

**OTHER**

# Salivary exRNA biomarkers to detect gingivitis and monitor disease regression

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**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 10 differentially expressed exRNAs were tested in a clinical cohort to determine whether 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 and 6 weeks and salivary exRNAs were assayed by means of reverse transcription quantitative polymerase chain reaction.

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**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, Inc-TET3-2:1, FAM25A, CRCT1] can detect gingivitis with a clinical performance of 0.91 area under the curve, with 71% sensitivity and 100% specificity.

**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.

#### KEYWORDS

biomarkers, exRNA, gingivitis, monitoring, saliva

## 1 | 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, Kim, & Camargo, 2013). If left untreated, gingivitis may progress to an irreversible form—periodontitis that 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, Thomas, Hegde, & Kumar, 2013). The common risk factors for periodontitis could be divided into inherited (e.g. genetic variants), and those that are acquired (e.g. socioeconomic 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 (Henskens, Van Der Velden, Veerman, & Nieuw Amerongen, 1993; Kinney et al., 2011; Lee et al., 2012; Morelli et al., 2014; Shaila, Pai, & Shetty, 2013), 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 pathological 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, Nagarajan, Akers, & Miller, 2015). Thus, it is desirable to develop objective and scientifically credible biomarkers for early detection and monitoring of periodontal diseases, especially

### Clinical Relevance

*Scientific rationale for the 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 panels 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.

that many of the affected cases are left undiagnosed and untreated (Albandar & Kingman, 1999; Tomar & Asma, 2000).

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

and 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 programmes as well as offers the ability to identify and monitor the patients at risk (Giannobile, 2012). Lastly, the dental, pharmaceutical and 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.

## 2 | MATERIALS AND METHODS

### 2.1 | 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.

### 2.2 | Discovery phase

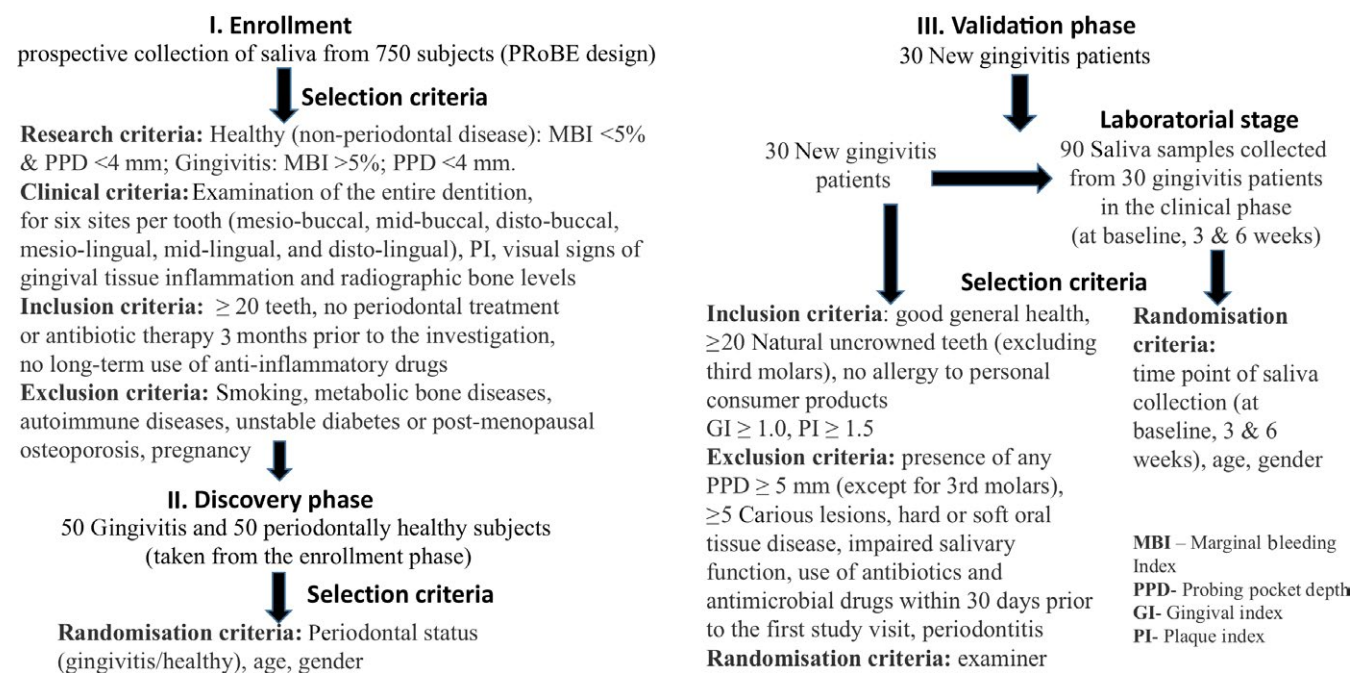
Based on the prospective-specimen-collection and retrospective-blinded-evaluation (PRoBE) study design (Pepe, Feng, Janes,

Bossuyt, & Potter, 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 hr 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), and kept on ice through the entire process. All samples were processed, around 1 hr after collection. Samples were firstly centrifuged in a refrigerated centrifuge at  $2,400 \times g$  for 15 min at  $4^{\circ}\text{C}$ , 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 [Na<sub>3</sub>VO<sub>4</sub>] [Sigma-Aldrich] RNase inhibitor (Invitrogen SUPERase-In RNase Inhibitor [Thermo Fisher Scientific, Austin, TX, USA]) based on our saliva standard operating procedure (Henson & Wong, 2010). These samples were aliquoted into smaller cryo-vials, labelled and frozen at  $-80^{\circ}\text{C}$ .

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  $\sim 1.8$ ) were profiled using the GeneChip Human Transcriptome Affymetrix HTA 2.0 expression arrays [Thermo Fisher Scientific].

## Study design



**FIGURE 1** Study design: flow chart

## 2.3 | Pilot clinical research study

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

### 2.3.1 | Clinical stage

For analysis of saliva samples, 30 volunteers (aged 18–65 years), 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 1,000 p.p.m. sodium monofluorophosphate (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  $-20^{\circ}\text{C}$  for about 3–4 hr until further analysis.

### 2.3.2 | Laboratory stage

#### 2.3.2.1 | Validation phase

**The direct saliva transcriptome analysis** 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<sup>®</sup> 480 Real-Time PCR System [Roche Life Science, Indianapolis, IN, USA] with a fixed thermal-cycling programme.

#### 2.3.2.2 | 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. 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 & Hein, 1962; Turesky, Gilmore, & Glickman, 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, inter-individual 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) and SAS V9.4 (SAS Institute Inc., Cary, NC, USA), whereas the randomization was performed using the online statistical computing web programming “Sealed envelope” (<https://sealedenvelope.com>).

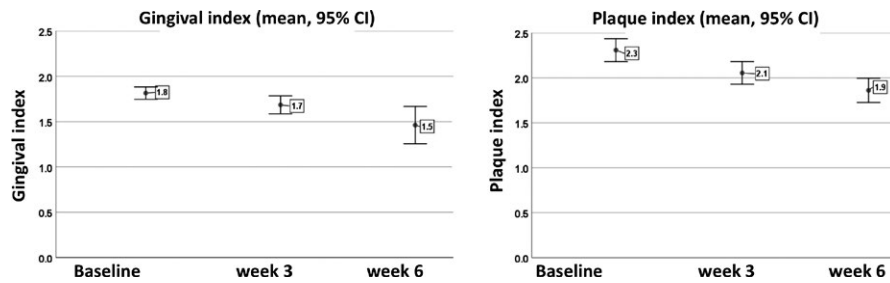
## 3 | RESULTS

### 3.1 | Clinical outcomes

In the discovery phase, the study group consisted of 100 subjects, including 50 gingivitis subjects (22 males and 28 females) aged  $26.4 \pm 6.77$  years, and 50 healthy individuals aged  $27.1 \pm 5.67$  years (23 males and 27 females). The scores for the gingivitis group were  $7.2 \pm 1.1\%$  for Marginal Bleeding Index (MBI) (Loe, 1967; Muhlemann & Son, 1971) and  $2.5 \pm 0.7$  mm for Probing Pocket Depth (PPD) (Ramfjord, 1959), while for the healthy group, the results were following:  $2.4 \pm 1.6\%$  for MBI and  $2.1 \pm 0.8$  mm 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 and 17 females), aged  $28.2 \pm 7.77$  years. 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)  $1.75 \pm 0.2$ , at B  $-1.814 \pm 0.18$ , at 3 weeks  $-1.684 \pm 0.26$  and at 6 weeks  $-1.46 \pm 0.55$ , while the average PI scores were following: at Scr  $2.293 \pm 0.44$ , at B  $-2.307 \pm 0.34$ , at 3 weeks  $-2.054 \pm 0.33$  and at 6 weeks  $-1.86 \pm 0.36$  (Supporting Information Table S1). 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.

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 enrolment of the patients into the study between the two stages. The MBI (Loe, 1967; Muhlemann & 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 & 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) ( $\text{PPD} < 4$  mm) and gingivitis ( $\text{PPD} < 4$  mm) from periodontitis ( $\geq 5$  mm) (apart from radiographic assessment). In the validation phase, only patients with diagnosed gingivitis were included. Study subjects could not have any PPD deeper than 5 mm (except for 3rd molars) to be enrolled in the research project.



**FIGURE 2** Comparison of the Gingival Index and the Plaque Index scores over time

### 3.2 | Investigation of potential clinical evaluators bias

There were no significant inter-examiner differences between the clinical evaluators, except for time point 6 weeks, which could potentially affect the GI and PI scores (Supporting Information Figure S1). This is despite the fact that all examiners were initially calibrated.

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

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.

### 3.4 | 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 four decreased exRNAs [SPRR1A (m1), LGALS3 (m4), FAM25A (m7), CRCT1 (m10)] and four 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  $< 0.001$ ) (Supporting Information Figure S2, Supporting Information Table S1). Figure 3 presents a plot with fold changes and 95% 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 inter-individual variability in exRNA patterns.

### 3.5 | Logistic regression analysis

The potential clinical discriminatory power of the developed salivary exRNA for gingivitis detection was evaluated. Logistic regression analysis of eight 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 area under the curve with 71% sensitivity and 100% specificity (Figure 4).

## 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-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, Zhou, St John, & Wong, 2004), followed by the intense investigations in the following years (Nussbaumer, Gharehbaghi-Schnell, & Korschineck, 2006). The revealed complex exRNA composition of saliva encompasses mostly mRNAs, but also ncRNAs such as long noncoding 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, Kiriakidou, Sharma, Maniatakis, & Mourelatos, 2003), while the current knowledge of lncRNAs is still largely uninvestigated (Kung, Colognori, & Lee, 2013).

The basis of biomarker research success in periodontology is to develop a diagnostic method that will have a potential to favourably affect clinical decision-making, patient outcomes and health care 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).



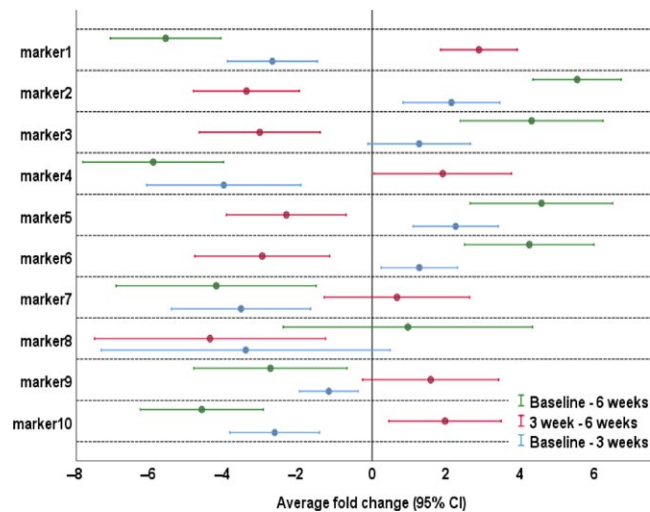
**TABLE 1** The list of 25 significantly differentially expressed salivary exRNA biomarker candidates between gingivitis and clinically periodontally healthy individuals (Affymetrix HTA 2.0 microarray analysis)

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_0011137548	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
25	BC127843	PET100	6.50932	6.99539	-0.48607	-1.40062	0.03033	mRNA

**TABLE 2** Concordance of the 10 salivary exRNA biomarker changes with initial microarray data

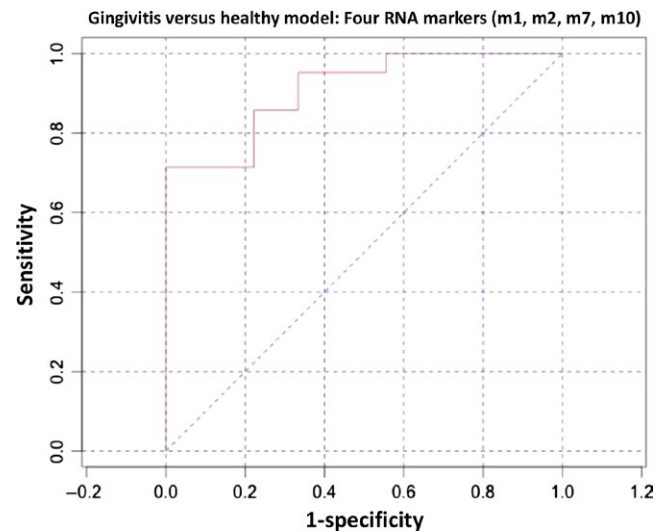
No.	Gene symbol	Gene accession	Association with disease			
			Microarray fold change		PCR slope	
			Direction	p-Value	Direction	p-Value
m1	SPRR1A	NONHSAT006501.2	-	0.044	-	<0.001
m2	lnc-TET3-2:1	NONHSAT071649	+	<0.001	+	<0.001
m3	RP5-965F6.2	NONHSAT005224	+	<0.001	+	<0.001
m4	LGALS3	NR_003225	-	0.025	-	<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

Notes. Values reported as direction of effect ± and p value for statistical significance. NS: nonsignificant.



**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

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).



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

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 aroused 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 and RP5-965F6.2). It is known only that SPRR1A (m1) is a regeneration-associated gene (Starkey et al., 2009), whereas lnc-TET3-2:1 (m2)

**TABLE 3** Description of functions and biological roles of validated exRNAs for gingivitis

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 ( <a href="http://www.genecards.org">http://www.genecards.org</a> ) (Raz et al., 1991) Association with increased risk for rheumatoid arthritis (Atabaki, Hashemi, Daneshvar, & Alijani, 2017)
m5	GALNT10 AJ156166	NM_198321	Homo sapiens putative tumour suppressor mRNA ( <a href="https://www.ncbi.nlm.nih.gov/nucleotide">https://www.ncbi.nlm.nih.gov/nucleotide</a> ) (Boulton 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 ( <a href="http://www.genecards.org">http://www.genecards.org</a> ) Obesity and type 2 diabetes (Ragvin et al., 2010) Airway inflammation (Kuwahara et al., 2012)
m7	FAM25A	NM_001146157	Inflammation and infectious disease, decrease in immune response (Deloukas et al., 2004; Mauritz et al., 2010; Wang et al., 2014)
m10	CRCT1	NM_019060	Linkage to oesophageal cancer (Wu, Song, Pang, & Chen, 2016) and oropharyngeal squamous cell carcinomas (Masterson et al., 2015) Early Alzheimer's disease-related stages (Parra-Damas et al., 2014) Promotion of tumour cell apoptosis and upregulation of the expression of apoptosis-related proteins (Wu et al., 2016)

acts as an epigenetic mediator (Langemeijer, Aslanyan, & Jansen, 2009) (Table 3).

In addition, our study also revealed five mRNA RNAs (LGALS3, GALNT10, SOX4, FAM25A and CRCT1) that are an emerging and flourishing field for non-invasive diagnostic applications. The identification of saliva-derived mRNA in normal and cancer

patients (Hu et al., 2008; Li et al., 2004) and other forensic applications (Juusola & 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, regulate T-helper type 2 (TH2) cell-driven airway inflammation (Kuwahara et al., 2012), whereas downregulation of FAM25A (m7) (observed also in our study) indicates increased risk for inflammation and infectious diseases (Deloukas et al., 2004; Mauritz et al., 2010; Wang, Kou, Wang, Cederbaum, & Wang, 2014). Lastly, CRCT1 (m10) is reported to be associated with early stages of Alzheimer's disease, in which transcriptome changes affecting cell signalling, 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 eight 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 (Kagiya, 2016; Ogata et al., 2014; Schmalz et al., 2016). 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 was the inter-individual differences in scoring of PI and GI, which 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 enrolment of the subjects into the project. Lastly, a comprehensive study of the variety of exRNAs and further increased sampling is needed 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 (IFN $\gamma$ ) and tumour necrosis factor alpha 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 is associated with gingivitis, aggressive and chronic

periodontitis (Vardar-Sengul et al., 2007). Also, the expression levels of Toll-like receptors (TLR)TLR2, -4, -7, -9, interferon-alpha1 (IFN- $\alpha$ 1) (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 and hsa-miR-3917) were significantly overexpressed, and seven (hsa-miR-1246, hsa-miR-1260, hsa-miR-141, hsa-miR-1260b, hsa-miR-203, hsa-miR-210 and hsa-miR-205) were underexpressed by > twofold in a diseased compared to healthy gingiva (Stoecklin-Wasmer et al., 2012). In turn, Bochenek et al. (2013) report about downregulation of lncRNA ANRIL associated with atherosclerosis, periodontitis and several forms of cancer.

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-1 $\beta$ ) can be considered as an indicator of periodontal disease progression (Nazar Majeed, Philip, Alabsi, Pushparajan, & Swaminathan, 2016), whereas matrix metalloproteinase-8 (MMP-8) may indicate response to therapy (Sexton et al., 2011). Additionally, macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) 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. 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.


## CONFLICT OF INTEREST

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. KEKU, HMT, JGM and DTWW have a pending patent related to this work [Attorney Docket No. 206030-0122-PI-US.606791]. None of the other authors have a conflict of interest to disclose related to this study.

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## SUPPORTING INFORMATION

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

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