Original Article

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Long-term effect of periodontal treatment on gingival crevicular fluid level of Cathepsin K in chronic periodontitis patients with type 2 diabetes mellitus

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Objective: Cathepsin K (CTSK), a cysteine protease, is predominantly expressed by osteoclasts. CTSK levels were monitored in gingival crevicular fluid (GCF) to observe osteoclastic activity in response to periodontal treatment and supportive periodontal therapy (SPT) in individuals with chronic periodontitis (CP) and type 2 diabetes mellitus (DM).

Materials and methods: Periodontal parameters and GCF were collected from 14 individuals with CP+DM and 14 individuals with CP but no DM at baseline (T1), 8 weeks after scaling and root planing (T2) and after SPT of 9-28 months, average 18.06 months (T3, n=9 in each group). Five participants with gingival health and no DM (GH group) were recruited at T3 to study CTSK levels in GCF and saliva. CTSK was detected by enzyme linked immunoassay (ELISA).

Results: At the baseline, there were no significant differences in periodontal parameters and CTSK between the CP+DM and CP groups. At T2, both groups showed significant clinical improvement ($p < 0.05$) and decreased CTSK in GCF ($p < 0.05$). Comparing between groups, CP+DM group showed a significantly higher amount of CTSK than CP group ($p < 0.01$). At T3, CP+DM and CP groups showed significant improvement in all periodontal parameters compared to baseline. By contrast, some GCF parameters rebounded. The CP+DM, CP and GH groups showed no significant differences in GCF and salivary fluid parameters of CTSK at T3. A strong positive correlation between the relative amounts of CTSK to total protein in GCF and saliva was found in the CP+DM group (r=0.94, p=0.005).

Conclusions: Periodontal treatment decreased CTSK in GCF; however, CP+DM group had a significantly higher amount of CTSK than CP group. However, good compliance with SPT in CP+DM and CP groups maintained good periodontal health, with comparable CTSK levels in GCF in both groups. All three groups had detectable CTSK in saliva.

Keywords: Cathepsin K, chronic periodontitis, gingival crevicular fluid, osteoclast, saliva, type 2 diabetes mellitus.

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Introduction

Periodontitis is a chronic inflammatory disease mediated by host immune responses resulting in soft tissue and supporting bone destruction leading to tooth loss [1]. In the resorptive process of alveolar bone, osteoclasts release an important cysteine protease, Cathepsin K (CTSK) [2], which has the ability to degrade extracellular bone matrix protein [3]. CTSK levels in the GCF of patients with chronic periodontitis were also higher than in periodontally healthy and gingivitis patients, while enzyme levels in the periodontitis group decreased after periodontal treatment [4].

Diabetes mellitus (DM) is a risk factor for periodontal diseases [5]. DM patients have higher prevalence, severity, and progression of periodontitis than non-diabetic patients [6]. Compared with non-diabetic patients, poorly controlled diabetic patients had 2.9 times higher risk of severe periodontitis

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development than non-diabetic patients [7]. Moreover, poorly controlled diabetic patients also had higher prevalence and severity of periodontitis than well-controlled diabetic patients [8, 9]. Mechanisms contributing to the increased risk of periodontitis development include abnormal neutrophil adherence, chemotaxis, phagocytosis, [10] and hyper-responsiveness of macrophages [11]. Hyperglycemia was reported to promote osteoclast differentiation/fusion in bone marrow cultures of type 2 diabetic mice. Human osteoclasts derived from the peripheral blood of individuals with type 2 DM were also found to be less responsive to regulation; they were refractive to lipopolysaccharide (LPS)-induced deactivation and more osteoclastic in response to LPS, as determined by CTSK expression in type 2 DM osteoclasts [12]. Catalfamo et al. suggested that in the presence of LPS, periodontal diseases in type 2 diabetic hosts showed uncontrolled osteoclast differentiation and activation, leading to excessive alveolar bone loss [12]. Furthermore, in a long-term study, DM increased the rate of alveolar bone loss progression [13], associated with attachment loss [14] and had considerable influence on the prognosis of teeth [15].

Whole saliva is a complex fluid containing many types of protein from salivary glands and GCF. Enzymes of bone metabolism associated with periodontal diseases have also been found in saliva [16]. Whole saliva is a fluid of interest for monitoring biomarkers of alveolar bone destruction in periodontal disease [17, 18]. Saliva sampling is also an easy, non-invasive method and well accepted by patients.

We hypothesized that individuals with both periodontitis and type 2 DM might have higher osteoclastic activities than individuals with only chronic periodontitis. Osteoclastic activity was observed with regard to CTSK for individuals with periodontitis and type 2 DM, and long-term responses to periodontal treatment were assessed. Several studies have assessed CTSK levels in GCF but none analyzed CTSK levels in saliva. The purposes of this study were to (i) compare CTSK in GCF for individuals with chronic periodontitis (CP) and those with chronic periodontitis and type 2 DM (CP+DM) at the baseline, 8 weeks after scaling and root planing and after supportive periodontal therapy (SPT), (ii) evaluate CTSK levels in saliva among groups with CP, CP+DM and a clinical gingival health group (GH) during SPT, and (iii) evaluate the relationships between CTSK levels in GCF and saliva.

Materials and methods

Study population

The study protocol was approved by the Institutional Review Board for human subjects at Mahidol University (COA.No.MU-DT/PY-IRB 2015/008.1702, COA.No.MU-DT/PY-IRB 2018/033.0606) and conducted in accordance with the Helsinki Declaration of 1975, revised in 2013. The study was registered to the Thai Clinical Trials Registry on the WHO International Clinical Trials Registry Platform (registration number: TCTR 20170424002, TCTR20190621003). All data and samples were obtained with informed consent.

The study population comprised three groups. The first two groups comprised 14 individuals with chronic periodontitis and type 2 diabetes mellitus (CP+DM) and 14 individuals with chronic periodontitis and no diabetes (CP). Participants in the CP+DM and CP groups were age-matched volunteers. The third group consisted of 5 individuals with clinical gingival health on an intact periodontium or a reduced periodontium and no DM (GH). The GH group was recruited and investigated along with the other two groups in SPT (T3) for study of CTSK in GCF and saliva in gingival health condition. All participants in the three groups had no previous history of periodontal treatment within 6 months of saliva or GCF sampling. The samples were volunteers from the Diabetic Clinic, Golden Jubilee Medical Center or the Periodontics Clinic, Faculty of Dentistry, Mahidol University.

Individuals with chronic periodontitis were examined and diagnosed according to the 2017 World Workshop on the Classification of Periodontal

and Peri-Implant Diseases and Conditions [19]. Individuals who had generalized periodontitis stage II or higher, with at least eight teeth from first molar to first molar area were eligible for participation in the study. Individuals diagnosed with chronic periodontitis who had no history of periodontal treatment, including subgingival scaling and root planing and/or adjunctive antibiotic treatment were recruited.

Individuals with diabetes mellitus were diagnosed by physicians at the Diabetic Clinic, Internal Medicine Department, Golden Jubilee Medical Center, Mahidol University. Only individuals with a current HbA1c >7% and no major systemic diabetic complications were included in the study.

Five individuals with clinical gingival health on an intact periodontium, or clinical gingival health on a reduced periodontium in a non-periodontitis patient, according to the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions [20] and with at least eight teeth from first molar to first molar area were recruited into the GH group.

Exclusion criteria included smoking, pregnancy, arthritis, osteoporosis and a history of taking antibiotics, nonsteroidal anti-inflammatory drugs, bisphosphonates, calcium, vitamin D or hormonal replacement.

Using Altman's nomogram [21], sample size in each group was determined to include at least 11 individuals having an 80% chance of detecting a difference in mean CTSK of 0.3 pg/μl at the 5% level of significance, assuming the standard deviation of CTSK to be 0.256 as reported in a previous study [4].

Treatment regimen

After baseline periodontal examination and GCF sampling (T1), both groups (CP+DM and CP) received non-surgical periodontal treatment including oral hygiene instruction, thorough scaling and root planing in 1-3 sessions. Eight weeks after completion of scaling and root planing, all participants were scheduled for post-treatment clinical evaluation and GCF collection (T2). Periodontal surgeries were performed as necessary; however, some patients declined treatment. After completion of active periodontal therapy, all patients were subjected to SPT every 3-6 months, according to their periodontal status, and conducted by postgraduate students in the residency training program in Periodontics. At T3, participants in the CP, CP+DM and GH groups were scheduled for clinical evaluation, GCF and saliva sampling.

Clinical assessment

Demographic information including age, blood pressure, weight, height, body mass index (BMI), fasting plasma glucose (FPG) and HbA1c level was recorded from medical records.

Full mouth (FM) periodontal examination was performed at T1 and T2 by one examiner (T.C.) and at T3 by another examiner (A.C.). Periodontal parameters were recorded on six sites per tooth as bleeding on probing (BOP), probing depth (PD), and distance from the cemento-enamel junction to gingival margin (CEJ-GM). Clinical attachment levels were calculated from PD+(CEJ-GM) measurements. Probing measurements were performed with a standard manual probe (PCPUNC 15, Hu-Friedy®, Chicago, IL, USA). Examination for BOP was performed by gentle probing into the orifice of the gingival crevice along the buccal and lingual surfaces; sites that bled within 10 seconds were recorded as BOP-positive (BOP+) [22]. Intraexaminer and interexaminer reliability were 0.89 and 0.90, respectively

Gingival crevicular fluid sampling: site selection and collection

GCF was collected one to two days after full mouth periodontal examination at T1 and T2 from four sites, one site per tooth, from each participant. The selected sites had a deepest probing depth of ≥5 mm and were BOP+ from the area between the first molars in the same arch. At T3, GCF was collected at the same sites. GCF collection was performed according to a previous study [23]. Briefly, the selected area was carefully polished with pumice and a rubber cup to remove supragingival plaque. During

polishing, care was taken to avoid touching the gingival margin. The area was rinsed, dried and isolated with gauze. An absorbent strip (Periopaper, ProFlowTM Amityville, NY, USA) was then inserted into the orifice of the gingival crevice at a 1-mm depth and left inside for 30 seconds. The volume of GCF was immediately determined by a Periotron 8000 (Periotron 8000, 14 Threepond Road Smithtown, NY 11787, USA). The absorbent strip was kept in an Eppendorf Tube and stored at -80°C for further laboratory analysis. In case of contamination with blood, saliva or plaque, the absorbent paper was excluded from the study.

Saliva collection

Five milliliters of non-stimulated whole saliva were collected during SPT visits by instructing the subjects to rest for 5 minutes to allow saliva to accumulate on the floor of the mouth and to spit it into a sterile tube every 60 seconds. Whole saliva was stored at - 80°C for further laboratory analysis [24]. Saliva collections were performed at T3 in the CP, CP+DM and GH groups.

Determination of Cathepsin K and total protein

CTSK samples were eluted from the pooled absorbent strips of the same individual by adding 300 µl of phosphate buffered saline containing 0.1 mM of phenylmethylsulfonylfluoride (PMSF) and centrifuging at 10,000 g for 5 minutes. The supernatants were collected for further analysis. CTSK in GCF at T1 and T2 was detected by enzyme linked immunoassay (ELISA) Kit, SensiZyme Cathepsin K Activity Assay Kit (CS1150 Sigma: 3050 Spruce Street, St. Louis, MO 63103, USA), while CTSK in GCF and saliva at T3 was detected by Human Cathepsin K ELISA Kit (Colorimetric) NBP2-62755, (Novus Biologicals: 10730 E. Briarwood Avenue, Centennial, CO 80112, USA), following the manufacturer's instructions. Each sample was assayed in duplicate and results were averaged per individual. Total protein from GCF and saliva was determined by the Bradford method using Coomassie® Brilliant Blue G-250 dye [25]. The dye was diluted with distilled water at 1:4 ratio, and filtered with Whatman #1 filter paper. Up to 200 µl of dye reagent was added to 10 µl of the eluted sample and then read with a spectrophotometer at 595 nm. Total protein was calculated as the average of duplicate experiments. The amount of CTSK (pg) per time of collection (30 s), concentration (pg/µl) per time of collection, and relative amounts of CTSK to total protein (pg/mg) per time of collection were also recorded for each individual.

Statistical analysis

Data were analyzed using SPSS for Windows version 20.0 (SPSS Inc., Chicago, IL, USA), with results presented as mean ± SD and median (P25, P75). Normality of data distribution was assessed using the Shapiro-Wilk test. At T1 and T2, independent *t*-test or Mann-Whitney U test for differences between the two groups, and paired *t*-test or the Wilcoxon signed-rank test were used to assess changes from T1. Pearson's correlation or Spearman's rank correlation were used to test the relationship between periodontal parameters of the selected sites for GCF collection and CTSK. At T3, the Kruskal-Wallis test was used to test for differences among the three groups. Spearman's rank correlation was used to test for the relationship between periodontal parameters and CTSK level (GCF and saliva) and Friedman's test was used to test for differences among T1, T2 and T3.

Results

After the periodontal examination and GCF collection at T1, all participants in the CP+DM and CP groups (14 in each group) completed full mouth scaling and root planing, post-treatment evaluation and GCF collection at T2. Two patients in the CP+DM group and three patients in the CP group underwent periodontal surgery (open flap debridement). After active periodontal treatment, all patients were scheduled for regular maintenance by SPT every 3-6 months. At T3, nine subjects in both groups complied with SPT and participated in periodontal evaluation and GCF collection.

The average number of appointments during SPT in both groups was 5.11±1.60 with an average of 3.45±0.76 visits per year. Average time of SPT for the CP+DM and CP groups was 18.06±4.98 months (Table 1). The number of appointments for SPT and SPT visits/year was comparable between the 2 groups. At T3, five additional subjects were included who had clinical gingival health and no diabetes (GH) for study of CTSK levels in saliva.

Table 1 shows demographic data of patients in the CP+DM, CP and GH groups. At baseline (T1), the CP+DM group comprised seven males and seven females and the CP group had eight males and six females. Ages ranged from 48 to 68 years old. No statistically significant differences regarding age, blood pressure, weight, height and BMI, except for fasting plasma glucose (FPG), were found between the groups. The CP+DM group had an average HbA1c level of 7.87 ± 0.69 and range 7.1% to 9%. Six out of 14 participants (42.86%) had an HbA1c level ≥8%. The approaches used to control DM of the participants in this group were oral medications and diet control. The GH group was significantly younger than the CP+DM and CP groups. In the CP+DM group, HbA1c levels were not significantly different among T1 (n=14), T2 (n=14) and T3 (n=9) with means of 7.87% (range 7.1%-9.0%), 8.01% (range 5.9%-11%) and 7.61% (range 6.6%-9.8%), respectively.

Table. 2 shows the periodontal parameters of the three groups. The CP+DM group and CP group had no difference in periodontal status at the baseline (T1) ($p > 0.05$). Comparing between the CP+DM and CP groups at 8 weeks after scaling and root planing (T2), the CP group had significantly deeper mean full mouth probing depth than the CP+DM group ($p=0.04$). When only the selected sites for GCF collection were considered, there was no significant difference in mean probing depths and clinical attachment levels between the CP+DM and CP groups at T1 and T2. For SPT (T3), the CP+DM group had deeper probing depth in selected sites than the GH group (post hoc test, $p=0.03$), while the CP+DM and CP groups had clinical attachment levels in selected sites more than the GH group (post hoc tests, $p < 0.01$ and $p=0.04$, respectively).

Group comparisons of full mouth and selected sites showed that short-term responses to scaling and root planing (T1-T2) in both groups showed significant improvement in all periodontal parameters ($p < 0.01$). Considering the long-term responses to periodontal treatment (T1-T2-T3) at SPT (T3), both the CP+DM and CP groups showed significant improvement of all periodontal parameters compared to the baseline (T1) (Table 3).

CTSK levels in GCF

GCF was collected from 40 and 42 single-rooted sites and 16 and 14 multi-rooted sites in the CP+DM and CP groups, respectively. At the baseline, no significant differences in all GCF parameters in the CP+DM and CP groups were observed ($p > 0.05$). After scaling and root planing, the CP+DM group had a significantly higher amount of CTSK than the CP group ($p < 0.01$), while both concentration of CTSK and relative amount of CTSK to total protein in the CP+DM group were higher than those of the CP group ($p < 0.01$). Between-group comparisons among the three groups in SPT showed no significant differences in GCF parameters ($p > 0.05$) (Table 4).

Within-group comparisons of GCF parameters found that after scaling and root planing (T1-T2), the CP+DM group showed no significant decrease in concentration of CTSK and relative amount of CTSK to total protein, while the CP group had a significant decrease in amount of CTSK, GCF volume, concentration of CTSK, total protein, and relative amount of CTSK to total protein. Long-term evaluation of GCF parameters from the baseline to supportive periodontal therapy (T1-T2-T3) showed that from T2-T3, the CP+DM group showed a significant increase in GCF volume and total protein at T3 compared to T2, while relative amount of CTSK to total protein a significantly decreased. The CP group had a significant increase in all GCF parameters, except for the relative amount of CTSK to total protein, while GCF volume at T3 was higher than for T1 (Table 5).

Table 1 Demographics of CP and CP+DM groups

Time	T1		T3		
Demographic data	CP group (n=14) (mean±SD)	CP+DM group (n=14) (mean±SD)	CP group (n=9) (mean±SD)	CP+DM group (n=9) (mean±SD)	GH group (n=5) (mean±SD)
Age (years)	58.21±6.99	61.00±5.19	62.89±7.10	63.33±5.83	46.40±4.04#
Systolic (mmHg)	128.14±10.42	132.07±13.74	128.56±11.08	123.78±14.44	122.20±5.59
Diastolic (mmHg)	78.21±8.28	79.86±5.89	73.78±7.16	74.78±9.11	75±6.56
Weight (kg)	66.07±13.07	67.87±9.57	68.18±12.63	63.28±7.09	54.0±6.67
Height (cm)	163.5±8.72	161.50±7.57	163.11±8.99	161.44±8.86	159.40±6.91
BMI (kg/m^2)	24.72±3.47	25.99±2.91	25.55±3.85	24.30±2.41	21.30±2.86
Fasting plasma glucose (mg/dl)	94.43±9.38	142.36±19.87*		143.22±12.96	
HemoglobinA1c (%)		7.87±0.69		7.61±1.02	
Number of appointments in SPT			5.33±1.80	4.89±1.45	
SPT visit/year			3.59±0.92	3.31±0.62	
SPT period (months)			18.44±6.36	17.66±3.46	

* Statistically significant difference between CP and CP+DM groups ($p < 0.05$)

Statistically significant difference between GH and CP, CP+DM groups ($p < 0.05$)

T1 = baseline

T3 = supportive periodontal therapy

CP = chronic periodontitis group

CP+DM = chronic periodontitis with type 2 diabetes mellitus group

GH = clinical gingival health with no diabetes group

Table 2 Comparison of clinical periodontal parameters (median (P25, P75)) between CP, CP+DM and GH groups

Time	T1			T2			T3			
Periodontal parameter	CP (n=14)	CP+DM (n=14)	*p*-value	CP (n=14)	CP+DM (n=14)	*p*-value	CP (n=9)	CP+DM (n=9)	GH (n=5)	*p*-value
FM probing depths (mm)	3.22 (2.91,3.54)	2.58 (2.58,3.31)	0.17	2.49 (2.4,2.86)	2.25 (2.12,2.54)	0.04*	2.39 (2.25,2.56)	2.25 (2.09,2.61)	2.21 (2.05,2.29)	0.15
FM percentages of bleeding sites (%)	48.66 (32.39,73.49)	43.09 (20.19,78.44)	0.78	18.0 (9.59, 22.0)	10.03 (4.69,13.83)	0.02*	9.03 (2.43,12.18)	5.95 (2.56,11.43)	2.98 (2.50,5.79)	0.42
FM clinical attachment levels (mm)	3.69 (3.14,4.46)	3.70 (3.21,4.42)	0.71	3.28 (2.63,4.19)	3.21 (3.06,3.83)	0.89	2.04 (1.55,2.37)	1.77 (1.25,2.34)	1.30 (0.96,1.63)	0.09
Probing depths (mm) in selected sites	6.00 (5.43,6.56)	6.12 (5.5,7.25)	0.43	3.25 (3.0,3.81)	3.25 (3.00,4.06)	1.71	3.00 (2.50,3.12)	3.00 (3.62,3.75)	0.27 (2.12,2.5)	0.03† 0.03:GH<CP+DM
Clinical attachment levels (mm) in selected sites	5.62 (5.18,7.00)	6.00 (5.75,7.75)	0.09	4.37 (3.68,5.62)	4.62 (4.18,6.00)	0.41	2.50 (2.37,4.50)	4.00 (3.12,6.12)	1.25 (1.00,1.75)	<0.01† 0.04: GH < CP <0.01: GH <CP+DM

* Statistically significant difference comparing CP and CP+DM groups ($p < 0.05$)

† Statistically significant difference comparing CP, CP+DM and GH groups ($p < 0.05$)

T1 = baseline

T2 = 8 weeks after initial periodontal treatment

T3 = supportive periodontal therapy

CP = chronic periodontitis group

CP+DM = chronic periodontitis with type 2 diabetes mellitus group

GH = clinical gingival health with no diabetes group

Table 3 Comparison of clinical periodontal parameters (median (P25, P75)) at T1, T2 and T3

Group	CP					CP+DM				
Periodontal parameters	T1 (n=14)	T2 (n=14)	T1-T2 *p*-value	T3 (n=9)	T1-T2-T3 *p*-value	T1 (n=14)	T2 (n=14)	T1-T2 *p*-value	T3 (n=9)	T1-T2-T3 *p*-value
FM probing depths (mm)	3.22 (2.91,3.54)	2.49 (2.4,2.86)	<0.01*	2.39 (2.25,2.56)	<0.01† <0.01: T1>T3 0.03: T1>T2	2.58 (2.58,3.31)	2.25 (2.12,2.54)	<0.01*	2.25 (2.09,2.61)	0.02† 0.03: T1>T3
FM percentages of bleeding sites (%)	48.66 (32.39,73.49)	18.0 (9.59,22.0)	<0.01*	9.03 (2.43,12.18)	<0.01† <0.01: T1>T3	43.09 (20.19,78.44)	10.03 (4.69,13.83)	<0.01*	5.95 (2.56,11.43)	<0.01† <0.01:T1>T3 0.01: T1>T2
FM clinical attachment levels (mm)	3.69 (3.14,4.46)	3.28 (2.63,4.19)	<0.01*	2.04 (1.55,2.37)	<0.01† 0.03: T2>T3 <0.01: T1>T3	3.70 (3.21,4.42)	3.21 (3.06,3.83)	<0.01*	1.77 (1.25,2.34)	<0.01† <0.01:T1>T3
Probing depths (mm) in selected sites	6.00 (5.43,6.56)	3.25 (3.0,3.81)	<0.01*	3.00 (2.50,3.12)	<0.01† <0.01:T1>T3 0.03: T1>T2	6.12 (5.50,7.25)	3.25 (3.00,4.06)	<0.01*	3.00 (3.62,3.75)	<0.01† <0.01:T1>T3 0.03: T1>T2
Clinical attachment levels (mm) in selected sites	5.62 (5.18,7.0)	4.37 (3.68,5.62)	<0.01*	2.50 (2.37,4.5)	<0.01† <0.01: T1>T3	6.00 (5.75,7.75)	4.62 (4.18,6.00)	<0.01*	4.00 (3.12,6.12)	<0.01† <0.01:T1>T3

*Statistically significant difference comparing T1 and T2 ($p < 0.05$)

†Statistically significant difference comparing T1, T2 and T3 ($p < 0.05$)

T1 = baseline

T2 = 8 weeks after initial periodontal treatment

T3 = supportive periodontal therapy

CP = chronic periodontitis group

CP+DM = chronic periodontitis with type 2 diabetes mellitus group

FM = full mouth

Table 4 Comparisons of gingival crevicular fluid parameters (median (P25, P75)) in CP, CP+DM and GH groups

Time	T1			T2			T3			
	CP (n=14)	CP+DM (n=14)	*p*-value	CP (n=14)	CP+DM (n=14)	*p*-value	CP (n=9)	CP+DM (n=9)	GH (n=5)	*p*-value
Amount of CTSK (pg)	2853.55 (1164.32, 4434.55)	2860.14 (2046.88, 6183.44)	0.57	283.56 (226.81, 407.11)	658.00 (479.57, 1232.67)	<0.01*	5040.22 (1305.20, 10631.97)	1945.28 (0.00, 3102.74)	2871.17 (33.10, 8404.90)	0.89
GCF volume (µl)	1721.78 (1400.8, 2268.52)	1991.48 (1639.17, 2411.73)	0.21	1077.04 (920.58, 1485.80)	1340.5 (1009.25, 1564.63)	0.56	5568.87 (4349.55, 7157.5)	5868.92 (3387.93, 6849.78)	4137.99 (3618.72, 5003.46)	0.36
Concentration of CTSK (pg/µl)	0.06 (0.04,0.22)	0.08 (0.05,0.13)	0.80	0.02 (0.01,0.04)	0.06 (0.03,0.09)	<0.01*	1.12 (0.24,2.30)	0.10 (0.00,0.49)	0.71 (0.01,1.67)	0.12
Total protein (mg)	0.40 (0.28,0.94)	0.77 (0.42,1.34)	0.24	0.15 (0.10,0.26)	0.18 (0.13,0.30)	0.48	1.82 (1.48,2.82)	4.08 (1.98,5.56)	0.68 (0.11,4.29)	0.07
Relative amounts of CTSK to total protein (pg/mg)	6407.8 (1826.49, 11425.63)	4900.86 (1948.14, 9633.17	0.74	1891.42 (1144.54, 2993.81)	4528.77 (2494.31, 6661.79)	<0.01*	3045.07 (1376.75, 13436.08)	764.43 (80.78, 2565.89)	2184.30 (741.16, 30483.62)	0.14

* Statistically significant difference comparing CP and CP+DM groups ($p < 0.05$)

† Statistically significant difference comparing CP, CP+DM and GH groups ($p < 0.05$)

T1 = baseline

T2 = 8 weeks after initial periodontal treatment

T3 = supportive periodontal therapy

CP = chronic periodontitis group

CP+DM = chronic periodontitis with type 2 diabetes mellitus group

GH = clinical gingival health with no diabetes group

Table 5 Comparisons of gingival crevicular fluid parameters (median (P25, P75)) at T1, T2 and T3

Group	CP					CP+DM				
Gingival crevicular fluid level of Cathepsin K	T1 (n=14)	T2 (n=14)	T1-T2 *p*-value	T3 (n=9)	T1-T2-T3 *p*-value	T1 (n=14)	T2 (n=14)	T1-T2 *p*-value	T3 (n=9)	T1-T2-T3 *p*-value
Amount of Cathepsin K (pg)	2853.55 (1164.32, 4434.55)	283.56 (226.81, 407.11)	<0.01*	5040.22 (1305.20, 10631.97)	<0.01 † 0.03:T1>T2 0.01:T3>T2	2860.14 (2046.88, 6183.44)	658.00 (479.57, 1232.67)	<0.01*	1945.28 (0.00, 3102.74)	0.45
GCF volume (μl)	1721.78 (1400.80, 2268.52)	1077.04 (920.58, 1485.80)	<0.01*	5568.87 (4349.55, 7157.50)	<0.01 † <0.01:T3>T2 0.03:T3>T1	1991.48 (1639.17, 2411.73)	1340.5 (1009.25, 1564.63)	<0.01*	5868.92 (3387.93, 6849.78)	<0.01 † 0.00: T3>T2
Concentration of Cathepsin K (pg/μl)	0.06 (0.04,0.22)	0.02 (0.01,0.04)	<0.01*	1.12 (0.24, 2.30)	<0.01 † <0.01:T3>T2	0.08 (0.05,0.13)	0.06 (0.03,0.09)	0.1	0.10 (0.00,0.49)	0.64
Total protein (mg)	0.40 (0.28,0.94)	0.15 (0.10,0.26)	<0.01*	1.82 (1.48, 2.82)	0.02 † 0.01:T3>T2	0.77 (0.42,1.34)	0.18 (0.13,0.30)	<0.01*	4.08 (1.98,5.56)	<0.01 † <0.01:T3>T2
Relative amounts of CTSK to total protein (pg/mg)	6407.8 (1826.49, 11425.63)	1891.42 (1144.54, 2993.81)	<0.01*	3045.07 (1376.75, 13436.08)	0.42	4900.86 (1948.14, 9633.17)	4528.77 (2494.31, 6661.79)	0.36	764.43 (80.78, 2565.89)	0.03 † 0.03: T2>T3

* Statistically significant difference comparing T1 and T2 ($p < 0.05$)

† Statistically significant difference comparing T1, T2 and T3 ($p < 0.05$)

T1 = baseline

T2 = 8 weeks after initial periodontal treatment

T3 = supportive periodontal therapy

CP = chronic periodontitis group

CP+DM = chronic periodontitis with type 2 diabetes mellitus group

CTSK levels in saliva

CTSK was recorded in all the saliva samples; however, there were no significant differences in CTSK levels in saliva among the CP+DM, CP and GH groups (Supplementary Table S1).

Relationship between CTSK levels in GCF and saliva

The relationship between CTSK levels in GCF and saliva was also investigated. A strong positive correlation between the relative amount of CTSK to total protein in GCF and saliva was found in the CP+DM group (r=0.94, p=0.005). However, in the GH group, a strong negative correlation between the relative amount of CTSK to total protein in saliva and total protein in GCF (r=-0.90, p=0.04) was recorded (Supplementary Table S2).

Relationship between CTSK levels and periodontal parameters

At the baseline, there was no relationship between the clinical periodontal parameters (probing depth, clinical attachment level, and bleeding on probing) of the selected sites for GCF collection and GCF parameters (amount of CTSK, GCF volume, concentration of CTSK, total protein, and amount of CTSK to total protein) (data not shown). At T2, a subgroup analysis was performed by dividing the CP+DM group into CP+DM with HbA1c ≥ 8% and <8%. When the collected sites of GCF in the subgroup CP+DM with HbA1c ≥ 8% (n=6) were considered, strong relationships were observed between periodontal parameters (probing depth and percentages of sites with bleeding on probing) and amount of CTSK (r=0.90, p=0.02 and r=0.94, p=0.01, respectively), and between probing depth and GCF volume (r=0.90, p=0.02) (Supplementary Table S3).

At T3, relationships between percentages of bleeding sites and GCF volume were found in the CP+DM and GH groups (r=0.71, p=0.03 and r=0.90, p=0.04, respectively). A strong positive correlation was also found between the percentage of bleeding sites and total protein in GCF in the GH group (r=0.90, p=0.04) (Table 6).

For saliva samples, relationships between saliva parameters and full mouth periodontal parameters were found in the CP+DM and GH groups (Table 6). In the CP+DM group, amount of total protein in 100 µl saliva and concentration of total protein had a positive correlation with probing depth (r=0.70, p=0.04 and r=0.70, p=0.04, respectively), while relative amounts of CTSK to total protein had a negative correlation with probing depth (r= -0.70, p=0.04). In the GH group, negative correlations were found between the amount of CTSK in 100 µl saliva and concentration of CTSK with probing depths and clinical attachment levels (r= -0.9, p=0.04 and r=-0.90, p=0.04, respectively), while positive correlations were found between amount of total protein in 100 µl saliva and concentration of total protein with percentages of bleeding sites (r=0.90, p=0.04 and r=0.90, p=0.04, respectively).

Discussion

One of the objectives of this study was to investigate osteoclastic activity, with regard to CTSK expression, in individuals with CP+DM compared to individuals with CP for short- and long-term evaluation after periodontal treatment. Previous studies showed no difference in response to periodontal treatment between individuals with no DM and individuals with well-controlled DM [26]. To examine the effect of non-surgical periodontal treatment in diabetic individuals, only diabetic patients with HbA1c >7% were included in the CP+DM group at the baseline. Serum levels of CTSK were reported to decrease with age in both men and women [27], and CTSK levels in GCF may also decrease with age. The ages and gender of our volunteers were not significantly different between groups; therefore, the confounding effect of age was eliminated regarding the outcomes of periodontal treatment in the CP and CP+DM groups. Regarding saliva cysteine cathepsin activity, previous studies reported no significant difference among age groups [28]. In this study, the GH group had significantly younger ages than the other groups at T3; however, no correlation was found between age and GCF levels, and salivary levels of CTSK in samples of CP, CP+DM, and GH groups (data not shown).

Table 6 Cathepsin K (CTSK) levels in GCF and saliva and full mouth periodontal parameters at T3

Correlation coefficient (*p*-value)					Correlation coefficient (*p*-value)				
Gingival crevicular fluid	Group	Full mouth periodontal parameters			Saliva	Group	Full mouth periodontal parameters		
		Probing depth	Percentage of bleeding sites	Clinical attachment level			Probing depth	Percentage of bleeding sites	Clinical attachment level
Amount of CTSK	CP	-0.02 (0.97)	0.07 (0.85)	0.30 (0.43)	Amount of CTSK in 100 µl	CP	0.20 (0.61)	0.40 (0.29)	0.25 (0.52)
	CP+DM	0.29 (0.45)	0.52 (0.15)	0.20 (0.60)		CP+DM	-0.40 (0.29)	0.05 (0.90)	0.18 (0.64)
	GH	0.20 (0.75)	0.70 (0.19)	0.80 (0.10)		GH	-0.90* (0.04)	-0.60 (0.29)	-0.90* (0.04)
GCF volume	CP	0.58 (0.10)	0.39 (0.30)	0.08 (0.83)	Concentration of CTSK	CP	0.20 (0.61)	0.40 (0.28)	0.25 (0.52)
	CP+DM	0.18 (0.64)	0.71* (0.03)	0.09 (0.81)		CP+DM	-0.40 (0.29)	0.05 (0.90)	0.18 (0.64)
	GH	0.10 (0.87)	0.90* (0.04)	0.60 (0.29)		GH	-0.90* (0.04)	-0.60 (0.29)	-0.90* (0.04)
Concentration of CTSK	CP	-0.12 (0.77)	0.07 (0.86)	0.23 (0.55)	Amount of total protein in saliva100 µl	CP	0.28 (0.46)	0.20 (0.60)	0.48 (0.19)
	CP+DM	0.20 (0.60)	0.49 (0.18)	0.19 (0.63)		CP+DM	0.70* (0.04)	0.01 (0.98)	0.03 (0.93)
	GH	0.20 (0.75)	0.70 (0.19)	0.80 (0.10)		GH	0.50 (0.39)	0.90* (0.04)	0.60 (0.29)
Total protein	CP	0.42 (0.27)	0.27 (0.47)	-0.20 (0.61)	Concentration of total protein	CP	0.28 (0.46)	0.20 (0.60)	0.48 (0.19)
	CP+DM	0.00 (1.00)	-0.50 (0.17)	-0.18 (0.64)		CP+DM	0.70* (0.04)	0.01 (0.98)	0.03 (0.93)
	GH	0.60 (0.29)	0.90* (0.04)	0.60 (0.29)		GH	0.50 (0.39)	0.90* (0.04)	0.60 (0.29)
Relative amounts of CTSK to total protein	CP	-0.33 (0.42)	-0.18 (0.67)	0.24 (0.57)	Relative amounts of CTSK to total protein	CP	-0.40 (0.29)	-0.22 (0.57)	-0.38 (0.31)
	CP+DM	-0.71 (0.11)	0.37 (0.47)	0.26 (0.62)		CP+DM	-0.70* (0.04)	0.16 (0.68)	0.12 (0.77)
	GH	0.40 (0.60)	-0.40 (0.60)	0.40 (0.60)		GH	-0.80 (0.10)	-0.80 (0.10)	-0.70 (0.19)

* Statistically significant (Spearman rank correlation, $p < 0.05$)

** Statistically significant (Spearman rank correlation, $p < 0.005$)

CP = chronic periodontitis group

CP+DM = chronic periodontitis with type 2 diabetes mellitus group

GH = clinical gingival health with no diabetes group

An *in vitro* study showed that human osteoclasts derived from type 2 DM participants responded aberrantly to LPS as determined by CTSK levels [12]. One would expect to find a higher GCF level of CTSK in the CP+DM group than in the CP group at baseline. However, baseline clinical periodontal parameters and GCF parameters of CTSK of both groups did not differ. It is possible that levels of periodontal inflammation, as determined in GCF at the baseline, overwhelmed the influence of hyperglycemia on osteoclastic activity [29].

In response to scaling and root planing, the CP+DM group demonstrated no significant reduction in concentration of CTSK and amount of CTSK to total protein, while the amount of CTSK in the CP+DM group was higher than that of the CP group. Liu *et al.* conducted a study in a ligature-induced model in type 2 diabetic rats. They reported that diabetic rats had a higher degree of inflammation and a more persistent inflammatory response following ligature removal than normoglycemic littermates. Prolonged inflammation after ligature removal resulted in higher osteoclast number and activity, while impaired new bone formation was also reported [30]. Response to scaling and root planing in the CP+DM group in our study may be impaired by subclinical prolonged inflammation, as suggested by Liu et al., resulting in higher osteoclast number and activity, thereby corresponding to higher amounts of CTSK in this group. However, decrease in concentration of CTSK after scaling and root planing in the CP group concurred with the results of a previous report [4].

At T2, a subgroup analysis of individuals with HbA1c ≥ 8% and <8% also revealed more details of the relationship between clinical periodontal parameters and CTSK levels in GCF. The strong correlation between probing depth, as well as percentages of bleeding on probing sites and the amount of CTSK in the CP+DM subgroup with HbA1c ≥ 8% is noteworthy. Our results demonstrated that in individuals with poorly controlled type 2 DM, deeper residual probing depths and higher percentages of bleeding sites were associated with higher amounts of CTSK at T2. Therefore, higher amounts of CTSK in deeper probing sites in individuals with poorly controlled type 2 DM may lead to progressive destruction of alveolar bone since CTSK mediates bone resorption and inflammation in periodontitis. Sites with residual deep probing depths after scaling and root planing in individuals with poorly controlled type 2 DM should be closely monitored or selected for further treatment with shallow probing depth as a goal.

When the CP+DM and CP groups were followed up for long-term maintenance by supportive periodontal therapy (T3), GCF parameters of the CP+ DM, CP and GH groups were found to be not significantly different. Based on the results at T2, the CP+DM group had higher CTSK levels than the CP group. We speculated that, at T3, the CP+DM group might show signs of periodontal disease progression compared to the CP group. However, at T3, clinical periodontal parameters and GCF parameters of the CP+ DM and CP groups were not significantly different. These outcomes at T3 may be explained by decreased resorptive activities of osteoclasts in diabetes [31, 32] and compliance with supportive periodontal therapy. The role of osteoclasts in type 2 DM and associated bone loss is still not well understood. Previous studies reported both increased and decreased resorptive activities under hyperglycemic conditions [30-32]. In this study, results of the CP+DM group, with mean HbA1c of 7.6 at T3, were possibly caused by the high glucose environment suppressing osteoclastogenesis and osteoclast resorptive activity. Participants in the CP+DM group also maintained good oral hygiene and complied with supportive periodontal therapy every 3 or 6 months, resulting in good periodontal health which reduced CTSK levels in GCF.

At T3, interestingly, the CP group recorded amounts of CTSK, GCF volume, concentration of CTSK and total protein that rebounded to the baseline level, while clinical periodontal parameters improved from the baseline. This requires further investigation. CTSK is strongly expressed by osteoclasts and considered a good marker for osteoclasts [33], while CTSK is crucial for bone remodeling during normal bone metabolism by degrading the protein components of the bone matrix [34]. Increased concentration of CTSK was detectable in the GCF

from patients with periodontitis [35]. while previous studies have demonstrated that CTSK is required for toll-like receptor (TLR) 9 functions in dendritic cell recognition of defense mechanisms [36]. Inhibition of CTSK impacted the expression of TLR4, TLR5, and TLR9 and their downstream cytokine signaling in gingival epithelial cells in periodontitis, demonstrating that CTSK was involved in immune response in periodontitis [37]. Beklen et al. reported that CTSK was expressed in osteoclasts and also in macrophage-like cells, fibroblast-like cells, vascular endothelial cells, and gingival epithelial cells in periodontal ligament cells (PDLs) and gingival tissues [38]. Current treatment of periodontitis focuses mainly on controlling inflammation by mechanical removal of bacterial pathogens, and whether the increased CTSK levels at T3 in this study result in bone resorption requires further investigation. When conventional treatment is unable to control bone destruction, a novo therapy should be considered. A Cathepsin-K specific inhibitor inhibited bone loss and immune response in the pathogenesis of periodontitis, indicating that CTSK showed promise for periodontitis treatment [37].

This is the first report on CTSK levels in salivary fluid of CP+DM, CP and GH groups. Comparable CTSK levels were found in treated and stable periodontitis patients with current gingival health (CP+DM, CP groups) and clinical gingival health (GH group). Further studies should be conducted to investigate the relationship between various stages of periodontal inflammation and CTSK levels in saliva.

The relationship between CTSK levels in GCF and saliva was also investigated. A strong positive correlation between the relative amounts of CTSK to total protein in GCF and saliva was found in the CP+DM group. This result was consistent with the composition of whole saliva, which consists of oral fluid from the salivary gland, whole cells of oral microorganisms and several constituents of non-salivary origin such as serum, blood derivatives and GCF [39]. High positive correlation was shown between the relative amounts of CTSK to total protein in GCF and saliva in the CP+DM group, CTSK level in saliva should be further investigated. Collection of the whole saliva is easy, non-invasive and repeatable without special training [40]. Negative correlations between saliva parameters of CTSK and full mouth clinical periodontal parameters found at T3 are difficult to interpret since the results were observed at specific points in time. More studies comprising larger sample size following longitudinal parameters are required to investigate CTSK activity in saliva in relation to periodontal inflammation and hyperglycemic condition.

We hypothesized that individuals with both periodontitis and type 2 DM might show higher osteoclastic activities than those with only chronic periodontitis. Results suggested that individuals with chronic periodontitis and type 2 DM who maintained good compliance with supportive periodontal therapy achieved good periodontal health. However, one drawback of this study was the small number of participants, while additional loss to follow up during SPT also reduced the numbers in each group. Further studies with larger sample sizes are needed to confirm our results to comprehensively investigate the role of osteoclasts in hyperglycemia in the pathogenesis and management of periodontitis.

Within the limits of this study, results suggested that (a) comparable levels of CTSK in GCF were observed in the CP and CP+DM groups at baseline, while after scaling and root planing the CP+DM group had a significantly higher amount of CTSK in GCF than the CP group, and after long-term maintenance of supportive periodontal therapy, the CP+DM and CP groups had comparable CTSK levels in GCF, (b) in poorly controlled type 2 DM, deeper residual probing depths after scaling and root planing were associated with higher amounts of CTSK in GCF; however, good compliance with supportive periodontal therapy maintained good periodontal health under hyperglycemia, (c) undifferenced levels of CTSK were detectable in the salivary fluid in all groups, and (d) a positive correlation between the relative amount of CTSK to total protein in GCF and saliva was found in the CP+DM group. Nevertheless, the role of CTSK in the pathogenesis of periodontitis is still not well understood and further studies are required to investigate the mechanisms of CTSK in periodontitis related to diabetes.

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Supplementary Table S1 Comparisons of Cathepsin K in saliva (median (P25, P75)), CP, CP+DM and GH groups

Salivary fluid level of Cathepsin K	CP (n=9)	CP+DM (n=9)	GH (n=5)	*p*-value
Amount of CTSK in 100 µl (pg)	348.0 (321.15,486.60)	716.7 (434.95,794.85)	474.3 (375.40,682.55)	0.09
Concentration of CTSK (pg/µl)	3.48 (3.21,4.86)	7.17 (4.35,7.95)	4.74 (3.75,6.83)	0.09
Amount of total protein in saliva 100 µl (mg)	8.71 (13.27,27.39)	13.17 (9.62,21.33)	15.72 (12.08,31.09)	0.44
Concentration of total protein (µg/ml)	87.1 (132.74,273.84)	131.76 (96.17.213.31)	157.21 (120.83,310.89)	0.44
Relative amounts of CTSK to total protein (pg/mg)	22.91 (14.48,37.83)	60.73 (21.23,77.36)	30.87 (13.54,56.91)	0.23

* Statistically significant difference comparing the three groups at T3 (Kruskal-Wallis test, $p < 0.05$)

Supplementary Table S2 Cathepsin K (CTSK) levels in GCF and saliva at T3

Correlation coefficient (p-value)						
		Gingival crevicular fluid level of Cathepsin K				
Salivary fluid level of Cathepsin K	Group	Amount of CTSK	GCF volume	Concentration of Cathepsin K	Total protein	Relative amount of CTSK to total protein
Amount of CTSK in 100 µl	CP	0.4 (0.286)	0.367 (0.332)	0.333 (0.381)	0.483 (0.187)	0.095 (0.823)
	CP+DM	0.322 (0.398)	-0.059 (0.881)	0.288 (0.452)	-0.467 (0.205)	0.771 (0.072)
	GH	-0.6 (0.285)	-0.5 (0.391)	-0.6 (0.285)	-0.8 (0.104)	-0.4 (0.6)
Concentration of Cathepsin K	CP	0.4 (0.286)	0.367 (0.332)	0.333 (0.381)	0.483 (0.187)	0.095 (0.823)
	CP+DM	0.322 (0.398)	-0.059 (0.881)	0.288 (0.452)	-0.467 (0.205)	0.771 (0.072)
	GH	-0.6 (0.285)	-0.5 (0.391)	-0.6 (0.285)	-0.8 (0.104)	-0.4 (0.6)
Amount of total protein in saliva 100 µl	CP	0.383 (0.308)	0.283 (0.46)	0.117 (0.765)	0.3 (0.433)	0.143 (0.736)
	CP+DM	0.186 (0.631)	0.19 (0.625)	0.102 (0.795)	-0.033 (0.932)	-0.714 (0.111)
	GH	0.6 (0.285)	0.55 (0.337)	0.6 (0.285)	0.8 (0.104)	-0.2 (0.8)
Concentration of total protein	CP	0.383 (0.308)	0.283 (0.46)	0.117 (0.765)	0.3 (0.433)	0.143 (0.736)
	CP+DM	0.186 (0.631)	0.19 (0.625)	0.102 (0.795)	-0.033 (0.932)	-0.714 (0.111)
	GH	0.6 (0.285)	0.55 (0.337)	0.6 (0.285)	0.8 (0.104)	-0.2 (0.8)
Relative amount of CTSK to total protein	CP	0.067 (0.865)	0.0 (1.0)	0.267 (0.488)	-0.05 (0.898)	0.119 (0.779)
	CP+DM	-0.017 (0.965)	0.084 (0.831)	0.017 (0.965)	-0.167 (0.668)	0.943** (0.005)
	GH	-0.5 (0.391)	-0.6 (0.285)	-0.5 (0.391)	-0.9* (0.037)	0.2 (0.8)

* Statistically significant (Spearman rank correlation, $p < 0.05$)

** Statistically significant (Spearman rank correlation, $p < 0.005$)

Supplementary Table S3 Periodontal parameters at selected sites and gingival crevicular fluid levels of Cathepsin K (CTSK) after scaling and root planing

		Correlation coefficient (p-value)				
		Amount of CTSK	GCF volume	Concentration of CTSK	Total protein	Relative amount of CTSK to total protein
Probing depth	CP group	0.23 (0.44)	0.18 (0.54)	0.20 (0.50)	0.50 (0.07)	-0.12 (0.68)
	CP+DM group	0.46 (0.10)	0.52 (0.05)	-0.11 (0.72)	0.41 (0.15)	-0.20 (0.48)
	Subgroup (HbA1c<8)	-0.14 (0.73)	0.20 (0.63)	-0.29 (0.49)	0.68 (0.06)	-0.65 (0.08)
	Subgroup (HbA1c≥8)	0.90** (0.02)	0.90** (0.02)	-0.23 (0.66)	0.49 (0.32)	0.17 (0.74)
Percentage of bleeding sites	CP group	0.43 (0.12)	0.10 (0.73)	0.22 (0.45)	0.22 (0.45)	0.04 (0.90)
	CP+DM group	0.48 (0.08)	0.35 (0.22)	-0.10 (0.73)	0.27 (0.34)	0.01 (0.98)
	Subgroup (HbA1c<8)	-0.57 (0.14)	-0.11 (0.79)	-0.15 (0.72)	-0.08 (0.84)	-0.52 (0.19)
	Subgroup (HbA1c≥8)	0.94* (0.01)	0.82* (0.045)	-0.15 (0.77)	0.33 (0.52)	0.33 (0.52)
Clinical attachment level	CP group	0.38 (0.19)	-0.08 (0.79)	0.52 (0.06)	0.31 (0.27)	-0.15 (0.62)
	CP+DM group	0.34 (0.23)	0.31 (0.29)	-0.22 (0.46)	0.40 (0.16)	-0.10 (0.73)
	Subgroup (HbA1c<8)	-0.49 (0.22)	0.22 (0.61)	-0.32 (0.44)	0.34 (0.42)	-0.66 (0.08)
	Subgroup (HbA1c≥8)	0.31 (0.54)	0.49 (0.33)	-0.49 (0.33)	0.26 (0.62)	0.09 (0.87)

* Statistically significant (Pearson's correlation, $p < 0.05$)

** Statistically significant (Spearman rank correlation, $p < 0.05$)