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Glycated albumin and calprotectin levels in gingival crevicular fluid from periodontitis patients with type 2 diabetes

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* Short running title: Glycated albumin level in GCF from diabetes patients
* Summary: High levels of GA and calprotectin in GCF from DM-associated periodontitis.
* Key words: diabetes mellitus-associated periodontitis; glycated albumin, calprotectin, gingival crevicular fluid, diagnosis.

ABSTRACT

Background: Diabetes mellitus (DM) patients have a high prevalence of periodontitis. DM-associated periodontitis (DM-P) is characterized by severe inflammation and tissue destruction. To diagnose DM-P is important for cures of periodontitis and DM. The purpose of this study was to investigate the levels of glycated albumin (GA), a DM marker, and calprotectin, an inflammatory marker, in gingival crevicular fluid (GCF) from patients with periodontitis and DM.

Methods: Seventy-eight subjects participated in this study were the patients with DM, chronic periodontitis (CP), DM-P and healthy individuals (H). GCF and blood were collected from four groups. GA and calprotectin in GCF were analyzed using western blotting and ELISA, and their levels were compared among H, DM, CP, and DM-P groups. GA and glycated hemoglobin (HbA1c) in blood were determined, and the correlation between GCF GA level and blood HbA1c or GA level was investigated. ROC analysis for GCF GA level to predict DM was performed.

Results: GA was identified in GCF, and its amount and concentration in GCF samples from DM and DM-P were significantly higher than those of non-DM

groups (H and CP). Calprotectin amount in GCF from CP and DM-P was significantly higher than that in H and DM groups. GCF GA level was positively correlated to blood HbA1c and GA level. ROC analysis of GCF GA level showed an optimal cut-off value to predict DM.

Conclusions: GA showed a high level in GCF from DM patients. GA and calprotectin in GCF may be useful markers to diagnose DM-associated periodontitis.

INTRODUCTION

Periodontitis is an oral inflammatory disease with a high incidence in middle-aged and elderly people. Diabetes mellitus (DM) is a major metabolic disorder with abnormal glucose metabolism; its prevalence is increasing in many countries. DM causes inflammatory complications including diabetic nephropathy, neuropathy and retinopathy, and is associated with cardiovascular diseases, vascular abnormalities in the brain, and atherosclerosis.[1-3] Periodontitis is the sixth common complication of DM and its incidence in DM patients is higher than in the general population.[4-6] DM-associated periodontitis shows edematous gingival swelling with severe inflammation and destruction of periodontal tissues. The hyperglycemia in DM induces the production of glycated proteins known as advanced glycation end-products (AGEs), and results in diabetic complications. AGEs cause vascular abnormalities, altered collagen metabolism, and dysfunction of immune cells, and regulate the expression of inflammatory cytokines and chemokines in periodontal tissues.[7-10] Overproduced AGEs accumulate in the periodontal tissues of DM patients and aggravate DM-associated periodontitis.[11,12] On the other hand,

periodontitis also appears to affect DM, and the treatment of periodontitis was shown to improve DM condition.[13,14] The diagnosis of DM-associated periodontitis is very important to arrest the inflammation and destruction of periodontal tissues in periodontitis, and may contribute to the prediction of DM.

DM is medically diagnosed by determining glycated hemoglobin (hemoglobin A1c: HbA1c), glycated albumin (GA), and blood glucose levels. HbA1c and GA are intermediate products called Amadori compounds, involved in the non-enzymatic glycation reactions of blood proteins, which finally change to AGEs.[15,16] HbA1c and GA levels reflect the status of glycemic control, and these variables were shown to be positively correlated; the level of GA or HbA1c was also significantly associated with plasma glucose levels in subjects with and without DM in epidemiological research.[17,18] HbA1c level represents the mean glycemic status over the previous 2-3 months, but does not reflect glycemic condition in the short term or provide an accurate measure in patients with anemia and hemolysis. In contrast, GA more exactly shows the glycemic condition in the short term (two to three weeks) because the half-life of albumin is approximately 17

days. GA is thus a useful DM marker to evaluate postprandial glycemic status and glycemic control after medication for DM treatment and accurately reflects the status of glycemic control in DM patients with certain diseases, including anemia, hemoglobin disorder, and renal failure, as well as in pregnancy,[19,20] suggesting that GA is an important marker for the diagnosis of DM.

Calprotectin is a major cytosolic protein in leukocytes and is also identified in monocytes/macrophages and epithelial cells.[21] Calprotectin level in body fluids, skin, or feces from patients with inflammatory diseases including rheumatoid arthritis, ulcerative colitis, and cystic fibrosis, is increased in comparison with that in healthy conditions.[22-24] Calprotectin was also detected in gingival crevicular fluid (GCF) and its level in GCF samples from cases of periodontitis was significantly higher than for those without periodontitis.[25-28] Calprotectin level in GCF from periodontitis patients was also decreased by periodontal treatments and predicted periodontal disease activity in patients with periodontitis,[29,30] suggesting that calprotectin in GCF is a useful marker for periodontal diseases.

We recently identified GA in GCF samples from patients with

DM-associated periodontitis. The purpose of the present study is to compare the levels of GA and calprotectin in GCF samples from subjects with or without periodontitis and DM, and to investigate the possibility that GA and calprotectin in GCF are markers that can diagnose DM-associated periodontitis.

MATERIALS AND METHODS

Subjects and clinical examinations

This clinical study was performed with the approval of the Ethics Committee of Tokushima University Hospital (approval No. 2773, 2775 and 2945) in accordance with the Helsinki Declaration of 1975, as revised in 2000. Seventy-eight subjects (33 males and 45 females) gave written-informed consent after hearing an explanation of the present study. Although fifty subjects among the total agreed to all procedures including the collection of GCF, blood, and medical information, twenty-eight subjects gave their consent to all procedures except drawing blood. Patients with periodontitis and type 2 diabetes as well as healthy volunteers without these two diseases were recruited from Tokushima University Hospital and the University of

Tokushima Graduate School. The participants were classified into four groups: diabetes mellitus without periodontitis (DM), chronic periodontitis without DM (CP), DM-associated periodontitis (DM-P), and no DM and no periodontitis (healthy: H); their characteristics are shown in Table 1. Periodontitis was evaluated by two clinical indicators: probing pocket depth (PD) of more than 4 mm and gingival index (GI) score of more than 1. GI score was examined according to the standard of Löe and Silness.[31] DM was diagnosed by physicians in patients who had HbA1c of more than 6.5% (National Glycohemoglobin Standardization Program value). Blood samples were collected from fifty subjects with or without DM using sterilized disposable needles and syringes, and HbA1c and GA percentages in blood samples were determined by a company specializing in clinical examinations*.

GCF sampling

GCF was collected using paper strips† according to our previous methods.[25-27] Briefly, oral sites for GCF sampling were isolated with cotton rolls and gently dried with air after the supra-gingival plaque had been removed. A

paper strip was inserted into a gingival crevice without periodontitis or a periodontal pocket with periodontitis and held there for 10 sec. GCF collection was sequentially repeated three times using three strips. The GCF volume in strip paper was determined using a calibrated unit‡, and the total volume from one site was calculated. GCF was extracted from a phosphate buffer solution (pH=7.4) with 0.2 μM phenylmethylsulfonyl fluoride by centrifugation according to a modified version of a previously described method.[32] After GCF sampling, the probing depth was measured and GI score was evaluated at the sampling sites. The prepared GCF samples were used for the assays of GA and calprotectin by western blotting and ELISA.

Western blotting

Human serum was prepared from the blood of healthy subjects. Western blotting was performed by a modified version of a previously described method.[32] Briefly, the extracted GCF solution and serum were dissolved in sampling buffer including 50 mM Tris-HCl (pH=6.8), sodium dodecyl sulfate (SDS), and β-mercaptoethanol for polyacrylamide gel electrophoresis. After

boiling, samples were applied to 10% SDS polyacrylamide gel and electrically separated and transferred to a polyvinylidene difluoride membrane§. After blocking of membrane with a blocking reagent‖ at room temperature for 1.5 hours, the membrane was incubated with monoclonal antibody (2 μg/ml) to human GA¶ at 4°C overnight. Bound primary antibody was then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG# (1/2000 dilution) for 1.5 hours at room temperature. The immunological signal of GA antigen on the membranes was detected using a detection system for western blot analysis** and exposed to a film†† for chemiluminescence.

ELISA

GA and calprotectin in GCF samples were determined using assay kits‡‡, §§ according to their instruction manuals. Briefly, the extracted GCF solution was added to wells coated with anti-human glycated albumin antibody, incubated for 1 hour at room temperature, and then reacted with HRP-conjugated human albumin for 1 hour. After the incubation with HRP substrate (3,3',5,5'-tetramethylbenzidine: TMB), the absorbance of the reacting solution in wells was determined at 450 nm. For calprotectin

determination, the extracted GCF solution was diluted from 200- to 400-fold in a dilution buffer in a kit, added to wells coated with anti-human calprotectin antibody, incubated for 1 hour at room temperature, and then reacted with a biotinylated tracer antibody and further with a streptavidin-peroxidase conjugate for 1 hour each. After TMB reaction, the absorbance of the reacting solution was determined at 450 nm. The concentrations of GA and calprotectin are expressed as micrograms per microliter GCF.

Statistical analysis

All data were analyzed using statistical analysis software‖‖,¶¶. The significance of differences in GA and calprotectin levels among GCF samples of H, DM, CP, and DM-P groups was evaluated using the Steel-Dwass test. The correlation between GCF GA level and blood HbA1c value or blood GA value was evaluated using Spearman's rank correlation test. P values < 0.01 were accepted as statistically significant. Receiver operating characteristic (ROC) curves were constructed for GA amount and concentration in GCF samples from H, DM, P and DM-P groups, and their

optimal cut-off values were determined by maximization of Youden's index.

RESULTS

Characteristics of subjects and GCF samples

The participants were classified into four groups and their numbers were as follows: H, 25; DM, 10; CP, 23; and DM-P, 20 (Table 1). The mean ages did not differ significantly among these four groups. One to four GCF samples were collected from each subject and the total number of GCF samples in the four groups was 219. The volumes of GCF samples from periodontitis sites (CP and DM-P) were significantly greater than those without periodontitis sites (H and DM). PD and GI at GCF collection sites with periodontitis were significantly higher than those without periodontitis.

Identification of GA in GCF and comparison of GA level

GCF samples from H, DM, CP, and DM-P groups were analyzed for GA by western blotting (Fig. 1A). GA was identified in GCF samples, and its band showed a molecular weight of 67kDa, which was similar to that of human serum. The intensity of GA bands of GCF samples from DM and DM-P was

greater than those of non-DM groups (H and CP). GA amount and concentration in GCF samples were determined using ELISA and their levels were compared among H, DM, CP, and DM-P groups (Fig. 1 B and C). The mean amounts of GCF GA were 4.24, 18.03, 5.89, and 28.44 μg per site, and the mean concentrations were 7.11, 37.25, 2.78, and 25.54 μg/μl GCF in H, DM, CP, and DM-P groups, respectively. These results suggest that the GA levels in GCF samples from DM patients (DM and DM-P) were significantly higher than those of subjects without DM (H and CP).

Comparison of calprotectin level in GCF

The calprotectin level in GCF was compared among the four groups of H, DM, CP, and DM-P (Fig. 2). The mean amounts of calprotectin in GCF samples of CP and DM-P groups were 257.4 and 236.4 ng/site, respectively, which were more than twofold higher than those of the groups without periodontitis, namely, H (108.5 ng/site) and DM (115.3 ng/site) (Fig. 2A). In contrast, the mean calprotectin concentrations in GCF samples from CP and DM-P were 0.123 and 0.159 μg/μl GCF, respectively, which were lower than those of the groups without periodontitis (H: 0.191 μg/μl and DM: 0.244 μg/μl

GCF).

Correlation between GCF GA and blood GA and HbA1c

GCF was collected as one to four samples in each of the subjects in the H, DM, CP, and DM-P groups (n=50). The mean amount and concentration of GA per person were calculated when multiple GCF samples had been taken. The mean GCF GA amount ranged from 0.22 to 97.84 μg/site and GCF GA concentration ranged from 0.28 to 84.72 μg/μl GCF. The blood GA percentage ranged from 12.0 to 29.9% and HbA1c percentage ranged from 4.2 to 9.8%. The blood GA value and GCF GA amount (Fig. 3A) or GCF GA concentration (Fig. 3B) showed a significant positive correlation (GCF GA amount: $\rho = 0.671$, $P < 0.001$; GCF GA concentration: $\rho = 0.598$, $P < 0.001$). The HbA1c value also significantly correlated to the GCF GA amount (Fig. 4A, $\rho = 0.587$, $P < 0.001$) and its GA concentration (Fig. 4B, $\rho = 0.499$, $P < 0.001$).

Determining the cut-off value of GCF GA for DM prediction

ROC curves for the GA level in GCF were plotted to predict DM (Fig. 5).

The areas under the ROC curve (AUC) for GCF GA amount and concentration were 0.842 (95% confidence interval (CI): 0.791-0.893, $P<$ 0.001) and 0.798 (95% CI: 0.740-0.856, $P<$ 0.001), respectively. According to the ROC analysis, the cut-off values of GA amount and concentration for a prediction of DM were 6.00 μg/site with a sensitivity of 82.5% and a specificity of 66.9%, and 5.13 μg/μl GCF with a sensitivity of 75.2% and a specificity of 71.1%, respectively. The median GCF GA amounts in DM and non-DM groups were 16.12 and 3.00 μg/site, respectively, and the concentrations were 9.57 and 2.00 μg/μl GCF.

DISCUSION

GCF is an exudate from periodontal tissues and blood vessels released into gingival crevices or periodontal pockets; it contains many proteins derived from the blood.[33-35] The present study first shows the existence of GA in GCF and a comparison of the GA levels in GCF samples between subjects with and without DM. GA is an Amadori compound that is produced in a process of AGE synthesis, the Maillard reaction, under hyperglycemic conditions in DM.[15,16] GA in the blood is clinically used as a DM marker to

evaluate the status of glycemic control after medication for DM and glycemic fluctuation in the short term.[19,20, 36,37]

The GCF levels of some proteins were found to differ among samples from patients with DM, with periodontitis, and healthy individuals. High-sensitivity C-reactive protein (CRP) level in GCF from DM patients with periodontitis was higher than those of non-DM patients with periodontitis and healthy individuals.[38] Prostaglandin E_2 (PGE_2) level in GCF samples from DM and periodontitis patients was higher than the level in healthy subjects.[39] However, CRP and PGE_2 reflect inflammatory activity, but not glycemic condition. Although Bulut *et al.*[40] reported that interleukin 1-β (IL-1β) concentration in GCF from DM patients was higher than that of periodontitis patients or healthy subjects, Kardşler *et al.*[41] showed that GCF IL-1β level in DM patients was lower than that of periodontitis patients. Apolipoprotein B (apoB), a constituent of very low-density lipoprotein, was identified in GCF and its concentration in GCF samples from DM patients who showed a mean HbA1c value of 11.4 NGSP% was significantly higher than that from subjects without DM.[42] However, the GCF apoB level in DM patients with HbA1c of more than

6.5%, a boundary value for DM, was not reported and, furthermore, GCF apoB level did not correlate to plasma apoB. In the present study, the mean GA amount/concentration in GCF from DM patients was approximately 4.5 to 6-fold higher than that of subjects without DM when the former group was defined as those with HbA1c of more than 6.5%; in addition, GCF GA level positively correlated to the levels of blood GA and HbA1c. Furthermore, the mean GCF GA concentration in non-DM groups was 4.94 μg/μl, and serum GA concentration in healthy males was 5.7 mg/ml,[42] showing that GA concentration in GCF is similar to that in serum. These results suggest that GA in GCF is an acceptable marker to predict DM.

Whereas only the GA amount of GCF samples was determined in the present study, GA level in the blood is generally expressed as a percentage of GA per albumin. The ratio of GA to albumin in biological samples more accurately reflects the glycemic condition. However, it is difficult to determine the two markers of GA and albumin in a GCF sample because the GCF volume is very low, at less than few microliters. If GA and albumin in a GCF sample can both be measured, the glycemic condition

may be precisely evaluated using GCF.

The total amount and concentration of GA in GCF samples from subjects with and without DM significantly correlated to the blood GA value, with correlation coefficients of 0.671 for GA amount and 0.598 for GA concentration, as well as to HbA1c value, with correlation coefficients of 0.587 for GA amount and 0.499 for GA concentration. Plasma GA level was strongly correlated to HbA1c level, with a correlation coefficient of 0.766.[17] Although the degree of correlation between GCF GA and HbA1c was weaker than that between blood GA and HbA1c, it was stronger than that between GCF apoB and HbA1c (r=0.333).[41] ROC analysis of GCF GA level for the prediction of DM showed relatively large AUC (total amount: 0.842, concentration: 0.798), and high sensitivity (total amount: 82.5%, concentration: 75.2%) and specificity (total amount: 66.9%, concentration: 71.1%), when the cut-off values of amount and concentration were 6.00 μg/site and 5.13 μg/μl GCF, respectively. These reports and results also suggest that GA in GCF is a useful marker to evaluate glycemic condition and predict DM.

Calprotectin is produced and secreted in neutrophils, monocytes/

macrophages, and oral epithelial cells, and its expression is increased by inflammatory cytokines and *Porphyromonas gingivalis* lipopolysaccharide[43-45]; therefore, calprotectin is contained in GCF at a high concentration. In the present study, the collected GCF was diluted by 200- to 400-fold for the determination of calprotectin level using a commercial ELISA kit. Calprotectin and GA in a GCF sample could be measured because of the high concentration of calprotectin in GCF. The total amount of calprotectin in GCF samples from periodontitis cases was significantly higher than that from cases without periodontitis, but the calprotectin concentration in periodontitis samples was slightly lower. We suppose that this difference between calprotectin amount and concentration was caused by the increase of GCF volume in periodontitis. We previously showed that calprotectin concentration in GCF from periodontitis sites was higher than that of non-periodontitis sites when GCF calprotectin concentration was measured using a different ELISA kit from the one used in the present study.[25,26] Kaner *et al.*[30,46] and Becerik *et al.*[47] also reported that calprotectin level in periodontitis sites were significantly higher than that of non-diseased sites. Furthermore, an increased calprotectin level was

decreased by periodontal treatments,[29] suggesting that GCF calprotectin level reflects the inflammatory activity in periodontitis. When the ROC curve for calprotectin amount was plotted, the AUC value was 0.970 (95% CI: 0.948-0.993, $P < 0.001$) and the cut-off value for the prediction of periodontitis was 165.1 ng/site, with a sensitivity of 94.4% and a specificity of 96.7% (data not shown). From this ROC analysis, calprotectin in GCF is thought to be a useful marker for the diagnosis of periodontitis. In contrast, calprotectin amount and concentration were not affected by DM and there was no significant correlation between GCF calprotectin level and HbA1c value in a preliminary study (data not shown).

The present study shows the possibility diagnosing DM-P using GA, a DM marker, and calprotectin, an inflammatory marker, in GCF. Determination of two specific markers for DM and periodontitis can lead to a reasonable diagnosis of DM-P and also the screening of DM using GCF. GCF is easily, non-invasively collected from gingival crevices and periodontal pockets using paper strips, in comparison with blood collection using a needle and syringe. GCF may be a useful biological sample to diagnose systemic diseases because it contains many proteins derived from the

blood.[33,34,48] On the other hand, the GCF volume is very low, at less than few microliters, so the amount of protein in GCF is also very small. It was difficult to determine GA, calprotectin and albumin in one GCF sample using conventional ELISA kits. We recently reported ELISA on a microchip to measure the amount of calprotectin in GCF.[49] In future, if a microdevice system to determine GA and albumin in GCF is developed, multiple disease markers in GCF will be easily measured at the same time, and such a system would contribute to more accurate diagnosis of diabetes-associated periodontitis.

FOOTNOTES

*: Bio Medical Laboratories Inc., Tokyo, Japan

†: Periopaper®, Oraflow Inc., Smithtown, NY, USA

‡: Periotron 8000, Harco Electronics, MB, Canada

§: Hybond-P, GE Healthcare Ltd., Amersham, Buckinghamshire, UK

‖: Starting Block™ Blocking Buffer, Pierce, Rockford, IL, USA

¶: Exocell, Philadelphia, PA, USA

#: GE Healthcare Ltd., UK

**: ECL™ Western Blotting Analysis System, GE Healthcare Ltd., UK

†† : Hyperfilm ECL, GE Healthcare Ltd., UK

‡‡ : GLYCABEN Glycated Albumin ELISA, Exocell, Philadelphia, PA, USA

§§ : Human Calprotectin ELISA KIT, HyCult Biotechnology, Uden, Netherlands

‖‖ : SPSS version 20.0, IBM, Chicago, IL, USA

¶¶ : Statcel 3, OMS Publishing Ltd., Saitama, Japan

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Figure legends

Figure 1: Analysis of GA in GCF. GCF samples were collected from H, DM, CP, and DM-P subjects. (A) GA in the extracted GCF was identified by western blotting, as described in the materials and methods. Human serum was a positive control. (B, C) GA amount in GCF samples (n=219) was determined by ELISA and its level was compared among the four groups. Total GA amount (B) and GA concentration (C) are expressed as μg/site and μg/μl GCF, respectively. Horizontal bars show the mean values of each

group.

* $P < 0.01$.

Figure 2: Comparison of calprotectin level in GCF. Calprotectin amount in GCF samples from H, DM, CP, and DM-P subjects was determined by ELISA. Total calprotectin amount (A) and calprotectin concentration (B) are expressed as ng/site and μg/μl GCF, respectively. Horizontal bars show the mean values of each group. * $P < 0.01$.

Figure 3: Correlation between GCF GA and blood GA. (A) Correlation between GA amount in GCF and blood GA was evaluated in subjects with and without DM (n=50). $\rho = 0.671$, $P < 0.001$. (B) Correlation between GCF GA concentration and blood GA was evaluated in subjects with and without DM. $\rho = 0.598$, $P < 0.001$.

Figure 4: Correlation between GCF GA and blood HbA1c. (A) Correlation between GA amount in GCF and blood HbA1c was evaluated in subjects with and without DM (n=50). $\rho = 0.587$, $P < 0.001$. (B) Correlation between

GCF GA concentration and blood HbA1c was evaluated in subjects with and without DM. $\rho = 0.499$, $P < 0.001$.

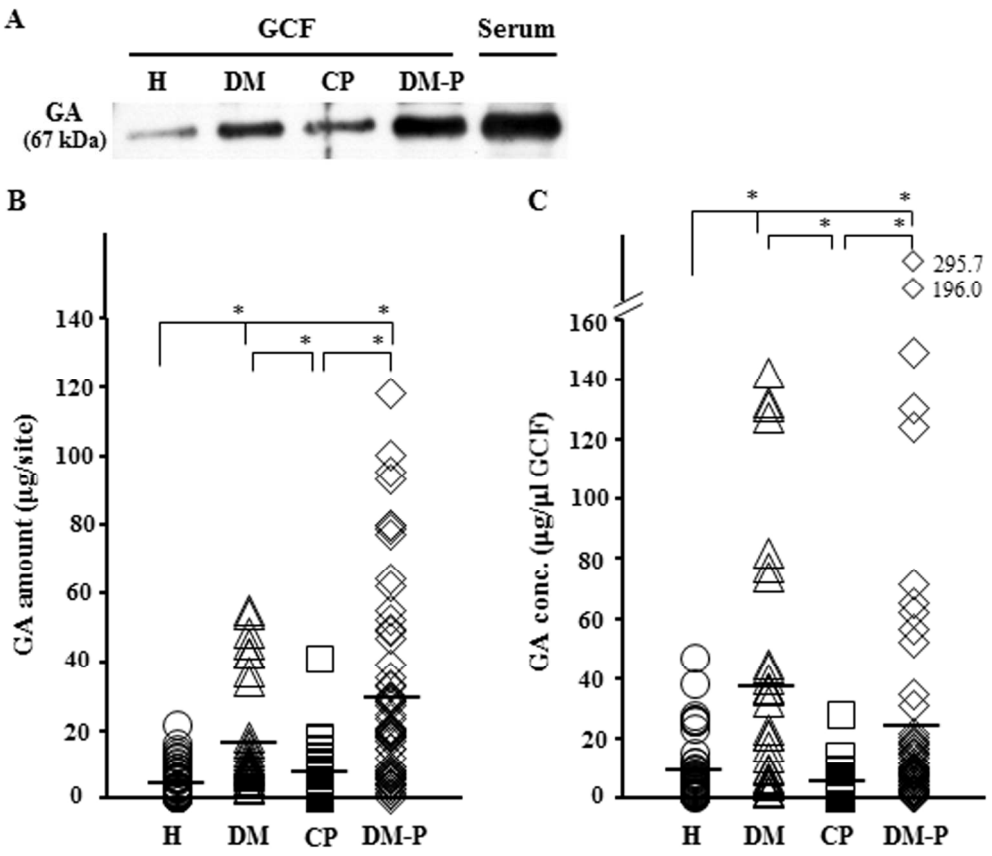
Figure 5: ROC analysis of GCF GA for the prediction of DM. GCF samples were collected from H, DM, CP, and DM-P subjects, and total GA amount (A) and concentration (B) in GCF samples (n=219) were subjected to ROC analysis. The AUC for GA amount and concentration were 0.842 (95% CI: 0.791-0.893, $P<0.001$) and 0.798 (95% CI: 0.740-0.856, $P<0.001$) when the cut-off values were set as 6.00 μg/site and 5.13 μg/μl GCF, respectively.

Table 1
Characteristics of subjects and GCF samples

Subjects	H	DM	CP	DM-P
Number	25	10	23	20
Gender (male:female)	8:17	5:5	11:12	9:11
Age	66.7±10.9	65.5±6.1	65.2±9.1	65.9±8.8
GCF sampling				
Number of samples	60	32	61	66
GCF volume (μl)	0.88±0.64	1.04±0.75	2.56±1.00*,**	2.16±1.15*,**
PD (mm)	2.02±0.68	2.19±0.59	5.44±1.73*,**	5.32±1.73*,**
GI	0	0	1.69±0.56*,**	1.61±0.60*,**

Statistically significant difference from H* and DM** ($P < 0.01$).

Characteristics of subjects and GCF samples
76x47mm (600 x 600 DPI)



Analysis of GA in GCF
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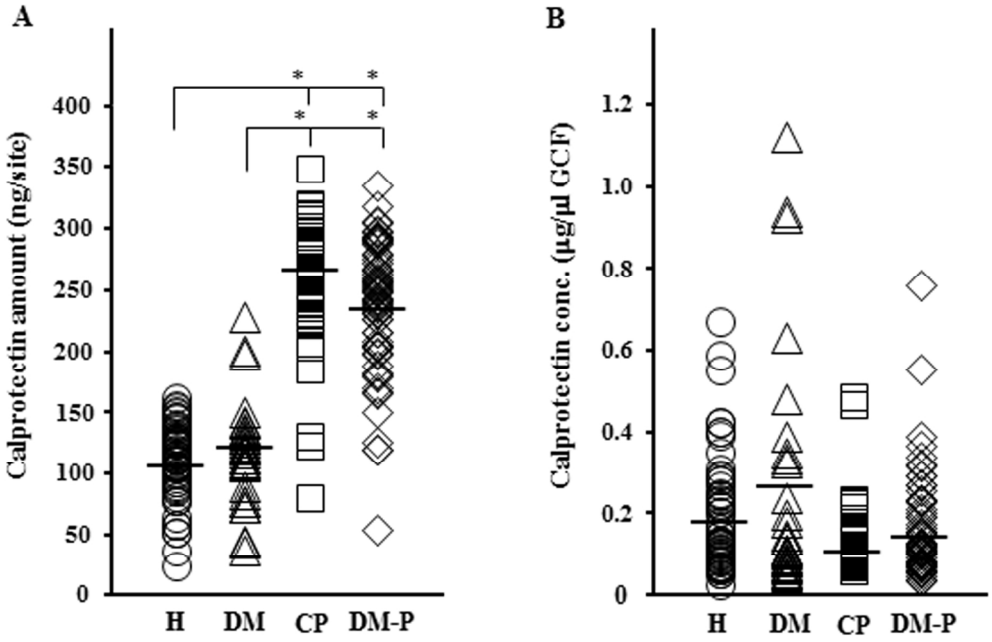

Comparison of calprotectin level in GCF
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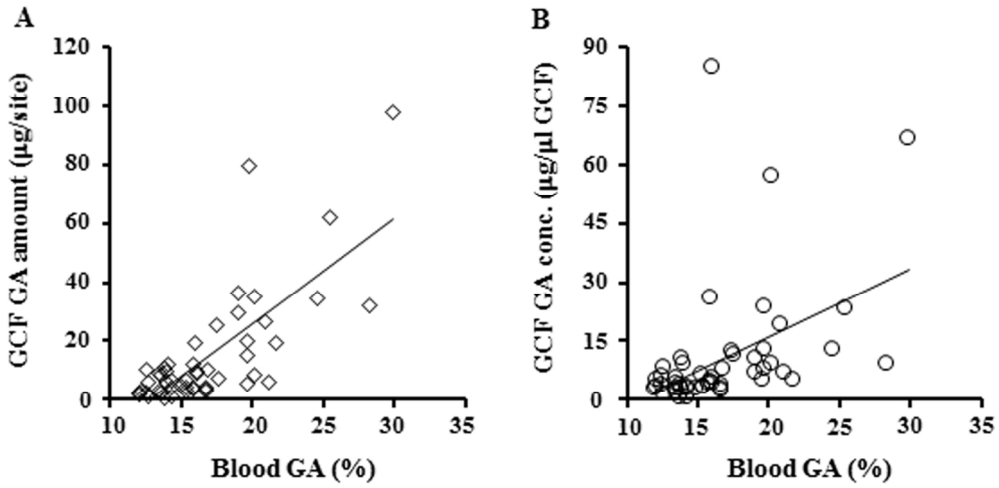
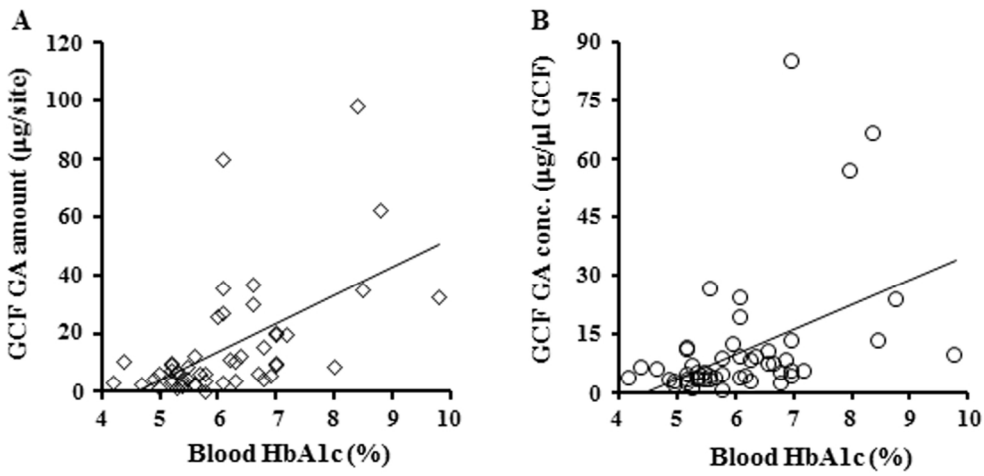


Correlation between GCF GA and blood GA
60x29mm (600 x 600 DPI)



Correlation between GCF GA and blood HbA1c
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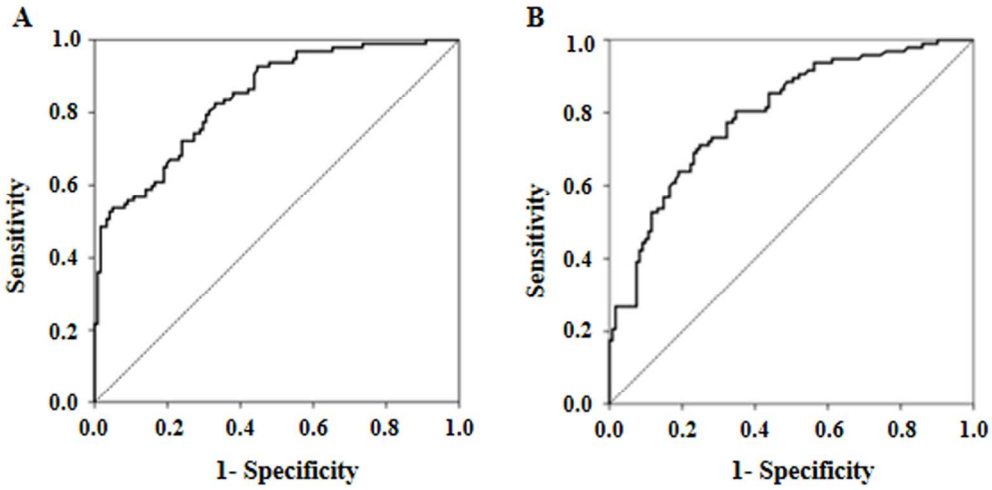

ROC analysis of GCF GA for the prediction of DM
60x29mm (600 x 600 DPI)