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Association between Aggregatibacter actinomycetemcomitans bacterial load and NLRP3 inflammasome activation in periodontitis patients with Diabetes

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Abstract:

Background: *Aggregatibacter actinomycetemcomitans* is one of the most common periodontal pathgens that has a direct effect on periodontium. Diabetes and periodontitis considered as chronic diseases with a bidirectional relationship between them. Evidence has shown that the Node like Receptor Pyrine-3 inflammasome, is crucial for both illnesses.

Objective: This study was conducted to observe the association between bacterial load of *A. actinomyctecomtans* and serum level of NLRP3 inflammasome in periodontitis patient with and without type 2 DM and control group.

Materials and Methods: This case-control study included 85 participants, whose ages ranged from (23-55) years. Split into three groups; the control group, which had a clinically and systemically healthy periodontium, and the two groups with periodontitis, one of which also had type 2 DM. Samples of the four most profound periodontal pockets were sampled for subgingival plaque, and from the gingival sulcus in the control group. The real-time (PCR) was utilized in this experiment. To quantify *A. actinomyctecomtans* DNA was isolated from samples of dental plaque. All subjects' serum was collected, and the concentration of NLRP3 was measured using an ELISA technique.

Results: The results showed that the bacterial count was higher in two groups of patient than the control group, but there were no-significant differences. On the other hand, there was a significant correlation between the bacterial count and periodontal parameters in periodontitis with type 2 DM group, while in periodontitis group there was positive correlation with clinical attachment loss and bleeding on probing. A significant positive correlation also noticed between NLRP3 inflammasome and periodontal parameters in periodontitis patient with type2 DM. Regarding the group of periodontitis patients without systemic disease there was no significance correlation between inflammasome and clinical parameters. Finally, there is a non-significant correlation of *A. actinomyctecomtans* with inflammasome.

Conclusion: *A. actinomyctecomtans* detection rate was strongly higher in patient groups compared to healthy subjects but statically non-significant. Moreover, the lack of correlation between *A. actinomyctecomtans* and the NLRP3 inflammasome indicates that NLRP3 activation is associated with inflammatory processes that are induced by a number of external factors, other than bacteria.

Keywords: *Aggregatibacter actinomycetemcomitans*, inflammasome, NLRP3, periodontitis, type 2 DM.

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Introduction:

Periodontal diseases include a lot of inflammatory situations that influence the gingiva, periodontal ligament and bone, that eventually cause tooth loss and involved in systemic inflammation. Oral microbita (dental plaque) cause initiation and proliferation of periodontal disease, later on inflammation and disease occur because of interaction between these microbiota and immune defenses (1). Just a few types of native Gram-negative periodontal bacteria are responsible for the onset of periodontal disease that provoke inflammatory, innate and adaptive immune responses. These processes cause tissue, bone, and tooth loss by destroying the tissues supporting and surrounding the teeth (2, 3, 4). The deterioration of periodontal health into periodontal disease is not caused by a single organism; rather, the subgingival microbiome community that exists in healthy gums changes from homeostasis to dysbiosis, a condition in which a community's biotic makeup and abundance take on more pathogenic characteristics (5). *A. actinomcytocommitance* and Red complex bacteria, which includes, *Tannerella forsythia*, *Porphyromonas gingivalis*, and *Treponema denticola*, was initially found in the microbiomes found underneath the gums of people with periodontal disease using culture-independent methods (6). *A. actinomycetemcomitans* has the ability to produce a number of virulence factors, and it exhibits a large genetic diversity (7). This organism's possible virulence factors are diverse and

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can be broken down into three categories: (I) factors that regulate the promotion of colonization and inflammation, (II) factors that cause host tissue damage, and (III) factors that suppress host tissue to heal. (8, 9). Diabetes mellitus is a chronic metabolic non-communicable disease (10) that happens when the pancreas does not produce sufficient insulin, or when the body is disabled of effectively using the insulin it produced or both (11, 12). There are a lot of studies on the connections between DM and periodontal disease, the DM has been notified as an important risk factor for periodontal disease (13, 14, 15). T2DM has been related to higher occurrence, distribution and severity of periodontitis when compared with non-diabetic adults. Actually, the risk for development of periodontal disease is highly seen in diabetic patients (both T1DM and T2DM) and became worse in patient with poor glycemic control (16, 17). The relation between periodontal condition and the glycemic control of diabetes is not easy to recognized absolutely (18), because of the heterogeneity seen in diabetic patients, according to that, patients with poor control DM suffering from highly destructive periodontal disease (19), others don't (20). Inflammasomes are caspase-1-dependent and -independent multiple-protein platforms that nucleate around an intracellular receptor belonging to the family of (NLRs). Components of the inflammasome are primarily expressed in monocytes and macrophages of the innate immune system (21). In response to microbial infection and cellular injury, the NLRP3 inflammasome causes caspase-1 activation and the production of proinflammatory cytokines IL-1 and IL-18. In addition to exogenous stimuli like extracellular ATP, uric acid, and cholesterol crystals, the NLRP3 can be activated by bacterial stimuli including lipopolysaccharide (LPS) and ribonucleic acid (RNA) (22). There is a two-step process necessary to activate the NLRP3 inflammasome. Signal 1 is priming, which is primarily mediated by TLRs and TNF- and attempts to upregulate pro-IL-1, pro-IL-18, and NLRP3 in an NF-B-dependent way. Caspase-8 and Fas-associated protein with death domain participate in the NF-B signaling pathway. The inflammasome can be primed through mechanisms other than transcription. Activation of the NLRP3 inflammasome requires signal 2, which initiates the recruitment and assembly of NLRP3, an apoptosis-associated speck-like protein with a Caspase-recruitment domain and pro-caspase-1 (23). This study was conducted to investigate the association between bacterial load of *A. actinomyctecomtans* and serum level of NLRP3 inflammasome in periodontitis patient with and without type 2 DM and control group.

Materials and Methods

Subjects: In this case-control study, 85 participants (44 men and 41 women) were included. They were recruited from Taji primary health care centers from November 2021 to January 2022. Ethical Clearance: The College of Dentistry and University of Baghdad's Ethical Review Committee authorized this research. (Ref. No. 379 in 21/11/2021). Inclusion and Exclusion Criteria: The participants enrolled in this study and considered eligible must have met the following criteria: Type 2 DM patients on oral hypoglycemic therapy only, the presence of at least 20 or more natural teeth, and patients with periodontitis had periodontal pockets equal or more than 3 millimeters in at least two non-adjacent teeth, with loss of attachment more than 3 mm. Exclusion criteria include any previous extensive periodontal therapy or being currently under active periodontal treatment. Patients were receiving antibiotic treatment and include individuals without systemic conditions rather than T2DM. Oral Hygiene Index: By analyzing clinical periodontal data, the periodontal health state was determined by using a periodontal probe of William's graduation (24). Dental Plaque Sampling: Samples of subgingival plaque were taken from the four periodontal pockets that were the deepest in each patient's mouth (periodontitis with type 2 DM group and periodontitis group) and gingival sulcus in control group. A single vertical stroke was used to collect samples from pocket, and these samples were then transferred to an eppendorf tube containing 0.5 ml of TE buffer (10 mM Tris- HCl, 1 mM EDTA, pH 7.6 [25] by vigorously agitating the tip. Afterward, the sample was kept at a deep freeze (-40 c^0) until DNA could be extracted. Extracting DNA and Running a Real-Time PCR Test: According to the Geneaid extraction methodology, genomic DNA was extracted from plaque samples. Lyophilized primers were provided by Macrogen Company. as shown in table (1). We prepared a stock solution of lyophilized primers, dissolved in nuclease-free water at a concentration of 100pmol/µl. These primers were diluted to a concentration of 10 pmol/µl by mixing 10 l of primer stock solution (stored at -20 C) with 90µl of nuclease-free water.

Table 1: Primers

Primer Name	Sequance	Annealing Temp.(°C)	Product Size (bp)
A.actinomycetemcomitans 16SrRNAA-F1	5'-CTTACCTACTCTTGACATCCGAA-3'	60	77
A.actinomycetemcomitans 16SrRNAA-R1	5'-ATGCAGCACCTGTCTCAAAGC-3'		

In the qPCR experiment, the standard curve method makes use of a dilution series of a known template copy number. The standard curve is obtained by doing a linear regression between log concentration (copy µl -1) and CT. in figure (1), and information is subsequently utilized in the calculation of the sample's template concentration

(copy µl-1). The melting curve that was made showed how specific the PCR products were, both for serial dilutions and for clinical samples.

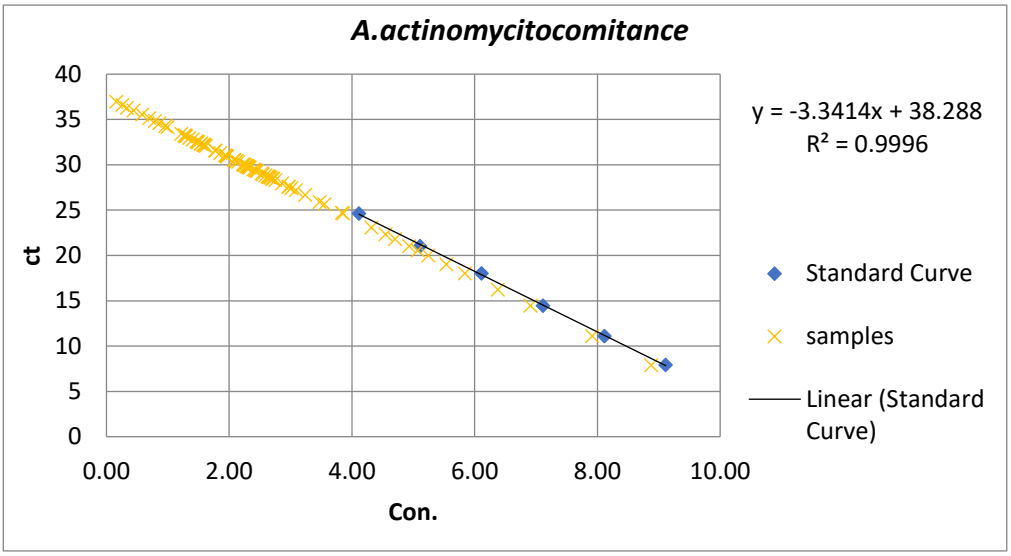

Figure 1: standard curve for *A.actinomycitocomitance*

RT- PCR amplifications were carried out using a magnetic induction cycler (Mic RT-qPCR), with 10µl volumes containing 0.5 µl for each primer; 2.5 µl nuclease free water; 1.5 µl template of DNA and finally 5 µl Master Mix, table (2).

Table 2: PCR component

Master mix components	Volume
qPCR Master Mix	5
Forward primer	0.5
Reverse primer	0.5
Nuclease Free Water	2.5
DNA	1.5
Total volume	10

Collection of Blood Samples: Under sterile conditions, three milliliters of the subject's venous blood will be collected from each individual. After transferring the blood into a sterile plain tube, the serum was separated from the blood by centrifugation at 3000 rpm for 10 minutes. The serum was then divided into small aliquots and stored at -20 C^0 until it was used for analysis. Measurement of NLRP3: The ELISA kit quantified NLRP3 concentration. Statistical analysis of data**:** Chi-square test (ANOVA) parametric test was used to determine and find difference between 3 or more independent groups, and Tukey honestly significant difference (HSD)/post hoc test was utilized to test whether the link between two data sets is statistically significant. For non-parametric data Kruskal-Wallis test. Correlation among different parameters was calculated by the Spearman and Pearson correlation coefficient test. The P-values of P<0.01 and P<0.05 have been regarded as significant.

Results:

The present study showed that the mean age for periodontitis+T2DM patients was (40±8.8) years, and for PD patients was (38±7.50) years, while for control subjects was (36±6.50) years. The distributions of subjects according to gender were shown in figure (2).

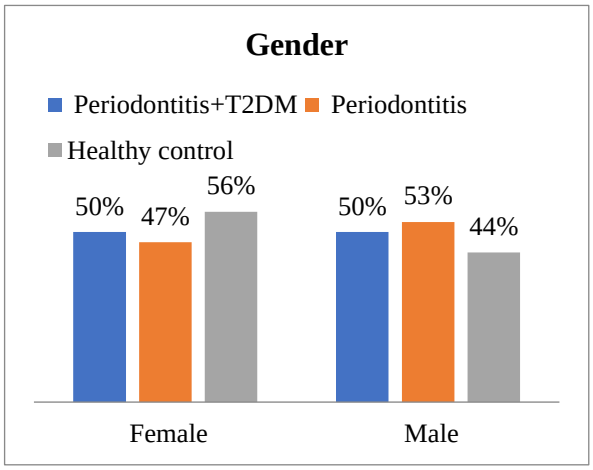

Figure 2: Sex distribution in study groups.

Table (3) showed the of quantification results for *A. actinomycetemcomitans* assessed by qRT-PCR, the mean rank value of bacterial count was higher in two patient's groups than control group but statically non significance (p>0.05). Moreover, this finding showed that there was no statistically significant difference between any of the three study groups when comparing the mean rank values of *A. actinomycetemcomitans*, (table 4).

Table 3: The difference in mean rank values of A.A bacterial load among study groups

A.A bacterial load	Study groups			Kruskal-Wallis test (*P*-value)
	Periodontitis +T2DM n=30	Periodontitis n=30	Healthy control n=25	
Minimum	1	9	6	
Maximum	756772000	2385151	20868	
Median	332.5	218	196	
Mean Rank	43.62	40.91	36.78	
Percentile 05	25.00	20.00	6.66	0.572^{NS}
Percentile 95	82424524.00	345516.00	3427.00	

Table 4: Inter groups comparisons of the mean rank values of A.A bacterial count between all pairs of groups.

Grouping	Mean Rank difference	Mann Whitney Test (*P*-value)
A.A bacterial load		
Periodontitis +T2DM vs. periodontitis	2.70	0.666^{NS}
Periodontitis +T2DM vs. Control	6.83	0.293^{NS}
Periodontitis vs. Control	4.13	0.514^{NS}

As noted in table (5) there was a significant correlation between the bacterial count and each of gingival index, periodontal pocket depth, clinical attachment level and bleeding on probing in PD+T2DM group, while no significant correlation with PI was observed. In regard the PD group, according to the results of this investigation, there is no significant correlation between bacterial count and each PI, GI and PPD. However, there was positive correlation with CAL and BOP.

Table 5: Correlation between A.A bacterial load and clinical periodontal parameters in periodontitis patients with T2DM

Periodontitis patients +T2DM	R-value	*P*-value
PI	0.027	0.236^{NS}
GI	0.475	0.007**
PPD	0.662	0.000**
CAL	0.613	0.000**
BOP	0.791	0.000**
Periodontitis patients		
PI	0.111	0.556^{NS}
GI	0.057	0.761^{NS}
PPD	0.094	0.619^{NS}
CAL	0.466	0.006**
BOP	0.547	0.001**

PI= Plaque Index,GI= Gingival Index, PPD= Periodontal Pocket Depth, CAL= Clinical Attachment Level, BOP= Bleeding On Probing

The results of this study showed a significant increase (P<0.05) in the mean rank of serum NLRP3 levels among PD+T2DM group (56.60pg/ml) compared to the PD group (35.82pg/ml) and the control group was (35.83pg/ml), as observed in figure (3). Comparisons between groups as shown in table (6) revealed a significant difference between PD+T2DM and control group, on the other hand, the control group did not show a significant difference with PD group.

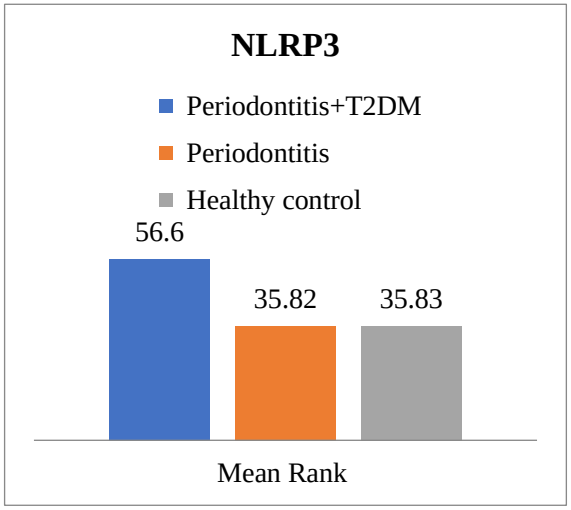

Figure- 3: The difference in mean rank values of serum NLRP3 among study groups

Table 6: Inter groups comparisons of the mean rank values of NLRP3 between all pairs of groups

Grouping	Mean Rank difference	Mann Whitney Test (*P*-value)
NLRP3		
Periodontitis +T2DM vs. periodontitis	20.87	0.001**
Periodontitis +T2DM vs. Control	21.03	0.001**

Periodontitis vs. Control	0.51	0.938^{NS}

Table (7) showed a significance correlation between the level of NLRP3 and each of GI, PPD, CAL and BOP in PD+T2DM patients, while no significance correlation was observed with PI. Regarding PD patients group this study indicates that there was no significance correlation of NLRP3 with all parameters.

Table 7: Correlation between NLRP3 level and clinical periodontal parameters in Periodontitis patients with T2DM

Periodontitis patients +T2DM	R-value	*P*-value
PI	0.093	0.623^{NS}
GI	0.794	0.000**
PPD	0.592	0.0005**
CAL	0.491	0.005**
BOP	0.447	0.013*
Periodontitis patients		
PI	0.100	0.598^{NS}
GI	0.120	0.525^{NS}
PPD	0.249	0.182^{NS}
CAL	0.040	0.833^{NS}
BOP	0.190	0.312^{NS}

The present results revealed no significant correlation between bacterial loads and NLRP3 inflammasome in two patient's groups. As shown in the table (8)

Table 8: Correlation of A.A bacterial load Counts with Serum NLRP3 in Periodontitis patients with and without T2DM.

Biomarkers	NLRP3
Periodontitis patients+T2DM	
A.A bacterial load	r=0.308 p=0.097
Periodontitis patients	
A.A bacterial load	r=0.061 p=0.745

Discussion:

In this research, *A. actinomycetemcomitans* was identified and quantified in subgingival microbial samples using the RT-PCR technique. Several research (26, 27, 28) establish that the statistical power to detect a predictive association between illness severity and bacterial quantification is increased when using quantitative data rather than dichotomous data (positive/negative or presence/absence). The result revealed that *A. actinomycetemcomitans* was detected in both periodontal healthy subjects and two groups of patients (PD +T2DM and PD). These results are in agreement with results reported by Socransky et al., who found that microbial complexes are repeatedly found together in subgingival biofilm with and without periodontal disease (6). Furthermore, Castrillon et al in (2013) compare frequency of detection for each types of bacteria relative to periodontal diagnosis, Patients with periodontitis had higher rates of *A. actinomycetemcomitans* and *P. gingivalis* with or without T2DM, respectively. On the contrary, the result of the current study disagrees with Castrillon and colleagues who indicate that the T2DM patients with periodontitis were more likely to have *A. actinomycetemcomitans* (25% vs. 18.7%) than those with periodontitis who did not have T2DM. Moreover, in individuals with type 2 diabetes and periodontitis, the prevalence of *A. actinomycetemcomitans* detection was greater than in systemically healthy people without periodontitis. (29). This variability could be attributed to several factors regarding the type of detection method, the specimen tested and sample size. Importantly, this study also discovered a strong relationship between bacterial count and clinical periodontal parameters in the patient group. These results coincide with Sanchez and colleague who found that a higher CAL value was associated with *A. actinomycetamcomitans* (30). While disagree with Tomita et al. (2013) (31) who prove that A. actinomycetamcomitans counts showed no significant correlation with PPD or CAL. Generally, as periodontal disease progresses, bone resorption can occur due to interactions between various virulence factors of *A. actinomycetemcomitans* and the host immune response (32). The present study showed an increase in the serum NLRP3 level in PD+T2DM group compared to PD patients group and control subjects. This consistent with Kim et al in (2016) (33) who approved that during T2DM, this confirms the regulatory effects induced by NLRP3 on micro- and macro-vascular endothelial cell activities and links increased NLRP3 concentrations to vascular and endothelial dysfunctions in T2DM patients. Furthermore, García-Hernández and colleague reported that periodontitis in patients with T2DM, through the chronic inflammatory/infectious impact of gingival biofilm pathogen load, may have added to the negative stimulation used to control NLRP3 levels in saliva and blood (34). On the other hand, they stated that Patients with periodontitis who received periodontal maintenance therapy had a significant decrease in ASC and IL-1 levels in their peripheral blood mononuclear cells (35). Furthermore, the present study also shows a significant positive correlation between NLRP3 and periodontal parameters, and these results are consistent with Aral et al in (2019) (36) who found positive correlation between NLRP3 and clinical periodontal parameters. Altogether, these data indicate that NLRP3 plays a significant role during periodontal disease pathogenesis. Shahbeik et al., showed that the possible association between the level of NLRP3 and clinical parameters indicates that it is a potential predictor of periodontal tissue degeneration (37).The current study found a non-significant correlation between bacterial loads and NLRP3, and this was in agreement with (38) who observed that Lack of association between NLRP3 and bacterial plaque

load. These results could be attributed to that the Periodontitis is not an infection, but a dysbiotic disease caused by an imbalance in the polymicrobial population (39, 40). Besides, Yilmaz et al. reported that when it was exposed to subgingival bacteria, the inflammasome's activity decreases (41).

Conclusion:

The number of *A. actinomycetemcomitans* increased in patients with type 2 DM and its counts were significantly correlated with clinical parameters in these patients. Moreover, the elevation levels of NLRP3 in periodontitis patients with DM, and their positive correlation with clinical parameters, indicates a critical role in the pathogenesis of Periodontitis with and without diabetes. The lack of correlation between *A. actinomyctecomtans* and the inflammatory biomarkers indicates that NLRP3 activation is associated with inflammatory processes that are induced by a number of external factors, other than bacteria.

Conflict of interest: The authors have disclosed no potential conflicts of interest.

Authors' Contributions:

All authors contributed equally to the study.

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العلاقة بين كمية بكتريا ال *Aggregatibacter actinomycetemcomitans* وتفعيل الجسيم الملتهب NLRP3 في مرضى التهاب دواعم السن المصابين بداء السكري

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الخلاصة:

الخلفية العلمية: *Aggregatibacter actinomycetemcomitans* هو أحد أكثر مسببات أمراض اللثة شيوعًا وله تأثير مباشر على اللثة. يعتبر مرض السكري والتهاب دواعم السن من الأمراض المزمنة التي لها علاقة ثنائية الاتجاه بينهما. وقد أظهرت الأدلة أن الجسيم الملتهب NLRP3 مهم لكلا المرضين. هدف الدراسة: تم إجراء هذا البحث لاكتشاف العلاقة بين الحمل البكتيري للبكتيريا *Aggregatibacter actinomyctecomtans* ومستوى المصل من التهاب اللثة NLRP3 في مرضى التهاب دواعم السن مع وبدون النوع الثاني من مرض السكري ومجموعة الاصحاء. **المواد والطرق:** شملت الدراسة خمسة وثمانون شخصا، بمعدل اعمار(23 - 55) عاما. تم تقسيمهم الى ثلاث مجموعا ت: مجموعتان مصابة بالتهاب دواعم الاسنان احدى هذه المجموعتان مصابة بداء السكري من النوع الثاني بينما الاخرى بدون امراض جهازية مزمنة، والمجموعة الثالثة كانت ذات لثة صحية سريريا وتتمتع بصحة جيدة. تم تسجيل معايير اللثة السريرية) مؤشر البلاك، النزف عند التسبير, فحص عمق الجيب اللثوي ومقدار انحسار اللثة(تم جمع عينات الدم وصفيحات الاسنان الجرثومية من جميع المرضى والاصحاء. كمية الحمض النووي الخاص ببكتريا ال *Aggregatibacter Actinomyctecomtans*والمستخرج من عينات الصفيحات الجرثومية السنية يتم اجراؤها عن طريق تفاعل البلمرة المتسلسل – الوقت المتسلسل. في حين اجريت تقنية الفحص المناعي المرتبط بالانزيم لتقدير مستوى للجسيم الملتهب.

النتائج: أظهرت الدراسة ان عدد البكتريا كان اعلى في مجموعتي المرضى من مجموعة الاصحاء. ولكن لم تكن هناك فروق ذات دلالة احصائية. من ناحية اخرى كان هناك ارتباط معنوي بين العدد البكتيري ومعايير اللثة السريرية في مجموعة التهاب دواعم الاسنان ويعانون من داء السكري من النوع الثاني، بالإضافة الى ذلك اظهرت النتائج زيادة معنوية في مستويات المصل للجسيم الملتهب في مرضى التهاب دواعم الاسنان ولديهم النوع الثاني من داء السكري مقارنة بالمجموعات الاخرى. كما ولوحظ ان هناك ارتباط ايجابي بين الجسيم الملتهب وكل من (مؤشر اللثة, عمق الجيب اللثوي, مقدار انحسار اللثة والنزيف عند التسبير)في مرضى التهاب دواعم الاسنان المصابين بداء السكري من النوع الثاني. اما فيما يتعلق بمجموعة مرضى التهاب دواعم الاسنان غير المصابين بامراض جهازية مزمنة لم يكن هنالك ارتباط معنوي بين الجسيم الملتهب والمعايير السريرية للثة. **الاستنتاجات:** كان معدل الكشف عن بكتريا ال*Aggregatibacter Actinomyctecomtans* اعلى بكثير في العينات التي تم استخلاصها من مجموعتي المرضى مقارنة بالاشخاص الاصحاء لكن دون وجود دلالة احصائية. علاوة على ذلك ارتبطت اعدادها بالمعايير السريرية للثة في مجموعتي المرضى. كما وتم ملاحظة ارتفاع مستويات مصل الجسيم الملتهب في مرضى التهاب دواعم الاسنان المصابين بداء السكري من النوع الثاني وارتباطهم الايجابي بمعايير اللثة السريرية، نتيجة لدورها الفعال في التهاب دواعم الاسنان مع وبدون داء السكري.

الكلمات المفتاحية: : التهاب دواعم السن، مرض السكري من النوع الثاني، الجسيم الملتهب، بكتريا *Aggregatibacter actinomycetemcomitans*