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ORIGINAL ARTICLE

Cumulative use of salivary markers with an adaptive design improves detection of periodontal disease over fixed biomarker thresholds

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ABSTRACT

Objective: Aim was to analyze the diagnostic ability of cumulative risk score (CRS), which uses salivary levels of *Porphyromonas gingivalis*, interleukin (IL)-1β, and matrix metalloproteinase (MMP)-8 in an adaptive design, compared to previously reported thresholds of each marker alone.

Materials and Methods: Oral and general health information of 463 participants were included in the analysis. Having the percentage of bleeding on probing (BOP) > 25%, having at least two sites with probing pocket depth (PPD) of 4–5 mm or having at least one tooth with alveolar bone loss (ABL) of at least 1/3 of the root length were accepted as outcome variables. Being above the salivary threshold concentrations of *P. gingivalis*, IL-1β, and MMP-8 and CRS values were used as explanatory variables. Receiver operating characteristics (ROC) producing an area under the curve (AUC) and multinomial regression analysis were used in statistical analysis.

Results: CRS provided AUCs larger than any other tested biomarker threshold. Sensitivity and specificity of CRS for detecting clinical markers of periodontitis were acceptable, and a strong association was observed between the highest CRS score and having at least two sites with PPD of 4–5 mm.

Conclusion: CRS brings additional power over fixed thresholds of single biomarkers in detecting periodontitis.

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Introduction

Periodontitis is a chronic infection-induced inflammatory disease, where bacteria or their by-products induce an inflammatory host response around the tooth. Chronic inflammation of tooth-supporting tissues progresses slowly and is usually symptomless. However, in susceptible subjects, uncontrolled chronic inflammation may cause extensive alveolar bone degradation and lead to tooth loss [1].

Specific host- or bacteria-derived biomarkers detected in saliva indicate the presence or progression/remission of periodontitis. With the aid of Omics Technologies, search for biomarkers with high sensitivity and specificity has broadened during the last decade [2]. The use of a single salivary marker in detection of periodontitis is, however, challenged by the episodic and multi-factorial characteristics of the disease [3]. In addition, diversities in sample collection, storage, and analysis techniques cause significant variations in the concentrations of selected biomarkers in saliva [4]. These variations limit the assignment of a fixed universal threshold for a salivary biomarker in detecting periodontitis. Indeed, a combinational use of salivary biomarker panels produces more robust outcomes in periodontal disease prediction [5]. Our group developed a novel diagnostic approach, namely Cumulative Risk Score (CRS), in which we analyze concentrations of three selected biomarkers representing periodontal pathogen burden (*Porphyromonas gingivalis)*, inflammation (interleukin (IL)-1β), and tissue destruction (matrix metalloproteinase (MMP)-8) [6]. In calculation of CRS, salivary concentrations of *P. gingivalis*, IL-1β, and MMP-8 are divided into tertiles and cumulative sub-score of each individual is calculated by the multiplication of the tertile values. Distribution of study population into tertiles allows CRS to take into account concentration ranges of the biomarkers and, in addition, to use an adaptive design to allocate study subjects in three risk groups (high, middle, low) for the presence of periodontitis. The CRS model was originally introduced in a subpopulation ($n = 165$) of the National Health 2000 Health Examination Survey in Finland. The CRS was later validated in a cohort study including 493 Finnish adults [7].

In determination of a cut-off threshold for a biomarker, receiver operating characteristics (ROC) curve is commonly used. According to this method, the point on the ROC curve, which has the minimum distance to the point where sensitivity and specificity is equal to one, is taken as the cut-off value. Another approach is to take the value, which maximizes the distance between the ROC curve and reference (random) line [8]. While these methods are commonly used in biomarker studies, the diagnostic power of the selected cut-off is highly dependent on the measured concentration range of the biomarker and the prevalence of the disease in the study population. Moreover, the diagnostic performance of the biomarker is usually exaggerated by the data-driven

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choice of a cut-off [9]. Therefore, there is an indisputable need of a validation study where the diagnostic power of the cut-off is tested in an independent population. *P. gingivalis*, IL-1β, and MMP-8 are among the most widely tested salivary biomarkers of periodontitis and several cut-off values are presented in different studies [10–14]. However, to our knowledge, none of these cut-off values are validated in an independent study. Here, we hypothesized that cumulative use of salivary markers with an adaptive design has superiority over fixed biomarker thresholds in detection of periodontal disease. To test this hypothesis, in the present study, the strength of CRS's diagnostic ability was tested against previously defined thresholds of *P. gingivalis*, IL-1β, and MMP-8.

Materials and methods

Definition for salivary thresholds of P. gingivalis*, IL-1β, and MMP-8*

A Medline search was performed using keywords '*P. gingivalis*', 'IL-1β', and 'MMP-8' together with 'salivary' or 'saliva', and 'threshold'. In the present analysis, the following studies defining the salivary thresholds of *P. gingivalis*, IL-1β, and MMP-8 to discriminate periodontally diseased subjects from healthy ones were included:

- *P. gingivalis*: 5×10^5 cells/ml [10]
- IL-1β: 28 pg/ml [11]; 212 pg/ml [12]; 235 pg/ml [13]
- MMP-8: 87 ng/ml [13]; 140 ng/ml [11]; 383 ng/ml [14].

Study participants

Oral and general health information of 463 dentate patients of Parogene study were included in the present analyses. The Parogene study was approved by the Helsinki University Central Hospital ethics committee (#106/2007). A detailed description of the study design can be found elsewhere [12]. Briefly, oral examination included the probing pocket depth (PPD) and bleeding on probing (BOP) measurements from all teeth. Alveolar bone loss (ABL) was measured 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; ABL in the cervical third of the root; ABL in the middle third of the root; and ABL from the apical third of the root to total ABL [15].

Paraffin-stimulated salivary samples were collected before the oral examinations and stored at −70 °C until laboratory analyses. Before the analysis, samples were thawed and centrifuged at 9300 g for 5 minutes. The pellet was used for the *P. gingivalis* measurement and the supernatant for the IL-1β and MMP-8 determinations. IL-1β concentrations were measured with a flow cytometry-based Luminex technique with commercially available kits (Milliplex Map Kit; MPXHCYTO-60k, Millipore, Billerica, MA, USA). MMP-8 concentrations detected with a time-resolved immunofluorometric assay and *P. gingivalis* concentrations with qPCR, as described previously in detail [7,16].

Cumulative risk score

The CRS score of each subject was calculated as follows:

P. gingivalis, 1: No *P. gingivalis*; 2: below the median value (12–1639 genome/ml); 3: above the median value (1678–1.2×10^7 genome/ml).

IL-1β, 1: the lowest tertile (0.01–12.21 pg/ml); 2: mid-tertile (12.22–50.43 pg/ml); 3: highest tertile (50.94–1351 pg/ml).

MMP-8, 1: the lowest tertile (1–641 ng/ml); 2: mid-tertile (643–1251 ng/ml); 3: highest tertile (1258–3580 ng/ml).

The cumulative sub-score of each subject was calculated by multiplication of the three tertile values. The final CRS groups were formed according to the sub-scores: CRS I, indicating the lowest risk of carrying periodontitis (the cumulative sub-scores of 1, 2, and 3); CRS II, indicating middle risk of carrying periodontitis (the cumulative sub-scores of 4, 6, 8, and 9); and CRS III, indicating the highest risk of carrying periodontitis (the cumulative sub-scores of 12, 18, and 27).

Statistical analysis

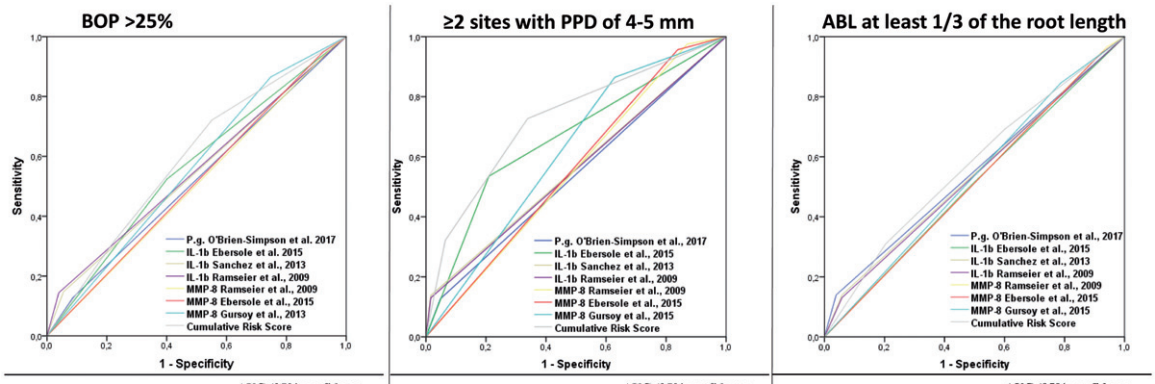
The statistical analysis was performed with IBM SPSS Statistics (version 22; IBM Corp. Armonk, NY, USA). Having BOP >25%, having at least two sites with PPD of 4–5 mm, or having at least one tooth with ABL of at least 1/3 of the root length were accepted as outcome variables. The CRS values and being above the thresholds of *P. gingivalis*, IL-1β, and MMP-8 were used as explanatory variables. When the selected thresholds (presented above) of these biomarkers were used in the ROC analysis as screening measures, continuous variables of *P. gingivalis*, IL-1β, and MMP-8 values were converted into a dichotomous variable of 'below the threshold' – '0', and 'above the threshold' – '1'.

Outcome variables are used in conjunction with explanatory variables in a diagnostic matrix to calculate specificity and specificity. ROC and area under the curve (AUC) analyses were calculated for the discriminative efficacy of each threshold and CRS.

Multinomial regression analyses were performed to describe the associations between outcome and explanatory variables, after adjusting for smoking, gender, and age. Statistical significance was defined as $p < .05$.

Results

Figure 1 presents the ROC curves and AUCs. The CRS distinguished the subjects having at least two sites with PPD of 4–5 mm and provided an AUC of 0.725. The IL-1β at concentrations of 28 pg/ml and 212 pg/ml distinguished subjects with BOP >25% with AUCs of 0.562 and 0.542, respectively. MMP-8 at concentration of 383 ng/ml and IL-1β at concentrations of 28 pg/ml distinguished subjects with having at least two sites with PPD of 4–5 mm with AUCs of 0.618 and 0.663, respectively. The sensitivity and specificity of CRS for detecting periodontitis were high, especially when CRS II (middle risk) and CRS III (high risk) were combined and compared to CRS I (low risk) (Table 1). The strongest association among the outcome and explanatory variables was observed between CRS III (high risk) and having at least two sites with



	AUC (95% confidence intervals), p
P.g. O'Brien-Simpson et al., 2017	0.541 (0.462-0.580), 0.499
IL-1b Ebersole et al., 2015	0.562 (0.502-0.621), 0.044
IL-1b Sanchez et al., 2013	0.546 (0.488-0.604), 0.029
IL-1b Ramseier et al., 2009	0.552 (0.495-0.610), 0.086
MMP-8 Ramseier et al., 2009	0.506 (0.446-0.566), 0.855
MMP-8 Ebersole et al., 2015	0.513 (0.453-0.573), 0.513
MMP-8 Gursoy et al., 2013	0.559 (0.498-0.620), 0.054
Cumulative Risk Score	0.591 (0.532-0.651), 0.003

	AUC (95% confidence intervals), p
P.g. O'Brien-Simpson et al., 2017	0.539 (0.466-0.613), 0.321
IL-1b Ebersole et al., 2015	0.663 (0.594-0.731), <0.001
IL-1b Sanchez et al., 2013	0.561 (0.490-0.632), 0.125
IL-1b Ramseier et al., 2009	0.557 (0.485-0.628), 0.152
MMP-8 Ramseier et al., 2009	0.552 (0.470-0.634), 0.187
MMP-8 Ebersole et al., 2015	0.559 (0.477-0.641), 0.137
MMP-8 Gursoy et al., 2013	0.618 (0.536-0.700), 0.003
Cumulative Risk Score	0.725 (0.661-0.789), <0.001

	AUC (95% confidence intervals), p
P.g. O'Brien-Simpson et al., 2017	0.550 (0.490-0.611), 0.122
IL-1b Ebersole et al., 2015	0.508 (0.444-0.572), 0.798
IL-1b Sanchez et al., 2013	0.541 (0.479-0.602), 0.211
IL-1b Ramseier et al., 2009	0.536 (0.474-0.598), 0.267
MMP-8 Ramseier et al., 2009	0.512 (0.448-0.577), 0.704
MMP-8 Ebersole et al., 2015	0.511 (0.447-0.576), 0.725
MMP-8 Gursoy et al., 2013	0.529 (0.464-0.594), 0.369
Cumulative Risk Score	0.568 (0.506-0.631), 0.037

Figure 1. Receiver operating characteristics (ROC) analysis of each salivary biomarker threshold and CRS. For each threshold published, a new dichotomous variable of 'below the threshold' – '0', and 'above the threshold' – '1' was calculated and used as a screening variable. Area under the curve (95% confidence intervals) and *p* values from the ROC analyses are given.

Table 1. Sensitivities and specificities of each threshold and CRS score, in diagnosis of having BOP >25%, having at least two sites with PPD of 4–5 mm or having at least one tooth with ABL of at least 1/3 of the root length.

		BOP >25%		≥2 Sites with 4–5 mm PPD		ABL at least 1/3 of the root	
	Threshold	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
P.g. (O'Brien-Simpson et al. [10]	5×10^5 cells/ml	12.8	91.3	12.7	95.6	13.6	95.5
IL-1β (Ebersole et al. [11]	28 pg/ml	52.5	59.8	53.5	79.0	49.2	52.4
IL-1β (Sánchez et al. [12]	212 pg/ml	14.8	94.5	13.8	98.4	14.0	94.2
IL-1β (Ramseier et al. [13]	235 pg/ml	14.4	96.1	13.0	98.4	13.1	94.2
MMP-8 (Ramseier et al. [13]	87 ng/ml	95.8	8.7	95.9	14.7	94.6	6.4
MMP-8 (Ebersole et al. [11]	140 ng/ml	93.9	14.1	93.4	20.6	91.8	10.1
MMP-8 (Gursoy et al. [14]	383 ng/ml	84.9	31.5	83.2	41.2	80.7	23.9
CRS	III	31.1	77.9	32.2	93.5	31.0	79.8
	II–III	72.2	44.9	72.7	66.1	69.3	39.8

Table 2. Associations of salivary biomarkers and CRS with clinical outcomes. All analyses are adjusted for age, gender, and smoking.

	Reference	BOP >25% OR (95% CI), *p*	≥2 Sites with 4–5 mm PPD OR (95% CI), *p*	ABL at least 1/3 of the root OR (95% CI), *p*
P.g. (O'Brien–Simpson et al. [10]	Below threshold	1.5 (0.8–2.9), .251	6.3 (0.8–47.4), .074	2.0 (0.7–5.5), .161
IL-1β (Ebersole et al. [11]	Below threshold	1.6 (1.1–2.5), .025	3.2 (1.5–6.9), .002	1.4 (0.8–2.3), .191
IL-1β (Sánchez et al. [12]	Below threshold	3.1 (1.3–7.0), .008	6.3 (0.8–47.4), .072	3.6 (1.4–9.4), .008
IL-1β (Ramseier et al. [13]	Below threshold	4.3 (1.6–11.1), .003	6.0 (0.8–44.9), .081	3.2 (1.2–8.1), .017
MMP-8 (Ramseier et al. [13]	Below threshold	2.3 (0.9–5.0), .052	2.9 (1.1–8.1), .036	2.1 (0.8–5.5), .130
MMP-8 (Ebersole et al. [11]	Below threshold	2.7 (1.4–5.3), .004	2.8 (1.1–6.8), .023	1.8 (0.8–4.1), .133
MMP-8 (Gursoy et al. [14]	Below threshold	2.6 (1.6–4.2), <.001	2.8 (1.4–5.4), .003	1.8 (1.0–3.2), .047
CRS III	CRS I	2.2 (1.2–3.8), .005	8.9 (2.6–30.4), <.001	2.3 (1.2–4.4), .015
CRS II–III	CRS I	2.1 (1.4–3.2), .001	4.2 (2.1–8.2), <.001	1.9 (1.1–3.2), .017

PPD of 4–5 mm presenting an odds ratio (95% CI) of 8.9 (2.6–30.4), $p < .001$ (Table 2).

Discussion

In the present analyses, CRS brings additional diagnostic power over single biomarker thresholds in detection of periodontitis. The CRS does not apply fixed thresholds to dichotomize the population as periodontitis patients and non-periodontitis individuals, instead, it distributes the subjects into the high-, middle-, and low-risk groups according to the variations of biomarker levels within the ranges of their concentrations. This approach takes into account the biomarker dynamics and minimizes discrepancies in the results due to methodological differences between studies.

On one hand, single cut-offs are commonly used in clinical studies to dichotomize the study population into healthy and diseased. On the other hand, recent evidence demonstrates that use of two cut-offs, one corresponding to certainty of diagnostic inclusion and one corresponding to certainty of diagnostic exclusion, is more useful in clinical setting [8]. According to the two cut-off method, when a value of a

biomarker falls into the gray zone between two cut-offs, the patient is directed to a physician to be diagnosed with additional tools [8]. The CRS method applies a similar approach and defines the subjects' periodontitis risk level to guide patients to get a clinical diagnosis and periodontal treatment.

The thresholds used in this study are not necessarily suggested in their original publications as functional thresholds to discriminate periodontitis patients from periodontally healthy individuals. The present study included all available thresholds in the literature regardless of their diagnostic strength. On the other hand, some thresholds with higher sensitivity and specificity values were presented in the original reports, in comparison to the present results. One reason behind this finding can be the definition of outcome variables and diagnostic criteria that defines periodontitis. In the study by O'Brien–Simpson et al. [10], the individuals with at least one deepened pocket (PPD $\geq$5mm) in each quadrant were recruited in chronic periodontitis group, while Ramseier and his co-workers [13] defined the periodontitis group as 'subjects with at least four sites with evidence of alveolar bone loss, at least four sites with attachment loss $>$3 mm, and at least four sites with PPD $>$4 mm'. Gursoy et al. [14] defined the generalized periodontitis group as subjects having at least 14 teeth with PPD $\geq$4 mm. For the present analyses, we grouped the subjects based on clinical measurements representing the important stages of periodontal disease pathogenesis, namely elevated gingival inflammation (BOP $>$25%), deepened periodontal pockets (at least two sites with PPD of 4–5 mm), and alveolar bone loss (ABL, at least 1/3 of the root length), as outcome variables. This approach allowed us to analyze the power of each biomarker and CRS at different stages of the disease.

The CRS method supports the idea of assigning study population-based thresholds with a more personalized approach, instead of using universal thresholds [17]. It was observed that, there are four- to nine-fold differences between the levels of given thresholds in studies using different methods for these biomarker determinations [10–14]. This observation clearly demonstrates that the application of a single universal threshold is cumbersome and not applicable. However, a chair-side test may solve the problem, as it will standardize the methods for saliva collection and biomarker analyses [18]. The main shortcoming of the CRS method is due to the requirement of heterogeneous study populations. For example, homogenous population cohorts, such as army recruits (males with age range of 18–20 years), will limit CRS's ability to create the high-, middle-, and low-risk groups.

Adaptive-threshold designs have gained interest in various fields of medicine as they may bring on increased power over fixed thresholds [17]. In the limits of the present study, we conclude that the cumulative use of salivary *P. gingivalis*, IL-1β, and MMP-8 levels with an adaptive design brings additional power over fixed thresholds of single biomarkers in detecting periodontitis.

Disclosure statement

The authors report no conflicts of interest.

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