DR. KAROLINA ELŻBIETA KACZOR-URBANOWICZ (Orcid ID : 0000-0001-9243-3652)
PROF. WILLIAM V. GIANNOBILE (Orcid ID : 0000-0002-7102-9746)

Article type : Other

Full TITLE:

"Salivary exRNA biomarkers to detect gingivitis and monitor disease regression"

Authors:

Karolina Elżbieta Kaczor-Urbanowicz[1*], Harsh M. Trivedi[11*], Patricia O. Lima[1,2], Paulo M. Camargo[3], William V. Giannobile[4], Tristan R. Grogan[5], Frederico O. Gleber-Netto[6], Yair Whiteman[7], Feng Li[1], Hyo Jung Lee[8], Karan Dharia[9], Katri Aro[1], Carmen Martin Carerras-Presas[10], Saarah Amuthan[9], Manjiri Vartak[9], David Akin[1], Hiba Al-adbullah[9], Kanika Bembey[9], Perry R. Klokkevold[3], David Elashoff[5], Virginia Monsul Barnes[12], Rose Richter[12], William DeVizio[12†], James G. Masters[11†,] David Wong[1†]

***** Joint first authors

†Joint corresponding authors

[1]Center for Oral/Head & Neck Oncology Research, UCLA School of Dentistry, University of California at Los Angeles, California, USA

[2]Department of Physiological Sciences, Piracicaba Dental School, University of Campinas, Piracicaba, São Paulo, Brazil

[3]Section of Periodontics, UCLA School of Dentistry, University of California at Los Angeles, California, USA

[4]Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA

[5] Department of Biostatistics, University of California at Los Angeles, California, USA

[6] Medical Genomics Laboratory, Centro Internacional de Pesquisa e Ensino (CIPE), AC Camargo Cancer Center, São Paulo, Brazil

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jcpe.12930

This article is protected by copyright. All rights reserved

Author Manuscript

[7] Center for Esthetic Dentistry, UCLA School of Dentistry, University of California at Los Angeles, California, USA
[8] Department of Periodontology, Section of Dentistry, Seoul National University Bundang Hospital, Seoul, Korea
[9] UCLA School of Dentistry, University of California at Los Angeles, California, USA
[10] Adult's Dentistry Department, Faculty of Biomedical Sciences, Universidad Europea de Madrid, Madrid, Spain.
[11]Colgate Palmolive Co., Early Research Oral Care, New Jersey, 909 River Road, Piscataway, New Jersey, USA 08855
[12]Colgate Palmolive Co., Clinical Research Oral Care, New Jersey, 909 River Road, Piscataway, New Jersey, USA 08855

Running title: "Salivary exRNA biomarkers for gingivitis"

Keywords: saliva; exRNA; gingivitis; biomarkers; monitoring

Correspondence authors:

David T.W. Wong
Felix & Mildred Yip Endowed and Distinguished Professor, Associate Dean for Research
Director for UCLA Center for Oral/Head & Neck Oncology Research (COOR)
UCLA School of Dentistry
10833 Le Conte Avenue
Los Angeles, CA 90095-1668
United States
Office: 73-017 CHS
Phone: (310) 206-3048
Fax: (310) 825-7609
dtww@ucla.edu

William DeVizio
Colgate Palmolive Co.
Advanced Technology Oral Care
New Jersey, 909 River Road, Piscataway, New Jersey, USA 08855
Office: (732) 878-7901
William_Devizio@colpal.com

James G. Masters
Colgate Palmolive Co.

This article is protected by copyright. All rights reserved

Author Manuscript

Advanced Technology Oral Care
New Jersey, 909 River Road, Piscataway, New Jersey, USA 08855
Office: 732 878 7633
jim_masters@colpal.com

Author Manuscript

Conflict of interest and sources of funding statement

DTWW is co-founder of RNAmeTRIX Inc., a molecular diagnostic company. He holds equity in RNAmeTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNAmeTRIX. Intellectual property that David Wong invented and which was patented by the University of California has been licensed to RNAmeTRIX. Additionally, he is a consultant to GlaxoSmithKlein, Wrigley, EZLife Bio Inc. and Colgate-Palmolive Company.

WVG has previously consulted for Colgate, but not on this specific investigation.

This article is protected by copyright. All rights reserved

KEKU, HMT, JGM and DTWW has a pending patent related to this work [Attorney Docket No. 206030-0122-Pl-US.606791].

None of the other authors have a conflict of interest to disclose related to this study.

This work was supported by a research grant from the Colgate-Palmolive Company – Protocol Number ERO-2015-SAL-03-HT and the Public Health Service (PHS) grants from the National Institutes of Health (NIH): UH3 TR000923 and R90 DE022734.

Author Manuscript

Abstract

Aim: This study tests the hypothesis that salivary extracellular RNA (exRNA) biomarkers can be developed for gingivitis detection and monitoring disease regression.

Materials and Methods: Salivary exRNA biomarker candidates were developed from a total of 100 gingivitis and non-gingivitis individuals using Affymetrix's expression microarrays. The top ten differentially expressed exRNAs were tested in a clinical cohort to determine if the discovered salivary exRNA markers for gingivitis were associated with clinical gingivitis and disease regression. For this purpose, unstimulated saliva was collected from 30 randomly selected gingivitis subjects, the gingival and plaque indexes scores were taken at baseline, 3 & 6 weeks and salivary exRNAs were assayed by means of reverse transcription quantitative polymerase chain reaction.

Results: Eight salivary exRNA biomarkers developed for gingivitis were statistically significantly changed over time, consistent with disease regression. A panel of four salivary exRNAs [SPRR1A, lnc-TET3-2:1, FAM25A, CRCT1] can detect gingivitis with a clinical performance of 0.91 area under the curve (AUC), with 71% sensitivity and 100% specificity.

This article is protected by copyright. All rights reserved

Conclusions: The clinical values of the developed salivary exRNA biomarkers are associated with gingivitis regression. They offer strong potential to be advanced for definitive validation and clinical laboratory development test (LDT).

Author Manuscript

Clinical Relevance

Scientific rationale for study: Presently, the gold standard for diagnosis of gingivitis includes mainly clinical examination. As a companion diagnostics, scientifically based evaluation of gingivitis would be very beneficial as it is the most prevalent inflammatory disease in humans.

Principal findings: Principles of prospective study design (PRoBE) and rigorous clinical classification of gingivitis led to the development and validation of salivary exRNA biomarkers for gingivitis detection, that are associated with disease regression.

Discriminatory salivary exRNA biomarkers were developed for gingivitis detection. Four salivary exRNA panel can discriminate gingivitis from healthy periodontium with a performance of 0.91 AUC, with 71% sensitivity and 100% specificity.

Practical implications: Salivary exRNA markers can be objective molecular indicators of periodontal health for disease detection as well as gingivitis regression.

This article is protected by copyright. All rights reserved

Author Manuscript

Introduction

Periodontal diseases are the most common inflammatory diseases in humankind (Kassebaum et al., 2017). Gingivitis, the reversible form of the disease, is induced by the accumulation of bacterial biofilm, that can be treated with professional biofilm removal and improvement in oral hygiene (Kim et al., 2013). If left untreated, gingivitis may progress to an irreversible form - periodontitis, which involves bone and attachment loss. Periodontitis can advance to the point that it induces tooth mobility and tooth loss (Kim et al., 2013). It may also adversely influence systemic health via cardiovascular diseases (Kim et al., 2013), ischemic stroke (Grau et al., 2004) or cancers (Binder Gallimidi et al., 2015, Mitsuhashi et al., 2015, Rajesh et al., 2013). The common risk factors for periodontitis could be divided into inherited (e.g. genetic variants), and those that are acquired (e.g. socio-economic factors, poor oral hygiene, cigarette smoking and diabetes) (Chapple et al., 2017).

The quest to develop salivary biomarkers for periodontal diseases has been elusive (Giannobile et al., 2009). Despite the scientific acceptance of salivary biomarkers for the detection of gingivitis (Kinney et al., 2011, Lee et al., 2012, Shaila et al., 2013, Morelli et al., 2014, Henskens et al., 1993), the absence of definitively validated biomarkers did not allow for such technology to receive regulatory approval and translating this diagnostic test to move into clinical practice. Presently, the gold standard for early detection and diagnosis for gingivitis includes a comprehensive periodontal exam along with clinical examination (i.e. redness, swelling or gingival bleeding) performed during dental examinations. Unfortunately, the absence of pain in gingivitis often renders individuals unaware of their pathologic gingival condition, particularly if they do not have to visit the dentist regularly (Kim et al., 2013). While very helpful, clinical parameters do not provide full information about the current status of the disease activity, patient-specific variations, do not predict non-responders for treatment therapy and those who are “at risk” for disease progression (Ebersole et al., 2015). Thus, it is desirable to develop objective and scientifically credible biomarkers for early detection and monitoring of periodontal diseases, especially that many of the affected cases are left undiagnosed and untreated (Albandar and Kingman, 1999, Tomar and Asma, 2000).

This article is protected by copyright. All rights reserved

The applications of such biomarkers could be multiple. Firstly, they might be used by community at large as self-administered point-of-care (POC) tests to screen for periodontal diseases, thus making people aware of the pathology in their oral cavities and to encourage them to search for a dental care. The chairside POC devices will require minimal clinical training and resources, lead to simpler, more cost-effective and less intensive treatment (Giannobile, 2012). Due to close proximity of saliva to periodontal tissues and its easy collection, saliva constitutes a valuable diagnostic source, specifically as its collection is easy, repeatable and can be performed by auxiliary personnel. In addition, the salivary biomarkers present an attractive tool for quantitative & qualitative objective evaluation and monitoring of gingivitis for both scientists and dentists. The use of salivary based-POCs would also enable the underserved communities an improved access to oral health programs as well as offers the ability to identify and monitor the patients at risk (Giannobile, 2012). Lastly, the dental, pharmaceutical & oral health care companies could use them for optimizing the pharmaceutical efficiency such as adequate selection of target patients, optimal dose selection, and to assess the performance of the current active compounds in the development of their new oral hygiene products such as toothpastes, mouthwashes, etc.

This study addresses the unmet clinical need of assessing host factors as a companion diagnostics to detect gingivitis and monitor its treatment response.

Materials and methods

Ethics statement

The sample collection was approved by the Institutional Review Board (IRB) at the University of California at Los Angeles (UCLA) for both the discovery phase (IRB#10-000505) and the clinical stage (IRB#15-001957) of the study. Each participant had to sign the informed consent.

Discovery phase

Based on the prospective-specimen-collection and retrospective-blinded-evaluation (PRoBE) study design (Pepe et al., 2008), saliva was collected prospectively from 750 human subjects at the UCLA School of Dentistry. The inclusion and exclusion criteria are presented in Figure 1. Subjects were classified as healthy or gingivitis according to research and clinical criteria (Figure 1).

Prior to a clinical evaluation, unstimulated whole saliva was collected for every subject. Subjects were asked to avoid oral hygiene measures, eating, drinking or gum chewing at least 1 h prior to saliva collection. All subjects rinsed with tap water (10 mL) for 30 s about 10 min prior to saliva collection and expectorated. Clinical samples were collected into sterile tubes, lasting 5-10 min per collection (at least 5 mL of saliva),

This article is protected by copyright. All rights reserved

Author Manuscript

and kept on ice through the entire process. All samples were processed, around 1 hour after collection. Samples were firstly centrifuged in a refrigerated centrifuge at 2400 x g for 15 min at 4°C, and the supernatant was processed immediately for the concurrent stabilization of proteins and RNA by the inclusion of a protease inhibitor cocktail [aprotinin, 3- phenylmethylsulfonyl fluoride (3- PMSF) (Sigma-Aldrich, St. Louis, MO, USA), sodium orthovanadate (Na3VO4) (Sigma-Aldrich, St. Louis, MO, USA)] and RNase inhibitor [Invitrogen SUPERase·In RNase Inhibitor (Thermo Fisher Scientific, Austin, TX, USA)] based on our saliva standard operating procedure (SOP) (Henson and Wong, 2010). These samples were aliquoted into smaller cryo-vials, labeled and frozen at –80°C.

Afterwards, salivary exRNA was extracted using the RNeasy Micro Kit (Qiagen, Germantown, MD, USA) including DNase I digestion. Samples with good purity QC (OD 260/280 ~1.8) were profiled using the GeneChip Human Transcriptome Affymetrix HTA 2.0 expression arrays (Thermo Fisher Scientific, Austin, TX, USA)].

Pilot Clinical Research Study

The pilot study consisted of two stages: clinical and laboratory.

Clinical stage

For analysis of saliva samples, 30 volunteers (aged 18-65), diagnosed with gingivitis were included in this study. Figure 1 presents the inclusion and exclusion criteria. The duration of this project was 7 weeks (including 1 week pretrial training “washout” period to check if there are any allergic reactions to toothpaste’s ingredients) and involved the same treatment protocol for gingivitis such as brushing with toothpastes containing 1000 ppm sodium monofluorophosphate (MFP) (Colgate-Palmolive Company, New York, NY, USA). Each volunteer was instructed to brush the whole mouth with a full ribbon of the toothpaste for 1 min twice daily. The subjects expectorated and rinsed with water. Subjects returned for a clinic visit at baseline (B), 3 and 6 weeks to donate saliva samples prior to the clinical examination. The protocol for collection and processing of saliva was the same as described previously (discovery phase), but saliva was collected only in the mornings and immediately frozen at -20°C for about 3-4 hours until further analysis.

Laboratory stage

Validation phase

The direct saliva transcriptome analysis (DSTA)

This article is protected by copyright. All rights reserved

Author Manuscript

For the validation phase, the direct saliva transcriptome analysis (DSTA) was performed that uses cell-free saliva supernatant instead of isolated messenger RNA (mRNA) for saliva transcriptomic detection (Lee et al., 2011). The validation was performed by means of reverse transcription quantitative polymerase chain reaction (RT-qPCR) in saliva taken from 30 gingivitis individuals in the clinical phase at B, 3 and 6 weeks. The qPCR associated with melting-curve analysis was conducted by the use of the LightCycler® 480 Real-Time PCR System (Roche Life Science, Indianapolis, Indiana, United States) with a fixed thermal-cycling program.

Statistical analysis

Kruskal-Wallis and chi-squared tests were performed to compare markers between healthy and gingivitis groups in the discovery phase of transcriptomic analysis. In the validation phase, the analysis of variance (ANOVA) was done at a significance level of $p < 0.05$. In addition, the logistic regression analysis in R was used to construct the final panel of biomarkers using the "best glm" function and the best Akaike Information Criterion (AIC)]. A paired t-test along with the 95% confidence interval (CI) was used to compare the Löe-Silness Gingival Index (GI) (Loe, 1967) and the Quigley and Hein Plaque Index (PI) (Turesky Modification) (Quigley and Hein, 1962, Turesky et al., 1970) scores over different time periods. Furthermore, generalized estimating equation (GEE) models were added to show the overall trends in biomarker changes over time. Finally, interindividual variability between two different clinical investigators was examined (the mean of each examiner's patients clinical scores at each time point). The examiners in the study followed the same patients through the investigation. The statistical analyses were performed using R V3.1.2 (R Core Team 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria) and SAS V9.4 (SAS Institute Inc., Cary, NC, USA), while the randomization was performed using the online statistical computing web programming "Sealed envelope" (https://sealedenvelope.com).

RESULTS

Clinical outcomes

In the discovery phase, the study group consisted of 100 subjects, including 50 gingivitis subjects (22 males & 28 females) aged 26.4 ± 6.77, and 50 healthy individuals aged 27.1 ± 5.67 (23 males & 27 females). The scores for the gingivitis group were: 7.2 ± 1.1% for Marginal Bleeding Index (MBI) (Loe, 1967, Muhlemann and Son, 1971) and 2.5 ± 0.7mm for Probing Pocket Depth (PPD) (Ramfjord, 1959), while for

This article is protected by copyright. All rights reserved

Author Manuscript

the healthy group, the results were following: 2.4 ± 1.6% for MBI and 2.1 ± 0.8mm for PPD. The participants were mainly of White origin (73%), but also Asian (17%), black or African American (10%).

The validation phase included 30 subjects (13 males & 17 females), aged 28.2 ± 7.77. The participants were mainly of White origin (75%), but also Asian (20%) and black or African American (5%). The average GI scores were at Screening (Scr) 1.75 ± 0.2, at B – 1.814 ± 0.18, at 3 weeks – 1.684 ± 0.26 and at 6 weeks – 1.46 ± 0.55, while the average PI scores were following: at Scr 2.293 ± 0.44, at B – 2.307 ± 0.34, at 3 weeks – 2.054 ± 0.33 and at 6 weeks – 1.86 ± 0.36 (Supplemental Table 1). Both GI and PI showed significantly decreased scores over time due to good oral hygiene regimens implemented by brushing with toothpastes (Figure 2). The GI scores improved for 60% and PI for 93.3% of subjects over the period of 6 weeks.

Measurements of clinical parameters

Due to the long-term period of the study and large time difference between performing the discovery and the validation phases, there was a change in the current protocol for the enrollment of the patients into the study between the two stages. The MBI (Loe, 1967, Muhlemann and Son, 1971) applied in the discovery phase, was replaced later, in the validation phase, with the more commonly used indices such as GI (Loe, 1967) and PI (Quigley and Hein, 1962, Turesky et al., 1970) for the determination of the gingivitis status. The PPD (Ramfjord, 1959) was measured in both phases of the study. In the discovery phase, PPD was used to differentiate between healthy (non-periodontal disease) (PPD < 4mm) and gingivitis (PPD < 4mm) from periodontitis (≥5mm) (apart from radiographic assessment). In the validation phase, only patients with diagnosed gingivitis were included. Study subjects could not have any PPD deeper than 5mm (except for 3rd molars) to be enrolled in the research project.

Investigation of potential clinical evaluators bias

The were no significant interexaminer differences between the clinical evaluators, except for time point 6 weeks, which could potentially affect the GI and PI scores (Supplementary Figure 1). This is despite the fact that all examiners were initially calibrated.

Discovery of salivary exRNA biomarker candidates for gingivitis by HTA Microarray profiling

This article is protected by copyright. All rights reserved

Author Manuscript

The 25 salivary exRNA biomarker candidates were identified from a discovery cohort of randomly selected 50 gingivitis and 50 age/gender-matched healthy controls (Table 1) (Figure 1). Of these, 10 differentially expressed exRNA biomarker candidates between gingivitis and healthy groups were advanced for validation by means of RT-qPCR using an independent cohort of 30 gingivitis subjects in the pilot clinical research study. Those exRNA biomarker candidates were selected based on their known association with inflammation and periodontal diseases, low p-value ($p < 0.05$) and high absolute fold change in their expression levels between gingivitis and healthy groups.

Validation of 10 salivary exRNA biomarker candidates for gingivitis by RT-qPCR

Eight out of 10 validated exRNA targets were concordant with the HTA 2.0 microarray data (Table 2), including 4 decreased exRNAs [SPRR1A (m1), LGALS3 (m4), FAM25A (m7), CRCT1 (m10)] and 4 increased exRNAs [lnc-TET3-2:1 (m2), RP5-965F6.2 (m3), GALNT10 (m5), SOX4 (m6)] over time (B – 3 weeks, 3 weeks – 6 weeks, B – 6 weeks) (GEE p-values < 0.001) (Supplemental Figure 2, Supplemental Table 1). Figure 3 presents a plot with fold changes and 95% confidence intervals (CIs) for each marker at B, 3 and 6 weeks. The increase in their alteration levels compared to the initial data might be a result of the interindividual variability in exRNA patterns.

Logistic regression analysis

The potential clinical discriminatory power of the developed salivary exRNA for gingivitis detection was evaluated. Logistic regression analysis of 8 validated exRNAs revealed that four exRNA marker model [SPRR1A (m1), lnc-TET3-2:1 (m2), FAM25A (m7), CRCT1 (m10)] could potentially provide a discriminatory performance of 0.91 AUC (area under the curve) with 71% sensitivity and 100% specificity (Figure 4).

Discussion

Saliva is a complex body fluid that is composed of unique molecular constituents (proteins, DNA, RNA, etc.) that can be discriminatory for oral and systemic disease screening, detection and monitoring (Kaczor-Urbanowicz et al., 2017). The development of non-invasive salivary test based on molecular biomarkers could have impactful clinical applications, providing accurate and real-time monitoring of periodontal disease activity for the general public at home or at the dental clinic. Although proteins are the essential effectors of cellular function, the eukaryotic complexity and phenotypic variation lies in a control of trans-

This article is protected by copyright. All rights reserved

Author Manuscript

acting RNAs - noncoding RNAs (ncRNAs) (Wong, 2015). NcRNAs regulate diverse biological functions, thus being very attractive for the development of new target therapies and discovery of new diagnostic biomarkers in human body fluids, including saliva (Ainsztein et al., 2015). The human salivary transcriptome was firstly described in 2004 (Li et al., 2004), followed by the intense investigations in the following years (Nussbaumer et al., 2006). The revealed complex exRNA composition of saliva encompass mostly mRNAs, but also ncRNAs such as long non-coding RNAs (lncRNAs), microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), etc. (Wong, 2015). Among all ncRNAs, small ncRNAs are the most exploited exRNAs in saliva, particularly miRNAs that play an important role in regulating various biological processes (Nelson et al., 2003), while the current knowledge of lncRNAs is still largely uninvestigated (Kung et al., 2013).

The basis of biomarker research success in periodontology is to develop a diagnostic method that will have a potential to favorably affect clinical decision-making, patient outcomes and healthcare providers (Ghallab, 2017). Periodontal disease is time consuming and expensive to treat, thus prevention and early detection constitutes significant health-care benefit (Ghallab, 2017). Thus, it would be desirable to develop biomarkers for early detection and monitoring of periodontal disease and its treatment response (Giannobile et al., 2009). In the present study, the objective was to validate exRNA biomarkers for gingivitis, and to evaluate their potential to monitor disease regression. Eight salivary exRNAs showed significant positive correlation of their expression levels with improving clinical measures (GI, PI) due to the introduced treatment (brushing teeth), thus making them potentially useful in monitoring the current activity of disease and its treatment response. The identified exRNAs are mostly involved in inflammatory response, antimicrobial activity, innate immune response, apoptosis, neutrophil, eosinophil & macrophage chemotaxis, and bone homeostasis, that is consistent with the current literature as periodontal diseases are mostly inflammatory in origin and involved in immune pathways (Ghallab, 2017) (Table 3). Interestingly, only very few of them had been previously reported in different biofluids, but none of them have been identified in human saliva. Specifically, lncRNAs have arousen a lot of widespread interest in recent years as potentially associated in inducing particular diseases or developmental processes, but knowledge of the particular pathogenetic mechanisms by which they act is still limited. Therefore, it is not surprising, that in the current literature, not much information can be found about the developed in this study three lncRNA biomarkers of gingivitis (SPRR1A, lnc-TET3-2, RP5-965F6.2). It is known only that SPRR1A (m1) is a regeneration-associated gene (Starkey et al., 2009), while lnc-TET3-2:1 (m2) acts as an epigenetic mediator (Langemeijer et al., 2009) (Table 3).

This article is protected by copyright. All rights reserved

Author Manuscript

In addition, our study also revealed 5 mRNA RNAs (LGALS3, GALNT10, SOX4, FAM25A, CRCT1) that are an emerging and flourishing field for noninvasive diagnostic applications. The identification of saliva-derived mRNA in normal and cancer patients (Li et al., 2004, Hu et al., 2008) and other forensic applications (Juusola and Ballantyne, 2005) have opened up a new avenue for further clinical usage. According to the literature, LGALS3 (m4) can be informative on the inflammatory and bacterial nature of gingivitis due to their connectivity to the disease-related features such as its involvement in specific cellular (apoptosis, neutrophil, eosinophil and macrophage chemotaxis, etc.) and biological functions (antimicrobial activity, etc.) (Raz et al., 1991) (Table 3). As expected, in gingivitis due to gum inflammation, decreased levels of LGALS3 can be observed, that is concordant with our findings. In turn, alterations in SOX4 (m6) expression, induced by transforming growth factor beta (TGF-β), regulate T helper type 2 (TH2) cell–driven airway inflammation (Kuwahara et al., 2012), while downregulation of FAM25A (m7) (observed also in our study) indicates increased risk for inflammation and infectious diseases (Wang et al., 2014, Mauritz et al., 2010, Deloukas et al., 2004). Lastly, CRCT1 (m10) is reported to be associated with early stages of Alzheimer's disease (AD), in which transcriptome changes affecting cell signaling, inflammation and neurotransmission pathways take place before the neuropathology in brains occur. In addition, the involvement of CRCT1 in protection against infection and inflammation is currently being explored (Parra-Damas et al., 2014) (Table 3). Thus, the potential role of the 8 validated in this study exRNAs in pathogenetic mechanisms of periodontal diseases can be explained through their biological roles and potential functions (Table 3).

The major advantage of the study was the use of saliva, collected in a prospective way, as a potential non-invasive diagnostic source for detection and monitoring of gingivitis. Secondly, our study was focused on long RNAs as potential salivary biomarkers of periodontal diseases, including novel finding of lncRNAs, while the current literature reports mostly on short RNAs (microRNAs) with rather unsatisfactory performance (Schmalz et al., 2016, Kagiya, 2016, Ogata et al., 2014). Lastly, the use of DSTA method, that omits the step of RNA extraction, seems to be much more convenient and practical way of performing the experimental stage, as RNA isolation from saliva is not easy to perform and requires diligent protocol.

The main limitation of the study were the interindividual differences in scoring of PI and GI, that suggest that a better system of clinical evaluation for gingivitis is largely needed, such as changes in expression levels of exRNA biomarkers, that can be used as companion diagnostic tool for periodontal and clinical examinations. In addition, the same inclusion and exclusion criteria for clinical evaluation of the periodontal status (the same periodontal indices for both discovery and validation phases) are recommended to be used for the enrollment of the subjects into the project. Lastly, a comprehensive study of the variety of exRNAs and

This article is protected by copyright. All rights reserved

Author Manuscript

further increased sampling is needed in order to establish the sensitivity and specificity of the final panel of salivary exRNA biomarkers for gingivitis.

In fact, different groups have studied the exRNA composition of saliva for detection of periodontal diseases. Inflammatory mRNA markers for interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) can be detected in whole saliva to monitor the status of periodontal disease in type II diabetes patients (Gomes et al., 2006). mRNA expression of human beta defensin-1 and -2 in the gingival tissue are associated with gingivitis, aggressive and chronic periodontitis (Vardar-Sengul et al., 2007). Also, the expression levels of Toll-like receptors (TRL) TLR2, -4, -7, -9, interferon-alpha1 (IFN-α1) (Kajita et al., 2007) and MYD88 mRNAs were significantly lower in gingivitis than in severe periodontitis lesions (Ghaderi et al., 2014). In addition, four miRNAs (hsa-miR-451, hsa-miR-223, hsa-miR-486-5p, hsa-miR-3917) were significantly overexpressed, and 7 (hsa-miR-1246, hsa-miR-1260, hsa-miR-141, hsa-miR-1260b, hsa-miR-203, hsa-miR-210, hsa-miR-205) were underexpressed by > 2-fold in a diseased compared to healthy gingiva (Stoecklin-Wasmer et al., 2012). In turn, Bochenek et al. reports about downregulation of lncRNA ANRIL associated with atherosclerosis, periodontitis and several forms of cancer (Bochenek et al., 2013).

Several studies in the last decades have focused on finding a precise method for the diagnosis of periodontal disease in its early stages, such as gingivitis, as well as to monitor its treatment and progression into periodontitis. Interleukin 1 beta (IL-1β) can be considered as an indicator of periodontal disease progression (Nazar Majeed et al., 2016), while matrix metalloproteinase-8 (MMP-8) may indicate response to therapy (Sexton et al., 2011). Additionally, macrophage inflammatory protein-1α (MIP-1α) appeared to increase prediction of periodontitis progression (Fine et al., 2009) and to decrease its levels following periodontal therapy (Al-Sabbagh et al., 2012, Sexton et al., 2011).

Recent advances in transcriptomic high-throughput technologies are shedding a new light on salivary biomarker discovery, which can advance salivary diagnosis of periodontal diseases to a higher level. Salivary biomarkers for periodontal diseases need to have the intended clinical context to obtain approval from the Food and Drug Administration (FDA). The PRoBE design, applied in our study, greatly facilitates the future translation of the biomarkers into the everyday dental practice (Kim et al., 2013). Our results indicate that salivary exRNAs represent a promising source of biomarkers for gingivitis as well as monitoring disease regression. Although challenges emerge, using saliva seems to have a bright future in early detection of periodontal diseases and monitoring of periodontal treatment outcomes.

This article is protected by copyright. All rights reserved

Author Manuscript

Author Manuscript

REFERENCES

Ainsztein, A. M., Brooks, P. J., Dugan, V. G., Ganguly, A., Guo, M., Howcroft, T. K., Kelley, C. A., Kuo, L. S., Labosky, P. A., Lenzi, R., Mckie, G. A., Mohla, S., Procaccini, D., Reilly, M., Satterlee, J. S., Srinivas, P. R., Church, E. S., Sutherland, M., Tagle, D. A., Tucker, J. M. & Venkatachalam, S. 2015. The NIH Extracellular RNA Communication Consortium. *J Extracell Vesicles,* 4**,** 27493.

Al-Sabbagh, M., Alladah, A., Lin, Y., Kryscio, R. J., Thomas, M. V., Ebersole, J. L. & Miller, C. S. 2012. Bone remodeling-associated salivary biomarker MIP-1alpha distinguishes periodontal disease from health. *J Periodontal Res,* 47**,** 389-95.

This article is protected by copyright. All rights reserved

Albandar, J. M. & Kingman, A. 1999. Gingival recession, gingival bleeding, and dental calculus in adults 30 years of age and older in the United States, 1988-1994. *J Periodontol,* 70**,** 30-43.

Atabaki, M., Hashemi, M., Daneshvar, H. & Alijani, E. 2017. Lectin, galactoside-binding, soluble, 3 rs4652 A/C gene variation and the risk for rheumatoid arthritis. *Biomed Rep,* 6**,** 251-255.

Binder Gallimidi, A., Fischman, S., Revach, B., Bulvik, R., Maliutina, A., Rubinstein, A. M., Nussbaum, G. & Elkin, M. 2015. Periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* promote tumor progression in an oral-specific chemical carcinogenesis model. *Oncotarget,* 6**,** 22613-23.

Bochenek, G., Hasler, R., El Mokhtari, N. E., Konig, I. R., Loos, B. G., Jepsen, S., Rosenstiel, P., Schreiber, S. & Schaefer, A. S. 2013. The large non-coding RNA ANRIL, which is associated with atherosclerosis, periodontitis and several forms of cancer, regulates ADIPOR1, VAMP3 and C11ORF10. *Hum Mol Genet,* 22**,** 4516-27.

Boultwood, J., Fidler, C., Strickson, A. J., Watkins, F., Kostrzewa, M., Jaju, R. J., Muller, U. & Wainscoat, J. S. 2000. Transcription mapping of the 5q- syndrome critical region: cloning of two novel genes and sequencing, expression, and mapping of a further six novel cDNAs. *Genomics,* 66**,** 26-34.

Chapple, I. L., Bouchard, P., Cagetti, M. G., Campus, G., Carra, M. C., Cocco, F., Nibali, L., Hujoel, P., Laine, M. L., Lingstrom, P., Manton, D. J., Montero, E., Pitts, N., Range, H., Schlueter, N., Teughels, W., Twetman, S., Van Loveren, C., Van Der Weijden, F., Vieira, A. R. & Schulte, A. G. 2017. Interaction of lifestyle, behaviour or systemic diseases with dental caries and periodontal diseases: consensus report of group 2 of the joint EFP/ORCA workshop on the boundaries between caries and periodontal diseases. *J Clin Periodontol,* 44 Suppl 18**,** S39-S51.

Deloukas, P., Earthrowl, M. E., Grafham, D. V., Rubenfield, M., French, L., Steward, C. A., Sims, S. K., Jones, M. C., Searle, S., Scott, C., Howe, K., Hunt, S. E., Andrews, T. D., Gilbert, J. G., Swarbreck, D., Ashurst, J. L., Taylor, A., Battles, J., Bird, C. P., Ainscough, R., Almeida, J. P., Ashwell, R. I., Ambrose, K. D., Babbage, A. K., Bagguley, C. L., Bailey, J., Banerjee, R., Bates, K., Beasley, H., Bray-Allen, S., Brown, A. J., Brown, J. Y., Burford, D. C., Burrill, W., Burton, J., Cahill, P., Camire, D., Carter, N. P., Chapman, J. C., Clark, S. Y., Clarke, G., Clee, C. M., Clegg, S., Corby, N., Coulson, A., Dhami, P., Dutta, I., Dunn, M., Faulkner, L., Frankish, A., Frankland, J. A., Garner, P., Garnett, J., Gribble, S., Griffiths, C., Grocock, R., Gustafson, E., Hammond, S., Harley, J. L., Hart, E., Heath, P. D., Ho, T. P., Hopkins, B., Horne, J., Howden, P. J., Huckle, E., Hynds, C., Johnson, C., Johnson, D., Kana, A., Kay, M., Kimberley, A. M., Kershaw, J. K., Kokkinaki, M., Laird, G. K., Lawlor, S., Lee, H. M., Leongamornlert, D. A., Laird, G., Lloyd, C., Lloyd, D. M., Loveland, J., Lovell, J., Mclaren, S., Mclay, K. E., Mcmurray, A., Mashreghi-Mohammadi, M., Matthews, L., Milne, S., Nickerson, T., Nguyen, M., Overton-Larty, E., Palmer, S. A., Pearce, A. V., Peck, A. I.,

This article is protected by copyright. All rights reserved

Author Manuscript

Pelan, S., Phillimore, B., Porter, K., Rice, C. M., Rogosin, A., et al. 2004. The DNA sequence and comparative analysis of human chromosome 10. *Nature,* 429**,** 375-81.

Ebersole, J. L., Nagarajan, R., Akers, D. & Miller, C. S. 2015. Targeted salivary biomarkers for discrimination of periodontal health and disease(s). *Front Cell Infect Microbiol,* 5**,** 62.

Fine, D. H., Markowitz, K., Furgang, D., Fairlie, K., Ferrandiz, J., Nasri, C., Mckiernan, M., Donnelly, R. & Gunsolley, J. 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**,** 106-13.

Ghaderi, H., Kiany, F., Razmkhah, M., Dadras, S., Chenari, N., Hosseini, A., Younesi, V. & Ghaderi, A. 2014. mRNA expression of pattern recognition receptors and their signaling mediators in healthy and diseased gingival tissues. *J Indian Soc Periodontol,* 18**,** 150-4.

Ghallab, N. A. 2017. Diagnostic potential and future directions of biomarkers in gingival crevicular fluid and saliva of periodontal diseases: Review of the current evidence. *Arch Oral Biol,* 87**,** 115-124.

Giannobile, W. V. 2012. Salivary diagnostics for periodontal diseases. *J Am Dent Assoc,* 143**,** 6S-11S.

Giannobile, W. V., Beikler, T., Kinney, J. S., Ramseier, C. A., Morelli, T. & Wong, D. T. 2009. Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontol 2000,* 50**,** 52-64.

Gibbs, S., Fijneman, R., Wiegant, J., Van Kessel, A. G., Van De Putte, P. & Backendorf, C. 1993. Molecular characterization and evolution of the SPRR family of keratinocyte differentiation markers encoding small proline-rich proteins. *Genomics,* 16**,** 630-7.

Gomes, M. A., Rodrigues, F. H., Afonso-Cardoso, S. R., Buso, A. M., Silva, A. G., Favoreto, S., Jr. & Souza, M. A. 2006. Levels of immunoglobulin A1 and messenger RNA for interferon gamma and tumor necrosis factor alpha in total saliva from patients with diabetes mellitus type 2 with chronic periodontal disease. *J Periodontal Res,* 41**,** 177-83.

Grau, A. J., Becher, H., Ziegler, C. M., Lichy, C., Buggle, F., Kaiser, C., Lutz, R., Bultmann, S., Preusch, M. & Dorfer, C. E. 2004. Periodontal disease as a risk factor for ischemic stroke. *Stroke,* 35**,** 496-501.

Henskens, Y. M., Van Der Velden, U., Veerman, E. C. & 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**,** 43-8.

Henson, B. S. & Wong, D. T. 2010. Collection, storage, and processing of saliva samples for downstream molecular applications. *Methods Mol Biol,* 666**,** 21-30.

Hu, Z., Zimmermann, B. G., Zhou, H., Wang, J., Henson, B. S., Yu, W., Elashoff, D., Krupp, G. & Wong, D. T. 2008. Exon-level expression profiling: a comprehensive transcriptome analysis of oral fluids. *Clin Chem,* 54**,** 824-32.

This article is protected by copyright. All rights reserved

Author Manuscript

Juusola, J. & Ballantyne, J. 2005. Multiplex mRNA profiling for the identification of body fluids. *Forensic Sci Int,* 152**,** 1-12.

Kaczor-Urbanowicz, K. E., Martin Carreras-Presas, C., Aro, K., Tu, M., Garcia-Godoy, F. & Wong, D. T. 2017. Saliva diagnostics - Current views and directions. *Exp Biol Med (Maywood),* 242**,** 459-472.

Kagiya, T. 2016. MicroRNAs: Potential Biomarkers and Therapeutic Targets for Alveolar Bone Loss in Periodontal Disease. *Int J Mol Sci,* 17.

Kajita, K., Honda, T., Amanuma, R., Domon, H., Okui, T., Ito, H., Yoshie, H., Tabeta, K., Nakajima, T. & Yamazaki, K. 2007. Quantitative messenger RNA expression of Toll-like receptors and interferon-alpha1 in gingivitis and periodontitis. *Oral Microbiol Immunol,* 22**,** 398-402.

Kassebaum, N. J., Smith, A. G. C., Bernabe, E., Fleming, T. D., Reynolds, A. E., Vos, T., Murray, C. J. L., Marcenes, W. & Collaborators, G. B. D. O. H. 2017. Global, Regional, and National Prevalence, Incidence, and Disability-Adjusted Life Years for Oral Conditions for 195 Countries, 1990-2015: A Systematic Analysis for the Global Burden of Diseases, Injuries, and Risk Factors. *J Dent Res,* 96**,** 380-387.

Kim, J. J., Kim, C. J. & Camargo, P. M. 2013. Salivary biomarkers in the diagnosis of periodontal diseases. *J Calif Dent Assoc,* 41**,** 119-24.

Kinney, J. S., Morelli, T., Braun, T., Ramseier, C. A., Herr, A. E., Sugai, J. V., Shelburne, C. E., Rayburn, L. A., Singh, A. K. & Giannobile, W. V. 2011. Saliva/pathogen biomarker signatures and periodontal disease progression. *J Dent Res,* 90**,** 752-8.

Kung, J. T., Colognori, D. & Lee, J. T. 2013. Long noncoding RNAs: past, present, and future. *Genetics,* 193**,** 651-69.

Kuwahara, M., Yamashita, M., Shinoda, K., Tofukuji, S., Onodera, A., Shinnakasu, R., Motohashi, S., Hosokawa, H., Tumes, D., Iwamura, C., Lefebvre, V. & Nakayama, T. 2012. The transcription factor Sox4 is a downstream target of signaling by the cytokine TGF-beta and suppresses T(H)2 differentiation. *Nat Immunol,* 13**,** 778-86.

Langemeijer, S. M., Aslanyan, M. G. & Jansen, J. H. 2009. TET proteins in malignant hematopoiesis. *Cell Cycle,* 8**,** 4044-8.

Lee, A., Ghaname, C. B., Braun, T. M., Sugai, J. V., Teles, R. P., Loesche, W. J., Kornman, K. S., Giannobile, W. V. & Kinney, J. S. 2012. Bacterial and salivary biomarkers predict the gingival inflammatory profile. *J Periodontol,* 83**,** 79-89.

Lee, Y. H., Zhou, H., Reiss, J. K., Yan, X., Zhang, L., Chia, D. & Wong, D. T. 2011. Direct saliva transcriptome analysis. *Clin Chem,* 57**,** 1295-302.

Li, Y., Zhou, X., St John, M. A. & Wong, D. T. 2004. RNA profiling of cell-free saliva using microarray technology. *J Dent Res,* 83**,** 199-203.

This article is protected by copyright. All rights reserved

Author Manuscript

Loe, H. 1967. The Gingival Index, the Plaque Index and the Retention Index Systems. *J Periodontol,* 38**,** Suppl:610-6.

Masterson, L., Sorgeloos, F., Winder, D., Lechner, M., Marker, A., Malhotra, S., Sudhoff, H., Jani, P., Goon, P. & Sterling, J. 2015. Deregulation of SYCP2 predicts early stage human papillomavirus-positive oropharyngeal carcinoma: A prospective whole transcriptome analysis. *Cancer Sci,* 106**,** 1568-75.

Mauritz, J. M., Tiffert, T., Seear, R., Lautenschlager, F., Esposito, A., Lew, V. L., Guck, J. & Kaminski, C. F. 2010. Detection of *Plasmodium falciparum*-infected red blood cells by optical stretching. *J Biomed Opt,* 15**,** 030517.

Mitsuhashi, K., Nosho, K., Sukawa, Y., Matsunaga, Y., Ito, M., Kurihara, H., Kanno, S., Igarashi, H., Naito, T., Adachi, Y., Tachibana, M., Tanuma, T., Maguchi, H., Shinohara, T., Hasegawa, T., Imamura, M., Kimura, Y., Hirata, K., Maruyama, R., Suzuki, H., Imai, K., Yamamoto, H. & Shinomura, Y. 2015. Association of *Fusobacterium* species in pancreatic cancer tissues with molecular features and prognosis. *Oncotarget,* 6**,** 7209-20.

Morelli, T., Stella, M., Barros, S. P., Marchesan, J. T., Moss, K. L., Kim, S. J., Yu, N., Aspiras, M. B., Ward, M. & Offenbacher, S. 2014. Salivary biomarkers in a biofilm overgrowth model. *J Periodontol,* 85**,** 1770-8.

Muhlemann, H. R. & Son, S. 1971. Gingival sulcus bleeding--a leading symptom in initial gingivitis. *Helv Odontol Acta,* 15**,** 107-13.

Nazar Majeed, Z., Philip, K., Alabsi, A. M., Pushparajan, S. & Swaminathan, D. 2016. Identification of Gingival Crevicular Fluid Sampling, Analytical Methods, and Oral Biomarkers for the Diagnosis and Monitoring of Periodontal Diseases: A Systematic Review. *Dis Markers,* 2016**,** 1804727.

Nelson, P., Kiriakidou, M., Sharma, A., Maniataki, E. & Mourelatos, Z. 2003. The microRNA world: small is mighty. *Trends Biochem Sci,* 28**,** 534-40.

Nussbaumer, C., Gharehbaghi-Schnell, E. & Korschineck, I. 2006. Messenger RNA profiling: a novel method for body fluid identification by real-time PCR. *Forensic Sci Int,* 157**,** 181-6.

Ogata, Y., Matsui, S., Kato, A., Zhou, L., Nakayama, Y. & Takai, H. 2014. MicroRNA expression in inflamed and noninflamed gingival tissues from Japanese patients. *J Oral Sci,* 56**,** 253-60.

Parra-Damas, A., Valero, J., Chen, M., Espana, J., Martin, E., Ferrer, I., Rodriguez-Alvarez, J. & Saura, C. A. 2014. Crtc1 activates a transcriptional program deregulated at early Alzheimer's disease-related stages. *J Neurosci,* 34**,** 5776-87.

Pepe, M. S., Feng, Z., Janes, H., Bossuyt, P. M. & Potter, J. D. 2008. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. *J Natl Cancer Inst,* 100**,** 1432-8.

This article is protected by copyright. All rights reserved

Author Manuscript

Quigley, G. A. & Hein, J. W. 1962. Comparative cleansing efficiency of manual and power brushing. *J Am Dent Assoc,* 65**,** 26-9.

Ragvin, A., Moro, E., Fredman, D., Navratilova, P., Drivenes, O., Engstrom, P. G., Alonso, M. E., De La Calle Mustienes, E., Gomez Skarmeta, J. L., Tavares, M. J., Casares, F., Manzanares, M., Van Heyningen, V., Molven, A., Njolstad, P. R., Argenton, F., Lenhard, B. & Becker, T. S. 2010. Long-range gene regulation links genomic type 2 diabetes and obesity risk regions to HHEX, SOX4, and IRX3. *Proc Natl Acad Sci U S A,* 107**,** 775-80.

Rajesh, K. S., Thomas, D., Hegde, S. & Kumar, M. S. 2013. Poor periodontal health: A cancer risk? *J Indian Soc Periodontol,* 17**,** 706-10.

Ramfjord, S. P. 1959. Indices for prevalence and incidence of periodontal disease. *Journal of Periodontology* 30**,** 51–59.

Raz, A., Carmi, P., Raz, T., Hogan, V., Mohamed, A. & Wolman, S. R. 1991. Molecular cloning and chromosomal mapping of a human galactoside-binding protein. *Cancer Res,* 51**,** 2173-8.

Schmalz, G., Li, S., Burkhardt, R., Rinke, S., Krause, F., Haak, R. & Ziebolz, D. 2016. MicroRNAs as Salivary Markers for Periodontal Diseases: A New Diagnostic Approach? *Biomed Res Int,* 2016**,** 1027525.

Sexton, W. M., Lin, Y., Kryscio, R. J., Dawson, D. R., 3rd, Ebersole, J. L. & Miller, C. S. 2011. Salivary biomarkers of periodontal disease in response to treatment. *J Clin Periodontol,* 38**,** 434-41.

Shaila, M., Pai, G. P. & 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**,** 42-6.

Starkey, M. L., Davies, M., Yip, P. K., Carter, L. M., Wong, D. J., Mcmahon, S. B. & Bradbury, E. J. 2009. Expression of the regeneration-associated protein SPRR1A in primary sensory neurons and spinal cord of the adult mouse following peripheral and central injury. *J Comp Neurol,* 513**,** 51-68.

Stemmler, S., Nothnagel, M., Parwez, Q., Petrasch-Parwez, E., Epplen, J. T. & Hoffjan, S. 2009. Variation in genes of the epidermal differentiation complex in German atopic dermatitis patients. *Int J Immunogenet,* 36**,** 217-22.

Stoecklin-Wasmer, C., Guarnieri, P., Celenti, R., Demmer, R. T., Kebschull, M. & Papapanou, P. N. 2012. MicroRNAs and their target genes in gingival tissues. *J Dent Res,* 91**,** 934-40.

Tomar, S. L. & Asma, S. 2000. Smoking-attributable periodontitis in the United States: findings from NHANES III. National Health and Nutrition Examination Survey. *J Periodontol,* 71**,** 743-51.

Turesky, S., Gilmore, N. D. & Glickman, I. 1970. Reduced plaque formation by the chloromethyl analogue of victamine C. *J Periodontol,* 41**,** 41-3.

This article is protected by copyright. All rights reserved

Author Manuscript

Vardar-Sengul, S., Demirci, T., Sen, B. H., Erkizan, V., Kurulgan, E. & Baylas, H. 2007. Human beta defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases. *J Periodontal Res,* 42**,** 429-37.

Wang, Y., Kou, Y., Wang, X., Cederbaum, A. & Wang, R. 2014. Multifactorial comparative proteomic study of cytochrome P450 2E1 function in chronic alcohol administration. *PLoS One,* 9**,** e92504.

Wong, D. T. 2015. Salivary extracellular noncoding RNA: emerging biomarkers for molecular diagnostics. *Clin Ther,* 37**,** 540-51.

Wu, N., Song, Y., Pang, L. & Chen, Z. 2016. CRCT1 regulated by microRNA-520 g inhibits proliferation and induces apoptosis in esophageal squamous cell cancer. *Tumour Biol,* 37**,** 8271-9.

Author Manuscript

This article is protected by copyright. All rights reserved

No.	Gene Accession	Gene Symbol	Log2 (gingivitis)	Log2 (healthy)	Log2 Fold change	Fold change	P-value	RNA type
1	NONHSAT006501.2	SPRR1A	12.89742	14.43722	-1.53979	-2.90754	0.04399	lncRNA
2	NM_001137548	FAM25C	9.449203	10.52453	-1.07532	-2.10720	0.02859	mRNA
3	NM_032488	CNFN	9.38666	10.45155	-1.06488	-2.09200	0.03430	mRNA
4	NM_005621	S100A12	7.61813	8.60196	-0.98382	-1.97770	0.01329	mRNA
5	NM_001146157	FAM25A	10.04811	10.99101	-0.94289	-1.92238	0.02341	mRNA
6	AJ243662	--------	6.83282	7.74533	-0.91250	-1.88231	0.02857	mRNA
7	NM_019060	CRCT1	6.65030	7.49772	-0.84741	-1.79928	0.04585	mRNA
8	NM_000700	ANXA1	7.64696	8.40568	-0.75872	-1.69199	0.02765	mRNA
9	NM_001010909	MUC21	6.73042	7.46782	-0.73739	-1.66715	0.03425	mRNA
10	NM_178435	LCE3E	6.76232	7.45423	-0.69191	-1.61542	0.00179	mRNA
11	NM_001010909	MUC21	6.80567	7.49384	-0.68817	-1.61124	0.02851	mRNA
12	NM_001145641	SRRM5	5.68198	6.32151	-0.63953	-1.55782	0.01259	mRNA
13	NM_198321	GALNT10	6.72602	6.10266	0.62336	1.54046	0.00012	mRNA
14	NONHSAT071649	lnc-TET3-2:1	5.88087	5.26156	0.61930	1.53613	0.00027	lncRNA
15	NM_003064	SLPI	6.70889	7.31280	-0.60391	-1.51983	0.04956	mRNA
16	NM_002272	KRT4	6.46975	7.07260	-0.60285	-1.51871	0.02572	mRNA
17	NM_001010909	MUC21	6.77905	7.37802	-0.59896	-1.51463	0.03135	mRNA
18	NR_003225	LGALS3	6.43733	7.01092	-0.57358	-1.48822	0.02500	mRNA
19	NM_001001435	CCL4L1	6.07940	6.65177	-0.57237	-1.48696	0.02471	mRNA
20	NM_001005234	OR1L3	6.00336	6.51749	-0.51412	-1.42812	0.00028	mRNA
21	AJ420500	SOX4	5.82488	5.31805	0.50683	1.42092	0.01984	mRNA
22	OTTHUMT0000003370	-------	5.49779	5.99039	-0.49259	-1.40697	0.00089	lncRNA
23	NONHSAT005224	RP5-965F6.2	5.95182	5.46229	0.48953	1.40398	0.00067	lncRNA
24	XM_294900.1	AL832615	5.93299	6.41925	-0.48626	-1.40080	0.00036	mRNA

Author Manuscript

This article is protected by copyright. All rights reserved

25	BC127843	PET100	6.50932	6.99539	-0.48607	-1.40062	0.03033	mRNA

Table 1. The list of 25 significantly differentially expressed salivary exRNA biomarker candidates between gingivitis and clinically periodontally healthy individuals (Affymetrix HTA 2.0 microarray analysis).

Author Manuscript

This article is protected by copyright. All rights reserved

No.	Gene Symbol	Gene Accession	Association with disease			
			Microarray Fold change		PCR slope	
			direction	p-value	direction	p-value
m1	SPRR1A	NONHSAT006501.2	-	0.044	-	<0.001
m2	lnc-TET3-2:1	NONHSAT071649	+	<0.001	+	<0.001
m3	RP5-965F6.2	NONHSAT005224	+	<0.001	+	<0.001
m4	LGALS3	NR_003225	-	0.025	-	<0.001
m5	GALNT10	NM_198321	+	<0.001	+	<0.001
m6	SOX4	AJ420500	+	0.020	+	<0.001
m7	FAM25A	NM_001146157	-	0.023	_	0.003
m8	AL832615	XM_294900.1	-	<0.001	NS*	0.867
m9	SLPI	NM_003064	-	0.049	NS*	0.088
m10	CRCT1	NM_019060	-	0.046	-	<0.001

*NS- nonsignificant

Values reported as direction of effect +/- and p-value for statistical significance

Table 2. Concordance of the 10 salivary exRNA biomarker changes with initial microarray data.

Author Manuscript

This article is protected by copyright. All rights reserved

Author Manuscript

No.	Gene symbol	Gene Accession	Function/Biological Role
m1	SPRR1A	NONHSAT006501.2	• epidermatitis development, keratinocyte differentiation and keratinization (Gibbs et al., 1993, Stemmler et al., 2009); • assessment of the regenerative potential of injured neurons (Starkey et al., 2009).
m2	lnc-TET3-2:1	NONHSAT071649	• a member of the ten-eleven translocation (TET) gene family, that plays a role in the DNA methylation process (Langemeijer et al., 2009).
m3	RP5-965F6.2	NONHSAT005224	• has not been reported yet in the publications.
m4	LGALS3	NR_003225	• a member of the galectin family of carbohydrate binding proteins that play a role in apoptosis, innate immunity, neutrophil, eosinophil & macrophage chemotaxis and negative regulation of endocytosis; • antimicrobial activity against bacteria and fungi (http://www.genecards.org) (Raz et al., 1991); • association with increased risk for rheumatoid arthritis (Atabaki et al., 2017).
m5	GALNT10	NM_198321 AJ156166	• Homo sapiens putative tumor suppressor mRNA (https://www.ncbi.nlm.nih.gov/nuccore), (Boultwood et al., 2000).
m6	SOX4	AJ420500	• apoptosis pathway leading to cell death as well as to tumorigenesis; • mediation of downstream effects of parathyroid hormone (PTH) and PTH-related protein (PTHrP) in bone development (http://www.genecards.org); • obesity and type 2 diabetes (Ragvin et al., 2010) • airway inflammation (Kuwahara et al., 2012).

This article is protected by copyright. All rights reserved

m7	FAM25A	NM_001146157	• inflammation and infectious disease, decrease in immune response (Wang et al., 2014, Mauritz et al., 2010, Deloukas et al., 2004).
m10	CRCT1	NM_019060	• linkage to esophageal cancer (Wu et al., 2016) and oropharyngeal squamous cell carcinomas (Masterson et al., 2015); • early Alzheimer's disease-related stages ((Parra-Damas et al., 2014); • promotion of tumor cell apoptosis and upregulation of the expression of apoptosis-related proteins (Wu et al., 2016).

Table 3. Description of functions and biological roles of validated exRNAs for gingivitis.

Author Manuscript

Figure legends

Figure 1. Study design - flow chart.

Figure 2. Comparison of the Gingival Index and the Plaque Index scores over time.

Figure 3. Validation of HTA 2.0 Affymetrix array profiling results by RT-qPCR. A plot presenting the fold changes and 95% confidence interval (CI) for each of 10 salivary exRNA biomarkers on the log base 2 scale for the following periods: baseline – 6 weeks, 3 week- 6 weeks and baseline – 3 weeks.

This article is protected by copyright. All rights reserved

Figure 4. Performance of salivary exRNA biomarkers to classify gingivitis from healthy subjects with no periodontal disease [baseline to 6 weeks], [4 exRNAs: SPRR1A (m1), lnc-TET3-2:1 (m2), FAM25A (m7), CRCT1 (m10)] [0.91 AUC, 71% sensitivity, 100% specificity].

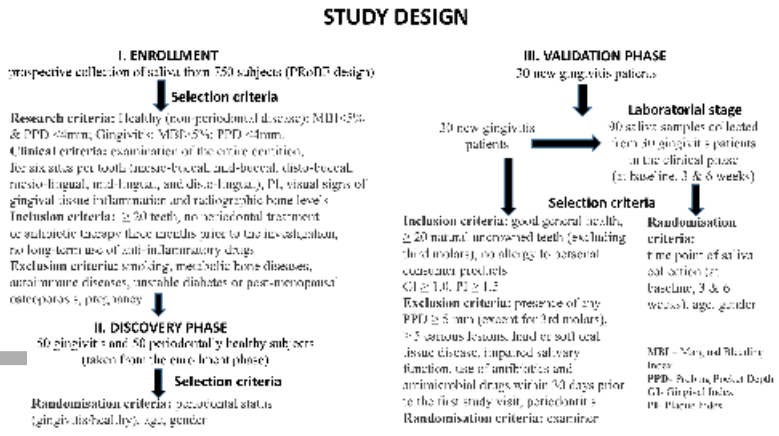
Supplementary Figure 1. Clinical evaluator investigation over time (at screening, baseline, 3 and 6 weeks).

Supplemental Figure 2. Statistically significant overall trends for each of the 8 validated salivary exRNAs across all 3 time points (at baseline, 3 & 6 weeks).

Author Manuscript

This article is protected by copyright. All rights reserved

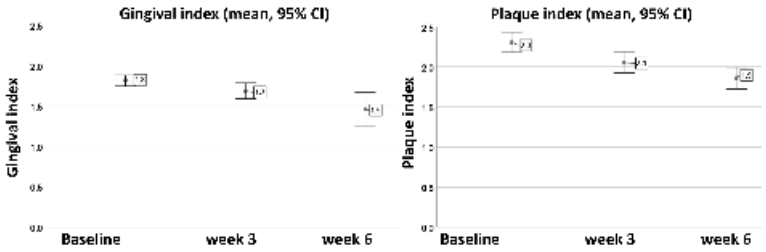
Author Manuscript



jcpe_12930_f1.tiff

This article is protected by copyright. All rights reserved

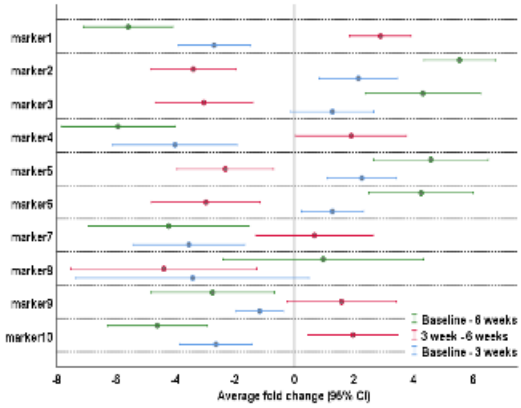
Author Manuscript



jcpe_12930_f2.tiff

This article is protected by copyright. All rights reserved

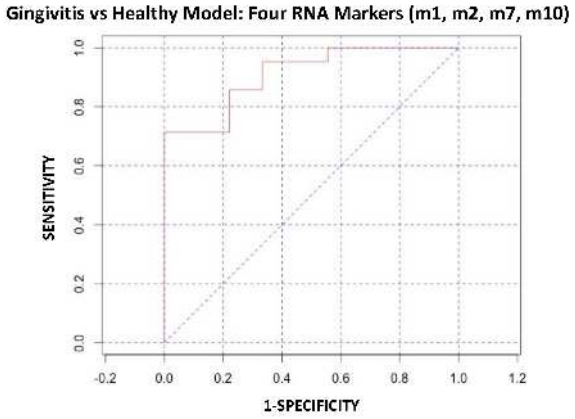
Author Manuscript



jcpe_12930_f3.tiff

This article is protected by copyright. All rights reserved

Author Manuscript



jcpe_12930_f4.tiff

This article is protected by copyright. All rights reserved