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ORIGINAL ARTICLE

Utility of a haemoglobin test of gingival crevicular fluid: A multicentre, observational study

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Abstract

Objective: The purpose of this study was to verify the accuracy and utility of clinical parameters (plaque index, gingival crevicular fluid volume, probing depth, clinical attachment level, bleeding on probing and gingival index) and biochemical parameters (aspartate aminotransferase, protein and haemoglobin) in a longitudinal analysis during the supportive periodontal therapy period.

Subjects and Methods: A total of 279 test sites of 128 patients were investigated clinically and biochemically. After the first examination of clinical and biochemical parameters, periodontal support treatments were administered immediately and performed once every three months up to the second examination.

Results: All of the clinical and biochemical parameters were significantly lower at the second examination than at the first, except for the plaque index and bleeding on probing. Of these parameters, in particular, aspartate aminotransferase and haemoglobin in the gingival crevicular fluid were significantly reduced compared to those of the first examination in both the ≤4 and ≥5 mm probing depth groups, and they clearly suggested that periodontitis tended to recover.

Conclusion: Adding the haemoglobin test to the bleeding on probing test strongly improves the accuracy of measurement of clinical parameters after periodontal treatment.

KEYWORDS
bleeding on probing, gingival crevicular fluid, haemoglobin, periodontitis, supportive periodontal therapy

1 | INTRODUCTION

Periodontal disease is one of the infectious diseases caused by oral bacteria, and the presence of biofilm, a bacterial plaque, is directly involved in the onset of the disease (Page & Kornman, 1997). Furthermore, periodontal disease is a highly recurrent disease, and mechanical oral hygiene treatments are the basis of periodontal disease prevention and treatment.

On the other hand, there are two possible reasons for its high prevalence and exacerbation, in addition to biofilm formation as the direct cause. First is that the accuracy of periodontal examination methods, such as probing depth (PD), bleeding on probing (BOP) and clinical attachment level (CAL), for evaluating the extent of periodontal tissue damage is not necessarily high, since examination skills differ among individual dentists (Fowler et al., 1982; Listgarten, 1980). There is a need for more sophisticated and precise tests in this regard. The other one is that scaling and root planing, a typical subgingival treatment, have limited success depending on the degree of periodontal disease and the affected area (Caffesse et al., 1986; Waerhaug, 1978). Therefore, it is necessary to determine the state of the periodontal tissue as accurately as possible in advance through high-precision tests.

We consider the first reason to be particularly important. The measurement of clinical parameters centred on the pocket probe has poor reproducibility due to differences in skill between examiners; thus, it may be difficult to understand the actual presence and degree of periodontal disease (Badersten et al., 1985). In addition, when the pain in the affected area increases with progression of the pathological condition of periodontal disease, it becomes difficult to carry out a periodontal examination using the probe (Heft et al., 1991). Therefore, there are limitations to the conventional periodontal examination, and the emergence and development of a new periodontal examination that compensates for these limitations is greatly needed. A requirement for a new next-generation periodontal examination is to be able to provide more accurate site-specific examination results, in addition to the results that can be provided using the conventional periodontal examination method using clinical parameters.

Therefore, we focused on gingival crevicular fluid (GCF) examination, which can be collected painlessly, unlike the probing test and whose component analysis results are highly correlated with the pathology of the collection site. To establish a new, next-generation, periodontal examination that uses this GCF test, we have continuously examined the limitations of conventional periodontal examination and the relevance of biochemical markers in GCF in cross-sectional studies. We previously quantified the biochemical parameters in GCF, aspartate aminotransferase (AST) activity and total protein amount, and compared them with the results of conventional periodontal examination including the BOP test (Ito et al., 2014). Because there was a discrepancy between the test results of the biochemical markers indicating tissue damage in GCF and the BOP test result, it appeared that the BOP test result does not always accurately reflect the condition of the periodontal tissue.

It was already known that haemoglobin (Hb) was present in the GCF of the inflamed periodontal pocket (Hanioka et al., 2005; Mäkinen et al., 1996). Therefore, to accurately diagnose the condition of inflamed periodontal tissue, we measured and assessed Hb levels in GCF using immune-chromatography (IC) (Ito et al., 2016) and found that invisible bleeding was already present in many periodontal pockets before the probing test. In addition, when the periodontal pocket in which Hb was detected in GCF in advance by this Hb test was subjected to the BOP test using a probe, a significant number of negative test results was observed (Ito et al., 2016). These results suggest that even healthy periodontal tissue pockets that show a negative result on the BOP test (BOP (−)) may have already been damaged. At the same time, in that study, there were also cases in which the BOP test was positive even in pockets in which Hb was not detected. These facts raise the issue that the BOP test is a method of visually recognizing the presence or absence of bleeding from the periodontal tissue by applying pressure to the tissue with the probe. At the same time, it also suggests that bleeding may be overlooked due to saliva.

In addition, we analysed in detail the results of cross-sectional periodontal examinations of patients receiving supportive periodontal therapy (SPT) with a cut-off value of Hb by BOP and PD (Ito et al., 2020) and found that the presence of Hb molecules within the GCF before probing showed that fine damage of the periodontal tissue with haemorrhage may have already occurred in the early stage of periodontitis.

Based on the above, the limitations of clinical parameter measurements were investigated, a search for markers that complement them was conducted, and from a series of reports, we found that Hb, which is evidence of previous bleeding in GCF, is an important marker (Ito et al., 2014, 2016, 2020). In these previous cross-sectional studies, the results of Hb and BOP tests were not always concordant. Therefore, in the present study, whether Hb test results correlated with clinical assessment over time was investigated.

In the present study, to further clarify the problems with the BOP test and the utility of the Hb test, periodontal examinations using clinical parameters (plaque index [PII], GCF volume, PD, CAL, gingival index [GI] and BOP) and biochemical parameters (AST, protein and Hb) were performed longitudinally in SPT patients. Since the BOP test in the periodontal examination can be prone to artificial discrepancies, the chronological changes of all these parameters including BOP were analysed statistically.

2 | MATERIALS AND METHODS

2.1 | Experimental design

This was a longitudinal study designed according to the STROBE guidelines. It was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 2013, with approval from the ethics committees of Nippon Dental University, Iwate Medical University School of Dentistry, Nihon University School of

TABLE 1 Study population.

Patients' information			Classification of periodontitis in patients (n = 128)												
	First examination	Second examination	Stage	I			II			III			IV		
			Grade	A	B	C	A	B	C	A	B	C	A	B	C
Patients, n	184	128													
Age, years (mean ± SD)	63.0 ± 11.3	62.4 ± 11.2	Localized chronic periodontitis	7	4	1	6	4	1	12	1	-	-	-	-
Sex	73 males and 111 females	53 males and 75 females	Generalized chronic periodontitis	-	-	1	3	1	-	62	12	13	-	-	-
Inspection sites, n	401	279													

Note: 128 subjects were classified according to the level of periodontal disease according to the new classification (Tonetti et al., 2018).

Note: Since 56 patients dropped out from the first periodontal examination to the second periodontal examination, a comparison of periodontal tissue conditions between the first and second examinations was performed using values (clinical and biochemical parameters) of 279 sites in 128 patients in the second examination. Clinical parameters were PII, GCF volume, PD, CAL, BOP, and GI. Biochemical parameters were AST, protein, and Hb.

Dentistry at Matsudo, and Tokushima University (Approval Nos. NDU-T 2017–12 and NDUH-RINRI 2018–07, 01179, EC 09–005 and 1293, respectively). Tokyo Medical and Dental University was included in the ethics application to Nippon Dental University. All patients provided their informed consent prior to inclusion in the study.

2.2 | Study population

Patients with chronic periodontitis who had completed periodontal treatment and entered the SPT stage while visiting the Nippon Dental University Hospital, Iwate Medical University School of Dentistry Hospital, Nihon University Hospital School of Dentistry at Matsudo, Tokyo Medical and Dental University Hospital, and Tokushima University Hospital were targeted. The selection criteria were non-smokers who had 12 or more remaining teeth and were generally healthy. The exclusion criteria were the following: (1) patients with endocrine metabolic diseases, malignant tumour, immune disease, liver disease, renal failure, heart disease or osteoporosis; (2) pregnant women or those taking contraceptives; and (3) patients who did not provide consent to participate in this study (Table 1). At the time of the first SPT examination, there was a mixture of patients who underwent periodontal surgery and patients who did not undergo periodontal surgery, but this study included both groups.

2.3 | Research protocol

The monitoring and sampling of this study were conducted at each facility from September 2009 to March 2016. The observation and SPT period for this study was 1 year from the first periodontal examination of clinical and biochemical parameters to the second examination (Figure 1). The SPT was performed four times (0, 3, 6, and 9 months), starting at the first periodontal examination. The SPT included oral hygiene instruction and professional tooth cleaning (Herbert & Thomas, 2006) during a single appointment. Subgingival debridement with ultrasonic devices was performed as necessary, such as when dental calculus deposition was observed. For the 279 sites of the 128 patients who did not drop out at the second periodontal examination, the clinical and biochemical test results were compared statistically between the first and second examinations (Table 1).

2.4 | Measurements of clinical parameters

All patients underwent periodontal examinations by specialist periodontists (HI, TY, DS, YO, YI, HW, YH and J-IK) certified by the Japanese Society of Periodontology belonging to each university hospital. In calibration, a 90% concordance rate (n = 10) within 1 mm for PD and CAL measurements between the first and second recordings with a 24-h interval was achieved.

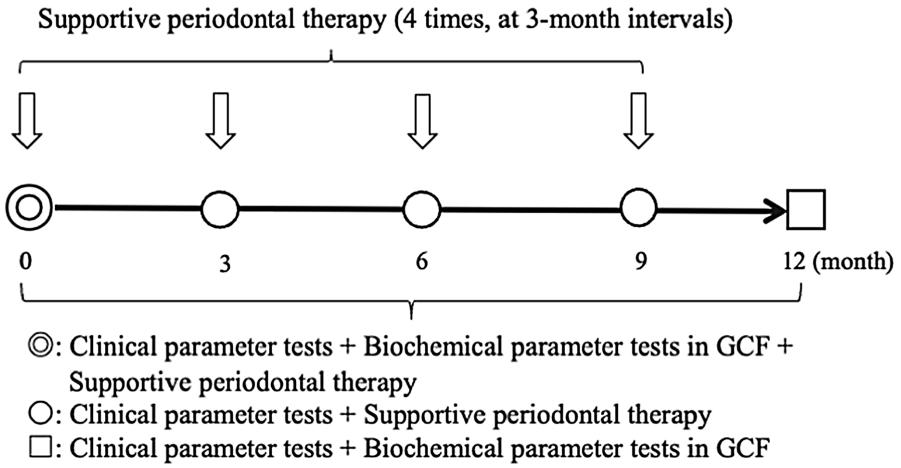

FIGURE 1 Research protocol. Clinical parameter tests are measurements of PII, PD, CAL, GI, and BOP. Biochemical parameter tests are measurements of AST activity, amount of protein and Hb amount in GCF. Supportive periodontal therapy includes oral hygiene instruction, subgingival debridement, and professional tooth cleaning. GCF sampling in the first and second periodontal examinations is performed between the measurements of PII and PD. The interval between the two periodontal examinations done as part of SPT is one year.

Each clinical periodontal examination for monitoring included the following items and was successively performed and recorded in the order of PII (Löe, 1967), GCF volume, PD, CAL, GI (Silness & Löe, 1964) and BOP. For measurements of PD, CAL, GI and BOP on the test tooth, a WILLIAMS PROBE (Hu-Friedy Inc.) with a tip diameter of 0.5 mm was used. The BOP test was done according to Ainamo and Bay (1975) and Armitage et al. (1977).

2.5 | GCF collection

The target tooth pocket was selected from the maxillary and mandibular single-rooted anterior teeth (central incisors, lateral incisors, canines and premolars) of the participants. Abutment teeth and implants of removable partial dentures were excluded.

GCF was sampled from a single tooth corresponding to each of the clinical parameters. Biochemical parameters in GCF were compared with the clinical parameters. To prevent contamination of GCF by blood due to probing, GCF was collected after PII measurement. Then, the clinical parameters of PD, CAL, GI and BOP were assessed in sequence.

To collect GCF as a sample, the pocket was simply dried using cotton rolls and air, and the overlying plaque was removed to the extent possible while avoiding touching the gingival margin. Subsequently, a blotting paper strip (PerioPaper, Oraflow Inc.) was inserted into the pocket until resistance was felt, and GCF was collected consecutively three times using three paper strips for 30 s per collection. That is, the GCF in target pockets was collected by inserting three paper strips into the same pocket three times, one at a time.

The amount of GCF collected was measured using a calibrated unit (Periotron 8000, Oraflow Inc.), expressed as μL and used as one of the clinical parameters. The three paper strips used to sample the GCF were then immediately soaked in 500 μL of phosphate-buffered saline (PBS), stirred for 5 min, and centrifuged for 5 min at 10,000 rpm. The supernatant was dispensed for biochemical analysis. The recovery rate of all biochemical parameters into the solvent from the paper strips that collected GCF was 98% or greater. If bleeding occurred due to insertion of the paper strip, the GCF sample was considered to be contaminated with blood and was excluded. In addition, the amount of GCF was actually small in some cases, but none of the biochemical parameters (AST, protein and Hb) extracted in the PBS was below detection limits. Each GCF sample was stored at −80°C until further analysis. For the biochemical analysis of GCF, the cryopreserved GCF sample was naturally thawed at room temperature (23°C) and used for the analysis of the biochemical parameters.

2.6 | Measurement of biochemical parameters

All GCF samples were analysed for various biochemical parameters (AST activity, protein amount and Hb amount) by the biochemist (SH).

AST activity was measured using the POP/TOOS method (Wako Pure Chemical Industries, Ltd.) and expressed as μU/pocket (Ito et al., 2014). The amount of protein was measured using the BCA Protein Assay Kit (Thermo SCIENTIFIC, Waltham, MA, USA) and expressed as μg/pocket (Ito et al., 2014, 2020). The amount of Hb was measured using the Hb detection kit (Check-Line Hemo, Wakamoto, Tokyo, Japan) that applies the IC method using a human monoclonal antibody. After chromatographic development for 15 min at 23°C, the amount of red latex-labelled human monoclonal primary antibody bound to Hb by the IC method was measured using a densitometer (GS-800 Calibrated Densitometer PC system, Bio-RAD, Tokyo, Japan) and expressed as ng/pocket (Ito et al., 2016, 2020).

2.7 | Statistical analysis

The results of the clinical parameters and biochemical parameters were compiled and sent to the statistician as coded data by the respective personnel for analysis.

TABLE 2 Changes in clinical and biochemical parameters from the first periodontal examination to the second periodontal examination for 279 sites.

		PII	GCF volume (μL)	PD (mm)	CAL (mm)	GI	BOP	AST (μU/pocket)	Protein (μg/pocket)	Hb (ng/pocket)
First examination	Mean	0.427	1.515	3.676	4.604	0.814	0.240	1083.0	25.3	50.6
	SD	0.537	2.061	1.782	2.406	0.745	0.428	942.4	34.1	84.1
p-value		0.179	<0.001	<0.001	<0.001	0.008	0.922	<0.001	0.019	<0.001
Second examination	Mean	0.358	0.925	3.145	4.194	0.667	0.244	280.4	20.4	9.6
	SD	0.594	1.464	1.623	2.421	0.744	0.430	317.2	29.0	29.7

Note: The differences between the values of the same test performed at the first and second measurements of clinical and biochemical parameters were analysed by the Wilcoxon test. *p*-values are truncated to three decimal places. For statistical analysis, the negative (−) and positive (+) results of the BOP test were quantified as 0 and 1, respectively.

The results of the first clinical and biochemical test items were compared with the results of the second tests. The results for the clinical test items and biochemical test items are expressed as means ± standard deviation (SD), and the statistical analysis software SPSS ver. 22.0J (IBM-SPSS, Inc.) was used for the analysis. The Kolmogorov–Smirnov test was performed to test whether the clinical test items and biochemical test items were normally distributed. In this study, correlations among the clinical and biochemical parameters were analysed using Spearman's correlation coefficient. The Wilcoxon signed-rank test was used for between-group comparisons. When the probability was less than 5% ($p < 0.05$), the difference was considered significant. For statistical analysis, the negative (−) and positive (+) results of the BOP test were quantified as 0 and 1, respectively. In previous reports, we analysed the BOP test in a similar way (Ito et al., 2016, 2020).

The sample size was determined according to a previous study (Ito et al., 2016), with the primary endpoint being the correlation between the BOP test and Hb amount. The power calculation showed that the sample size necessary to perform this research was $n = 32$ (coefficient of correlation: 0.2; a-error: 0.05; power: 0.8). However, 50 or more GCF samples from each facility were collected at baseline.

Claffey et al. (1990) have already discussed the condition of periodontal tissues in SPT patients by dividing them into groups with PD values of 4 mm or less and of 5 mm or more. In the present study, the state of progression of periodontal disease in SPT was thus classified into two groups with PD ≤4 and PD ≥5 mm. In each group, clinical and biochemical parameters were compared between the first and second periodontal examinations, with an interval of one year.

3 | RESULTS

3.1 | Patients

A total of 184 (mean age 63.0 ± 11.3 years, 73 male and 111 female) patients with 401 GCF collection sites in the first periodontal examination were included. At the second periodontal examination, 56 patients dropped out; thus, 128 (mean age 62.4 ± 11.2 years, 53 male and 75 female) patients with 279 GCF collection sites were included (Table 1). The 56 patients who could not be monitored after one year included 41 victims of the Great East Japan Earthquake, 14 patients who could not visit the hospital for personal reasons, and one patient whose target teeth had fallen out naturally. Therefore, these 279 test sites in 128 subjects who could be monitored and sampled both at the first visit and the second visit one year later were the periodontal examination targets. The breakdown of the 279 sites for which GCF was collected for the 128 subjects was as follows: 20 subjects with 1 site, 76 subjects with 2 sites, 25 subjects with 3 sites, 5 subjects with 4 sites, and 2 subjects with 6 sites. In addition, Table 1 shows the classification of periodontal disease (Tonetti et al., 2018) for 128 patients. At baseline, the mean PD was 3.15 mm, the mean CAL was 4.45 mm, and the BOP-positive rate was 12.22%.

The Kolmogorov–Smirnov test showed that all clinical and biochemical parameters were not normally distributed.

3.2 | Clinical and biochemical parameter values in the first and second periodontal examinations

During SPT, after the first periodontal examination including tests of clinical and biochemical parameters, four periodontal support treatments were performed, and one year later, the second periodontal examination was performed (Table 2). Comparing the results of the first and second periodontal examinations, all clinical and biochemical parameter test values, except the BOP test-positive rate, decreased in the second periodontal examination compared to the first examination. In particular, the reduction rates in the GCF volume, PD, CAL, GI, AST, protein and Hb values were significant. Of these, the values of the GCF volume, AST and Hb tests in the second periodontal examination were decreased to 61.0%, 25.9% and 19.0% of the values in the first examination, respectively. There was almost no change and no significant difference in the BOP test results between the two periodontal examinations.

3.3 | Correlations among clinical and biochemical parameter values of the first and second periodontal examinations

Test results of clinical and biochemical parameters in each of the first and second periodontal examinations were compared. In

the first periodontal examination, there were significant correlations among the results of the parameter tests, except between the BOP and AST test results (Table 3). Furthermore, the second periodontal examination also showed significant correlations among the results of parameters, except between the PII and Hb test results (Table 4).

TABLE 3 Correlations of clinical and biochemical parameters in the first periodontal examination of 279 sites.

		GCF volume	PD	CAL	GI	BOP	AST	Protein	Hb
PII	CC	0.302	0.364	0.370	0.393	0.169	0.275	0.284	0.326
	p-value	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	<0.001	<0.001
GCF volume	CC	-	0.580	0.525	0.336	0.303	0.438	0.631	0.489
	p-value	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PD	CC		-	0.855	0.611	0.464	0.355	0.495	0.399
	p-value		-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
CAL	CC			-	0.587	0.417	0.337	0.450	0.357
	p-value			-	<0.001	<0.001	<0.001	<0.001	<0.001
GI	CC				-	0.499	0.161	0.383	0.351
	p-value				-	<0.001	<0.001	<0.001	<0.001
BOP	CC					-	0.099	0.294	0.187
	p-value					-	0.100	<0.001	0.002
AST	CC						-	0.355	0.255
	p-value						-	<0.001	<0.001
Protein	CC							-	0.607
	p-value							-	<0.001

Abbreviation: CC, coefficient of correlation.

Note: In the first periodontal examination, the correlations among the values of the clinical and biochemical parameter tests were analysed using Spearman's correlation coefficient. There were significant correlations among the values of these tests, except between the BOP and AST tests. *p*-values are truncated to three decimal places.

TABLE 4 Correlations of clinical and biochemical parameters in the second periodontal examination of 279 sites.

		GCF volume	PD	CAL	GI	BOP	AST	Protein	Hb
PII	CC	0.127	0.311	0.282	0.398	0.289	0.244	0.379	0.090
	p-value	0.034	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.132
GCF volume	CC	-	0.385	0.254	0.320	0.238	0.420	0.408	0.268
	p-value	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PD	CC		-	0.707	0.434	0.455	0.290	0.391	0.280
	p-value		-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
CAL	CC			-	0.273	0.296	0.142	0.241	0.157
	p-value			-	<0.001	<0.001	0.018	<0.001	0.009
GI	CC				-	0.597	0.358	0.489	0.279
	p-value				-	<0.001	<0.001	<0.001	<0.001
BOP	CC					-	0.258	0.324	0.268
	p-value					-	<0.001	<0.001	<0.001
AST	CC						-	0.569	0.223
	p-value						-	<0.001	<0.001
Protein	CC							-	0.415
	p-value							-	<0.001

Abbreviation: CC, coefficient of correlation.

Note: The second periodontal examination, like the first in Table 3, showed significant correlations among the values of these tests, except between PII and Hb. *p*-values are truncated to three decimal places.

3.4 | Changes in clinical or biochemical parameter values from the first periodontal examination to the second examination in the PD ≤4 mm and PD ≥5 mm groups

There were 158 sites with PD ≤4 mm and 121 sites with PD ≥5 mm among the 279 sites in the first periodontal examination. Table 5 shows the changes in clinical parameters from the first periodontal examination to the second periodontal examination in each of the two groups with PD ≤4 mm and with PD ≥5 mm.

In the clinical parameters of the PD ≤4 mm group, the GCF volume of the second periodontal examination was significantly lower (by 12.8%, $p < 0.001$) than that of the first examination. On the other hand, the PII, PD, CAL and GI values of the second periodontal examination showed a tendency to increase compared to the first examination, but none of these increases was significant. Of these parameters, only the BOP value was significantly increased from 0.089 to 0.171 from the first to the second periodontal examination.

In the clinical parameters of the PD ≥5 mm group, the PII, GCF, PD, CAL and GI values of the second periodontal examination were significantly decreased from those of the first examination; the reduction rates of these parameter values were 28.8%, 48.3%, 23.2%, 16.9% and 34.8%, respectively. In contrast, the BOP value decreased by 22.6% compared to the first periodontal examination, but no significant difference was observed.

In Table 6, the changes in biochemical parameters in GCF from the first periodontal examination to the second examination in each of the groups with PD ≤4 mm and with PD ≥5 mm are shown.

In the biochemical parameters of the PD ≤4 mm group, the AST and Hb values in GCF were significantly lower at the second periodontal examination than at the first examination; the reduction rates were 76.3% and 74.4% ($p < 0.001$), respectively. The protein value of the second periodontal examination was increased by 14.6% compared to that of the first examination, but the increase was not significant.

For the biochemical parameters of the PD ≥5 mm group, all of the AST, protein and Hb values of the second periodontal examination were significantly decreased compared to those of the first examination, by 72.3%, 31.7% and 82.7%, respectively.

4 | DISCUSSION

In the present study, after the first periodontal examination of SPT patients was performed, the second examination was performed one year later after four SPTs. The periodontal examination included measuring PII, GCF volume, PD, CAL, GI and BOP as clinical parameters and AST activity, protein amount and Hb amount as biochemical parameters in GCF. In particular, of these biochemical parameters, Hb is known to be present in the pockets of inflamed periodontal tissue, and Hb peptides have been previously detected in the gingival inflammatory exudates within gingival pockets by high-performance liquid chromatography (Mäkinen et al., 1996). Hanioka et al. (2005)

TABLE 5 Comparisons of clinical parameters at the first and second periodontal examinations by PD value (158 sites ≤4 mm and 121 sites ≥5 mm in the first examinations).

		PII		GCF volume (μL)		PD (mm)		CAL (mm)		GI		BOP	
		≤4 mm	≥5 mm	≤4 mm	≥5 mm	≤4 mm	≥5 mm	≤4 mm	≥5 mm	≤4 mm	≥5 mm	≤4 mm	≥5 mm
First examination	Mean	0.241	0.661	0.696	2.589	2.326	5.455	2.968	6.707	0.443	1.281	0.089	0.438
	SD	0.457	0.525	0.753	2.653	0.794	0.975	1.390	1.665	0.682	0.520	0.285	0.498
p-value		0.051	0.011	<0.001	<0.001	0.794	<0.001	0.321	<0.001	0.129	<0.001	0.029	0.109
Second examination	Mean	0.272	0.471	0.607	1.339	2.345	4.190	3.139	5.570	0.538	0.835	0.171	0.339
	SD	0.541	0.659	0.964	1.855	0.953	1.724	1.684	2.549	0.762	0.687	0.378	0.475

Note: *p*-values are truncated to three decimal places. For statistical analysis, the negative (−) and positive (+) results of the BOP test were quantified as 0 and 1, respectively.

		AST (μU/pocket)		Protein (μg/pocket)		Hb (ng/pocket)	
		≤4 mm	≥5 mm	≤4 mm	≥5 mm	≤4 mm	≥5 mm
First examination	Mean	867.3	1364.0	12.0	42.7	19.9	90.1
	SD	890.5	937.5	13.3	43.8	47.1	103.3
p-value		<0.001	<0.001	0.487	<0.001	<0.001	<0.001
Second examination	Mean	205.3	378.4	13.7	29.1	5.1	15.6
	SD	210.6	397.5	18.8	36.7	20.4	37.9

Note: *p*-values are truncated to three decimal places.

TABLE 6 Comparison of biochemical parameters in GCF at the first and second periodontal examinations by PD value (158 sites ≤4 mm and 121 sites ≥5 mm in the first examinations).

evaluated the amount of Hb in GCF of patients with periodontal disease by ELISA and showed that the amount of Hb tended to increase as periodontitis progressed. We also reported that the amount of Hb in GCF was closely related to the periodontal disease condition (Ito et al., 2016, 2020). These findings show that Hb in GCF is an important marker of the progression of periodontitis.

When the results of the first periodontal examination were compared with the results of the second examination one year later after four SPTs, all other clinical and biochemical parameters, except the BOP tests, were decreased significantly from the first examination to the second (Table 2). Changes in these values indicate that the periodontal disease tended to improve with the SPT.

These 9 parameters were correlated with each other at each time point, not only at the first periodontal examination but also at the second examination (Tables 3 and 4). This suggests that the values of these parameters reflect the state of the periodontal tissue to some extent. We have already reported in another experiment that biochemical parameters such as AST correlate well with clinical parameters (Ito et al., 2014).

In addition, the test results for each of the clinical and biochemical parameters were divided into two periodontal pocket groups with PD ≤4 mm and with PD ≥5 mm, and the changes in each parameter from the first periodontal examination to the second were analysed (Tables 5 and 6).

In the case of the 6 clinical parameters (Table 5), only the GCF volume decreased in the PD ≤4 mm group, and all the parameter values except the BOP decreased in the PD ≥5 mm group. This indicates that the examination of Hb and AST in GCF can detect tissue damage due to the extravasation of erythrocytes and leukocytes, respectively. At the same time, the BOP value tended to increase from the first examination to the second, opposite to the other clinical and biochemical parameter values. This is thought to be because the BOP test is a visual inspection for the presence or absence of bleeding and is prone to misdiagnosis. On the other hand, of the 3 biochemical parameters in the PD ≤4 mm group (Table 6), the AST and Hb values decreased significantly. Furthermore, in the PD ≥5 mm group, all biochemical parameter values also decreased. These results suggest that tissues with deep periodontal tissue pockets are more likely to be significantly improved by the SPT, consistent with the reports of Badersten et al. (1981 and 1984).

To summarize these results, it became clear that, after the SPT, the clinical and biochemical parameters, except for BOP, were mostly decreased in all target SPT patients and even in each state of periodontal disease progression classified by PD, and the periodontal disease improved. In particular, the reduction rates of clinical and biochemical parameter values except for AST values after one year were significantly greater in periodontal pockets with PD ≥5 mm than in those with PD ≤4 mm. Therefore, it was shown that inflamed periodontal tissue having a deep periodontal pocket is more likely to improve than inflamed tissue having a shallow periodontal pocket.

Of these parameters, the reductions in values of Hb and AST released into GCF from injured tissues strongly suggest improvement of the inflamed periodontal tissue. Takeuchi-Hatanaka et al. (2016) reported that the amounts of cytokines in GCF on periodontal examination over time in SPT patients were decreased by SPT, and periodontal disease was improved. Therefore, the present study confirmed that the condition of periodontal tissue can be inferred by comprehensively analysing the clinical and biochemical parameters used, and that SPT is effective.

However, the change in the BOP test result from the first periodontal examination to the second periodontal examination was different from the changes seen in other clinical and biochemical parameters. Lang et al. (1986) have already reported that the negative predictive value of the BOP test is low. We have also observed many negative results of the BOP test in severely inflamed periodontal tissue classified by the cut-off values of BOP and PD (Ito et al., 2014). This peculiar phenomenon with respect to BOP may occur because the BOP test, unlike other parameter tests, is a method in which an external force is artificially applied to the periodontal tissue to observe the presence or absence of bleeding, leading to a diagnosis. In fact, we previously reported that many periodontal tissues diagnosed as BOP (−) are slightly damaged because of the presence of Hb in GCF (Ito et al., 2016, 2020).

The results of the present research showed that changes of AST and Hb in GCF, especially, are extremely strongly reflected in clinical parameters and are powerful biochemical parameters that complement conventional periodontal examination. From these many findings, it became clear that the BOP test results differed from those for GCF components, including Hb and AST. Therefore, it is possible that the values of Hb and AST tests in GCF, which can be used to evaluate the invasion history of erythrocytes and leukocytes from

fragile inflamed periodontal tissues, reflect the state of periodontal tissue more accurately than the BOP test.

From these cross-sectional and longitudinal studies of clinical and biochemical parameters of the periodontal examination, we believe that, if BOP test results show significant discrepancies with other clinical parameter test results, Hb and AST should be measured in the GCF as complementary tests. In particular, the Hb test using the IC method is simpler and quicker to perform than the AST test and is suitable for chairside implementation.

In conclusion, the accuracy of periodontal examination after periodontal therapy may be improved by adding the haemoglobin test to the BOP test, which applies artificial external force.

AUTHOR CONTRIBUTIONS

Hiroshi Ito: Funding acquisition; investigation; methodology; validation; writing – original draft; writing – review and editing. **Yukihiro Numabe:** Funding acquisition; investigation; methodology; validation; writing – original draft; writing – review and editing. **Shuichi Hashimoto:** Methodology; writing – original draft; writing – review and editing. **Satoshi Sekino:** Investigation; methodology; validation. **Etsuko Murakashi:** Investigation; methodology; validation. **Hitomi Ishiguro:** Investigation; methodology; validation. **Daisuke Sasaki:** Writing – original draft; writing – review and editing. **Takashi Yaegashi:** Methodology; writing – original draft; writing – review and editing. **Hideki Takai:** Writing – original draft; writing – review and editing. **Masaru Mezawa:** Writing – original draft; writing – review and editing. **Yorimasa Ogata:** Methodology; validation; writing – original draft; writing – review and editing. **Hisashi Watanabe:** Writing – original draft; writing – review and editing. **Yuichi Izumi:** Methodology; writing – original draft; writing – review and editing. **Jun-ichi Kido:** Writing – original draft; writing – review and editing. **Yuka Hiroshima:** Writing – original draft; writing – review and editing. **Toshihiko Nagata:** Methodology; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

PATIENT CONSENT STATEMENT

All patients provided their informed consent prior to inclusion in the study.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

CLINICAL TRIAL REGISTRATION

Not applicable.

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