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ت	فهرس البحوث	الصفحة
1	**The Relationship Between Periodontitis Severity and MCP-1, IL-6 Levels in Gingival Crevicular Fluid** Mohammed Faisal Ali Ghada Ibrahim Taha	15 – 1
2	**Organizational Reflection and Its Impact on Strategic Performance: An Analytical Research in the General Company for Electrical and Electronic Industries** Ayman Abdul Sattar Jasim Aamer Fadous Azib Al-Lami	29 – 16
3	**Convolutional Neural Networks in Detection of Plant Diseases** Shaymaa Adnan Abdulrahman	42 - 30
4	**Gestural and Facial Expression Feedback in Motivating EFL Learners to Learn Grammar** Inas Kamal Yaseen	57 - 43
5	**The Effect of Titanium Oxide Nanotubes on the Surface Hardness of a Three-Dimensional Printed Denture Base Material** Anwr Hasan Mhaibes Ihab Nabeel Safi	67 - 58
6	**Myofunctional Appliance for Class III Malocclusion: A review** Maryam S. Al-Yasari Layth M. Kareem Ihab N. Safi Mustafa S. Tukmachi Zahra S. Naji	81 - 68
7	**The Intertwined Trajectory between Gender and Psychic Anxiety in Chimamanda Ngozi Adichie's Americanah** Tahseen Ali Mhodar Hayder Ali Abdulhasan	91 - 82
8	**The Role of Digital Human Resource Management Practices in Achieving Employee Well-being: An Analytical Study within the Civil Aviation Authority** Ayman Kadhum Al-Qaraghouli Ali Hasson Al-Taee Sinan Fadhel Hamad	104 - 92
9	**Employing the Frontload Vocabulary Strategies in Enhancing Iraqi EFL Students' Vocabulary Retrieval Abilities** Aswan Fakhir Jasim	113 - 105
10	**Assessment of the surface hardness of high-impact polymethylmethacrylate following long-term dipping in clove oil solution** Karrar Salah Al-Khafagi Wasmaa Sadik Mahmood	122 - 114
11	**Improved Machine Learning Techniques for Precise DoS Attack Forecasting in Cloud Security** Yasir Mahmood Younus Ahmed Salman Ibraheem Murteza Hanoon Tuama wahhab Muslim mashloosh	133 - 123
12	**The Impact of Using Menus Strategy on the Performance of Iraqi University Students in English as a Foreign Language in Writing Composition** Ansam Ali Sadeq	147 - 134
13	**Attitudes of students in the Department of General Science in the College of Basic Education towards electronic tests** Shaimaa Jasim Mohammed	167 - 148

188 - 168	**The Systemic Heterogeneity in Adnan Al-Sayegh's Poetry – with Reference to Group (Text Dice)** Abdulrahman Abdullah Ahmed	**14**
198 - 189	**Legal Means Employed by the Iraqi and French Legislators to Deter Abuse of Office: A Comparative Study** Mahdi Khaghani Isfahani Jaafar Shakir Hussein	**15**
208 - 199	**Challenges of the social and structural identity in the Middle East (Iraq as a model)** Yousif Radhi Kadhim	**16**
226 - 209	**Evaluation of the Susceptibility of some Eggplant Varieties and the Role of Their Biochemical Compounds in Resistance to the Leafhopper *Amrasca biguttula*** Fayroz T. Lafta Aqeel Alyousuf Hayat Mohammed Ridhe Mahdi	**17**
241 - 227	**The Effect of Using Modern Technologies on the Interaction of Middle School Students in Geography** Ali Fakhir Hamid	**18**
252 - 242	**Narrative themes in the papers of Atyaf Sanidah, the novel (I may be me) as a mode** Raad Huwair Suwailem	**19**
272 - 253	**The Role of Composing the Soundtrack for the Dramatic Film (Psychopath): An Analytical Study** Seerwan Mohammad Mustafa Abdulnaser Mustafa Ibrahim	**20**
287 - 273	**The psychological connotations of poetic images in the poetry of Rahim Al-Gharabawi** Salam Radi Jassim Al-Amiri Mehdi Nasseri Haider Mahallati	**21**
311 - 288	**Pedagogical Knowledge Competencies Among Students/Teachers in the Mathematics Department and Their Relationship to Professional Motivation** Duha Hamel Hussei Haider Abdel Zahra Alwan	**22**
323 - 312	**Derivatives in douaa Alahad: a semantic morphologicalstudy** Zahraa shehab Ahmed	**23**
330 - 324	**The effect of biological control agents in controlling the larval stages of *Spodoptera littoralis* in Basra Governorate** Zahraa J. Khadim and Ali Zachi Abdulqader	**24**

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The Relationship Between Periodontitis Severity and MCP-1, IL-6 Levels in Gingival Crevicular Fluid

Mohammed Faisal Ali[1], Ghada Ibrahim Taha[2]*

[1]Department of Basic Sciences, College of Dentistry, University of Baghdad, Baghdad, Iraq. Email: mohammed.ali2307m@codental.uobaghdad.edu.iq
https://orcid.org/0009-0006-3772-7758

[2]Department of Basic Sciences, College of Dentistry, University of Baghdad, Baghdad, Iraq. Email: ghada_ibraheem@codental.uobaghdad.edu.iq
ORCID: https://orcid.org/0000-0002-1958-1939

*Correspondence Authors: Ghada Ibrahim Taha,
Email: ghada_ibraheem@codental.uobaghdad.edu.iq
ORCID: https://orcid.org/0000-0002-1958-1939

Abstract:

Periodontitis is an inflammatory disorder that affects the teeth's supporting structures, leading to attachment loss and bone resorption. Cytokines produced by immune and non-immune cells regulate the immune response, acting as both pro-inflammatory and anti-inflammatory agents. This complex pathogenesis presents a significant public health challenge, emphasizing the need for early and accurate diagnostics. This study aimed to measure volume of gingival crevicular fluid (GCF) using Periotron® and assess potential biomarkers (MCP-1 and IL-6) to predict periodontal disease severity and progression using the Human Enzyme-linked immunosorbent Assay (ELISA). GCF samples were collected from 30 patients with stage 1 periodontitis, 30 patients with stage 3 periodontitis, and 30 healthy subjects. The level of GCF recorded using Periotron® was 0.04 µl for the healthy control group, 0.10 µl for the stage 1 periodontitis group, and 0.70 µl for the stage 3 periodontitis group. MCP-1 and IL-6 levels in the GCF were significantly higher in the stage 3 periodontitis group compared to the stage 1 periodontitis group and healthy subjects.

Keywords: Periotron, gingival crevicular fluid, periodontitis, monocyte chemoattractant protein-1, interleukin-6

Introduction:

Periodontitis is a multifactorial and complex inflammatory disorder that affects the supporting structures of the teeth, leading to progressive attachment loss and bone resorption. This inflammatory disease, if left untreated, can culminate in tooth loss. It is well-recognized that the pathogenesis of periodontitis is modulated by the interacttion between microbial agents and the host immune response (Talib & Taha, 2024). While bacteria are fundamental initiators of the disease, the damage to the periodontal tissues is primarily mediated by the host's inflammatory and immune responses (Al-Duboni, 2013; Salahuddin Jasim & Ghada Ibrahim Taha, 2023).

The recent classification system categorizes periodontitis into four stages: Stage I—Initial Periodontitis; Stage II—Moderate Periodontitis; Stage III—Severe Periodontitis with potential tooth loss; and Stage IV—Very Severe Periodontitis with potential loss of the entire dentition, with each stage further classified based on the extent and severity of the disease (Gupta et al., 2024).

To craft an efficacious treatment strategy, understanding the epidemiology of periodontitis is crucial, as it underscores both the probability of the disease's presence and the diagnostic test's utility. Current data suggests a tiered prevalence: approximately 10% of adults grapple with "severe" periodontitis (stage III or IV), another 10% maintain periodontal health, and the remaining 80% exhibit symptoms of either gingivitis or mild to moderate periodontitis (Stage I or II). Notably, while the diagnosis of stages III and IV periodontitis can be straightforward, differentiating between gingivitis and milder forms of periodontitis is more nuanced, necessitating an evaluation of interdental Clinical Attachment Loss (CAL). Such differentiation is paramount, as it aids in distinguishing gingivitis from periodontitis. Thus, the cornerstone of any periodontal health assessment should be the early detection of attachment or bone loss (Tonetti & Sanz, 2019).

The immune system responds to subgingival microorganisms and their by-products, implicated in periodontitis. This response, while targeting the microbes, also damages the periodontium. A network of cytokines and chemokines secreted by various immune cells drives the inflammatory process (Gupta et al., 2024).

Gingival crevicular fluid (GCF), an inflammatory exudate produced in excess, is a hallmark of the host response in periodontitis. GCF originates from the vascular network of the gingival corium (Fageeh et al., 2021; Talib & Mohammed, 2023). Its composition reflects the inflammation state of gingival and periodontal tissues, providing insights into the pathogenesis of periodontal disease (Wassall & Preshaw, 2016). Numerous biomarkers are found in GCF, with cytokines playing a significant role (Stadler et al., 2016). Cytokines, produced by immune and non-immune cells, regulate and modulate the immune response, acting as both pro-inflammatory and anti-inflammatory agents (Sumbayak et al., 2023).

The Periotron is a sophisticated electronic device designed to measure gingival crevicular fluid (GCF), periodontal pocket fluid, salivary flow, and saliva thickness. It operates using micro-moisture metering, where an electronic transducer detects the moisture level of a paper strip, called Periopaper, and converts it into a digital readout (Asikainen et al., 1985; Garnick et al., 1979). The electrical conductivity changes with the moisture level of the strip, allowing the Periotron to convert these readings into clinical conditions and scores, which are then recorded using the gingival index (Fernández-Reyes et al., 2023).

Monocyte chemoattractant protein-1 (MCP-1), also known as CC Chemokine Ligand 2 (CCL-2) is one of the most potent chemoattractant for monocytes (He et al., 2023). It is produced by a variety of cell types in response to different signals such as tumour necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1b), and interferon-gamma (IFN-γ) (Ledo et al., 2023). Marked expression of MCP-1 gene has been observed in gingival tissue of adult periodontitis patients, suggesting its predominant role in monocyte chemotactic activity in the gingival crevicular fluid (GCF) (Gupta et al., 2024). Heightened MCP-1 expression has been observed in several chronic inflammatory diseases such as atherosclerosis, osteoarthritis, rheumatoid arthritis, tumors, and delayed type hypersensitivity

reactions (Villiger et al., 1992). Thus, any increase in plasma levels of MCP-1 in patients with chronic periodontitis who are otherwise clinically healthy may be hypothesized to be a risk indicator for systemic illnesses. Till date, only one study has assessed serum levels of MCP-1 in patients of chronic periodontitis and found these levels to be significantly elevated (Pradeep et al., 2009).

Cytokines are small, soluble proteins that play vital roles in modulating the immune response and inflammation. Periodontal tissue components such as fibroblasts and epithelial and endothelial cells also participate in cytokine formation during inflammatory responses (Santos et al., 2021). Periodontal health depends on the local balance among reactive and suppressor immune cells, their cytokines, and mediators. Cytokines IL-6, IL-8, and IL-12 have proinflammatory functions, and induce bone reabsorption while IL-10 exerts anti-inflammatory effects (Maddah & Taha, 2024). IL-6, a pro-inflammatory cytokine, plays a significant role in the pathophysiology of periodontal disease. Its production is greatly influenced by TNF-α and IL-1β, which are produced by various immune cells such as dendritic cells, T cells, B cells, and macrophages. IL-6 accelerates B-cell differentiation, T-cell proliferation, and bone resorption, making it a valuable marker for periodontal disease research (Pai et al., 2021; TAHA, 2023). Studies have shown that IL-6 release correlates with the severity of periodontal disease, with high levels detected in gingival crevicular fluid (GCF) from patients with gingivitis and periodontitis (Musskopf et al., 2018). Additionally, increased IL-6 levels regulate the transition from acute to chronic inflammation and induce MCP-1 synthesis (Kaplanski, 2003).

Marital and methods:

In this case-control study conducted at the Periodontics Department of the Dental College Teaching Hospital at Baghdad University, samples were collected from November 2023 to May 2024. A total of 90 participants were included, divided into two main groups: the study group and the healthy control group. The study group, consisting of 60 patients, was further divided into two subgroups, with 30 patients diagnosed with stage 1 Periodontitis and another 30 with stage 3 Periodontitis. Additionally, 30 individuals with healthy gingiva comprised the healthy control group.

The inclusion criteria for this study require participants to be aged 18-60 and to have a clinical diagnosis of either mild or severe periodontitis, confirmed by periodontist. Additionally, participants must be free from autoimmune or systemic diseases. For the control group, individuals must not exhibit clinical signs of periodontal disease, also verified by periodontist. The exclusion criteria specify that individuals with systemic diseases affecting periodontal health, those who have used antibiotics or anti-inflammatory drugs recently, pregnant or lactating women, individuals with recent periodontal treatment or surgery, smokers or users of tobacco products, those with other oral conditions such as ulcers or tumors, and those undergoing orthodontic treatment are not eligible for the study. These criteria are designed to ensure a focused and reliable assessment of periodontitis and its effects.

Ethical approval:

The study protocol was approved by the scientific committee at the Basic Science Department/College of Dentistry/University of Baghdad, (Reference number: 862, Project number: 862823, Date: 23/11/2023), and all patients were given a piece of detailed information about the study's objectives and informed consent was signed to represent the patient's acceptance in order to indicate their agreement for involved in the study.

Diagnostic Categories and Oral Examination:

Periodontitis is categorized based on ***Ababneh et al., (2019)*** (Ababneh et al., 2019), into two stages: Stage 1 Periodontitis, which typically presents with 1-2 mm of Clinical Attachment Loss (CAL) and probing depths between 4-5 mm, and Stage 3 Periodontitis, which involves more than 5 mm of CAL with probing depths greater than 6 mm. During oral examinations, several clinical parameters are utilized to assess the presence, severity, and progression of periodontitis. Bleeding on Probing (BOP) occurs when a gentle insertion of a periodontal probe into the sulcus or pocket around a tooth lead to bleeding in inflamed tissues, indicating inflammation and active disease (Lang et al., 1986). Probing Depth (PD) is measured using a calibrated periodontal probe to determine the depth of the sulcus or pocket from the gingival margin to the base of the pocket, with depths greater than 3 mm considered pathological and indicative of advancing periodontitis (Armitage, 2004). Clinical Attachment Loss (CAL) is measured from a fixed point on the tooth, typically the cementoenamel junction (CEJ), to the base of the pocket, incorporating both gingival recession and probing depth to provide a comprehensive assessment of tissue loss (Cairo et al., 2011).

Sample collection:

Patients were prepared ninety minutes prior to the sample collection, which took place between 9:00 and 11:00 a.m. They were instructed to refrain from eating and tooth brushing before the procedure. The targeted sites underwent a cleansing process involving rinsing with water, isolation with cotton rolls, and a gentle air spray. "Perio Paper" strips were used to collect fluid samples from the test groups. Supragingival plaque was removed using dry gauze before placing standard paper strips in the sulcus, where they remained for 30 seconds. Any strips that showed signs of blood staining were excluded from the sample set. To maintain the integrity of the samples, the paper strips were immediately placed into sterile Eppendorf tubes containing 0.5 ml of preservative phosphate Buffer Saline (PBS) (Figure 1). The tubes were then centrifuged at 3000 rpm for 10 minutes and stored at -80°C until they were ready for laboratory analysis (Bhardwaj & Prabhuji, 2013; Jasim & Taha, 2023; TAHA, 2023). The detection of biomarkers was conducted using the Human Enzyme-linked immunosorbent Assay (ELISA) By lab technicians.

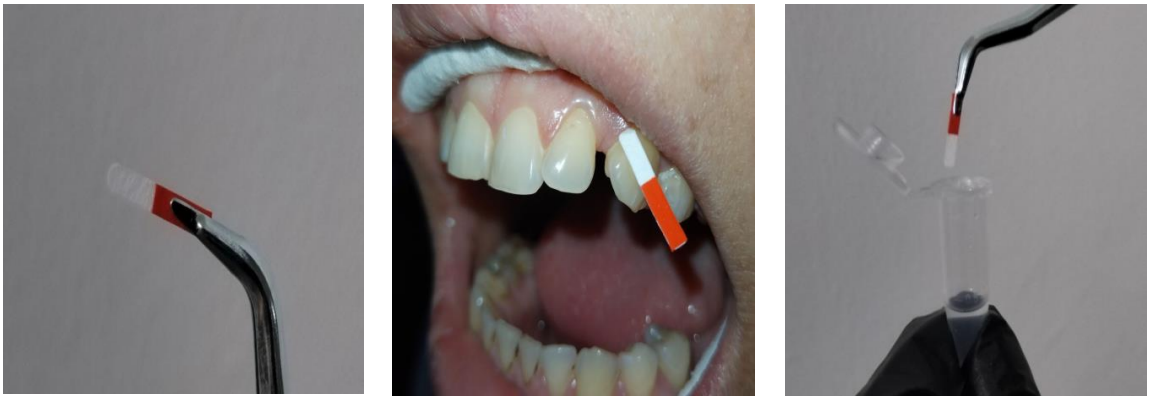

Figure 1: Gingival crevicular fluid collection at baseline

Enzyme-linked immunosorbent Assay (ELISA): was employed for the detection of pro-inflammatory biomarkers using the Human ELISA quantitative immunoassay kit (Human Neutrophil Extracellular Traps (NET)Lot No: E23XMB286, Feiyuo company, China) and (Human IL-23/Lot No: E23UMS852, Feiyuo company, China) in saliva samples. The readings were obtained using an ELISA reader from BioTek (USA).

Principle of the Procedure: This kit uses sandwich enzyme immunoassay. This package includes a microtiter plate pre-coated with either Neutrophil Extracellular Traps (NET) and IL-23 antibody. Microtiter plate wells containing standards or samples get a biotin-conjugated NET or IL-23 antibody. Incubate microplate wells with avidin-HRP. TMB substrate solution only colors wells containing NET or IL-23, biotin-conjugated antibodies, and enzyme-conjugated avidin. Sulfuric acid blocks the enzyme-substrate reaction, and the color change is measured at 450 nm±10 nm. The concentration of biomarkers in the samples is then determined by comparing the optical density (OD) of the samples to the standard curve.

Gingival crevicular fluid (GCF) and calculate quantification of mediator concentration by Periotron:

An electronic method has been devised for measuring the fluid collected on a "blotter" (Periopaper, PerioPaper®, Oraflow Inc., New York, USA) (Figure 2) with the use of an electronic transducer (Periotron, Oraflow Inc., New York, USA). The wetness of the paper strip affects the flow of an electric current and provides a digital readout. The readings obtained by Periotron can be converted into corresponding clinical conditions and scores recorded by gingival index (Figure 3).

Figure 2: PerioPaper®, Oraflow Inc., New York, USA)

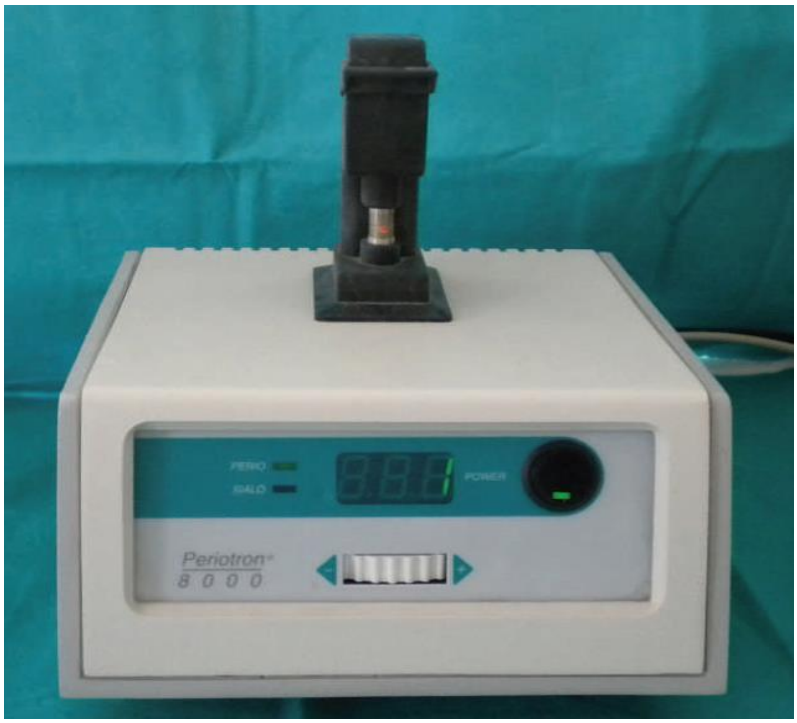

Figure 3. The Periotron 8000 device (Oraflow Inc., New York, USA).

Statistical analysis:

The data processing utilized Statistical Package for Social Sciences (SPSS) version 26 and Microsoft Excel 2019. Statistical analyses included Chi-square and Mann-Whitney U test, Analysis of Variation-ANOVA as well as Kruskal-Wallis H tests. The significance level was set at $p>0.05$ for non-significant results and $p<0.05$ for significant results.

Results:

Thirty patients with stage 1 Periodontitis with a mean age (36.37±9.197), and Thirty patients with stage 3 Periodontitis with a mean age (36.60±11.047), in addition to Thirty healthy control group with mean age (33.60±9.171). Furthermore, there was a non-significant male predominance (56.7%) among the study groups, no statistically significant differences (p=0.426), (p=1.000) in age or sex existed between study groups. As demonstrated in Table 1 and figure 4 , respectively.

Table 1: Frequency and percentages of Demographical Characteristics variables for sex in the studied groups

		Groups			Total	Chi-Square (*p-value*)
		Control group	Stage 1	Stage 3		
Sex	Female	13 (33.3%)	13 (33.3%)	13 (33.3%)	39 (43.3%)	1.000 NS
	Male	17 (33.3%)	17 (33.3%)	17 (33.3%)	51 (56.7%)	
Total		30 (33.3%)	30 (33.3%)	30 (33.3%)	90 (100.0%)	
NS: Non-significant						

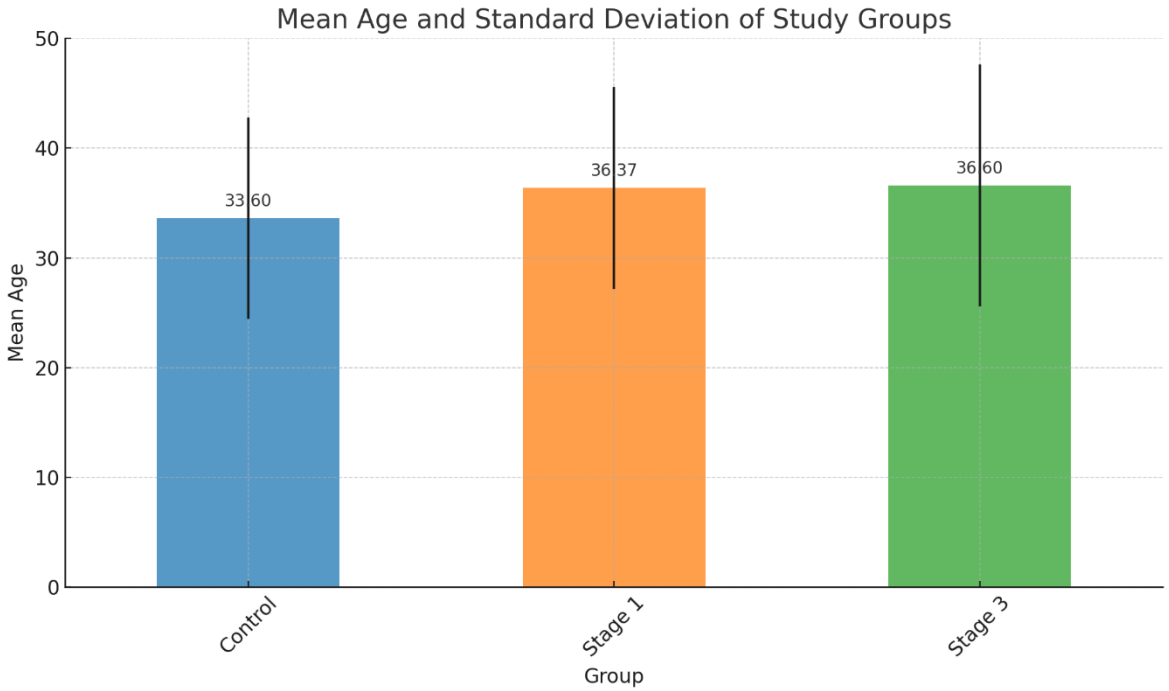

Figure 4: demographic data for age between groups

The results of this study revealed a significant elevation in the median levels of Gingival crevicular fluid (GCF) value among Stage 3 Periodontitis (0.70 pg/ml) followed by Stage 1 Periodontitis (0.10 pg/ml) compared to the control group (0.04 pg/ml). The statistical analysis revealed a significant difference (P=0.000), as observed in Table 2.

Table 2: Summary Statistics for the studied groups concerning of GCF value

GCF	Control group	Stage 1	Stage 3	Kruskal-Wallis H	*p- value*
Median	0.04	0.10	0.70	79.293	0.0001
SD	0.01	0.02	0.16		
SE	0.00	0.00	0.03		
Significant, p<0.05, non-signifcant, (p>0.05)., SD: Standard Deviation, SE: Standard Erarr, df=2					

The percentage of BOP in the stage 3 periodontitis group and stage 1 periodontitis group which is non-significant (p>0.05). While the result of both CAL & PD parameters showed statistically significant differences (p=0.0001), (p=0.003) between the stage 1 and stage 3 periodontitis. As demonstrated in Figure 5.

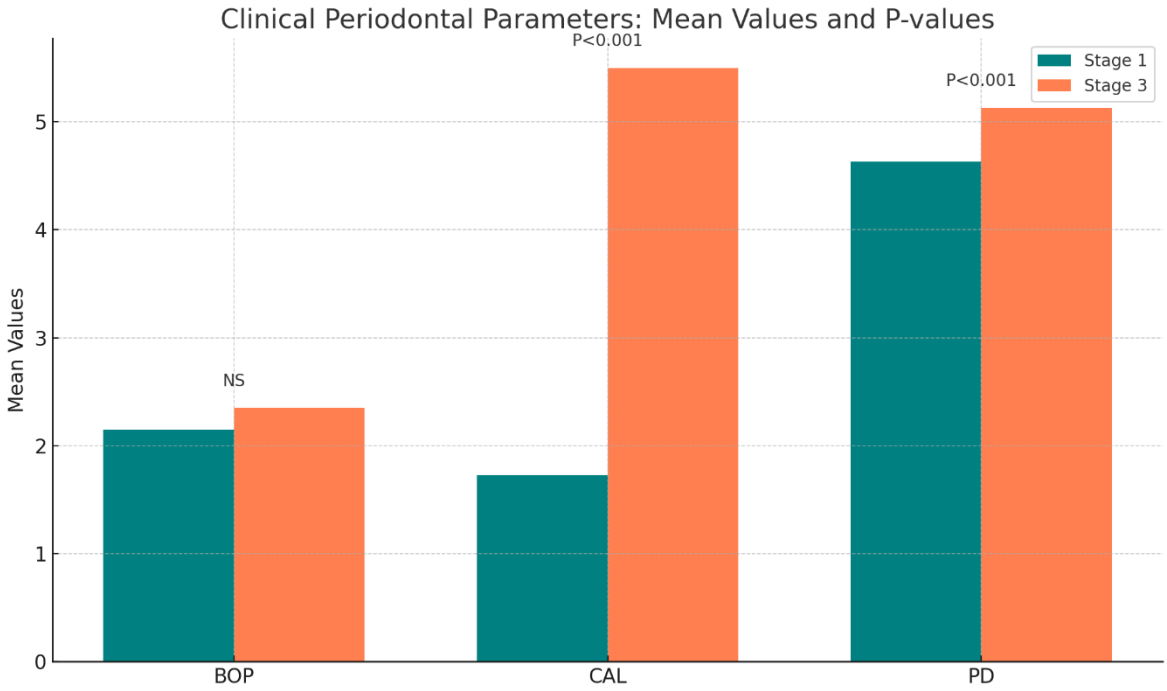

Figure 5: chart for the mean values for BOP, CAL, and PD in both Stage 1 and Stage 3 periodontitis groups.

The descriptive statistics of MCP-1, as observed in Table 3 and analyzed using the Kruskal-Wallis H test, revealed statistically significant differences in the median concentrations of MCP-1 across the study groups (Stage 1 and Stage 3) compared to the control group. The highest median concentration was found in Stage 3 (711.13), followed by Stage 1 (611.32), and then the control group (538.98), with a p-value highly significant different (p=0.0001).

Table 3: Summary Statistics for the studied groups concerning of MCP-1

MCP-1	Control group	Stage 1	Stage 3	Kruskal-Wallis H	p- value
Median	538.98	611.32	711.13	54.717	0.0001
SD	31.60	89.46	129978.53		
SE	5.77	16.33	23730.72		
Significant, p<0.05, non-signifcant, (p>0.05)., SD: Standard Deviation, SE: Standard Erarr, df=2					

Spearman correlation between MCP-1 (Monocyte Chemoattractant Protein-1) levels and Gingival Crevicular Fluid (GCF) value in Stage 1 and Stage 3 periodontitis groups shown in Table 4. In the Stage 1 periodontitis group, the correlation coefficient between MCP-1 levels and GCF is a very weak and non-significant inverse correlation (r=-0.027), (p= 0.889). While in the Stage 3 periodontitis group, the correlation coefficient is -0.480 with a p-value of 0.007, indicating a moderate negative correlation that is statistically significant.

Table 4: Correlation coefficient between MCP-1 and GCF in Study groups

Parameters	Stage 1		Stage 3	
	r	p.value	r	p. value
MCP-1 Vs GCF	-0.027	0.889	-0.480**	0.007
r= Spearman correlation, NS: Non-Significant. **= Significant				

Table 5 presents descriptive statistics for IL-6 (Interleukin-6) levels, measured in pg/ml, across the control group, Stage 1 periodontitis group, and Stage 3 periodontitis group, analyzed with the Kruskal-Wallis H test. The result showed highly significant differences in IL-6 levels among these groups. The highest median concentration was found in Stage 3 (50.96), followed by Stage 1 (20.35), and then the control group (15.36), with a p-value less than 0.05 (p=0.0001).

Table 5: Summary Statistics for the studied groups concerning of IL-6

IL-6	Control group	Stage 1	Stage 3	Kruskal-Wallis H	p- value
Median	15.36	20.35	50.96	72.247	0.0001
SD	1.16	11.89	21.88		
SE	0.21	2.17	3.99		
Significant, p<0.05, non-signifcant, (p>0.05).,SD: Standard Deviation, SE: Standard Erarr, df=2					

Table 6 shows the correlation between IL-6 (Interleukin-6) levels and Gingival Crevicular Fluid (GCF) in both Stage 1 and Stage 3 periodontitis groups. The analysis employs Spearman correlation to assess the relationship, providing correlation coefficients (r) and p-values to gauge the strength and significance of this association. In the Stage 1 periodontitis group, the correlation between IL-6 levels and GCF is remarkably strong and positively significant, with a correlation coefficient (r) of 0.933 and a p-value of 0.000. Similarly, in the Stage 3 periodontitis group, the correlation remains strong and positively significant, with a correlation coefficient (r) of 0.915 and a p-value of 0.000.

Table 6: Correlation coefficient between IL-6 and GCF in Study groups

Parameters	Stage 1		Stage 3	
	r	p.value	r	p. value
IL-6 Vs GCF	0.933**	0.000	0.915**	0.000
r= Spearman correlation, **: Significant, NS: Non-Significant.				

Discussion:

The result of the current study shows a progression-dependent increase in GCF levels from control to Stage 1 and Stage 3 periodontitis, with a marked elevation in Stage 3. This may be because GCF could serve as a biomarker for periodontitis severity. The elevation in GCF levels is likely due to an enhanced inflammatory response and immune activity within the periodontal tissue as the

disease progresses. GCF is known to contain inflammatory cytokines and biomarkers that reflect the host's response to periodontal pathogens (Ayoob & Abdulbaqi, 2024; Nair et al., 2022).

According to Preianò et al. (2020) and Nair et al. (2022) highlighted the potential of GCF as a source for discovering biomarkers for periodontal diseases, noting that various antimicrobial peptides and proteins in GCF play roles in the immune-inflammatory response (Nair et al., 2022; Preianò et al., 2020). In addition to a study by Andronovici et al. (2022) that developed a gingival crevicular fluid collagenase-2 (MMP-8) test stick for chair-side monitoring of periodontitis (Andronovici et al., 2022). In addition to Alamri et al. (2023) found that scaling and root planning on interleukin-1β, interleukin-8, and MMP-8 levels in gingival crevicular fluid from chronic periodontitis patients (Alamri et al., 2023). These studies further emphasize the importance of GCF as a potential biomarker for periodontal diseases. However, conflicting studies have reported opposite results, such as Romano et al. (2022) reporting lower GCF levels in periodontitis compared to gingivitis (Romano et al., 2022). This discrepancy may be due to differences in study design, sample size, and patient populations. Khurshid et al. (2017) conclude that GCF might be a source of new biomarkers of periodontitis/gingivitis. The oral cavity is indeed a reserve of the microbiome and, when adverse changes occur within the oral cavity, it results in pathological changes, such as gingivitis, periodontitis, and dental caries (Khurshid et al., 2017). Majeed et al., (2016) reported that GCF may play a key role both in the diagnosis and in the prognosis of oral diseases, in particular for periodontal diseases. It can be included among the most non-traumatic proximal fluid to obtain information about periodontal tissue status, including the conditions of the connective tissue and the level of hard destruction (Nazar Majeed et al., 2016). The result of the present study revealed statistically significant differences in the concentrations of Monocyte Chemoattractant Protein-1 (MCP-1) across the study groups (Stage 1 and Stage 3) compared to the control group. The highest median concentration was found in Stage 3, followed by Stage 1, and then the control group, with a statistically highly significant difference ($p=0.0001$). MCP-1/CCL2 is a potent chemoattractant for monocytes and macrophages. It recruits these cells to sites of inflammation where they differentiate into macrophages, which play a crucial role in the immune response by releasing pro-inflammatory cytokines such as TNF-α, IL-1, and nitric oxide. These cytokines exacerbate the inflammatory response and contribute to tissue destruction in periodontal disease (Graves et al., 2011; Mohammed Hussein & Ali, 2024). Actually, MCP-1/CCL2-induced macrophages promote the differentiation and activation of osteoclasts, leading to bone resorption, a hallmark of periodontal disease. This process is mediated by the release of pro-inflammatory cytokines and other factors that enhance osteoclast activity (Graves et al., 2011; Mohammed Hussein & Ali, 2024). According to Tonetti et al., (1994) and Yu and Graves, (1995) MCP-1/CCL2 is preferentially expressed in diseased periodontal sites, localized along the basal layer of the oral epithelium and expressed by endothelial cells, fibroblasts, and mononuclear phagocytes in the inflammatory infiltrate (Tonetti et al., 1994; Yu & Graves, 1995). A previous study by Öngöz Dede et al. (2023) have shown that MCP-1/CCL2 levels in gingival crevicular fluid (GCF) increase with the severity of periodontal disease, suggesting its significant role in the pathogenesis and progression of the disease (Öngöz Dede & Bozkurt Doğan, 2023). Similarly higher numbers of macrophages, attracted by MCP-1/CCL2, are found in active sites of periodontitis, further supporting this correlation (Graunaite et al., 2012). While Gemmell and Seymour, (2004)

found that while MCP-1/CCL2 levels in GCF correlate with disease severity, other chemokines such as MIP-1α/CCL3 did not show significant differences between healthy and diseased sites in GCF (Gemmell & Seymour, 2004). This indicates that while MCP-1/CCL2 is a critical factor, other chemokines may play more nuanced roles in different stages or aspects of the disease.

Regarding IL-6, the result showed highly significant differences in IL-6 levels among these groups. The highest median concentration was found in Stage 3 (50.96), followed by Stage 1 (20.35), and then the control group (15.36), with a statistically significant difference (p=0.0001).

IL-6 is a cytokine with both pro-inflammatory and anti-inflammatory actions. It plays a key role in various biological events such as B and T cell activation and differentiation, the occurrence of local acute phase events, and stimulation of osteoclast development. In the context of periodontal disease, IL-6 is primarily involved in the inflammatory response and bone resorption processes (Gündogar et al., 2021).

IL-6 is a major mediator of the acute phase response and is produced by various cells, including macrophages, T cells, and fibroblasts. It promotes the differentiation of B cells and the activation of T cells, enhancing the immune response to bacterial infection in periodontal tissues. Elevated IL-6 levels are indicative of ongoing inflammation, which is characteristic of periodontitis (Jassim et al., 2023; Offenbacher et al., 2007).

The result of current finding is consistent with Offenbacher et al. (1993) showed that patients with severe periodontitis had high levels of IL-6, which were associated with severe bleeding on probing (Offenbacher et al., 1993). Similarly, Engebretson et al. (2002) demonstrated that IL-6 levels in GCF were significantly higher in periodontitis patients compared to healthy controls, supporting the use of IL-6 as a biomarker for periodontal disease progression (Engebretson et al., 2002). Yücel et al. (2008) noted that while IL-6 levels were elevated in periodontitis, other cytokines like IL-10 also played a significant role, indicating the complexity of the cytokine network in periodontal disease (Yücel et al., 2008).

Conclusion:

Volumetric analysis of GCF is a reliable method for assessing periodontal inflammation. Increased levels of MCP-1 and IL-6 in the GCF of subjects with stage 3 periodontitis, compared to those with stage 1 periodontitis and healthy individuals, correlate positively with disease severity. This correlation is significant because the role of chemokine MCP-1 in recruiting macrophages, promoting inflammation, and contributing to bone resorption provides a robust mechanistic basis for its increased levels in more severe stages of periodontal disease. Additionally, this chemokine's expression and activity are closely linked to disease severity, making it a potential biomarker for periodontal disease progression and a target for therapeutic intervention.

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