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Interleukin-32 levels in gingival crevicular fluid and saliva of patients with chronic periodontitis after periodontal treatment

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Background and Objective: The cytokine, interleukin (IL)-32, is a relatively new discovery. However, it is very powerful for stimulating tumor necrosis factor-alpha (TNF-α) under inflammatory conditions. The objective of this research was to explore fluctuations in the levels of TNF-α, IL-32 and IL-10, in both saliva and gingival crevicular fluid. The focus was on measurements taken before and after clinical treatment of chronic periodontitis.

Material and Methods: For the purposes of the study, a total of 27 patients with chronic periodontitis and 27 controls (periodontally healthy) were recruited. Important clinical periodontal criteria were established before and 4 wk after the start of the research. The chronic periodontitis group was given an initial form of periodontal care. Samples of saliva and gingival crevicular fluid were collected exactly 4 wk preceding and 4 wk following the care. The levels of IL-10, IL-32 and TNF-α present in saliva and gingival crevicular fluid were recorded via the use of an ELISA.

Results: At baseline, the levels of TNF-α and IL-32 in the gingival crevicular fluid and saliva were significantly higher among patients in the chronic periodontitis group than among patients in the control group ($p < 0.05$). On the other hand, at baseline the levels of IL-10 were significantly lower in the gingival crevicular fluid and saliva of the chronic periodontitis group than the control group ($p < 0.05$). A significantly positive link was found between the TNF-α and IL-32 levels in the two study groups ($p < 0.05$). After treatment, the levels of TNF-α and IL-32 in saliva and gingival crevicular fluid were significantly lower in the chronic periodontitis group when compared with the baseline readings. However, the levels of IL-10 were significantly higher ($p < 0.05$).

Conclusion: Ultimately, the level of IL-32 present in saliva and gingival crevicular fluid might be useful as an indicator of the condition and the expectations for its treatment and care. According to the results of the research, the proinflammatory impact of IL-32 could potentially be linked to the intensity and progression of periodontitis.

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The degenerative condition, chronic periodontitis, takes the form of a bacterial inflammation in the mouth. As such, its clinical pathogenesis is defined by contact between the immune and inflammatory reactions of a host and the periodontal bacteria that cause it (1,2). The periodontal bacterium that is responsible for periodontitis – namely *Porphyromonas gingivalis* – turns on immunity-driven reactions in the host (3). These reactions are designed to safeguard cells against the impact of bacterial antigens (3). They lead to the accelerated production of a range of different cytokines, for example, interleukin (IL)-1, IL-8, IL-6 and tumor necrosis factor-alpha (TNF-α) (3). However, an imbalance between anti- and proinflammatory cytokines is likely to cause damage to collagen and bone tissue, particularly in the presence of periodontitis (4). In periodontitis, proinflammatory cytokines are important for the control and regulation of inflammation (5). Consequently, it is essential that clinical experts have the ability to recognize these control agents as they are an important part of calculating the effectiveness of therapy for patients with periodontitis (2).

One of the most influential cytokines is TNF-α (6). It is believed to play a key part in the pathogenesis of several serious and persistent inflammatory ailments, including periodontitis (6). It provides both an immunoregulatory and a proinflammatory contribution (6). Interestingly, a number of earlier studies have discussed the possibility that measurement of TNF-α levels, in saliva and gingival crevicular fluid, could be an effective way to test for periodontitis (5–7). Higher levels of TNF-α were observed within the saliva (6) and gingival crevicular fluid (5,7) of individuals diagnosed with periodontitis than in those with no diagnosis of this condition. Furthermore, the powerful anti-inflammatory cytokine, IL-10, might also play a part in the control and development of periodontal inflammation (1,4). This could be important when trying to treat and mitigate symptoms (1,4). The periodontitis group had lower levels of IL-10 in their saliva (8) and gingival crevicular fluid (9) compared with the healthy group.

One of the newest discoveries is the cytokine IL-32 (10,11). It has proinflammatory properties and was initially referred to by the name natural killer cell transcript 4 (NK4) (10,11). It is stimulated by activated T-lymphocytes and activated natural killer cells (10,11). IL-32 is produced by T-cells, epithelial cells, natural killer cells, monocytes and endothelial cells (12). A total of six isoforms of IL-32 (α, β, γ, δ, ε and ζ) may be created via the use of alternative mRNA splicing (13). Each isoform is produced by both nonimmune and immune cells (13). IL-32 also exerts a pleiotropic impact on the way in which cells operate (11). It stimulates the production of inflammatory agents and controls IL-6, prostaglandin E2 and TNF (14,15). It stimulates cell differentiation, production of anti- and proinflammatory cytokines and the destruction of cells (particularly as part of apoptosis) (14,15). It also stimulates the macrophage inflammatory protein-2 and the anti-inflammatory cytokine, IL-10, by interacting with p38 mitogen-activated protein kinase and nuclear factor-κB signaling channels (14,15). IL-32 is also believed to be a powerful stimulator of TNF-α (16). It is known to play a role in a number of inflammatory conditions. These include, but are not limited to, inflammatory bowel disease (17), atherosclerosis (18) and rheumatoid arthritis (RA) (16). IL-32 also stimulates the production of osteoclasts, without the need for autonomous RANKL generation (16).

Currently, there is a distinct lack of data on the association of periodontitis with TNF-α, IL-32 and IL-10. This means that the precise nature of the regulation of IL-32 production, throughout episodes of chronic periodontitis inflammation, is not yet clear. In earlier research, *P. gingivalis*-based lipopolysaccharide was found to significantly up-regulated the production of IL-32, in contrast with the same production for unprovoked cells in monocytes (also known as THP-1 cells) (19). The researchers argued that IL-32 could play a role in the pathogenesis of periodontitis, as the bacterium *P. gingivalis* is known to be strongly connected to progression of chronic periodontitis (19). However, in a different study it was discovered that the amount of IL-32 present in gingival tissue is lower among patients with chronic periodontitis than it is among periodontally healthy individuals (20).

In this study, it is theorized that the gingival crevicular fluid and/or saliva levels of TNF-α, IL-32 and IL-10 might be different in patients with chronic periodontitis than they are in periodontally healthy individuals. As such, the objectives of the research were clear. The first objective was to identify the role of IL-32 in the progression of chronic periodontitis. This was achieved by comparing and contrasting the levels of IL-10 and TNF-α in saliva and gingival crevicular fluid, in people with and without symptoms of chronic periodontitis. The second objective was to explore the impact of nonsurgical periodontal therapy on the levels of IL-32 in saliva and gingival crevicular fluid of individuals with chronic periodontitis. The implication is that a thorough comparison of the levels of newly discovered cytokines could be an appropriate way in which to further our understanding of the role of IL-32 in the progression of periodontitis.

Material and methods

Study population and study design

The research design for this particular study was fully assessed and verified by the Ethics Committee of the Faculty of Medicine, at Bülent Ecevit University, Zonguldak, Turkey. All ethical and design assessments were carried out in line with the 1975 Helsinki Declaration (updated in 2002). For more details on the research design, see Fig. 1. The research is registered with ClinicalTrials.gov and has the identification number, NCT02632981. A total of 54 study participants (28 women and 26 men) were selected after attending clinical appointments at the Periodontology Department of

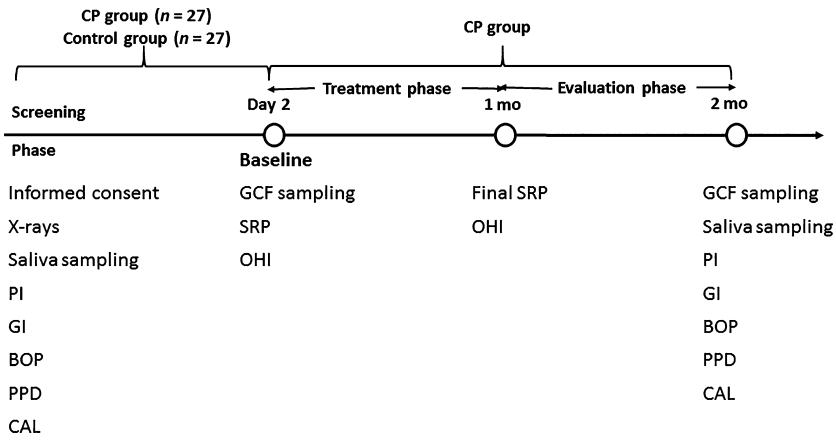

Fig. 1. Study design. BOP, bleeding on probing; CAL, clinical attachment loss; CP, chronic periodontitis; GCF, gingival crevicular fluid; GI, gingival index; OHI, oral health indicators; PI, periodontal index; PPD, periodontal probing depth; SRP, scaling and root planing.

Bulent Ecevit University (located in the Faculty of Dentistry) (Protocol ID: 2014-10-14/01). All of these individuals received either a clinical treatment or a routine dental examination, between February 2014 and October of the same year. They were all between 30 and 48 years of age. Each participant provided a written document of consent before taking part in the study.

Their respective diagnoses were made according to the severity of periodontal symptoms. The typical chronic periodontitis sufferer shows radiographic demonstrations of tissue attachment and bone loss. They have a gingival index (GI) rating of > 1 and at least six teeth with a probing pocket depth of ≥ 5 mm. Furthermore, the gums show bleeding on probing (BOP) within a minimum of two seperate regions. In the control group, the entire mouth probing pocket depth had to be ≤ 3 mm, with a GI rating of zero. This indicates a complete lack of clinical inflammation. Healthy participants showed no indications of clinical attachment loss and no radiographic demonstrations of alveolar bone degradation. This latter measurement is taken by recording the distance between the bone crest and the cementoenamel junction. In a healthy mouth, this distance is < 3 mm at $> 95\%$ of the surrounding tooth regions. All participants in the study – regardless of their assigned study group – had to have a minimum of 20 teeth, not including teeth with severe decay or wisdom teeth. After recruitment, the participants were allocated to one of two categories: those with generalized chronic periodontitis ($n = 27$; 14 men and 13 women; 39.44 ± 3.15 years of age); and those with a clinically healthy periodontium (control; $n = 27$; 12 men and 15 women; 37.30 ± 3.80 years of age). The industry guide for such diagnoses is the 1999 International World Workshop for the Classification of Periodontal Diseases and Conditions (21).

Participants were only selected if they had mild to moderate chronic periodontitis (those with severe chronic periodontitis were not eligible to participate in the study). Individuals with periapical symptoms were also deemed unsuitable. Additional exclusion criteria were: other systemic conditions; mechanical forces resulting from occlusal and orthodontic forces; and treatment with noninflammatory and nonsteroidal medicines or antibiotics within the 6-mo period preceding the study. Individuals who had been given invasive chronic periodontitis care within the year leading up to the study were also excluded. Other eligibility criteria were a requirement for antibiotic prophylaxis (as part of dental care), pregnancy, allergies to medication, breastfeeding and smoking.

Clinical measurements and intra-examiner reproducibility

The participants underwent a clinical assessment. The aforementioned rules and guidelines for diagnosis were used. The test was carried out using a Williams Periodontal Probe (Hu-Friedy, Chicago, IL, USA) device. These tools take measurements in millimeters. They also calculate clinical attachment loss, probing pocket depth, plaque index (PI) (22), GI (23) and BOP (24). The same investigator (S.B.D.), who was blinded with respect to the study design, carried out every clinical test. This included collection of gingival crevicular fluid and saliva, selection of sample site and assignment of patients to the appropriate study group. A total of six tooth regions were investigated: midlingual, distobuccal, mesiolingual, mesiobuccal, midbuccal and distolingual. Before the readings were taken, 10 participants were randomly chosen and used to calibrate the researcher. The researcher evaluated all clinical measurements on two distinct occasions with 48 h apart. Calibration of the researcher was deemed to be admissible if two sets of measurements were $> 90\%$ similar at the millimetre level (25).

Periodontal treatment

The subjects with chronic periodontitis were given nonsurgical periodontal therapy. This therapy was delivered on a number of occasions, according to the severity of the diagnosis. All treatments were delivered by the same individual (S.B.D.) responsible for the initial and subsequent readings. The treatments involved instruction in high-quality cleaning and hygiene, complete scaling and root planing (across all four tooth regions) and the mitigation and tracking of dental conditions. The clinical measurements and saliva and gingival crevicular fluid samples were taken again for the subjects with chronic periodontitis, around 4 wk after the last scaling and

root planing session. The average participant with chronic periodontitis attended a total of four care appointments over the course of a month. All study participants (chronic periodontitis and healthy) were encouraged to perform, and guided in, regular plaque control, every day.

Collection of samples

The sampling site selections and the radiological and clinical assessments were all carried out by a single person (S.B.D.). To stop blood, stimulated by the probing of inflamed regions, from compromising gingival crevicular fluid samples, the samples of gingival crevicular fluid were collected 48 h after the clinical tests, after taking saliva samples from the sampling sites and after selection of the sampling sites. The gingival crevicular fluid samples were gathered from the mesiobuccal region on every tooth (molars, premolars and canines/incisors). In total, three samples were taken from every participant. Among the chronic periodontitis group, the samples were gathered from subjects at regions with 30% bone loss, ≥ 6 mm probing pocket depth and ≥ 5 mm clinical attachment loss. Among the control group, gingival crevicular fluid samples were collected from the teeth that demonstrated probing pocket depth < 3 mm, but did not present BOP or clinical attachment loss. To retrieve a sample, the targeted region was first sectioned off using cotton buds. Then, it was allowed to dry naturally, before paper indicators were used to take the sample. The paper strips (Periopaper; Ora Flow Inc., Amityville, NY, USA) were positioned inside the crevice until a small degree of resistance (or 'push') was experienced. Then, the paper was left in place for a further 30 s. This is what is known as the 'intracrevicular' technique (26). The amount of gingival crevicular fluid present on the strips was determined carefully with the use of measuring tools. The fluid thus collected was weighed. The paper strips were then put into clearly labeled and sealed plastic microcentrifuge containers and weighing was instantly repeated after the collection to clarify any evaporation (27). Any paper indicators compromised with saliva or blood were eliminated from the study. The gingival crevicular fluid samples were collected at baseline and again 4 wk after the final care session for the participants with chronic periodontitis. For the healthy participants, only baseline measures were made. All three samples, from every study subject, were put into a single disposable container. They were then combined to form a single large sample (28). Finally, the containers were stored at a temperature of −40°C until required for assessment and evaluation.

Before any clinical assessment could be carried out, a whole saliva sample was taken. This involved swilling and cleaning the mouth using distilled water. Then, the unstimulated saliva samples were collected by instructing the participants to spit into a plastic container (29). Once collected, a volume of 2 mL of saliva was instantly placed in a centrifuge tube and centrifuged (10,000 ***g***, 10 min, 4°C). This process is designed to eliminate cell debris. The remaining supernatants (50 μL each) were stored at −40°C until the next phase of the study was due to begin.

Biochemical analysis

For biochemical analysis, 400 μL of phosphate-buffered saline (pH 7.4) was added to every container filled with participant samples. Following this, the containers were homogenized and vortexed for approximately 60 s. They were then centrifuged (15 min, 3000 ***g***, 4°C). Afterwards , the supernatants were collected. The levels of TNF-α, IL-10 (Boster, Pleasanton, CA, USA) and IL-32 (BlueGene, Shanghai, China) present in gingival crevicular fluid and saliva could then be analysed. The readings were recorded in picograms. This analysis was achieved with the use of an ELISA. This equipment is widely available and can be accessed by anyone who is interested in analysing clinical samples. The maker of the assay kit advises users to test samples in duplicate, so this is what was done. The calibration span for IL-32 was as much as 5000 pg/mL, with an analytical sensitivity of 1 pg/mL. The within- and total-run coefficient of variation outcomes were $< 10\%$. For the TNF assessment, the calibration parameter was as high as 1000 pg/mL, with an analytical sensitivity of 1 pg/mL. The within- and total-run coefficient of variation outcomes were $< 5\%$ and $< 10\%$, respectively. The calibration parameter for the IL-10 assessment was as high as 500 pg/mL, with an analytical sensitivity of 0.5 pg/mL. The within- and total-run coefficient of variation outcomes were $< 5\%$ and $< 10\%$, respectively. The concentrations of TNF-α, IL-32 and IL-10 (pg) in gingival crevicular fluid were determined by dividing the total amount of TNF-α, IL-32 and IL-10 by the volume of gingival crevicular fluid (microliters). The measurements of concentration are presented in the form of picograms per microliter (pg/μL).

Statistical analyses

The sample-size calculations were formulated using the primary outcome variables (gingival crevicular fluid IL-32 levels). All predictions were made according to the results of pilot research. This initial research involved a total of 10 participants, from both the control and the chronic periodontitis categories. It was predicted that a sample population of 21 participants (42 in total) would be sufficient to provide a type I error reading of $\alpha = 0.05$ (5% probability) and a type II error reading of $\beta = 0.20$ (80% power).

Every statistical analysis or assessment involved in this research was carried out with the support of a sophisticated statistical program (SPSS version 19.0; SPSS Inc., Chicago, IL, USA). For all tests, a reading of $p < 0.05$ is deemed to be statistically notable and worthy of further investigation and consideration. As a way to verify and confirm that the results were accurate, a Shapiro–Wilk assessment was carried out. Following this, comparisons

between the clinical and biochemical criteria were evaluated with the use of a Mann–Whitney *U* nonparametric assessment. This was conducted after the normality measure for the outcomes had failed to reach the required level. A Wilcoxon signed-rank assessment (designed for dual readings) was carried out as a way to compare the baseline readings with the measurements recorded after treatment. Comprehensive chi-square analysis was an effective way to contrast BOP proportions with the spread of genders across the two study categories. Finally, a Spearman rank correlation assessment was conducted to identify the relationships of correlations shared by the levels of TNF-α, IL-32 and IL-10 with clinical periodontal parameters.

Results

Clinical findings

Refer to Tables 1 and 2 for a full breakdown of the clinical results. The findings make it clear that the distribution of gender and age has no notable impact on the results; neither for participants with chronic periodontitis nor for control subjects ($p = 0.067$). The BOP, GI, probing pocket depth, PI and clinical attachment loss measures for the whole mouth and for samples from individual regions were significantly higher among the chronic periodontitis group than among the control subjects ($p < 0.001$). It was predicted that, following targeted care, the clinical measures and symptoms would present a marked decline, and this was observed ($p < 0.001$).

Biochemical findings

The levels of saliva and the total amount and concentration of IL-32 in gingival crevicular fluid were both significantly higher among the chronic periodontitis group than the control group, at baseline ($p < 0.001$). However, the levels of IL-32 in the participants with chronic periodontitis fell following periodontal therapy, particularly in comparison with the baseline

Table 1. Full-mouth periodontal examination at baseline and after treatment in the study groups

	Baseline					After treatment				
Group	PPD[a] (mm)	CAL[a] (mm)	GI[a]	PI[a]	BOP[a] (%)	PPD[b] (mm)	CAL[b] (mm)	GI[b]	PI[b]	BOP[b] (%)
Chronic periodontitis ($n = 27$)	3.31 (2.92–4.47)	3.57 (3.04–4.68)	1.77 (1.11–2.43)	1.70 (0.90–2.43)	68.00 (34.57–90.35)	2.11 (1.43–3.00)	2.41 (1.69–3.29)	0.43 (0.22–1.10)	0.44 (0.21–0.83)	15.94 (4.67–46.16)
Controls ($n = 27$)	1.43 (1.37–1.78)	1.43 (1.37–1.78)	0.18 (0.00–0.68)	0.33 (0.00–0.82)	0.00 (0.00–0.00)					

Data are expressed as median (range: minimum–maximum).
[a]Significant difference between groups, $p < 0.05$. (Mann–Whitney *U* nonparametric test.)
[b]Statistically significant difference from baseline, $p < 0.05$. (Wilcoxon signed-rank test.)
BOP, bleeding on probing; CAL, clinical attachment loss; GI, gingival index; PI, periodontal index; PPD, periodontal probing depth.

Table 2. Periodontal examination of sample sites at baseline and after treatment in the study groups

	Baseline					After treatment				
Group	PPD[a] (mm)	CAL[a] (mm)	GI[a]	PI[a]	BOP[a] (%)	PPD[b] (mm)	CAL[b] (mm)	GI[b]	PI[b]	BOP[b] (%)
Chronic periodontitis ($n = 27$)	6.00 (5.00–7.00)	6.67 (5.33–7.67)	2.00 (2.00–3.00)	2.00 (1.00–3.00)	100.00 (100.00–100.00)	3.00 (2.00–3.00)	3.67 (3.00–4.33)	1.00 (0.33–1.33)	0.83 (0.33–1.33)	0.00 (0.00–33.33)
Controls ($n = 27$)	1.67 (1.33–2.33)	1.67 (1.33–2.33)	0.00 (0.00–0.00)	0.33 (0.00–1.00)	0.00 (0.00–0.00)					

Data are expressed as median (range: minimum–maximum).
[a]Significant difference between groups, $p < 0.05$. (Mann–Whitney *U* nonparametric test.)
[b]Statistically significant difference from baseline, $p < 0.05$. (Wilcoxon signed-rank test.)
BOP, bleeding on probing; CAL, clinical attachment loss; GI, gingival index; PI, periodontal index; PPD, periodontal probing depth.

measures ($p < 0.001$). The levels of saliva and the total amount and concentration of TNF-α in gingival crevicular fluid were also significantly higher among the chronic periodontitis group than the control group, at baseline ($p < 0.001$). There was a statistically notable decrease of TNF-α levels in saliva and gingival crevicular fluid among the chronic periodontitis group following targeted treatment and, particularly, when compared with baseline readings ($p < 0.001$). The levels of saliva and the total amount and concentration of IL-10 in gingival crevicular fluid were significantly lower among the chronic periodontitis group than the control group, at baseline ($p < 0.001$). However, the readings after treatment were higher compared with the baseline measures ($p < 0.001$). For more details, refer to the total amounts and concentrations of gingival crevicular fluid TNF-α, IL-10 and IL-32, which are clearly presented in Tables 3 and 4. Also, see Table 5 for the salivary levels of TNF-α, IL-10 and IL-32.

Correlations

For the chronic periodontitis group, there was a significantly positive link between IL-32 and TNF-α ($p < 0.05$). However, there was a clearly negative connection shared by IL-10 and TNF-α ($p < 0.05$). Both IL-32 and TNF-α shared a positive link with GI and clinical attachment loss, among the chronic periodontitis group ($p < 0.05$). When all clinical subjects were analysed as one group, IL-32 exhibited a statistically notable and positive link with clinical attachment loss, GI and TNF-α ($p < 0.05$). On the other hand, IL-10 shared a negative link with these same values ($p < 0.05$). See Tables 6 and 7 for more details on the correlation coefficients.

Among the participants with chronic periodontitis, there was a notably positive link between the number of regions with probing pocket depth ≥ 6 mm or 4–5 mm and TNF-α ($p < 0.05$). On the other hand, IL-32 was positively linked only with probing pocket depths of ≥ 6 mm ($p < 0.05$).

Discussion

Within the development and progression of periodontal disease, it is clear that cytokines make an important contribution. Yet, the precise nature of this contribution is not quite as transparent. Specifically, the individual roles of separate cytokines remain a little unclear. There needs to be much more research into the role that cytokines, such as IL-32, play in the progression of periodontal disease. This research explored IL-32 levels in the saliva and gingival crevicular fluid of participants with a diagnosis of chronic periodontitis. It also examined the shifts across these levels following nonsurgical targeted treatments. The study compared the levels of IL-32 in saliva and gingival crevicular fluid with the levels of TNF-α. This is because earlier studies have shown that TNF-α has a predictable impact on the development of periodontitis (5). It also compared the levels of IL-32 with the levels of IL-10, as the latter is an established and powerful anti-inflammatory agent during periodontal episodes (9). The findings of the research demonstrate that the levels of IL-32 in saliva and gingival crevicular fluid are substantially higher in people with chronic periodontitis than they are in a non-periodontitis control group. However, the levels drop dramatically if nonsurgical periodontal therapy is provided. For both study categories, the levels of TNF-α and IL-32 were found to be positively correlated.

For this research, both whole unstimulated saliva and gingival crevicular fluid samples were collected. It is possible that biochemical evaluations of gingival crevicular fluid could be used to identify tissue developments across the periodontium (5,10). They may also be helpful for improving our understanding of the pathogenesis that defines periodontal disease (5,10). The collection of gingival crevicular fluid samples is a nonintrusive process (30). It is fast, simple and pain free (30). Furthermore, it can be achieved in around 30 s, with nothing more than paper strips (30). In earlier studies, the total amount of cytokines present in gingival crevicular fluid, as opposed to their concentration, is presented as potentially more influential for disease progression (30). As such, this research was structured primarily around the total amount of gingival crevicular fluid. It is important to note that the individual gingival crevicular fluid samples (three for each study subject) used in this research were later combined. This move was designed to make it easier to identify all of the biochemical signs present in all of the participants. The combined samples may also be valuable for periodontitis diagnoses and examinations in the future. The downside to this method is the fact that, while the merged samples can demonstrate the overall periodontal status of the study subject, they cannot do the same for each single tooth region that was tested and sampled. A number of researchers have, in the past, chosen to utilize saliva as their diagnostic liquid (6,8). This is because it can be used to identify various agents and stimulators of tissue degradation and persistent inflammation among patients with chronic periodontitis. Plus, there is the added benefit of easy access and availability. Saliva is always there and can always be sampled. It can be collected in a nonintrusive manner and no sophisticated tools or devices are required. The only obstacle is the tendency of probing and touching to actually raise the volume of saliva to an abnormal level (31). If the volume is abnormal, a representative and accurate sample cannot be obtained (31). To avoid this, completely unstimulated saliva samples were collected in the present study. Evaluation of the saliva samples helped to establish valuable insights into all the regions tested in the mouth (even the inactive regions). In addition, the biochemical assessment of gingival crevicular fluid provided comprehensive information on the molecular indicators found in the periodontal pockets sampled (5).

Within earlier research, both the total amount and concentration of TNF-α in gingival crevicular fluid were found to be substantially higher

Table 3. Total amounts of tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10) and interleukin-32 (IL-32) in gingival crevicular fluid, at baseline and after treatment

	Baseline			After treatment		
Group	TNF-α[a]	IL-10[a]	IL-32[a]	TNF-α[b]	IL-10[b]	IL-32[b]
Chronic periodontitis (n = 27)	22.27 (9.55–42.78)	2.60 (1.32–10.58)	29.78 (15.35–46.99)	5.48 (2.48–10.25)	21.11 (13.86–50.89)	12.11 (6.46–26.95)
Controls (n = 27)	5.00 (2.34–11.72)	31.98 (8.18–49.52)	4.04 (2.42–9.34)			

Data are given as pg/site and are expressed as median (range: minimum–maximum).
[a]Significant difference between groups, $p < 0.05$. (Mann–Whitney U nonparametric test.)
[b]Statistically significant difference from baseline, $p < 0.05$. (Wilcoxon signed-rank test.)

Table 4. Concentrations of tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10) and interleukin-32 (IL-32) in gingival crevicular fluid, at baseline and after treatment

	Baseline			After treatment		
Group	TNF-α[a]	IL-10[a]	IL-32[a]	TNF-α[b]	IL-10[b]	IL-32[b]
Chronic periodontitis (n = 27)	28.23 (11.74–59.29)	2.35 (1.18–11.52)	56.53 (28.48–92.87)	17.25 (4.59–35.23)	37.38 (21.86–102.61)	39.11 (20.97–82.91)
Controls (n = 27)	10.72 (4.26–26.36)	58.85 (13.54–100.54)	13.14 (7.71–36.48)			

Data are given as pg/μL and are expressed as median (range: minimum–maximum).
[a]Significant difference between groups, $p < 0.05$. (Mann–Whitney U nonparametric test.)
[b]Statistically significant difference from baseline, $p < 0.05$. (Wilcoxon signed-rank test.)

Table 5. Salivary levels of tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10) and interleukin-32 (IL-32), at baseline and after treatment

	Baseline			After treatment		
Group	TNF-α[a]	IL-10[a]	IL-32[a]	TNF-α[b]	IL-10[b]	IL-32[b]
Chronic periodontitis (n = 27)	45.00 (34.00–88.00)	12.40 (8.38–34.00)	511.38 (227.02–857.22)	11.80 (8.30–25.00)	47.30 (35.76–84.00)	197.79 (105.94–378.29)
Controls (n = 27)	18.00 (11.20–26.80)	82.00 (42.50–140.30)	227.02 (103.22–324.92)			

Data are given as pg/mL and are expressed as median (range: minimum–maximum).
[a]Significant difference between groups, $p < 0.05$. (Mann–Whitney U nonparametric test.)
[b]Statistically significant difference from baseline, $p < 0.05$. (Wilcoxon signed-rank test.)

in individuals diagnosed with periodontal disease than those in the control group (7). The total TNF-α levels were positively linked with every one of the clinical parameters and measures (7). The total amount of TNF-α was discovered to be notably higher within the gingival crevicular fluid of affected (but untreated) disease regions than in the nonaffected regions of those without the condition (2). Statistically significant reductions in the total amount of gingival crevicular fluid TNF-α were observed among the chronic periodontitis group, following nonsurgical periodontal therapy, particularly when compared with baseline readings (32). For this research, the total amount and concentration of TNF-α in gingival crevicular fluid were both notably higher among the chronic periodontitis group than the control group. Plus, the findings show that the levels of saliva-based TNF-α were notably larger among the chronic periodontitis group than among the control subjects. This is important because the discovery supports the results reported in previous research (6). Furthermore, there was a strongly positive link shared by the GI and clinical attachment loss and TNF-α for both groups. This finding may also be used to support the discoveries made by earlier researchers (2,6,32), particularly regarding the idea that TNF-α levels in saliva and gingival crevicular fluid are valuable for analyzing and understanding the pathophysiological mechanisms involved in periodontal disease and the consequences of relevance after therapy.

A number of researchers observed that the levels of IL-10 in gingival crevicular fluid are lower among

Table 6. Spearman's rank correlation (ρ) among groups for the total amount of tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10) and interleukin-10 (IL-32), and sample site clinical attachment loss (CAL) and gingival index (GI)

Group	TNF-α/IL-10	TNF-α/IL-32	IL-10/IL-32	TNF-α/CAL	TNF-α/GI	IL-10/CAL	IL-10/GI	IL-32/CAL	IL-32/GI
Chronic periodontitis (n = 27)									
r	−0.394*	0.397*	−0.363	0.549*	0.485*	−0.485*	−0.350	0.502*	0.394*
p	0.042*	0.040*	0.063	0.003*	0.010*	0.010*	0.074	0.008*	0.042*
Controls (n = 27)									
r	−0.327	0.395*	−0.361	0.414*	NA	−0.201	NA	0.350	NA
p	0.096	0.042*	0.065	0.032*	NA	0.315	NA	0.074	NA
All groups (n = 54)									
r	−0.825*	0.847*	−0.835*	0.871*	0.869*	−0.832*	−0.846*	0.857*	0.862*
p	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

NA, not applicable.
*Statistically significant ($p < 0.05$).

Table 7. Spearman's rank correlation (ρ) among groups with respect to tumour necrosis factor-α (TNF-α), interleukin-10 (IL-10) and interleukin-32 (IL-32) and number of deep sites in the chronic periodontitis group

Group	TNF-α/PPD 4–5	TNF-α/PPD ≥ 6	IL-10/PPD 4–5	IL-10/PPD ≥ 6	IL-32/PPD 4–5	IL-32/PPD ≥ 6
Chronic periodontitis (n = 27)						
r	0.403*	0.462*	−0.237	−0.356	0.331	0.385*
p	0.037*	0.015*	0.235	0.069	0.092	0.047*

*Statistically significant ($p < 0.05$).

subjects with chronic periodontitis than in control subjects (4,9). They argue that the reduced levels could potentially contribute to the stimulation and development of inflammatory responses throughout chronic periodontitis episodes (33). Within this research, the total amount and concentration of IL-10 in the gingival crevicular fluid of subjects with chronic periodontitis were notably smaller than they were for control subjects. Crucially, the levels did rise following targeted treatment, particularly when compared with baseline values. Some recent research shows that salivary levels of IL-10 are lower in subjects with chronic periodontitis than they are in control subjects (8,34). In addition, a rise in salivary IL-10 levels was demonstrated when tested again, around 6 wk after a targeted scaling and root planing treatment (particularly when compared with baseline readings) (8). Moreover, negative links were identified between probing pocket depth, BOP, clinical attachment loss and IL-10 (34). Ultimately, the findings of the present research are very similar, and have much in common with the results of previous studies (8,34). Together, they make it clear that gingival crevicular fluid and saliva samples are a valuable diagnostic tool for understanding the impact of IL-10 levels.

Research has shown that the level of IL-32 is lower among the gingival tissues of subjects with chronic periodontitis than among control subjects (20). This same research also argued that limiting the production of IL-8 within human gingival fibroblasts – IL-8 is stimulated by *P. gingivalis* and down-regulated by the expression of every *IL32* mRNA isoform – leads to enhancement of these anti-inflammatory qualities. Unfortunately, the relatively small number of participants meant that it was not possible, in this study, to perform an accurate comparison of the differences between healthy and chronic periodontitis tissues regarding the influence of cytokines on gene transcription or of single-nucleotide polymorphisms. However, IL-32 does appear to have a clear anti-inflammatory impact on healthy gingival tissues (20). In contrast, other research investigated inflammation of the airways and discovered that TNF-α substantially up-regulates the production and amounts of IL-32α, β, γ and δ within human lung fibroblasts (15). According to Ouhara *et al*. (20), this conflicting view could be explained by the influence of bacterial variables. They may have an impact on human gingival fibroblasts and limit the formation of IL-32 after creating a tool for lowering the production of *IL32* mRNA. It is possible that IL-10 might then try to change this process and influence to the bacteria-mediated suppression in the IL-32 creation via human gingival fibroblasts. It may also be the case that there are two key types of proinflammatory catalyst (or tool) and that the IL-32 receptors are those that send out anti-inflammatory indicators to try to stimulate these interactions. There is compelling evidence then to prove that IL-32 has proinflammatory qualities, in response to a number of different conditions (15–18,35).

The results show that the levels of IL-32 in saliva and gingival crevicular fluid are notably higher among subjects with chronic periodontitis than among control subjects. These levels of IL-32, however, decreased substantially following targeted treatment for the condition, particularly when compared with baseline readings. Plus, the saliva-based levels of IL-32 among

the chronic periodontitis group, following treatment, were lower than the levels of the control group. We also discovered a positive link between GI, clinical attachment loss and IL-32 among the subjects in the chronic periodontitis group. It is worth mentioning the strong correlation between periodontitis and salivary levels of IL-32. Earlier studies provide evidence that IL-32 is substantially up-regulated within the oral epithelial cell line, H400, by periodontal bacteria (*Fusobacterium nucleatum* and *P. gingivalis*) (36). Alternatively, it was argued, in different research, that IL-32 is produced by monocytes in response to *P. gingivalis* lipopolysaccharide (19). They also claimed that IL-32 probably plays a part in the progression of periodontitis (19). In light of these discoveries (19,36), it could be theorized that IL-32 functions as a proinflammatory cytokine within the saliva and gingival crevicular fluid of individuals with and without symptoms of the condition. Thus far, apart from this research, there has been no other attempt to investigate the saliva and gingival crevicular fluid levels of IL-32 and the probable consequences of nonsurgical periodontal therapy on these specific amounts of the cytokine.

It is well known that proteinase 3 (PR3) has a substantial contribution to periodontal inflammation (37). It is an important IL-32α binding protein, regardless of the rest of its enzymatic functions (38). According to Kim *et al.* (39), varying functions of IL-32 control – each leading to a precise consequence of PR3 – can develop in different people. Therefore, the dissimilarities in the results found by Ouhara *et al.* (20) and the results of this research could be explained by the varying functions of IL-32 isoform regulation via PR3 and also the binding and cleavage of IL-32. The restricted cleavage of IL-32α, via the use of PR3, accelerates and strengthens the functions of this cytokine (38). The prolonged cleavage of IL-32, when driven by PR3, actually results in the destruction of this cytokine (38). Ultimately, the targeted reduction of PR3, particularly regarding its influence on IL-32 (for creation or control, via the use of inactive PR3 and related fragments) probably weakens the impact of IL-32 on the progression of immune-based conditions (38).

A number of researchers have shown that IL-32 is very powerful for stimulating the production of TNF-α throughout inflammatory episodes (especially RA) (10,15,40,41). The studies demonstrated high levels of IL-32 and TNF-α in peripheral blood (40) and synovial tissues (10) of patients with RA, particularly when compared with healthy controls. Also, TNF-α resistance treatments among subjects with RA actually lowered the production of IL-32 in the synovial tissues (10,41). If considered in tandem, the findings indicate that IL-32 shares a close relationship with TNF-α. They also suggest that it plays a role in the stimulation and progression of TNF-α-based inflammatory arthritis. However, according to Moon *et al.* (16), IL-17 has an impact on the production of IL-32 within the fibroblast-like synoviocytes of subjects with RA. They also found that IL-17 and IL-32 play closely related and similar roles in disease pathogenesis. According to another earlier study, IL-17 plays a valuable role in the synergistic and additive influences created by TNF-α, among subjects with RA (42). As demonstrated by one researcher, there is a strong link between TNF-α and IL-17 within the saliva of individuals diagnosed with Type 2 diabetes mellitus (43). This research showed a substantially positive link between TNF-α and IL-32, within saliva and gingival crevicular fluid. It also offered evidence of a strongly negative link between IL-10 and IL-32, within the saliva and gingival crevicular fluid of both study categories. The IL-32 and TNF-α within the saliva and gingival crevicular fluid were substantially and positively linked with probing pocket depth of ≥ 6 mm, for the chronic periodontitis group. Ultimately, periodontitis, as an inflammatory condition, and RA exhibit closely related pathological qualities, for example, cell invasion, the production of inflammatory agents and controls in weakened spots, and accelerated tissue degradation (44). They also possess quite similar cytokine structures and mechanisms; for instance, high levels of proinflammatory cytokines (TNF-α) and lower levels of anti-inflammatory cytokines (IL-10) (32).

Therefore, it is likely that IL-32 contributes to the progression of periodontitis via accelerated and increased levels of TNF-α. It is just as likely that IL-17 is the clear and immutable connection shared by TNF-α and IL-32 among subjects diagnosed with periodontitis. For the research to be truly comprehensive, there needs to be a more in-depth exploration of the interaction functions between IL-32 and TNF-α. This communication must be better understood if peridontal treatments are to be refined and improved in the future. According to earlier research, IL-32β up-regulates the production of IL-10 because it controls and regulates the creation/function of protein kinase C within myeloid cells. In addition, IL-32δ reduces the production of this particular cytokine, along a dose incremental system, within U937 cells (14). In view of these findings, the contribution and impact of IL-32 on peridontal disease is probably demonstrated most efficiently by exploring the individual contributions of the various isoforms. The amounts of IL-32 and TNF-α in blood, and the levels of IL-17, were not investigated in this study. This is a clear downside to the research design and it may potentially limit the applicability of the findings.

To summarize, the findings of this research indicate that the proinflammatory impact of IL-32 is likely to be linked with the progression and intensity of periodontitis. However, nonsurgical periodontal therapy for the treatment of periodontitis inflammation can substantially reduce the levels of IL-32 in subjects with chronic periodontitis. This implies that the amounts of IL-32 are linked to the intensity and progression of the disease. The results demonstrate that the amount of IL-32 present in saliva and gingival crevicular fluid could be a

valuable tool for identifying the presence of periodontal disease. On the other hand, IL-32 has no less than six separate subtypes. The precise contribution of these subtypes is not yet clear. Further research should utilize knockout and knockdown techniques on IL-32 and the associated cytokines, both *in vivo* and *in vitro*, to provide a better understanding of how IL-32 contributes to the development of chronic periodontitis. The hope is that such studies will lead to the discovery of new approaches to treatment. If future research is carried out, it will need to use much larger sample sizes and a broader selection of subject types (for example, smokers). There is also a need to gain a clearer understanding of the link shared by the production of IL-32 and the progression of chronic periodontitis inflammatory episodes.

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