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Original Article

Glycated hemoglobin influence on periodontal status, pathogens and salivary interleukins in type II diabetic Tunisian subjects with chronic periodontitis

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Abstract *Background/purpose:* Studies have shown that there is a possible correlation between the amount of glycated hemoglobin and the periodontal status. The goal of this study was to investigate the relationship between glycated hemoglobin (HbA1c) and the prevalence of gingival pathogens and circulating interleukin levels in type II diabetic Tunisian subjects.
Material and methods: The research included four groups; 30 healthy subjects (H group), 30 non-diabetic subjects suffering from chronic periodontitis (CP group). Type-II diabetic patients were divided according to HbA1c level into 30 adequately-controlled type-II diabetes subjects (HbA1c $\leq$ 7 percent (ATIID&CP group)) and 30 inadequately-controlled type-II diabetes

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subjects and HbA1c > 7 percent (ITIID&CP group). Clinical periodontal condition parameters and assessment of salivary interleukin IL-1beta, IL-6 and IL-10 were assessed. Quantitative Polymerase Chain Reaction used for detection of Subgingival biofilm of periodontal pathogens.
Results: Clinical parameters analyzed were positively associated with HbA1c levels ($p < 0.05$). *A. Actinomycetemcomitans* were found in 80 percent of ITIID&CP, 65 percent of CP and almost absent in H group. *Porphyromonas gingivalis* was present in 100 percent of CP, 85 percent of ITIID&CP, 50 percent of ATIID&CP and 3 percent of H group. *T. Denticola* had an equivalent occurrence. While *Tannerella forsythia* was scarce in ITIID&CP groups, but abundant in the H group. ITIID&CP had the highest IL-6 and IL-1beta/IL-10 ratios.
Conclusion: HBA1c levels affect periodontal status, pathogens and salivary interleukins in Type-II diabetic Tunisians with chronic periodontitis, compared with stable and chronic periodontitis groups and can interact with periodontal infections and increase the inflammatory state.

Introduction

Type II diabetes (TIID), dramatically increasing in its incidence and is estimated to affect 15% of Tunisians according to Achour et al.[1] TIID is a chronic metabolic disease arising from insulin secretion defect and body resistance to insulin. Diabetes also followed by an elevated risk of complications including cardiovascular disorders, retinopathy, neuropathy, nephropathy and periodontitis. The latter is more common in diabetic people than non-diabetics.[2]

Periodontitis is a polymicrobial teeth-supporting tissues infection that results in periodontium and alveolar bone loss.[3] Chronic hyperglycemia leads to molecules created by biological mechanisms called advanced glycated products. The presence of these molecules and the oral pathogens initiate immune response in the epithelial cells of periodontium and exacerbate the periodontal inflammation.

The host's immune-inflammatory response mediates the periodontal destruction. In periodontal sites, the host immune response is dysregulated and is therefore ineffective to restrain bacterial outgrowth and overt pathogenicity.[4]

Several studies have highlighted the evidence of increasing rates of interleukins and other mediators in TIIDM patients, in the presence or absence of Periodontitis.[5–7] In addition, serum IL-6 levels in TIIDM patients were reported to both increase and decrease after periodontal therapy in different studies.[8,9]

Bidirectional relationship between Diabetes and periodontitis have overlap.[9] Diabetes mellitus is considered as significant potential risk factor for periodontal diseases. In fact, hyperglycemia leads to abnormal oral pathogen-dominated biofilms.[10] Moreover, periodontitis leads to an increase response in systemic inflammation and enhance the insulin resistance onset.[11]

Several studies have also shown the important significant effect that glycated hemoglobin levels have on the periodontal disease severity. Studies have shown that well-glycemic controlled subjects have less severe periodontal diseases than poorly controlled ones.[12–14] To date, none in Tunisia has studied potential associations between glycated hemoglobin levels in type II diabetic patients suffering from chronic periodontitis.

Therefore, the present study aimed to investigate the effect of uncontrolled glycemia on type II diabetic Tunisian patients with chronic periodontitis and comparing the periodontal status of these patients with healthy and chronic periodontitis subjects. For that, we evaluated the prevalence of subgingival periodontal pathogens biofilm and interleukins (IL-)s in chronically periodontitis patients with and without TIID compared to healthy subjects. The state of periodontitis was evaluated in terms of increased probing pocket depth (PPD), loss of clinical attachment (CAL) and bleeding on the check (BoP). The presence of *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* was described in the Subgingival biofilm. Assessment of ILs and the inflammatory status were also explored.

Material and methods

Study design and sample

TIID patients with chronic periodontitis were recruited from the Fatouma Bourguiba hospital's endocrinology clinic in Monastir. The Faculty of Dentistry clinic recruited patients with periodontitis and healthy volunteers. This research was carried out in complete compliance with Declaration of Helsinki. The Faculty of Medicine's Ethical Committee, University of Monastir (Tunisia), approved this study and a signed consent from all subjects was obtained.

Inclusion criteria comprised subjects older than 35 years of age, confirmed diagnosis of type II diabetes over than 3 years; affected by chronic periodontitis. Exclusion criteria consisted of the use of antibiotics in the previous 3 months, pregnancy, smoking status, associated other systematic disease.

This study enrolled 120 subjects divided into 4 groups as following:

- Healthy group (H): 30 volunteers, systemically and periodontally healthy, considered as negative control.
- Chronic periodontitis group (CP): 30 subjects systemically healthy and diagnosed with chronic periodontal disease.

- Adequately controlled type II diabetic group (ATIID&CP): 30 selected type II diabetes patients with HbA1c $\leq$ 7% and diagnosed with chronic periodontitis.[15]
- Inadequately controlled type II diabetes (ITIID&CP): 30 selected type II diabetes patients with HbA1c >7% and diagnosed with chronic periodontitis. Diabetic complications such as cardiovascular disease, retinopathy, neuropathy and nephropathy were assessed.

Periodontal examination

PPD (probing pocket depth) was determined using a William periodontal probe, which was defined, as the distance to the nearest whole millimeter between the gingival margin and the bottom of the likely pocket. CAL (loss of clinical bonding) was measured by subtracting the distance from the clear cemento-enamel junction from the entire probing depth to the gingival edge. CAL measurements were determined at six separate sites of all present teeth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) except for third molars. After measurement of PPD, all sites were observed for probing bleeding (BoP). All measurements of the study variables were done by the same periodontist for each subject of our dental clinic population.

Biofilm samples

The supra gingival biofilm sample was collected with a sterile gauze for the microbiological identification by PCR. Subgingival biofilm samples were removed using a sterile periodontal curette from the sites where PD was about 5 mm and from the dental furcation area. Samples were placed in sterile eppendorf tubes and kept at $-80\,^{\circ}C$.

Salivary interleukins measure

For measurement of interleukins, all participants obtained unstimulated collection of saliva. Samples were immediately put on ice and then transferred to the laboratory. After sampling, aliquots were prepared and the samples were frozen at-80 °C until analysis was carried out.

For interleukin measurement, saliva samples were tested in duplicate: IL-1beta, IL-6 and IL-10 using commercial ELISA cytokine multiplex kits (R&D Systems, Inc. Minneapolis, MN, USA) and analyzed using a Luminex system (Luminex corporation, Austin, TX, USA) Absorbance was converted to concentration, using standard curves (in pg/ml).

Pathogens assessment

Table 1 lists the primers evaluated and utilized in this analysis. The samples were added sterile distilled water, and then boiled for 10 min. The PCR was processed using a sample of 5.0 ml applied to a 45 ml reaction buffer containing 1.5 mm MgCl2 (Taq DNA polymerase), 200 mm of deoxynucleotide triphosphate mixture, 2 mm of each primary, and 2U TaqDNA polymerase. In addition to the samples, each experiment employed positive and negative controls. Table 2 summarizes the conditions for the PCR amplification. The PCR products were analyzed in 1.5 percent agarose gel using electrophoresis. Data were expressed as either positive or negative.

Glycated hemoglobin

HbA1c was measured in Biochemistry laboratory of Fattouma Bourguiba hospital. HbA1c was determined by immunoturbidimetric assay (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

In all, a statistical analysis of the SPSS statistical software was used. The quality variables or categorical values have been described as frequencies, and the quantitative variables have been described as the mean and standard deviation (SD). Continuous variables among four groups were evaluated using a one-way variance analysis (ANOVA), and those between two groups were evaluated in the bivariate analysis using t test. Chi-square test evaluated the categorical values between the two groups. The Data for continuous variables and percentages for categorical variables were expressed as mean $\pm$ SD. P-values smaller than $p < 0.05$ were deemed statistically significant.

Result

An overview of the demographics and clinical parameters of the study population were summarized in Table 3. The study enrolled 90 subjects diagnosed for periodontitis with or without TIID compared to 30 healthy individuals. ITIID&CP had significantly fewer remaining teeth than ATIID&CP ($p < 0.001$). In addition, CP exhibited the highest BoP but moderate PPD.

Oral examination results are shown in Table 4. PPD, CAL and BoP significantly varied between all groups. PPD and CAL were significantly the highest in ITIID&CP. Consequently, ITIID&CP presented higher tooth loss. PPD and CAL were significantly higher in ITIID&CP than in patients with ATIID&CP. However, CP showed significantly the highest BoP. BoP was about 60% in ATIID&CP whereas; BoP was about 70% in ITIID&CP.

Table 1 PCR primers used.

Species	Sequence	Base position
Aa	5′AAACCCATCTCTGACTTCTTCTTC3′	557
	5′ATGCCAACTTGACGTTAAT3′	
Pg	5′AATCGTAACGGGCGACACAC3′	593
	5′GGGTTGCTCCTTCATCATAC3′	
Tf	5′GCGTATGTAACCTGCCCGCA3′	641
	5′TGCTTCAGTGTCAGTTATACCT3′	
Td	5′TAA TAC CGT ATG TGC TCA TTT ACA T3′	316
	5′TCA AAG AAG CAT TCC CTC TTC TTC TTA3′	

Aa: *Aggregatibacter actinomycetemcomitans*, Pg: *Porphyromonas gingivalis*, Tf: *Tannerella forsythia*, Td: *Treponema denticola*.

Table 2 Conditions for PCR amplification.

	I	Dcn	Extension	Annealing	Extension	F
Aa	95 °C for 2 min	36	95 °C for 30s	60 °C for 60s	72 °C for 60s	72 °C for 120s
Pg	94 °C for 5 min	30	94 °C for 60s	70 °C for 60s	72 °C for 60s	72 °C for 120s
Tf	95 °C for 2 min	36	95 °C for 30s	60 °C for 60s	72 °C for 60s	72 °C for 120s
Td	94 °C for 5 min	36	94 °C for 60s	70 °C for 60s	72 °C for 60s	72 °C for 120s

I: initial denaturation, Dcn: number of cycles of denaturation, F: final elongation, Aa: *Aggregatibacter actinomycetemcomitans*, Pg: *Porphyromonas gingivalis*, Tf: *Tannerella forsythia*, Td: *Treponema denticola*.

Biofilm species distribution of the study population were illustrated in Table 4. ITIID&CP showed significantly the highest *A. actinomycetemcomitans* prevalence (80%) followed by ATIID&CP (70%) then CP (65%, $p < 0.05$). However, CP showed significantly the highest *P. gingivalis* presence (100%) followed by ITIID&CP (85%) then ATIID&CP (50%, $p < 0.05$). Periodontally healthy individuals showed the highest *T. forsythia and T. denticolla* prevalence followed by CP then ATIID&CP and ITIID&CP. ITIID&CP showed the highest association of *A. actinomycetemcomitans, P. gingivalis, T. denticola,* and *T forsythia* while CP showed the highest association of *P. gingivalis, T. denticola*. Means of saliva cytokines quantities across the study groups presented in Table 4.

We evaluated three salivary biomarkers accompanied with inflammatory and destructive tissue conditions in periodontitis; IL-1beta, IL-6 and IL-10. A find worth noting is that ITIID&CP, who had the highest association of *A. actinomycetemcomitans, P. gingivalis, T. denticola, and T. forsythia* showed significantly the highest IL-1beta and IL-6. However, *P. gingivalis and T. denticola* were more frequent in CP than in ATIID&CP, in both group, CP and ATIID&CP, there were no significant difference in the overall amount of IL-1beta and IL-6. Our result showed an amount of IL-10 different from one another. ITIID&CP showed the lowest level of IL-10 while H subjects had the highest mean value. The type II diabetic groups showed a significantly higher total amount of IL-1beta/IL-10 than the other studied groups (CP and healthy groups). However, ITIID&CP characterized by the highest amount of the ratio of IL-1beta/IL-10. P-value <0.05 indicates differences between groups by the Student's t-test ($P < 0.05$). There were no differences between groups for gender by Fisher's exact test.

Discussion

The main objective of the current study was to investigate the relationship between unregulated glycated hemoglobin and clinical periodontal conditions, the prevalence of gingival pathogens and the circulating interleukins levels in type II diabetic Tunisian patients with chronic periodontitis and to compare the findings with healthy and chronic periodontitis subjects.

Concerning the single measurement of hemoglobin, subjects with TIID were divided into two groups, those with HbA1c $< 7\%$ which are adequately or well controlled TIID patients and the second group consist of those with HbA1c $\geq 7\%$ which are poorly or inadequately controlled individuals.[15] Professional organizations conclusively determine that setting Hba1c below 7 decreases microvascular complications.[16] However, studies evaluating the impact of HbA1c on periodontitis complication remain unclear.

Many studies consider periodontitis as a potential risk factor for tooth loss.[17–19] The first clinical parameter studied, in our research, was the number of remaining

Table 3 Population characteristics of the study.

PARAMETRES	Healthy N = 30	CP N = 30	ATIID&CP N = 30	ITIID&CP N = 30
Age (years/Mean+SD)	40 + 8	48 + 4	45 + 5	47 + 3
Gender (F/M)	15/15	15/15	15/15	15/15
Duration of diabetes (years)	–	–	6 + 3.2	8 + 3.5
Number of remaining teeth (Mean+SD)	30 + 1	25.2 + 3.3	23.1 + 4.8	20 + 3
Personal history				
Macroangiopathy (%)			3	18
Retinopathy (%)			3	25
Neuropathy (%)			6	50
Hypoglycemic agents				
Metformin (%)			30	12
Sulphonylureas (%)			30	19
Metformin + Sulphonylureas (%)			30	29
Insulin (%)			10	40

CP: chronic periodontitis patients, ATIID&CP: appropriately controlled type II diabetes (HbA1c ≤ 7 Percent) with chronic periodontitis, ITIID&CP: Inadequately controlled diabetes (HbA1c > 7 Percent) with chronic periodontitis. SD: automatic deflection.

Table 4 Periodontal levels, prevalence of Biofilm organisms and interleukins in the population studied.

	HEALTHY N = 30	CP N = 30	ATIID&CP N = 30	ITIID&CP N = 30
Periodontal Indices				
PPD (MM;MEAN+SD)	2.20 ± 1.5	4.7 ± 0.3	5.9 ± 0.35	6.3 ± 0.6
CAL (MM;MEAN+SD)	0	4.9 ± 0.5	6.1 ± 0.4	7.36 ± 0.4
BOP (%;MEAN ± SD)	4.06 ± 2	75 ± 10.7	60 ± 11.85	70 ± 10.05
Biofilm Species Prevalence				
AA	1%	65%	70%	80%
TD	3%	90%	45%	80%
PG	3%	100%	50%	85%
TF	100%	75%	45%	30%
AA + TD + PG + TF %	0%	75%	65%	95%
Interleukins				
IL-1BETA (PG/ML;MEAN+SD)	180 ± 20	268 ± 46.7	261 ± 50	357 ± 60
IL-6 (PG/ML;MEAN+SD)	7 ± 2	11 ± 6	12 ± 6	16 ± 8
IL-10 (PG/ML;MEAN+SD)	14.67 ± 3.2	11 ± 2.4	10 ± 3.47	9 ± 2.5
IL-1BETA/IL-10	12 ± 8	24 ± 11	26 ± 14	40 ± 18

CP: chronic periodontitis patients, ATIID&CP: adequately controlled type II diabetes (HbA1c ≤ 7%) with chronic periodontitis, ITIID&CP: poorly controlled diabetes (HbA1c >7%) with chronic periodontitis. PPD: Sampling pocket depth, CAL: loss of clinical connection, BoP: Bleeding on sampling.

teeth. We noticed that there was a tooth loss in both groups with chronic periodontitis with and without diabetes. The highest number of tooth lost was seen in ITIID&CP patients. Kaur et al., 2009 demonstrated type I and type II Diabetes mellitus impact on dental loss.[19] The correlation of poor glycemic control and tooth lost was reported by Renata et al., 2012.[14]

Our analysis of the effect of glycemic control on the clinical periodontal indices expressed by PPD and CAL showed that ITIID&CP exhibited the worst periodontal status compared to ATIID&CP and CP in terms of the highest PPD and CAL as presented in Table 4. These findings are in accordance with those of several earlier studies reporting elevated periodontal parameters among HbA1c in poorly controlled TIID patients.[20,21]

In this study, gingival biofilm were processed by means of PCR to detect four periodontal pathogens (*A. actinomycetemcomitans, P. gingivalis, T. denticola, and T. forsythia*). The prevailing of *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, and *T. forsythia* was substantially different for each group studied (ITIID&CP, ATIID&CP, CP and peridontally healthy individuals). Demmer et al. showed the existence of associations between abnormal glucose metabolism and periodontal microbiota.[10] There are conflicting findings which have shown that ITIID&CP may have elevated levels of microbiota,[22] lower prevalence of pathogenic species,[23] or even a microbiota similar to that of non-diabetic mellitus individuals.[24]

We found that *A. actinomycetemcomitans* were present in 80 percent of the ITIID&CP population with the highest CAL, while they were present in 75 percent of the ATIID&CP community, 65 percent of the CP group and almost absent in periodontally healthy individuals. Several separate studies proved the existence of *A. actinomycetemcomitans* in the periodontal pocket plaque which increase the risk of initiation and progression of the attachment loss.[25] *A. actinomycetemcomitans* prevalence was positively correlated with the increased HbA1c. Castrillon et al., published similar results suggesting the presence of the red complex comprising *P. gingivalis*, *T. denticola*, and *T. forsythia*.[22] However, a different trend was observed, *A. actinomycetemcomitans* counts was not positively correlated with increased HbA1c.[26] The same study found that other periodontal pathogen bacteria counts such as *P. gingivalis, T. denticola, and T. forsythia* were positively correlated with HbA1c.[26] Additionally, studies among patients with chronic periodontitis from Sudan reported the absence of HbA1c influence on the prevalence of *A. actinomycetemcomitans*.[27] Recently, literature has emerged that contradictory findings about periodontal microbiota may vary according to the geographical areas, diet, as well as life style and genetic predisposition. These factors which determine the outcome of the disease activity make comparisons in that regard between studies difficult.[28]

We found that *P. gingivalis* was present in 100 percent of CP, 85 percent of ITIID&CP, 50 percent of ATIID&CP and 3 percent of periodontally healthy individuals. The current observation may suggest that of *P. gingivalis* multiplication may be more associated with periodontitis than with insulin resistance. In this regard, recent studies reported that periodontitis patients had the highest prevalence of *P. gingivalis* followed by periodontitis-diabetes and TIID patients.[29] In line with our findings, Castrillon et al., showed that in non-diabetic patients *P. gingivalis* has been associated with periodontitis, while *A. actinomycetemcomitans* in diabetic patients have been associated with periodontitis.[30] A Chinese study revealed that in TIID patients with chronic periodontitis the distribution of *P. gingivalis* FimA genotype was significantly higher than in systemically healthy individuals. Interestingly, Arimatsu showed that *P. gingivalis* administered mice showed an elevated insulin resistance and systemic inflammation.[30]

We found that *T. Denticola* showed fairly the same prevalence as *P. gingivalis* in the population groups studied.

It has been shown that together in culture, these species combine synergistically to produce more biomass than the monoculture additive amounts.[31] This nutritional cross-feeding involves the *P. gingivalis* use of the produced succinate by *T. Denticola*, and, the development in turn, of *T. denticola* which is stimulated by the isobutyric acid produced by *P. gingivalis* as a metabolic end product.[31] Moreover, *P. gingivalis* produces proteinaceous substrates boosting *T. denticola* growth.[32]

In the present study, whereas in *T. forsythia* was copious in ITIID&CP, and abundant in periodontally healthy individuals. It was present in 75% of CP, 45% of ATIID&CP and 30% of ITIID&CP group. We noted that *T. forsythia* prevalence decrease was in connection with increased HbA1c, similar results were reported.[33] Earlier, it was shown that the quantity of *T. forsythia* was lower in ITIID&CP than that in non-diabetic mellitus group.[24] The same study reported that the quantity of *T. forsythia* was declining with the worsening of glucose control.[24] Our finding is different from a previous study reporting high prevalence of *T. forsythia* in periodontitis groups with or without diabetes.[29]

Our analysis revealed that the highest association of *A. actinomycetemcomitans, P. gingivalis, T. Denticola, and T. forsythia* has been found in ITIID&CP. This finding may be considered as causative agents of the deep PPD and large CAL characterizing ITIID&CP. Our study indicated that poor glycemic control is associated with an increase in the presence of red complex bacteria. A salivary presence of *A. actinomycetemcomitans, P. gingivalis, Porphyromonas intermedia, and T. Denticola*, has been shown to contribute to deepened pockets.[34] Interspecies co-adhesion and binding Mechanisms are well documented.[35]

Our result revealed that the patients ITIID&CP had the highest saliva IL-1beta and IL-6 levels. Indeed, the high *A. actinomycetemcomitans* prevalence observed in ITIID&CP may be a cause of IL-1beta induction.[36] In patients with diabetes, IL-1 beta is one of the key cytokines in inflammatory periodontal tissue destruction. IL-1 beta is present in pathological conditions that cause bone loss through osteoclast survival and activation. Moreover, diabetes produces a hyperinflammatory response in certain cells due to the action of advanced glycation end products.[37]

Although *P gingivalis* prevalence was the highest in CP, a high level of IL-1beta was observed also. Hajishengallis et al. demonstrated that the prevalence of *P. gingivalis*, is considered pathogenic due to its ability to cause dysbiotic microbial populations even at a low concentrations, and thus acts as a keystone pathogen[38] and trigger inflammatory periodontal bone loss. ITIID&CP were characterized by the highest IL-1beta/IL-10 ratio suggesting an imbalance between pro- and anti-inflammatory cytokines. Interleukin-10 is In fact a potent anti-inflammatory cytokine that suppresses both immunoproliferative and inflammatory responses by preventing of TNF-alpha, IL-6 production and other mediators.[20] In our research, the serum IL-6 levels were elevated on both chronic periodontitis groups with and without diabetics.

It has been shown that IL-6 levels have been involved in cross-susceptibility between T1DM and periodontitis,[39] Indeed, some studies report IL-6 increases and many other mediators in TIIDM patients, with or without periodontitis.[8]

A Mexican study found, unlike our results, that TIID subjects with generalized periodontitis had a lower percentage of the red complex than non-TIID subjects.[40] A British survey showed no impact of diabetes on the prevalence of Aa and Pg.[29] In addition to a Chinese report, according to our results, Tf are considered to be periodontitis-related bacteria.[41] Conversely, a Brazilian study also found that poor glycemic regulation in TIID subjects is correlated with increased Tf frequency.[42]

Similar to our findings, a Colombian study found that higher colonization levels of particular periodontal microbiota are correlated with a higher prevalence of diabetes.[10] In addition, levels of Pg, Td and Tf in Thailandian subjects with TIIDM[26] were positively associated with HbA1c. Taking all the data into account, we propose that some colonizing genes of Gram-negative bacteria in periodontal pockets express environmental and geographic factors differently.

Our findings are consistent with the hypothesis that elevated HbA1c is associated with amplified numbers of *P. gingivalis*, *T. denticola*, *T. forsythia* and *A. actinomycetemcomitans* accompanied by an increased inflammatory response. ITIID&CP showed the highest prevalence of red complex in subgingiva, and had higher levels of IL-1 beta, IL-6 and IL-1 beta/IL-10 ratio than the patients with good control of HBA1c and with chronic periodontitis alone.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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