against infection and thus could function as an early diagnostic marker of periodontitis. However, in the use of antimicrobial peptides as biomarkers of periodontitis there are significant limitations to consider, and the majority of these limitations are not fully characterized (**Figure 1**). Firstly, antimicrobial peptides can aggregate in a concentration dependent manner (Brogden, 2005), and this may weaken the sensitivity of test methods, such as an enzyme-linked immunoassay (ELISA). It is also possible that host-derived and bacterial enzymes degrade antimicrobial peptides, decreasing the sensitivity of the methods depending on the antibody of choice. Further, binding to bacterial lipopolysaccharides and DNA may force salivary antimicrobials to accumulate in the pellet at the initial centrifugation of the sample. Finally, some antimicrobial peptides do not get out of the tissue, but accumulate in the cell cytoplasm and cell nucleus as recently described by Yilmaz et al. (2015). They may still be protective against invading bacteria, however, their accumulation tendency affects their concentrations in saliva that will not reflect the degree of inflammation.

CONCLUSION

Despite the limitations, it can be concluded that salivary antimicrobial peptides have potential to be considered as early markers of periodontitis. The entire human salivary proteome was reported by a consortium of three research groups, revealing that 1166 proteins are present in human saliva (Denny et al., 2008). In the pathogenesis of periodontitis, antimicrobial peptides cooperate with other inflammatory proteins and regulate distinct inflammatory pathways. Thus, a combinational approach, in which antimicrobial peptides are measured together

with their activators or target proteins, will increase their value as diagnostic biomarkers. This aim may be achieved by newly developed omics technologies.

AUTHOR CONTRIBUTIONS

All authors contributed to the preparation of the manuscript.

REFERENCES

Abiko, Y., and Saitoh, M. (2007). Salivary defensins and their importance in oral health and disease. *Curr. Pharm. Des.* 13, 3065–3072. doi: 10.2174/138161207782110417

Bartold, P. M., and Narayanan, A. S. (2006). Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 40, 29–49. doi: 10.1111/j.1600-0757.2005.00140.x

Beckloff, N., and Diamond, G. (2008). Computational analysis suggests beta-defensins are processed to mature peptides by signal peptidase. *Protein Pept. Lett.* 15, 536–540. doi: 10.2174/092986608784567618

Bonass, W. A., High, A. S., Owen, P. J., and Devine, D. A. (1999). Expression of beta-defensin genes by human salivary glands. *Oral Microbiol. Immunol.* 14, 371–374. doi: 10.1034/j.1399-302X.1999.140607.x

Bowdish, D. M., Davidson, D. J., and Hancock, R. E. (2006). Immunomodulatory properties of defensins and cathelicidins. *Curr. Top. Microbiol. Immunol.* 306, 27–66. doi: 10.1007/3-540-29916-5_2

Brancatisano, F. L., Maisetta, G., Barsotti, F., Esin, S., Miceli, M., Gabriele, M., et al. (2011). Reduced human beta defensin 3 in individuals with periodontal disease. *J. Dent. Res.* 90, 241–245. doi: 10.1177/0022034510385686

Bratthall, D., and Hänsel Petersson, G. (2005). Cariogram–a multifactorial risk assessment model for a multifactorial disease. *Community Dent. Oral Epidemiol.* 33, 256–264. doi: 10.1111/j.1600-0528.2005.00233.x

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 3, 238–250. doi: 10.1038/nrmicro1098

Christodoulides, N., Floriano, P. N., Miller, C. S., Ebersole, J. L., Mohanty, S., Dharshan, P., et al. (2007). Lab-on-a-chip methods for point-of-care measurements of salivary biomarkers of periodontitis. *Ann. N. Y. Acad. Sci.* 1098, 411–428. doi: 10.1196/annals.1384.035

Cuevas-Córdoba, B., and Santiago-García, J. (2014). Saliva: a fluid of study for OMICS. *OMICS* 18, 87–97. doi: 10.1089/omi.2013.0064

Dale, B. A., and Krisanaprakornkit, S. (2001). Defensin antimicrobial peptides in the oral cavity. *J. Oral Pathol. Med.* 30, 321–327. doi: 10.1034/j.1600-0714.2001.300601.x

Darveau, R. P. (2010). Periodontitis: a polymicrobial disruption of host homeostasis. *Nat. Rev. Microbiol.* 8, 481–490. doi: 10.1038/nrmicro2337

Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., et al. (2008). The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *J. Proteome Res.* 7, 1994–2006. doi: 10.1021/pr700764j

De Smet, K., and Contreras, R. (2005). Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnol. Lett.* 27, 1337–1347. doi: 10.1007/s10529-005-0936-5

Diamond, G., Beckloff, N., and Ryan, L. K. (2008). Host defense peptides in the oral cavity and the lung: similarities and differences. *J. Dent. Res.* 87, 915–927. doi: 10.1177/154405910808701011

Dommisch, H., Açil, Y., Dunsche, A., Winter, J., and Jepsen, S. (2005). Differential gene expression of human beta-defensins (hBD-1, -2, -3) in inflammatory gingival diseases. *Oral Microbiol. Immunol.* 20, 186–190. doi: 10.1111/j.1399-302X.2005.00211.x

Dommisch, H., and Jepsen, S. (2015). Diverse functions of defensins and other antimicrobial peptides in periodontal tissues. *Periodontol. 2000* 69, 96–110. doi: 10.1111/prd.12093

Dunsche, A., Açil, Y., Siebert, R., Harder, J., Schroder, J. M., and Jepsen, S. (2001). Expression profile of human defensins and antimicrobial proteins in oral tissues. *J. Oral Pathol. Med.* 30, 154–158. doi: 10.1034/j.1600-0714.2001.300305.x

Ebersole, J. L., Schuster, J. L., Stevens, J., Dawson, D. III, Kryscio, R. J., Lin, Y., et al. (2013). Patterns of salivary analytes provide diagnostic capacity for distinguishing chronic adult periodontitis from health. *J. Clin. Immunol.* 33, 271–279. doi: 10.1007/s10875-012-9771-3

Ganz, T., Selsted, M. E., Szklarek, D., Harwig, S. S., Daher, K., Bainton, D. F., et al. (1985). Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* 76, 1427–1435. doi: 10.1172/JCI112120

Giannobile, W. V., Beikler, T., Kinney, J. S., Ramseier, C. A., Morelli, T., and Wong, D. T. (2009). Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol. 2000* 50, 52–64. doi: 10.1111/j.1600-0757.2008.00288.x

Goebel, C., Mackay, L. G., Vickers, E. R., and Mather, L. E. (2000). Determination of defensin HNP-1, HNP-2, and HNP-3 in human saliva by using LC/MS. *Peptides* 21, 757–765. doi: 10.1016/S0196-9781(00)00205-9

Gomes Pde, S., and Fernandes, M. H. (2010). Defensins in the oral cavity: distribution and biological role. *J. Oral Pathol. Med.* 39, 1–9. doi: 10.1111/j.1600-0714.2009.00832.x

Goodson, J. M. (1992). Diagnosis of periodontitis by physical measurement: interpretation from episodic disease hypothesis. *J. Periodontol.* 63, 373–382. doi: 10.1902/jop.1992.63.4s.373

Gorr, S. U. (2009). Antimicrobial peptides of the oral cavity. *Periodontol. 2000* 51, 152–180. doi: 10.1111/j.1600-0757.2009.00310.x

Gorr, S. U. (2012). Antimicrobial peptides in periodontal innate defense. *Front. Oral Biol.* 15, 84–98. doi: 10.1159/000329673

Gorr, S. U., and Abdolhosseini, M. (2011). Antimicrobial peptides and periodontal disease. *J. Clin. Periodontol.* 38(Suppl. 11), 126–141. doi: 10.1111/j.1600-051X.2010.01664.x

Gursoy, U. K., and Könönen, E. (2012). Understanding the roles of gingival beta-defensins. *J. Oral. Microbiol.* 4:1527. doi: 10.3402/jom.v4i0.15127

Gursoy, U. K., Könönen, E., Luukkonen, N., and Uitto, V. J. (2013). Human neutrophil defensins and their effect on epithelial cells. *J. Periodontol.* 84, 126–133. doi: 10.1902/jop.2012.120017

Gursoy, U. K., Könönen, E., Pradhan-Palikhe, P., Tervahartiala, T., Pussinen, P. J., Suominen-Taipale, L., et al. (2010). Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *J. Clin. Periodontol.* 37, 487–493. doi: 10.1111/j.1600-051X.2010.01563.x

Gursoy, U. K., Könönen, E., Pussinen, P. J., Tervahartiala, T., Hyvärinen, K., Suominen, A. L., et al. (2011). Use of host- and bacteria-derived salivary markers in detection of periodontitis: a cumulative approach. *Dis. Markers* 30, 299–305. doi: 10.1155/2011/621484

Gursoy, U. K., Könönen, E., Uitto, V. J., Pussinen, P. J., Hyvärinen, K., Suominen-Taipale, L., et al. (2009). Salivary interleukin-1beta concentration and the presence of multiple pathogens in periodontitis. *J. Clin. Periodontol.* 36, 922–927. doi: 10.1111/j.1600-051X.2009.01480.x

Gursoy, U. K., Pöllänen, M., Könönen, E., and Uitto, V. J. (2012). A novel organotypic dento-epithelial culture model: effect of *Fusobacterium nucleatum* biofilm on B-defensin-2, -3, and LL-37 expression. *J. Periodontol.* 83, 242–247. doi: 10.1902/jop.2011.110177

Hosokawa, I., Hosokawa, Y., Komatsuzawa, H., Goncalves, R. B., Karimbux, N., Napimoga, M. H., et al. (2006). Innate immune peptide LL-37 displays distinct expression pattern from beta-defensins in inflamed gingival tissue. *Clin. Exp. Immunol.* 146, 218–225. doi: 10.1111/j.1365-2249.2006.03200.x

Jaeger, S. U., Schroeder, B. O., Meyer-Hoffert, U., Courth, L., Fehr, S. N., Gersemann, M., et al. (2013). Cell-mediated reduction of human β-defensin 1: a major role for mucosal thioredoxin. *Mucosal Immunol.* 6, 1179–1190. doi: 10.1038/mi.2013.17

Jaradat, S. W., Hoder-Przyrembel, C., Cubillos, S., Krieg, N., Lehmann, K., Piehler, S., et al. (2013). Beta-defensin-2 genomic copy number variation and chronic periodontitis. *J. Dent. Res.* 92, 1035–1040. doi: 10.1177/0022034513504217

Ji, S., and Choi, Y. (2015). Point-of-care diagnosis of periodontitis using saliva: technically feasible but still a challenge. *Front. Cell. Infect. Microbiol.* 5:65. doi: 10.3389/fcimb.2015.00065

Kai-Larsen, Y., and Agerberth, B. (2008). The role of the multifunctional peptide LL-37 in host defense. *Front. Biosci.* 13, 3760–3767. doi: 10.2741/2964

Keskin, M., Zeidán-Chuliá, F., Gursoy, M., Könönen, E., Rautava, J., and Gursoy, U. K. (2015). Two cheers for Crohn's disease and periodontitis: beta-defensin-2 as an Actionable target to intervene on two clinically distinct diseases. *OMICS* 19, 443–450. doi: 10.1089/omi.2015.0077

Kinane, D. F., Preshaw, P. M., Loos, B. G., and Working Group 2 of Seventh European Workshop On Periodontology (2011). Host-response: understanding the cellular and molecular mechanisms of host-microbial interactions–consensus of the Seventh European Workshop on Periodontology. *J. Clin. Periodontol.* 38(Suppl. 11), 44–48. doi: 10.1111/j.1600-051X.2010.01682.x

Krisanaprakornkit, S., Kimball, J. R., Weinberg, A., Darveau, R. P., Bainbridge, B. W., and Dale, B. A. (2000). Inducible expression of human beta-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect. Immun.* 68, 2907–2915. doi: 10.1128/IAI.68.5.2907-2915.2000

Kuula, H., Salo, T., Pirilä, E., Hagström, J., Luomanen, M., Gutierrez-Fernandez, A., et al. (2008). Human beta-defensin-1 and -2 and matrix metalloproteinase-25 and -26 expression in chronic and aggressive periodontitis and in peri-implantitis. *Arch. Oral Biol.* 53, 175–186. doi: 10.1016/j.archoralbio.2007.09.010

Lehrer, R. I. (2011). Immunology: peptide gets in shape for self-defence. *Nature* 469, 309–310. doi: 10.1038/469309a

Li, Y., St John, M. A., Zhou, X., Kim, Y., Sinha, U., Jordan, R. C., et al. (2004). Salivary transcriptome diagnostics for oral cancer detection. *Clin. Cancer Res.* 10, 8442–8450. doi: 10.1158/1078-0432.CCR-04-1167

Lu, Q., Samaranayake, L. P., Darveau, R. P., and Jin, L. (2005). Expression of human beta-defensin-3 in gingival epithelia. *J. Periodont. Res.* 40, 474–481. doi: 10.1111/j.1600-0765.2005.00827.x

Marsh, P. D., Moter, A., and Devine, D. A. (2011). Dental plaque biofilms: communities, conflict and control. *Periodontol. 2000* 55, 16–35. doi: 10.1111/j.1600-0757.2009.00339.x

Mathews, M., Jia, H. P., Guthmiller, J. M., Losh, G., Graham, S., Johnson, G. K., et al. (1999). Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infect. Immun.* 67, 2740–2745.

Miller, C. S., Foley, J. D., Bailey, A. L., Campell, C. L., Humphries, R. L., Christodoulides, N., et al. (2010). Current developments in salivary diagnostics. *Biomark. Med.* 4, 171–189. doi: 10.2217/bmm.09.68

Miller, C. S., King, C. P. Jr., Langub, M. C., Kryscio, R. J., and Thomas, M. V. (2006). Salivary biomarkers of existing periodontal disease: a cross-sectional study. *J. Am. Dent. Assoc.* 137, 322–329. doi: 10.14219/jada.archive.2006.0181

Mineshiba, F., Takashiba, S., Mineshiba, J., Matsuura, K., Kokeguchi, S., and Murayama, Y. (2003). Antibacterial activity of synthetic human B defensin-2 against periodontal bacteria. *J. Int. Acad. Periodontol.* 5, 35–40.

Mizukawa, N., Sugiyama, K., Ueno, T., Mishima, K., Takagi, S., and Sugahara, T. (1999). Defensin-1, an antimicrobial peptide present in the saliva of patients with oral diseases. *Oral Dis.* 5, 139–142. doi: 10.1111/j.1601-0825.1999.tb00078.x

Niyonsaba, F., Ushio, H., Nakano, N., Ng, W., Sayama, K., Hashimoto, K., et al. (2007). Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *J. Invest. Dermatol.* 127, 594–604. doi: 10.1038/sj.jid.5700599

Ohnishi, T., and Daikuhara, Y. (2003). Hepatocyte growth factor/scatter factor in development, inflammation and carcinogenesis: its expression and role in oral tissues. *Arch. Oral Biol.* 48, 797–804. doi: 10.1016/S0003-9969(03)00180-8

Page, R. C., and Kornman, K. S. (1997). The pathogenesis of human periodontitis: an introduction. *Periodontol. 2000* 14, 9–11. doi: 10.1111/j.1600-0757.1997.tb00189.x

Pereira, A. L., Franco, G. C., Cortelli, S. C., Aquino, D. R., Costa, F. O., Raslan, S. A., et al. (2013). Influence of periodontal status and periodontopathogens on levels of oral human beta-defensin-2 in saliva. *J. Periodontol.* 84, 1445–1453. doi: 10.1902/jop.2012.120321

Preshaw, P. M., and Taylor, J. J. (2011). How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis? *J. Clin. Periodontol.* 38(Suppl. 11), 60–84. doi: 10.1111/j.1600-051X.2010.01671.x

Salazar, M. G., Jehmlich, N., Murr, A., Dhople, V. M., Holtfreter, B., Hammer, E., et al. (2013). Identification of periodontitis associated changes in the proteome of whole human saliva by mass spectrometric analysis. *J. Clin. Periodontol.* 40, 825–832. doi: 10.1111/jcpe.12130

Silva, N., Dutzan, N., Hernandez, M., Dezerega, A., Rivera, O., Aguillon, J. C., et al. (2008). Characterization of progressive periodontal lesions in chronic periodontitis patients: levels of chemokines, cytokines, matrix metalloproteinase-13, periodontal pathogens and inflammatory cells. *J. Clin. Periodontol.* 35, 206–214. doi: 10.1111/j.1600-051X.2007.01190.x

Sorsa, T., Suomalainen, K., and Uitto, V. J. (1990). The role of gingival crevicular fluid and salivary interstitial collagenases in human periodontal diseases. *Arch. Oral. Biol.* 35(Suppl), 193S–196S. doi: 10.1016/0003-9969(90)90156-5

Sorsa, T., Tjäderhane, L., and Salo, T. (2004). Matrix metalloproteinases (MMPs) in oral diseases. *Oral Dis.* 10, 311–318. doi: 10.1111/j.1601-0825.2004.01038.x

Soruri, A., Grigat, J., Forssmann, U., Riggert, J., and Zwirner, J. (2007). beta-Defensins chemoattract macrophages and mast cells but not lymphocytes and dendritic cells: CCR6 is not involved. *Eur. J. Immunol.* 37, 2474–2486. doi: 10.1002/eji.200737292

Streckfus, C., and Bigler, L. (2005). The use of soluble, salivary c-erbB-2 for the detection and post-operative follow-up of breast cancer in women: the results of a five-year translational research study. *Adv. Dent. Res.* 18, 17–24. doi: 10.1177/154407370501800105

Takeuchi, Y., Nagasawa, T., Katagiri, S., Kitagawara, S., Kobayashi, H., Koyanagi, T., et al. (2012). Salivary levels of antibacterial peptide (LL-37/hCAP-18) and cotinine in patients with chronic periodontitis. *J. Periodontol.* 83, 766–762. doi: 10.1902/jop.2011.100767

Taylor, J. J. (2014). Protein biomarkers of periodontitis in saliva. *ISRN Inflamm.* 2014:593151. doi: 10.1155/2014/593151

Teles, R. P., Haffajee, A. D., and Socransky, S. S. (2006). Microbiological goals of periodontal therapy. *Periodontol. 2000* 42, 180–218. doi: 10.1111/j.1600-0757.2006.00192.x

Uitto, V. J., Suomalainen, K., and Sorsa, T. (1990). Salivary collagenase. Origin, characteristics and relationship to periodontal health. *J. Periodontal. Res.* 25, 135–142. doi: 10.1111/j.1600-0765.1990.tb01035.x

Van Dyke, T. E. (2007). Control of inflammation and periodontitis. *Periodontol. 2000* 45, 158–166. doi: 10.1111/j.1600-0757.2007.00229.x

Vardar-Sengul, S., Demirci, T., Sen, B. H., Erkizan, V., Kurulgan, E., and Baylas, H. (2007). Human beta defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases. *J. Periodont. Res.* 42, 429–437. doi: 10.1111/j.1600-0765.2006.00964.x

Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., López-Boado, Y. S., Stratman, J. L., et al. (1999). Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* 286, 113–117. doi: 10.1126/science.286.5437.113

Woo, J. S., Jeong, J. Y., Hwang, Y. J., Chae, S. W., Hwang, S. J., and Lee, H. M. (2003). Expression of cathelicidin in human salivary glands. *Arch. Otolaryngol. Head Neck Surg.* 129, 211–214. doi: 10.1001/archotol.129.2.211

Yilmaz, D., Güncü, G. N., Könönen, E., Baris, E., Çaglayan, F., and Gursoy, U. K. (2015). Overexpressions of hBD-2, hBD-3, and hCAP18/LL-37 in gingiva of diabetics with periodontitis. *Immunobiology* 220, 1219–1226 doi: 10.1016/j.imbio.2015.06.013

Zhang, L., Henson, B. S., Camargo, P. M., and Wong, D. T. (2009). The clinical value of salivary biomarkers for periodontal disease. *Periodontol. 2000* 51, 25–37. doi: 10.1111/j.1600-0757.2009.00315.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 11 January 2016
doi: 10.3389/fcimb.2015.00102

A Systems Biology Approach to Reveal Putative Host-Derived Biomarkers of Periodontitis by Network Topology Characterization of MMP-REDOX/NO and Apoptosis Integrated Pathways

Fares Zeidán-Chuliá[1,2], Mervi Gürsoy[2], Ben-Hur Neves de Oliveira[1], Vural Özdemir[3,4†], Eija Könönen[2,5†] and Ulvi K. Gürsoy[2†]*

[1] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, [2] Department of Periodontology, Institute of Dentistry, University of Turku, Turku, Finland, [3] Faculty of Communications and Office of the President, International Technology and Innovation Policy, Gaziantep University, Gaziantep, Turkey, [4] Amrita School of Biotechnology, Amrita Vishwa Vidyapeetham (Amrita University), Kollam, India, [5] Oral Health Care, Welfare Division, Turku, Finland

OPEN ACCESS

Edited by:
Yongqun He,
University of Michigan, USA

Reviewed by:
Arzucan Ozgur,
Bogazici University, Turkey
Junguk Hur,
University of Michigan, USA
Jie Zheng,
University of Pennsylvania, USA

***Correspondence:**
Fares Zeidán-Chuliá
fzchulia.biomed@gmail.com

† These authors are designated as co-senior authors.

Received: *18 August 2015*
Accepted: *15 December 2015*
Published: *11 January 2016*

Citation:
Zeidán-Chuliá F, Gürsoy M, Neves de Oliveira B-H, Özdemir V, Könönen E and Gürsoy UK (2016) A Systems Biology Approach to Reveal Putative Host-Derived Biomarkers of Periodontitis by Network Topology Characterization of MMP-REDOX/NO and Apoptosis Integrated Pathways. Front. Cell. Infect. Microbiol. 5:102. doi: 10.3389/fcimb.2015.00102

Periodontitis, a formidable global health burden, is a common chronic disease that destroys tooth-supporting tissues. Biomarkers of the early phase of this progressive disease are of utmost importance for global health. In this context, saliva represents a non-invasive biosample. By using systems biology tools, we aimed to (1) identify an integrated interactome between matrix metalloproteinase (MMP)-REDOX/nitric oxide (NO) and apoptosis upstream pathways of periodontal inflammation, and (2) characterize the attendant topological network properties to uncover putative biomarkers to be tested in saliva from patients with periodontitis. Hence, we first generated a protein-protein network model of interactions ("BIOMARK" interactome) by using the STRING 10 database, a search tool for the retrieval of interacting genes/proteins, with "Experiments" and "Databases" as input options and a confidence score of 0.400. Second, we determined the centrality values (*closeness*, *stress*, *degree* or *connectivity*, and *betweenness*) for the "BIOMARK" members by using the Cytoscape software. We found Ubiquitin C (UBC), Jun proto-oncogene (JUN), and matrix metalloproteinase-14 (MMP14) as the most central hub- and non-hub-bottlenecks among the 211 genes/proteins of the whole interactome. We conclude that UBC, JUN, and MMP14 are likely an optimal candidate group of host-derived biomarkers, in combination with oral pathogenic bacteria-derived proteins, for detecting periodontitis at its early phase by using salivary samples from patients. These findings therefore have broader relevance for systems medicine in global health as well.

Keywords: gelatinases, oxidative stress, saliva, drug target, omics, computational biology, *in silico*, network

INTRODUCTION

Periodontitis is a chronic infection with a progressive inflammatory process of the tooth-supporting tissues and characteristically causes gingival recession, alveolar bone loss, and mobility of the teeth. If the necessary treatment is not performed, this disease can lead to the loss of affected teeth. Several pathways, such as apoptosis, matrix metalloproteinase (MMP)-REDOX/nitric oxide (NO) activation, toll-like receptor, and nuclear factor-kB (NF-kB) signaling, cytokine and chemokine network, complement cascade, and osteoclastogenesis, play a role in the pathogenesis of this chronic disease with remissions and exacerbations (Cekici et al., 2014).

The two host cell types that mainly come across with periodontopathogenic bacteria during inflammation in periodontal tissues are epithelial cells and polymorphonuclear leukocytes (PMNL). The epithelium expresses cytokines, chemokines, proteases, and natural antimicrobial peptides against infectious stimuli (Gursoy and Könönen, 2012). Once bacteria bind to the PMNL surface, phagocytosis results in entrapment of the bacterial cell into a membrane-delimited structure, also known as phagosome. The phagosome undergoes maturation by fusion with endosomes and finally with lysosomes. Lysosomal vesicles include reactive oxygen species (ROS). ROS activate the redox sensitive NF-kB signaling pathway, which induces the expression of cell adhesion receptors, proinflammatory cytokines and chemokines, involved in the production of free radicals and persistence of inflammation (Scott and Krauss, 2012). On one hand, ROS destroy pathogenic bacteria and other phagocytosed material within the safe confines of the phagolysosome. On the other hand, the extracellular release of oxygen intermediates leads to significant destruction in periodontal tissues. This damage is mainly an outcome of elevated MMP activation and increased apoptosis of gingival resident cells (Zeidán-Chuliá et al., 2013, 2014a).

Biomarkers of periodontitis are sorely needed to intervene early in the course of the disease. Periodontitis causes little, if any, discomfort at its initial stages thereby allowing the clinical diagnosis not until alveolar bone loss has already commenced or materialized (Gursoy et al., 2011). Over the past decade, research on salivary diagnostics received attention by virtue of an easy sample access and low cost, while the search for biomarkers broadened in scope to characterize both the human host and resident bacteria using systems science and omics technology (e.g., genomics and/or proteomics) approaches (Cuevas-Córdoba and Santiago-García, 2014; Gürsoy et al., 2014).

It is now widely accepted that periodontitis is a consequence of complex host-environment and biological pathway interactions, rather than a product of a single gene or protein (Hajishengallis and Sahingur, 2014). Network modeling by systems biology-based approaches is increasingly employed as versatile effective tools to unravel the pathogenesis of periodontal disease by integration of multi-omics data (Zhu et al., 2007; Zeidán-Chuliá et al., 2013, 2014a; Gürsoy et al., 2014). Moreover, by studying the topological network properties within an interactome, one might identify nodes (e.g., genes/proteins) with a biologically critical position (bottlenecks) in the overall network architecture and thus putative early disease biomarkers and drug targets. In general, the word "bottleneck" refers to nodes with high *betweenness* values, indicating that those nodes are central points that control the communication between other nodes within the network. These nodes are "between" highly interconnected subgraph clusters and by removing them, the network could be divided (Yu et al., 2007).

In previous studies, we reported the deregulated expression of apoptosis and MMP-REDOX/NO-related genes in periodontitis samples when compared to those of healthy controls (Zeidán-Chuliá et al., 2013, 2014a). Most common histological findings in early periodontitis are related to neutrophil migration and activation and weakened wound healing in resident cells (Biasi et al., 1999). Neutrophils are one main source of tissue degrading MMPs, while oxidative stress-induced apoptosis of resident cells (gingival epithelia and fibroblasts) is one main outcome of increased bacterial invasion and decreased tissue regeneration (Nussbaum and Shapira, 2011). Therefore, our aims for the present study were (1) to simultaneously analyze these two initial pathways of periodontal inflammation (MMP-REDOX/NO and apoptosis) by systems biology, and (2) to define their functional interconnections as putative biomarkers of early periodontitis.

MATERIALS AND METHODS

Interaction Network Development, Analysis of Topological Network Properties and Landscape Visualization of Centrality Values

The "BIOMARK" interactome was developed by using the STRING 10 database (http://string-db.org/; Szklarczyk et al., 2011, 2015) with "Experiments" and "Databases" as input options and a confidence score of 0.400. STRING is a search tool for the retrieval of interacting genes/proteins extracted from diverse curate and public databases with information on direct and indirect functional associations/interactions. Interactions are derived from different sources (1) primary databases, (2) manually-curated databases, (3) Medline abstracts and a large collection of full-text articles, (4) algorithms and co-expression analysis using genomic information, and (5) interactions observed in one organism that are systematically transferred to others via pre-computed orthology relations (Szklarczyk et al., 2015).

As a starting point, we selected two published *in silico* network models to get the list of genes/proteins that would be part of the interactome (**Figure 1**): the "MRN" model with MMP and REDOX/NO-related genes/proteins (Zeidán-Chuliá et al., 2013) and the "APOP" model with apoptosis-related genes/proteins (Zeidán-Chuliá et al., 2014a). The criteria to select these models (subnetworks) were based on biological processes typically altered in periodontitis, such as (1) increased production and activity of MMPs by host cells, (2) increased NO production and NOS activity by human oral neutrophils, (3) oxidative stress, as well as (4) increased apoptosis and tissue destruction induced by periodontal pathogens. *In silico* integration of the two subnetworks onto one interactome

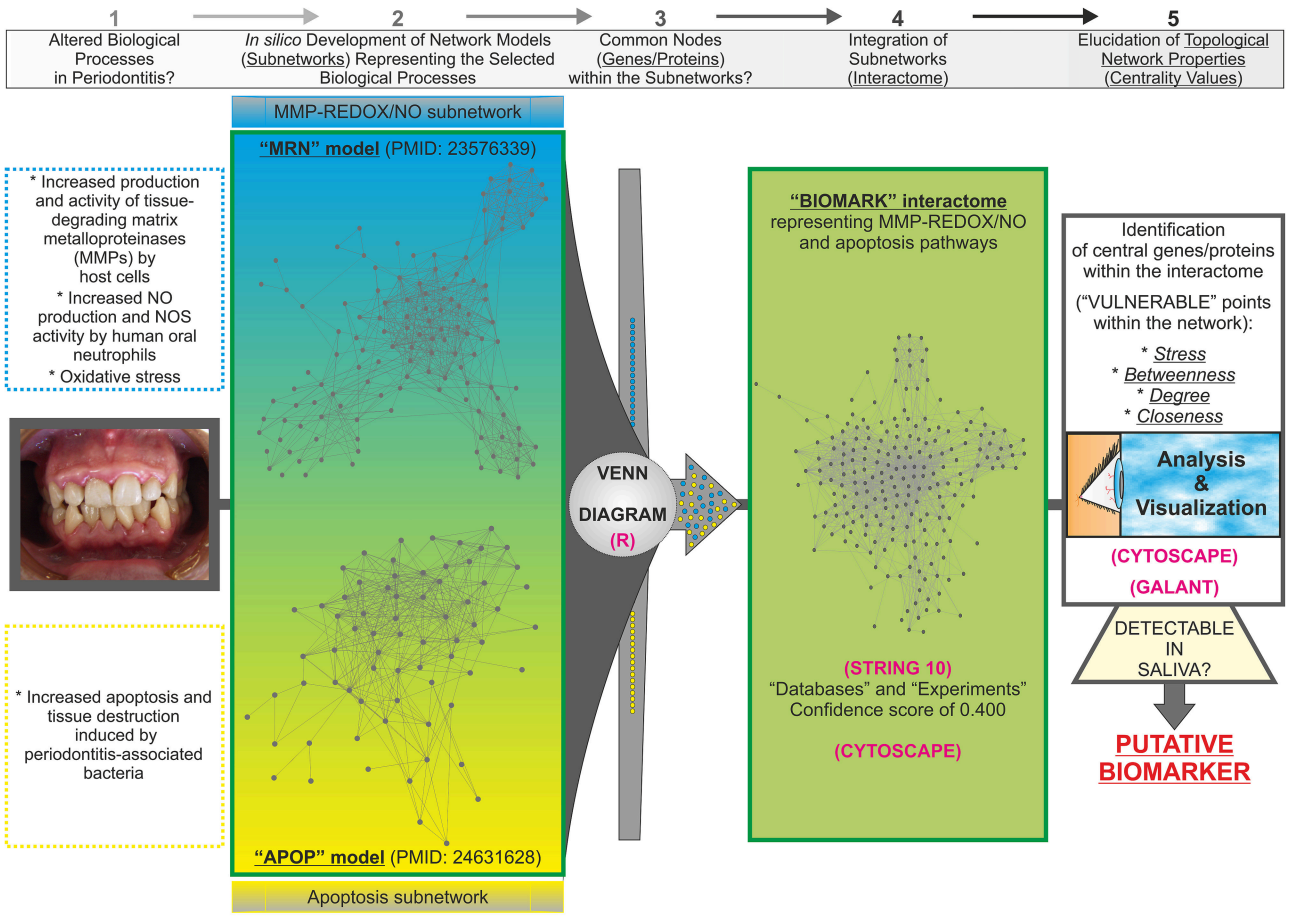


FIGURE 1 | Abstract workflow summarizing the criteria and tools used for the present study. The "MRN" and "APOP" *in silico* models, already available in the literature, were used as the starting material to develop the "BIOMARK" integrating MMP-REDOX/NO and apoptosis related-genes/proteins into one single network of interactions. The Venn diagram, performed by using the software system R, revealed the existence of common nodes (genes/proteins). Thereafter, the interactome was developed for integrating these subnetworks into one single gene/protein interaction network model ("BIOMARK") by using the STRING 10 and Cytoscape tools. Further elucidation of the topological network properties reveals "vulnerable" points within the *in silico* model representing candidate diagnostic biomarkers and also potential molecular targets for therapeutics. With GALANT, a Cytoscape plugin, we project numerical data from the topological network properties (centrality measurements) onto "BIOMARK" interactome to create a smoothed data map resembling the network layout. Central members of "BIOMARK," easily detectable in saliva, represent optimal candidates to be tested as periodontitis biomarkers.

would characterize above-mentioned biological processes at the molecular level for the search of potential biomarkers of periodontitis. Thereafter, a Venn diagram was constructed by using the freely available software system R (http://www.r-project.org; Gentleman et al., 2004) in order to visualize the grade of molecular relation (common genes/proteins) between the "MRN" and "APOP" subnetworks. The genes/proteins that integrated the "BIOMARK" interactome were identified by using the Human Genome Organization (HUGO) Gene Symbol (Wain et al., 2004) and Ensembl protein ID (Birney et al., 2006). The selected list (Supplementary Table 1) was applied into the STRING database and the links (interaction strength) between two different nodes (genes/proteins) were saved in data files and handled by utilizing the Cytoscape open source software platform. Cytoscape is used for visualizing complex networks and integrating these with any type of attribute data (Smoot et al., 2011). The original Cys file of "BIOMARK" model is additionally provided as Supplementary Material (Supplementary Data Sheet 1). Topological network properties (Yu et al., 2007) such as *closeness*, *stress*, *degree* or *connectivity*, and *betweenness* centralities (Supplementary Table 2) were also analyzed by using the NetworkAnalyzer plugin from the Cytoscape software. Values of centralities above one standard deviation (+1 SD) of the mean were selected to identify potential candidate host-derived biomarker/s.

Of note, *closeness* measures the grade of proximity of a node to the rest of nodes. The larger the value, the faster the information spreads through this node. *Stress* measures the number of times a given node is traversed by ideal routes or "shortest paths" within a network. Nodes that are traversed by higher numbers of short paths are by definition more stressed. *Degree* measures the local topology of each node by summing up the number of its adjacent nodes. Nodes with high values of *degree* over the thresholds values are named as "hubs." *Betweenness*, which is similar to *stress*

as a topological network property, measures how frequently the shortest path, connecting every pair of nodes, is going through a third given node. Therefore, both *stress* and *betweenness* provide information about the influence of a node over the spread of information throughout the interactome. All nodes with high values of *betweenness* over the thresholds values were named as "bottlenecks" (non-hub- and hub-bottlenecks are represented by NH-B and HB, respectively).

For the network-level visualization of centrality values in the "BIOMARK" interactome, we utilized GALANT (GrAph LANdscape VisualizaTion). GALANT is a Cytoscape plugin that builds functional landscapes onto biological networks (Camilo et al., 2013). GALANT projects any kind of numerical data (e.g., centrality measurements) onto a network in order to create landscapes resembling the network layout, and also offers a friendly interface fully integrated with the Cytoscape where users can easily build their landscapes of interest by using one of the previously mentioned functions.

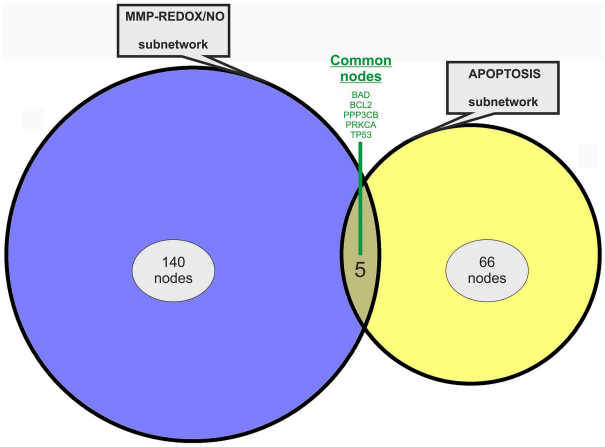


FIGURE 2 | Venn diagram showing information integration from the studied subnetworks. The "MRN" (MMP-REDOX/NO) and "APOP" (apoptosis) network models contain five common nodes (genes/proteins).

RESULTS

"BIOMARK" is an Integrative Network Model for MMP-REDOX/NO and Apoptosis Subnetworks

Two previously published subnetworks of gene/protein interactions characterizing the molecular landscape of MMP-REDOX/NO ("MRN") and apoptosis ("APOP") pathways were selected (**Figure 1**). The Venn diagram, which visualizes the level of subnetwork information integration, shows that both subnetworks contain five common nodes (genes/proteins) allowing communication with one another for constructing the "BIOMARK" interactome (**Figure 2**). In the case of non-existing direct crosstalk through common subnetwork nodes, it is possible to find common neighboring nodes (genes/proteins) to members of both subnetworks by the use of mathematical algorithms in the R environment. By using the STRING 10 ("Experiments" and "Databases"; confidence score of 0.400) and plotting with the Cytoscape software, we then developed the *in silico* network model "BIOMARK" (**Figure 3**) composed of 211 genes/proteins interconnecting through 1634 interactions.

Identification of Three Key Hub Genes/Proteins within the "BIOMARK" Interactome Suggests UBC, JUN, and MMP14 as Putative Host-Derived Biomarkers of Periodontitis

For describing the global characteristics of the newly-developed interactome, we elucidated the topological network properties or centrality values (Supplementary Table 2). The measurements uncover the most central nodes (genes/proteins) of our model, representing "vulnerable" points within the "BIOMARK" interactome. Central genes/proteins can be thus considered putative biomarkers, because variations in these members can trigger more intense changes in the rest of the interactome than those in non- or less central members. After calculating each centrality value for the 211 "BIOMARK" gene/protein members and visualizing the data as a functional landscape projected onto the interactome, we identified network areas with high centrality values and, more specifically, Ubiquitin C (UBC) and Jun proto-oncogene (JUN) as hub-bottlenecks (HBs) of our model (**Figure 4** and **Table 1**). These genes/proteins also displayed the highest values of *stress* and *closeness* centralities. Moreover, only a third gene/protein, MMP14 (an activator of host gelatinases), was identified as non-hub-bottleneck (NH-B) with a *betweenness* value over the threshold (**Figure 5** and **Table 1**).

DISCUSSION

In the present study, our principal aim was to test the feasibility of creating an *in silico* model able to provide a molecular landscape of putative salivary biomarkers of periodontitis that could be later analyzed by wet laboratory techniques from patient samples. This model should be able to integrate a maximum number of genes/proteins from biological processes that are typically altered in periodontal inflammation (e.g., oxidative stress and apoptosis) into one single interactome.

According to our results, UBC, JUN, and MMP14 control the flow of biological information within the interactome integrating the MMP-REDOX/NO and apoptosis pathways. Their up- or down-regulation would critically affect the entire network, since they are the most central proteins of the "BIOMARK" interactome in comparison to the rest of network members. In a similar manner, if UBC, JUN, or MMP14 is disrupted by drug interaction, the entire network would be destroyed into small components. Thus, they represent three optimal therapeutic targets *in silico*, to be tested *in vitro* and/or *in vivo*. By applying the same criteria, the cumulative use of JUN, UBC, and MMP14 together with oral pathogenic bacteria-derived proteins

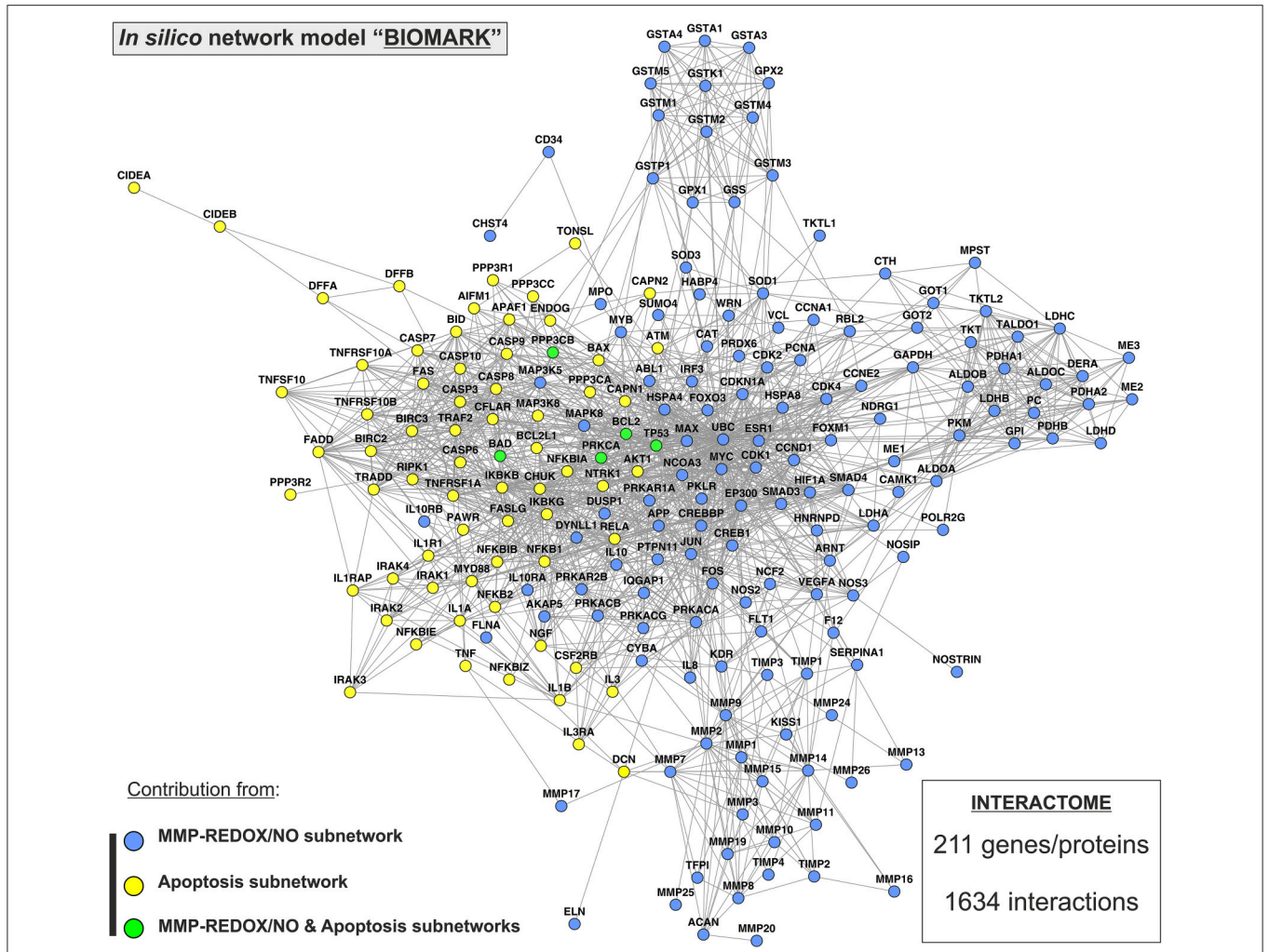


FIGURE 3 | "BIOMARK" interactome. The network model of interactions between genes/protein belonging to "MRN" (MMP-REDOX/NO) and "APOP" (apoptosis) subnetworks or "BIOMARK" interactome was developed by using the STRING 10 database resource search tool, under a confidence score of 0.400, and by using "Databases" and "Experiments" as input options and visualized by plotting it with Cytoscape software. The subnetwork contribution of each gene/protein within the network is represented in the figure with blue-colored (MMP-REDOX/NO), yellow-colored (apoptosis), or green-colored nodes (contributing to both MMP-REDOX/NO and apoptosis subnetworks).

(Gursoy et al., 2011) could be used as representative biomarkers of susceptibility to periodontitis for early intervention, if these central members are detectable in saliva from diseased individuals at the RNA or protein level (Kousvelari et al., 1990; Fábián et al., 2008; Cuevas-Córdoba and Santiago-García, 2014; Rall et al., 2015). UBC is a highly connected protein in the whole human protein-protein interaction network and it is involved in pathogenesis of different diseases (e.g., neurodegenerative diseases; Ullrich et al., 2010). Its use for the diagnosis of periodontitis may lack sensitivity and this could be considered as a potential limitation.

As a general conceptual framework, protein-protein interactions offer the opportunity to analyze the functional relationships among biological molecules (Gursoy et al., 2008). The more processes are integrated in the form of subnetworks in the interactome, the more accurate will our *in silico* model be to reflect the molecular pathogenesis of periodontal inflammation. An alternative possibility is, however, that other markers could also act as central members over the threshold upon the integration of additional subnetworks in the model, representing deregulated biological processes in periodontitis (e.g., epithelial cell adhesion; Haapasalmi et al., 1995; Gürsoy et al., 2015). These newly developed subnetworks might also be considered in the future as part of a systems biology approach utilized in different fields of medicine such as cancer, neurodegenerative, and psychiatric diseases (Rosado et al., 2011; Santana-Codina et al., 2013; ElRakaiby et al., 2014; Podder and Latha, 2014; Zeidán-Chuliá et al., 2014b; Ebhardt et al., 2015).

Saliva has a major importance in the maintenance of oral health, and, during the past two decades, it has been considered a potential specimen to detect oral and systemic

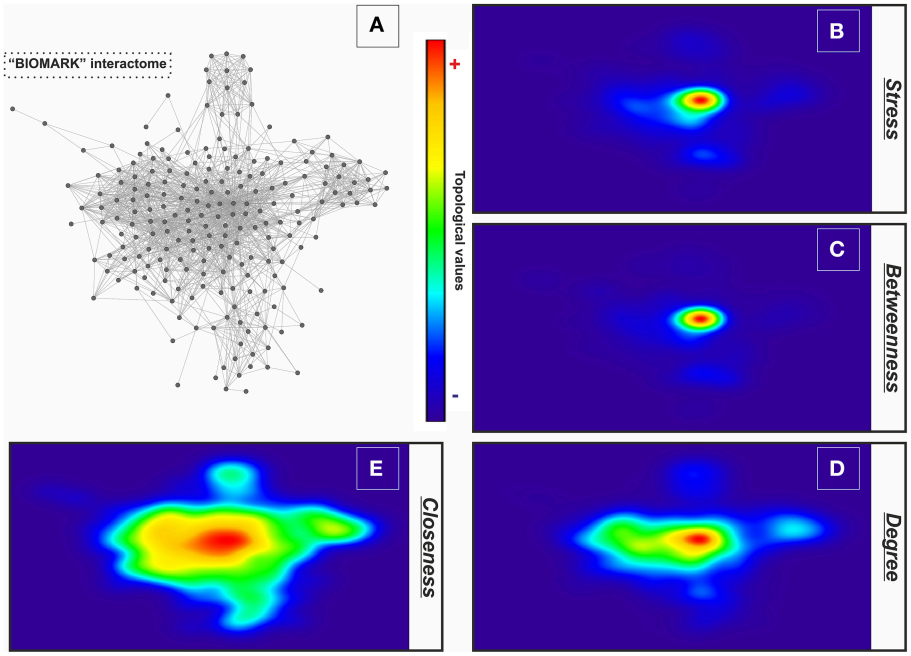


FIGURE 4 | GALANT plots of centrality values over the "BIOMARK" interactome. Analysis of the topological network properties by Cytoscape and its 2D projection by the GALANT software over the "BIOMARK" interactome **(A)** show the areas of maximum values (red color) of stress **(B)**, betweenness **(C)**, degree **(D)**, and closeness centralities **(E)** of each node in this color-grading representation.

diseases (Ji and Choi, 2015). Specific biomarkers have been identified from saliva, reflecting the three key features of pathogenic processes in periodontal disease, i.e., infection-induced inflammation, collagen degradation, and bone turnover (Zhang et al., 2009). Host- and bacteria-derived enzymes, proteins, and other inflammatory mediators appear to hold great promise as salivary biomarkers for the diagnosis of periodontal disease (Ramseier et al., 2009; Haigh et al., 2010; Salazar et al., 2013).

Saliva represents a non-invasive and safe study specimen, being especially useful in large-scale studies. During the onset and progress of periodontitis, inflammatory markers are released from cells present in the periodontium. Elevated levels of enzymes, cytokines, and biomarkers of connective tissue destruction and bone turnover can be found in saliva of periodontitis patients in comparison to their controls (Gursoy et al., 2009, 2010, 2013; Kinney et al., 2011; Lee et al., 2012). Furthermore, there is a considerable interest in applying sequencing and genotyping studies in various human populations. It has been demonstrated that saliva samples, in comparison to cheek swabs, provide a substantial increase in the amount of human DNA (Fábián et al., 2008). Although saliva may be used as a diagnostic tool for detecting periodontitis, there is only a subtle consensus on few salivary molecules to be used as putative markers of periodontitis (Gursoy et al., 2011; Salminen et al., 2014). The search for a novel biomarker is a costly and long-term process. With the aid of systems biology, however, it is possible to simultaneously analyze multiple candidate biomarkers within a network of interactions representing different biological processes (e.g., apoptosis, oxidative stress, and MMP secretion), which are characteristic of a given disease, such as periodontitis.

Up to our knowledge, our study is the first one combining MMP-REDOX/NO and apoptosis pathways that are well-known pathways in inflammatory diseases, including periodontitis, in an attempt to find regulative biomarkers. These two pathways that represent diminished tissue regeneration were taken as an example, since periodontitis is an outcome of a disrupted balance between tissue degeneration and regeneration. The selected pathways, however, form only a part of the whole cascade of events, and applying other additional models may give different results. Therefore, testing different underlying mechanisms in a combined manner would be beneficial to design putative biomarker groups for periodontitis.

In general, pathogenic pathways involved in the imbalance of connective tissue homeostasis in periodontitis are complex. Regarding our proposed candidate biomarkers, polyubiquitin-C is known as a precursor protein encoded by the *UBC* gene that is cleaved into the active ubiquitin monomer (Hanna et al., 2007). Among other processes, the ubiquitin conjugation system regulates protein degradation and signal transduction, playing a critical role in the regulation of innate and adaptive immunological responses (Liu et al., 2005). It seems that a decreased ubiquitin level can reduce the activation threshold of cells to environmental stressors, and UBC has been thus proposed as one promising candidate biomarker together with calmodulin-like protein 5 for the identification of newborns predisposed to develop atopic eczema (Holm et al., 2014).

TABLE 1 | Nodes (genes/proteins) with the highest *betweenness* values from the "BIOMARK" interactome that integrates MMP-REDOX/NO ("MRN") and apoptosis ("APOP") subnetworks.

NAME/SYMBOL	—/HB/NH-B	*Closeness*	*Stress*	*Degree*	*Betweenness*
UBC	HB	0.74204947	111,970	146	0.50695465
JUN	HB	0.53571429	17,440	45	0.04948189
MMP14	NH-B	0.47727273	10,116	18	0.04427346
TP53	—	0.53164557	12,626	50	0.03929094
MMP2	—	0.42510121	10,940	24	0.03761779
MYB	—	0.47191011	5590	17	0.02991401
MMP9	—	0.41666667	10,146	24	0.02747875
RELA	—	0.52369077	10,756	47	0.02417225
EP300	—	0.52631579	8960	48	0.01898303
DFFB	—	0.43659044	2568	4	0.01857965
APP	—	0.48498845	4704	25	0.01581576
NFKB1	—	0.5060241	7870	38	0.01551606
CASP3	—	0.48387097	5000	34	0.01418542
TIMP1	—	0.47511312	3998	14	0.01402902
CHUK	—	0.50724638	7092	47	0.01394735
AKT1	—	0.49065421	5388	34	0.01363533
DCN	—	0.37366548	2994	8	0.01291272
MAP3K5	—	0.47619048	3706	27	0.01285955
TRAF2	—	0.48498845	4716	28	0.01225608
IKBKB	—	0.5060241	5976	44	0.01139259
Average		0.4278415	2297.54	15.583	0.006697052
+1 SD		0.492552	10277.8	29.4	0.042061202
+2 SD		0.5572626	18,258	43.217	0.077425351

Closeness, stress, and degree values are also shown. Centralities over the thresholds with value/s above one (+1 SD) or two (+2 SD) standard deviations of the mean are color-marked. Nodes identified as non-hub-bottlenecks and hub-bottlenecks (based on degree and betweenness centralities), with values above +1 SD of the mean, are represented as NH-B and HB, respectively.

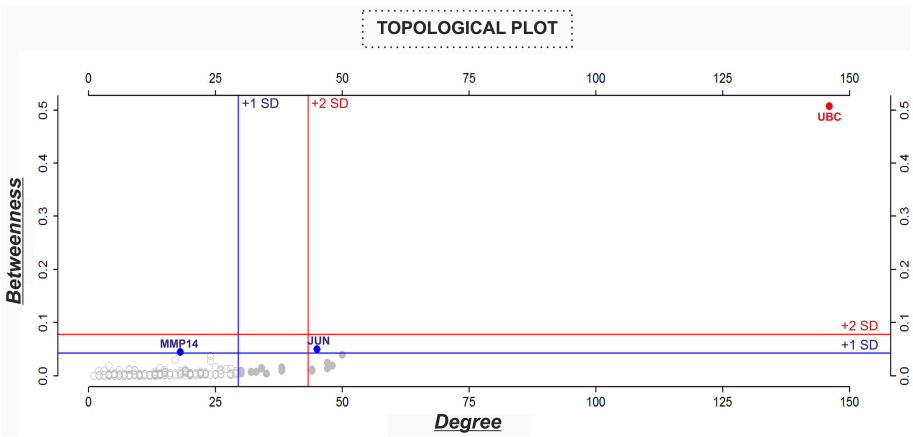


FIGURE 5 | Analysis of the topological properties (*betweenness* vs. *degree*) of genes/proteins belonging to the "BIOMARK" interactome. Blue and red lines indicate the threshold value for each property or value/s above one (+1 SD, blue color) or two (+2 SD, red color) standard deviations of the mean. In the graphs, genes/proteins are represented by circles and plotted in blue or red when value/s fall within +1 or +2 SD, respectively. Values of centralities above +1 SD of the mean were selected to identify potential candidate host-derived biomarker/s.

The Jun proto-oncogene (*JUN*) is a component of the AP-1 transcription factor, which is activated by several extracellular stimuli, such as proinflammatory cytokines and UV radiation, and plays a role in mediating the cellular response (Wisdom et al., 1999). For instance, c-Jun confers protection against UV-induced cellular apoptosis (Wisdom et al., 1999). It was

recently shown that several genes involved in cellular apoptosis are deregulated in diseased gingival samples from periodontitis patients (Zeidán-Chuliá et al., 2014a). In fibroblasts, c-Jun has been suggested to activate cell death by acting as a transcriptional regulator (Bossy-Wetzel et al., 1997), likely having a similar role in periodontal inflammation. Finally, it is well-known that MMP14 is able to degrade collagen and gelatin, activate other MMPs relevant in periodontal inflammation progression, such as MMP2 and MMP13, shed cell surface proteins, and prevent collagen-induced apoptosis (Maquoi et al., 2012; Albrechtsen et al., 2013; Zeidán-Chuliá et al., 2013). To the best of our knowledge, there are no previous data reporting UBC and JUN as putative biomarkers of periodontitis. Our study group has previously analyzed salivary MMP14 concentrations as a potential biomarker for advanced periodontitis but failed to show any significant difference between periodontitis and non-periodontitis/gingivitis subjects (Gursoy et al., 2010). Instead, as an activator of main collagen degrading enzymes of gingiva like MMP2 and MMP9, which are known to be up-regulated in periodontitis (Zeidán-Chuliá et al., 2013; Haage et al., 2014), MMP14 may be a potential marker of inflammatory processes at early phases of periodontal pathogenesis.

On the basis of our findings, we propose the cumulative use of UBC, JUN, and MMP14, possibly in combination with oral pathogenic bacteria-derived proteins, as putative biomarkers for early detection of periodontitis to be tested either at the RNA or protein level in salivary samples. For example, high-throughput omics data found in other publicly available databases (e.g., Gene Expression Omnibus, Reactome, or BioGRID) could be used to validate our candidate biomarkers or further expand the BIOMARK interactome. Besides, the present systems biology-based approach may be used as an objective tool to identify, with measurable parameters, candidate molecular targets to treat this disease.

AUTHOR CONTRIBUTIONS

FZ and UG conceived of the study; FZ, UK, EK, VO, and MG participated in its design and coordination; FZ and BN performed the *in silico* analyses; FZ and UG wrote the manuscript; EK, VO, and MG provided the critical revision of the manuscript; all authors read and approved the final manuscript.

ACKNOWLEDGMENTS

Fares Zeidán-Chuliá holds a PNPD postdoctoral position (Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul) funded by CAPES.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2015.00102

REFERENCES

Albrechtsen, R., Kveiborg, M., Stautz, D., Vikeså, J., Noer, J. B., Kotzsh, A., et al. (2013). ADAM12 redistributes and activates MMP-14, resulting in gelatin degradation, reduced apoptosis and increased tumor growth. *J. Cell Sci.* 126, 4707–4720. doi: 10.1242/jcs.129510

Biasi, D., Bambara, L. M., Carletto, A., Caramaschi, P., Andrioli, G., Urbani, G., et al. (1999). Neutrophil migration, oxidative metabolism and adhesion in early onset periodontitis. *J. Clin. Periodontol.* 26, 563–568. doi: 10.1034/j.1600-051X.1999.260901.x

Birney, E., Andrews, D., Caccamo, M., Chen, Y., Clarke, L., Coates, G., et al. (2006). Ensembl 2006. *Nucleic Acids Res.* 34, 556–561. doi: 10.1093/nar/gkj133

Bossy-Wetzel, E., Bakiri, L., and Yaniv, M. (1997). Induction of apoptosis by the transcription factor c-Jun. *EMBO J.* 16, 1695–1709. doi: 10.1093/emboj/16.7.1695

Cekici, A., Kantarci, A., Hasturk, H., and Van Dyke, T. E. (2014). Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontol. 2000* 64, 57–80. doi: 10.1111/prd.12002

Cuevas-Córdoba, B., and Santiago-García, J. (2014). Saliva: a fluid of study for OMICS. *OMICS* 18, 87–97. doi: 10.1089/omi.2013.0064

Camilo, E., Bovolenta, L. A., Acencio, M. L., Rybarczyk-Filho, J. L., Castro, M. A., Moreira, J. C., et al. (2013). GALANT: a Cytoscape plugin for visualizing data as functional landscapes projected onto biological networks. *Bioinformatics* 29, 2505–2506. doi: 10.1093/bioinformatics/btt377

Ebhardt, H. A., Root, A., Sander, C., and Aebersold, R. (2015). Applications of targeted proteomics in systems biology and translational medicine. *Proteomics* 15, 3193–3208. doi: 10.1002/pmic.201500004

ElRakaiby, M., Dutilh, B. E., Rizkallah, M. R., Boleij, A., Cole, J. N., and Aziz, R. K. (2014). Pharmacomicrobiomics: the impact of human microbiome variations on systems pharmacology and personalized therapeutics. *OMICS* 18, 402–414. doi: 10.1089/omi.2014.0018

Fábián, T. K., Fejérdy, P., and Csermely, P. (2008). Salivary Genomics, transcriptomics and proteomics: the emerging concept of the oral ecosystem and their use in the early diagnosis of cancer and other diseases. *Curr. Genomics* 9, 11–21. doi: 10.2174/138920208783884900

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 5, R80. doi: 10.1186/gb-2004-5-10-r80

Gursoy, A., Keskin, O., and Nussinov, R. (2008). Topological properties of protein interaction networks from a structural perspective. *Biochem. Soc. Trans.* 36, 1398–1403. doi: 10.1042/BST0361398

Gürsoy, M., Zeidán-Chuliá, F., Könönen, E., Moreira, J. C., Liukkonen, J., Sorsa, T., et al. (2014). Pregnancy-induced gingivitis and OMICS in dentistry: *in silico* modeling and *in vivo* prospective validation of estradiol-modulated inflammatory biomarkers. *OMICS* 18, 582–590. doi: 10.1089/omi.2014.0020

Gursoy, U. K., and Könönen, E. (2012). Understanding the roles of gingival beta-defensins. *J. Oral Microbiol.* 4. doi: 10.3402/jom.v4i0.15127

Gursoy, U. K., Könönen, E., Huumonen, S., Tervahartiala, T., Pussinen, P. J., Suominen, A. L., et al. (2013). Salivary type I collagen degradation end-products and related matrix metalloproteinases in periodontitis. *J. Clin. Periodontol.* 40, 18–25. doi: 10.1111/jcpe.12020

Gursoy, U. K., Könönen, E., Pradhan-Palikhe, P., Tervahartiala, T., Pussinen, P. J., Suominen-Taipale, L., et al. (2010). Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *J. Clin. Periodontol.* 37, 487–493. doi: 10.1111/j.1600-051X.2010.01563.x

Gursoy, U. K., Könönen, E., Pussinen, P. J., Tervahartiala, T., Hyvärinen, K., Suominen, A. L., et al. (2011). Use of host- and bacteria-derived salivary markers in detection of periodontitis: a cumulative approach. *Dis. Markers.* 30, 299–305. doi: 10.1155/2011/621484

Gursoy, U. K., Könönen, E., Uitto, V. J., Pussinen, P. J., Hyvärinen, K., Suominen-Taipale, L., et al. (2009). Salivary interleukin-1beta concentration and the

presence of multiple pathogens in periodontitis. *J. Clin. Periodontol.* 36, 922–927. doi: 10.1111/j.1600-051X.2009.01480.x

Gürsoy, U. K., Zeidán-Chuliá, F., Yilmaz, D., Özdemir, V., Mäki-Petäys, J., de Oliveira, B. N., et al. (2015). Analyses of Gingival Adhesion Molecules in periodontitis: theoretical *in silico*, comparative *in vivo*, and explanatory *in vitro* models. *J. Periodontol.* doi: 10.1902/jop.2015.150361. [Epub ahead of print].

Haage, A., Nam, D. H., Ge, X., and Schneider, I. C. (2014). Matrix metalloproteinase-14 is a mechanically regulated activator of secreted MMPs and invasion. *Biochem. Biophys. Res. Commun.* 450, 213–218. doi: 10.1016/j.bbrc.2014.05.086

Haapasalmi, K., Mäkelä, M., Oksala, O., Heino, J., Yamada, K. M., Uitto, V. J., et al. (1995). Expression of epithelial adhesion proteins and integrins in chronic inflammation. *Am. J. Pathol.* 147, 193–206.

Haigh, B. J., Stewart, K. W., Whelan, J. R., Barnett, M. P., Smolenski, G. A., and Wheeler, T. T. (2010). Alterations in the salivary proteome associated with periodontitis. *J. Clin. Periodontol.* 37, 241–247. doi: 10.1111/j.1600-051X.2009.01525.x

Hajishengallis, G., and Sahingur, S. E. (2014). Novel inflammatory pathways in periodontitis. *Adv. Dent. Res.* 26, 23–29. doi: 10.1177/0022034514526240

Hanna, J., Meides, A., Zhang, D. P., and Finley, D. (2007). A ubiquitin stress response induces altered proteasome composition. *Cell* 129, 747–759. doi: 10.1016/j.cell.2007.03.042

Holm, T., Rutishauser, D., Kai-Larsen, Y., Lyutvinskiy, Y., Stenius, F., Zubarev, R. A., et al. (2014). Protein biomarkers in vernix with potential to predict the development of atopic eczema in early childhood. *Allergy* 69, 104–112. doi: 10.1111/all.12308

Ji, S., and Choi, Y. (2015). Point-of-care diagnosis of periodontitis using saliva: technically feasible but still a challenge. *Front. Cell. Infect. Microbiol.* 5:65. doi: 10.3389/fcimb.2015.00065

Kinney, J. S., Morelli, T., Braun, T., Ramseier, C. A., Herr, A. E., Sugai, J. V., et al. (2011). Saliva/pathogen biomarker signatures and periodontal disease progression. *J. Dent. Res.* 90, 752–758. doi: 10.1177/0022034511399908

Kousvelari, E., Yeh, C. K., Mertz, P. M., and Chinchetru, M. (1990). Regulation of proto-oncogenes and salivary gland cell proliferation. *Adv. Dent. Res.* 4, 61–68.

Lee, A., Ghaname, C. B., Braun, T. M., Sugai, J. V., Teles, R. P., Loesche, W. J., et al. (2012). Bacterial and salivary biomarkers predict the gingival inflammatory profile. *J. Periodontol.* 83, 79–89. doi: 10.1902/jop.2011.110060

Liu, Y. C., Penninger, J., and Karin, M. (2005). Immunity by ubiquitylation: a reversible process of modification. *Nat. Rev. Immunol.* 5, 941–952. doi: 10.1038/nri1731

Maquoi, E., Assent, D., Detilleux, J., Pequeux, C., Foidart, J. M., and Noël, A. (2012). MT1-MMP protects breast carcinoma cells against type I collagen-induced apoptosis. *Oncogene* 31, 480–493. doi: 10.1038/onc.2011.249

Nussbaum, G., and Shapira, L. (2011). How has neutrophil research improved our understanding of periodontal pathogenesis? *J. Clin. Periodontol.* 38, 49–59. doi: 10.1111/j.1600-051X.2010.01678.x

Podder, A., and Latha, N. (2014). New insights into schizophrenia disease genes interactome in the human brain: emerging targets and therapeutic implications in the postgenomics era. *OMICS* 18, 754–766. doi: 10.1089/omi.2014.0082

Rall, K., Eisenbeis, S., Barresi, G., Rückner, D., Walter, M., Poths, S., et al. (2015). Mayer-Rokitansky-Küster-Hauser syndrome discordance in monozygotic twins: matrix metalloproteinase 14, low-density lipoprotein receptor-related protein 10, extracellular matrix, and neoangiogenesis genes identified as candidate genes in a tissue-specific mosaicism. *Fertil. Steril.* 103, 494–502. doi: 10.1016/j.fertnstert.2014.10.053

Ramseier, C. A., Kinney, J. S., Herr, A. E., Braun, T., Sugai, J. V., Shelburne, C. A., et al. (2009). Identification of pathogen and host-response markers correlated with periodontal disease. *J. Periodontol.* 80, 436–446. doi: 10.1902/jop.2009.080480

Rosado, J. O., Henriques, J. P., and Bonatto, D. (2011). A systems pharmacology analysis of major chemotherapy combination regimens used in gastric cancer treatment: predicting potential new protein targets and drugs. *Curr. Cancer Drug Targets* 11, 849–869. doi: 10.2174/156800911796798977

Salazar, M. G., Jehmlich, N., Murr, A., Dhople, V. M., Holtfreter, B., Hammer, E., et al. (2013). Identification of periodontitis associated changes in the proteome of whole human saliva by mass spectrometric analysis. *J. Clin. Periodontol.* 40, 825–832. doi: 10.1111/jcpe.12130

Salminen, A., Gursoy, U. K., Paju, S., Hyvärinen, K., Mäntylä, P., Buhlin, K., et al. (2014). Salivary biomarkers of bacterial burden, inflammatory response, and tissue destruction in periodontitis. *J. Clin. Periodontol.* 41, 442–450. doi: 10.1111/jcpe.12234

Santana-Codina, N., Carretero, R., Sanz-Pamplona, R., Cabrera, T., Guney, E., Oliva, B., et al. (2013). A transcriptome-proteome integrated network identifies endoplasmic reticulum thiol oxidoreductase (ERp57) as a hub that mediates bone metastasis. *Mol. Cell. Proteomics* 12, 2111–2125. doi: 10.1074/mcp.M112.022772

Scott, D. A., and Krauss, J. (2012). Neutrophils in periodontal inflammation. *Front. Oral Biol.* 15, 56–83. doi: 10.1159/000329672

Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P. L., and Ideker, T. (2011). Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics* 27, 431–432. doi: 10.1093/bioinformatics/btq675

Szklarczyk, D., Franceschini, A., Kuhn, M., Simonovic, M., Roth, A., Minguez, P., et al. (2011). The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res.* 39, 561–568. doi: 10.1093/nar/gkq973

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., et al. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43, 447–452. doi: 10.1093/nar/gku1003

Ullrich, C., Mlekusch, R., Kuschnig, A., Marksteiner, J., and Humpel, C. (2010). Ubiquitin enzymes, ubiquitin and proteasome activity in blood mononuclear cells of MCI, Alzheimer and Parkinson patients. *Curr. Alzheimer Res.* 7, 549–555. doi: 10.2174/156720510792231766

Wain, H. M., Lush, M. J., Ducluzeau, F., Khodiyar, V. K., and Povey, S. (2004). Genew: the human gene nomenclature database, 2004 updates. *Nucleic Acids Res.* 32, 255–257. doi: 10.1093/nar/gkh072

Wisdom, R., Johnson, R. S., and Moore, C. (1999). c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *EMBO J.* 18, 188–197. doi: 10.1093/emboj/18.1.188

Yu, H., Kim, P. M., Sprecher, E., Trifonov, V., and Gerstein, M. (2007). The importance of bottlenecks in protein networks: correlation with gene essentiality and expression dynamics. *PLoS Comput. Biol.* 3:e59. doi: 10.1371/journal.pcbi.0030059

Zeidán-Chuliá, F., de Oliveira, B. H., Salmina, A. B., Casanova, M. F., Gelain, D. P., Noda, M., et al. (2014b). Altered expression of Alzheimer's disease-related genes in the cerebellum of autistic patients: a model for disrupted brain connectome and therapy. *Cell Death Dis.* 5, e1250. doi: 10.1038/cddis.2014.227

Zeidán-Chuliá, F., Gursoy, M., de Oliveira, B. H., Gelain, D. P., Könönen, E., Gursoy, U. K., et al. (2014a). Focussed microarray analysis of apoptosis in periodontitis and its potential pharmacological targeting by carvacrol. *Arch. Oral Biol.* 59, 461–469. doi: 10.1016/j.archoralbio.2014.01.007

Zeidán-Chuliá, F., Neves de Oliveira, B. H., Gursoy, M., Könönen, E., Fonseca Moreira, J. C., Gursoy, U. K., et al. (2013). MMP-REDOX/NO interplay in periodontitis and its inhibition with *Satureja hortensis* L. essential oil. *Chem. Biodivers.* 10, 507–523. doi: 10.1002/cbdv.201200375

Zhang, L., Henson, B. S., Camargo, P. M., and Wong, D. T. (2009). The clinical value of salivary biomarkers for periodontal disease. *Periodontol. 2000* 51, 25–37. doi: 10.1111/j.1600-0757.2009.00315.x

Zhu, X., Gerstein, M., and Snyder, M. (2007). Getting connected: analysis and principles of biological networks. *Genes Dev.* 21, 1010–1024. doi: 10.1101/gad.1528707

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on