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ROLE OF SALIVARY MATRIX METALLOPROTEINASE-8 (MMP), TISSUE INHIBITOR OF MATRIX METALLOPROTEINASE-1 (TIMP-1) AND ZINC (ZN^{+}) LEVELS IN MILD, MODERATE & SEVERE PERIODONTITIS PATIENTS

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Abstract
Periodontitis is disorder of periodontium characterized by inflammation around teeth. Imbalance between matrix metalloprotienase8, tissue inhibitor of matrix metalloproteinase-1 & Zn+ levels have an important part in periodontal destruction. A comparative cross-sectional study was conducted on 144 individuals in 4 groups having 36 number in each group. Group I included mild periodontitis patients, group II include moderate & group III include severe periodontitis patients. Group IV included healthy controls. Saliva samples were taken. Samples were processed and stored for analysis of MMP-8, TIMP-1 & Zn+. Data was analyzed by SPSS version 25.0. Normality of data was checked and student t-test was applied. A p-value of <0.05 was considered statistically significant. Levels of MMP-8 were high in PD patients compared to healthy controls. The levels of TIMP-1 & Zn+ were markedly reduced in patients with periodontitis compared to healthy controls. The correlation of MMP-8 with TIMP-1 & TIMP-1 with Zn+ has significant role in developing periodontitis.

Keywords: Periodontitis, Matrix Metalloproteinase – 8(MMP8, Tissue Inhibitor of Matrix Metalloproteinase – 1 (TIMP-1). & Zn+.

Introduction
Periodontitis is defined as host-mediated inflammation that results in loss of periodontal attachment (Alhassani, 2023). According to WHO, it is 30% prevalent in Pakistani population (Khan et al., 2016). 57% in men compared to women 39% (Ioannidou, 2017) . A group of enzymes considered to play an important role in this degradation process is the MMP family. MMPs can degrade the vast majority

of extracellular matrix (ECM) proteins. Tissue inhibitors of MMPs suppress MMPs, hence limiting excessive ECM degradation.

Periodontitis has been connected to other diseases including heart disease, cancer of head, neck, and a lung in chronic obstructive pulmonary disorder (COPD) is especially worrying. This emphasizes the need of early detection and treatment for periodontitis (Shi et al., 2018). Periodontitis classified in four different stages. Stage1 is less severe periodontitis, also called gingivitis. Stage 2 is observed as moderate severe periodontitis. Stage 3 is severe periodontitis with chances of tooth loss. 2 and 3 stages are irreversible. Stage 4 is observed as aggressive periodontitis with definite tooth loss and potential to affect masticatory function (Marini et al., 2022)

Since saliva may be swiftly and painlessly collected, saliva-derived biomarkers are used for the early diagnosis of periodontitis are promising. Salivary biomarkers have been studied to determine their associations with periodontal disease symptoms. Enzyme activity was increased among individuals with Periodontitis patient (PD) compared to healthy controls. Salivary enzymes may arise from cells in the salivary glands, bacteria, neutrophils, epithelial cells and play a vital part in the periodontium death (Freitas et al., 2017) . It has been established that many different types of salivary biomarkers are associated to both systemic and oral illnesses, detections of these biomarkers are noninvasive, easily accessible and inexpensive (Beck et al., 2019). Patients with periodontitis have been demonstrated to have significantly increased levels of salivary biomarkers such interleukins, and MMP-8 when compared to healthy controls. Cells of the immune system that have been triggered produce destructive enzymes that eat away at connective tissue, ultimately leading to the loss of periodontal tissue. The role of metalloproteinases in the ECM has been hypothesized to be important in these events bec(Borges et al., 2013) . MMPs are zinc-dependent proteolytic enzymes dysregulation of MMP promote oral Diseases. They could be used as a biomarker in saliva (Alotaibi et al., 2020). MMPs are key enzymes for protein degradation involved in PD. There are many different forms of MMPs, however 90% to 95% of lysis activity in saliva is originates from MMP-8. It has ability to breakdown the type I and type III collagens, which are predominant in supporting structures of teeth. Tissue destruction is directly proportional to the concentration of MMPs in saliva and blood. TIMPs glycoprotein has inhibitory effect on MMPs. The levels of TIMPs are generally higher in healthy periodontal tissues than in inflammatory periodontal tissues. The levels of TIMPs are generally higher in healthy periodontal tissues than in inflammatory periodontal tissues (Costa et al., 2022). The health and vitality of periodontal tissue is highly dependent on the zinc (Zn^{+}) essential for the metabolism of macromolecules.The imbalance of Zn^{+} levels in saliva can predispose an individual to the risk of developing periodontitis (Taru et al., 2017). The lower levels of Zn^{+} lead to deterioration of periodontium. Zn^{+} acts as cofactor for matrix metalloproteinases and vital for regulating their biochemical activity (Kritikou et al., 2022).

Our objective of research is to measure and compare salivary MMP-8 (matrix metalloproteinase 8), TIMP-1 (tissue inhibitor of matrix metalloproteinase-1) & Zn^{+} levels in mild, moderate, severe periodontitis patients & healthy controls.

Materials and Methods

All the samples were collected from Rashid Latif Dental College & Hospital Lahore. This research was carried out in the Biochemistry Department at University of Health Sciences Lahore. All participants were individually informed about the purposes of the study, written informed consent was obtained. This cross-sectional study was approved by the Advanced Studies & Review Board of University of Health Sciences, Lahore, Pakistan. The study was conducted in full accordance with the Declaration of Helsinki. Our study targeted 144 individuals (n=144) in 4 groups having 36 participants each. Group I, II & III had mild, moderate & severe periodontitis patients respectively and Group IV had healthy controls. All were in between 30-50 years of age both male & female having minimum 20 teeth present with history of no periodontal treatment in past. Individuals having any kind of periodontal therapy, systemic illness like diabetes, smoking habits or any kind of periodontal effecting drugs (phenytoin, calcium channel blockers, cyclosporine) were excluded.

Measuring and distribution of periodontitis patient in all groups.
Clinical attachment loss (CAL) was measured by periodontal probe to categorize patients in three groups. Patients having CAL 1 or 2 mm were placed in mild, with CAL 3 to 4 mm were placed in moderate & with CAL ≥ 5 mm were placed in severe periodontitis group (Graetz et al., 2019).

Sample preparation.
A 2-3 ml of un-stimulated saliva was collected in sterile graduated flasks, the saliva was placed in Eppendorf tubes and centrifuged at 15,000 rpm for 15 mins at 4 °C. The supernatant was transferred to new sterile Eppendorf tubes and was stored at − 80 °C for biochemical tests.

Biochemical analysis
Salivary MMP-8 & TIMP-1 was measured by using commercially available ELISA Kit. Salivary $Zn+^{+}$ was measured by using zinc Kits on Micro lab 300.

Statistical analysis
The data was entered and analyzed using SPSS version 25.0. Mean + SD was given for normally distributed quantitative variables. The normal distribution of the data was checked by the Shapiro-Wilk test. A p-value < 0.05 will be considered statistically significant.

RESULTS.
Demographic analysis
We observed significant difference in socioeconomic status, oral hygiene, brushing, junk food intake, clinical attachment loss , & bleeding on probing (p value <0.005) (p value <0.001), (p value <0.001) (p value <0.001) & <0.001) respectively in table 1 as compare to control.

Biochemical Estimation
Estimation of Matrix Metalloproteinase -8 (MMP-8)
MMP-8 was found significant increased by 16.8% in mild periodontitis, 52.9% in moderate periodontitis and 66.4% in severe periodontitis patients as compare to control. Mean ± SD MMP-8 is 1.26 ± 0.53 in mild periodontitis, 1.99 ± 0.26 in moderate periodontitis and 2.96 ± 0.85 in severe periodontitis patients. (p <0.001 Fig 1)

Estimation of Tissue Inhibitor of Matrix Metalloproteinase -1 (TIMP-1).
TIMP-1 was found significant decreased by 13.6% in mild periodontitis, 33.3% in moderate periodontitis and 56.5% in severe periodontitis patients as compare to control. Mean ± SD is 326.6 ± 150.0 in mild periodontitis, 252.0 ± 114.9 in moderate periodontitis and 164.4 ± 117.9 in severe periodontitis patients. (p <0.001 Fig. 2)
Estimation of Zinc levels
Zn^{+} was found significant decreased by 68.9% in mild periodontitis, 95.9% in moderate periodontitis and 97.4% in severe periodontitis patients as compare to control. Mean ± SD of Zn^{+} is 0.90 ± 0.95 in mild periodontitis, 0.12 ± 0.01 in moderate periodontitis and 0.07 ± 0.03 in severe periodontitis patients. (p <0.001 Fig. 3)

Correlations
MMP-8 & TIMP-1
There was negative correlation between MMP-8 & Timp-1 levels as compared to control (p=0.001 Fig 4).

MMP-8 & Zn^{+}
There was negative correlation between MMP-8 & Zn^{+} levels as compared to control (p=0.001 Fig 5).

TIMP-1 & Zn^{+}

There was positive correlation between TIMP-1 & Zn^{+} levels as compared to control (p=0.001 Fig 6).

Table 1: Comparison of demographic and clinical characteristics among groups

Variable	Categories	Group-I	Group-II	Group-III	Group-IV	p-value
Socioeconomic Status	Low	17 (47.2%)	11 (30.6%)	16 (44.4%)	26 (72.2%)	0.005*
	Middle	19 (52.8%)	25 (69.4%)	20 (55.6%)	10 (27.8%)	
	High	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Oral Hygiene	Poor	6 (16.7%)	23 (63.9%)	25 (69.4%)	0 (0.0%)	< 0.001*
	Fair	9 (25.0%)	0 (0.0%)	0 (0.0%)	24 (66.7%)	
	Average	21 (58.3%)	13 (36.1%)	11 (30.6%)	12 (33.3%)	
Brushing	Yes	17 (47.2%)	6 (16.7%)	21 (58.3%)	20 (55.6%)	0.001*
	No	19 (52.8%)	30 (83.3%)	15 (41.7%)	16 (44.4%)	
Junk Food	Yes	10 (27.8%)	27 (75.0%)	14 (38.9%)	6 (16.7%)	0.001*
	No	26 (72.2%)	9 (25.0%)	22 (61.1%)	30 (83.3%)	
Clinical Attachment Loss	0.0	0 (0.0%)	0 (0.0%)	0 (0.0%)	36 (100.0%)	< 0.001*
	3.0	36 (100.0%)	35 (97.2%)	0 (0.0%)	0 (0.0%)	
	3.5	0 (0.0%)	0 (0.0%)	11 (30.6%)	0 (0.0%)	
	4.0	0 (0.0%)	1 (2.8%)	25 (69.4%)	0 (0.0%)	
Bleeding on Probing	Yes	27 (75.0%)	36 (100.0%)	36 (100.0%)	9 (25.0%)	0.001*
	No	9 (25.0%)	0 (0.0%)	0 (0.0%)	27 (75.0%)	

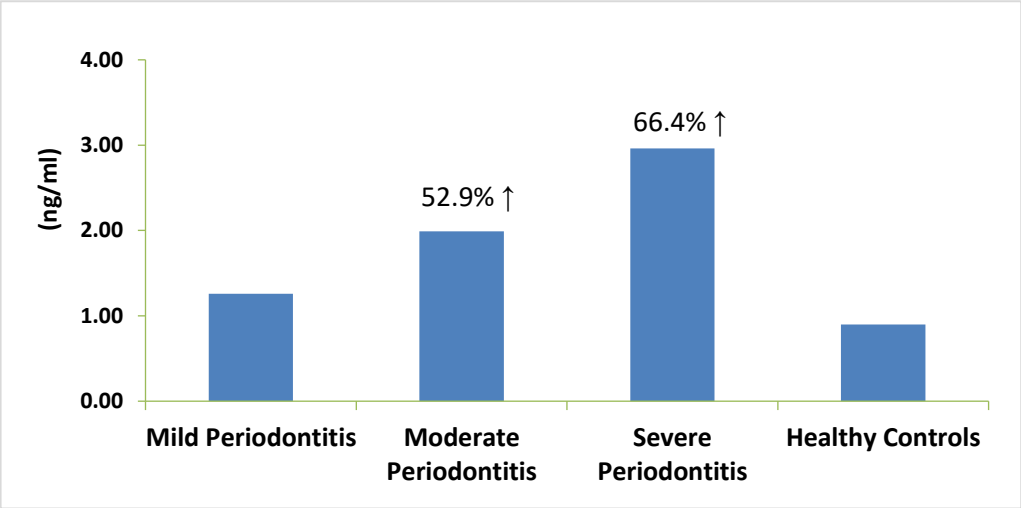

Fig.1. There is significant difference in MMP-8 levels among four groups (p value < 0.001).

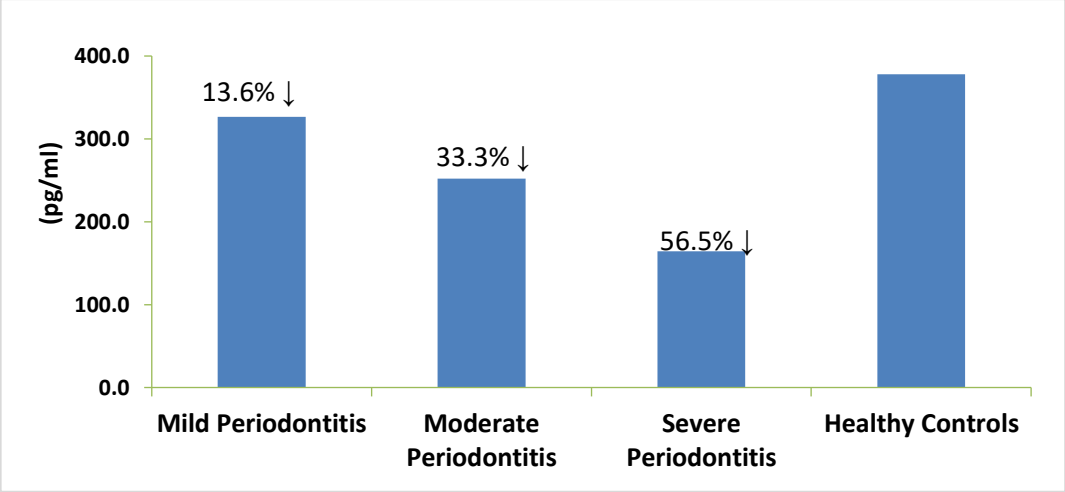

Fig.2 There is significant difference in the mean TIMP-1 levels among study groups, (p-value 0.01).

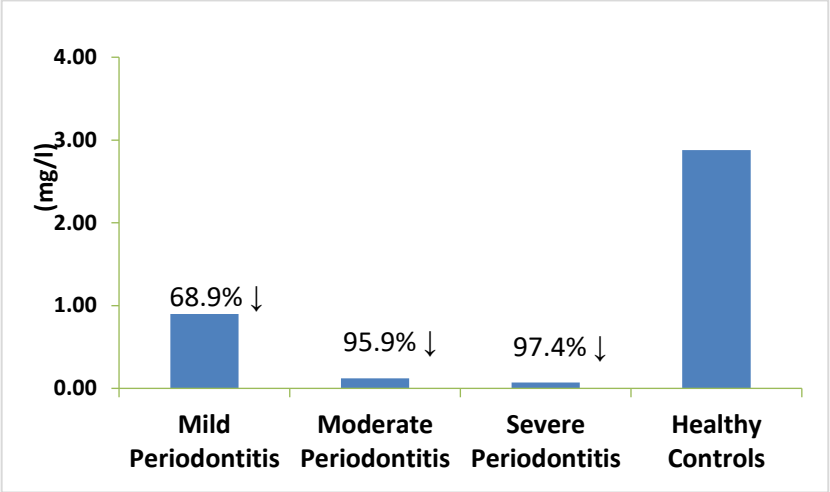

Fig.3 Significant difference in mean zinc levels in our study groups, (p-value <0.001)

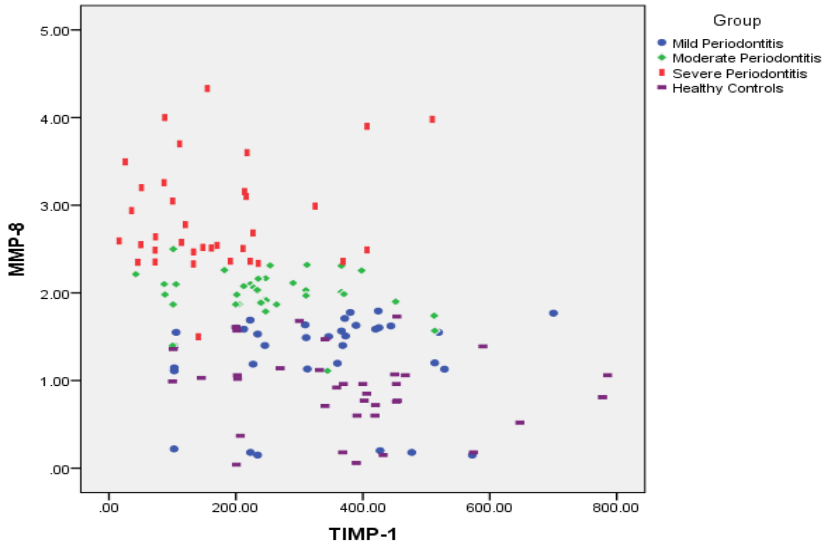

Fig 4: Negative Correlations between salivary MMP-8 & TIMP-1 levels. among study groups students i.e., (r= -0.467). The p-value shows that this correlation was statistically significant.

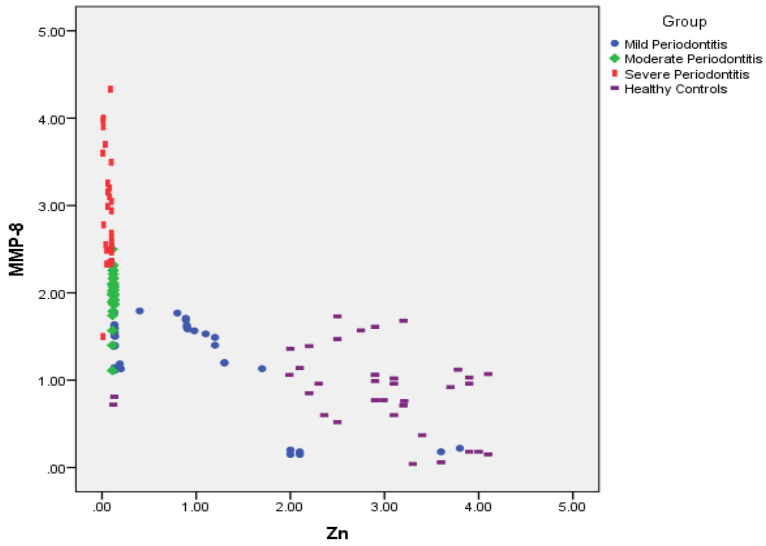

Fig 5: Negative Correlations between salivary MMP-8 & Zn+ levels among study groups i.e., (r= -0.884). The p-value shows that this correlation was statistically significant.

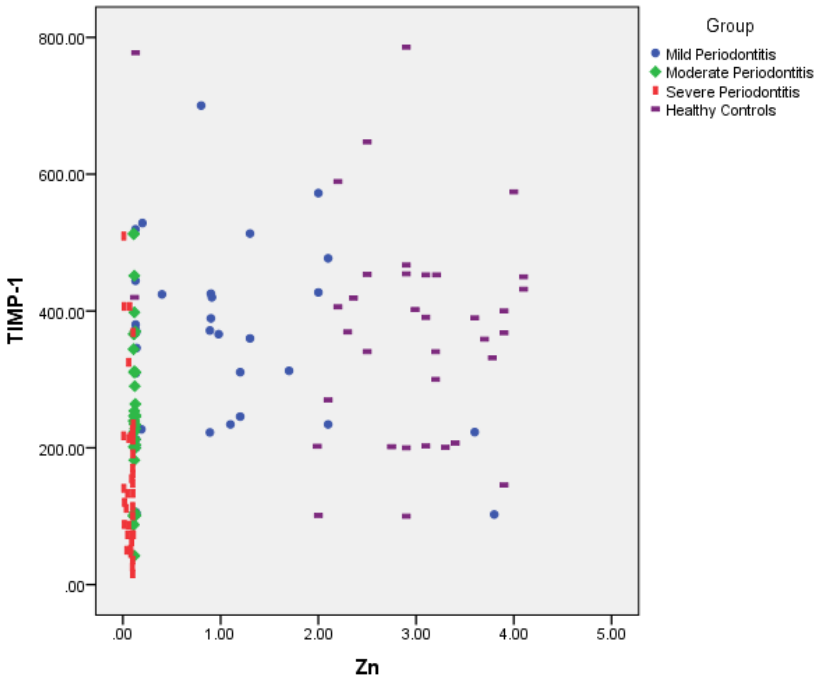

Fig 6: positive Correlation between TIMP-1 & Zn+ levels among study groups i.e., (r = 0.468). The p-value shows that this correlation was statistically significant.

Discussion

Periodontitis is defined as host-mediated inflammatory process which results in loss of periodontal attachment (Tonetti et al., 2018). This disease ultimately activates proteases that degrade periodontal tissue which results in poor teeth health leading to absence of teeth which causes inability to chew food and social embarrassment (Ray, 2023).

The disease is both affected by local and systemic etiological factors. Unawareness for cleaning teeth, improper brushing techniques, accumulation of yellow plaque layer on teeth, poor oral hygiene & untreated gingival inflammation i.e. gingivitis leads to periodontitis.(Stepaniuk, 2019). In PD the inflammation is triggered by bacteria & secretion of inflammatory mediators such as cytokines, growth factors and matrix metalloproteinases (MMPs). (Checchi et al., 2020). In this disease, there is increased gingival redness, bleeding gums with swelling, presence of neutrophils & activation of the body defense against infection by increasing the pro-inflammatory mediators (Interleukin 1-beta, prostaglandins, tumor necrosis factor) and MMPs. MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs), which restrict periodontal breakdown. The balance in MMPs and TIMPs has important role in maintaining the periodontal health. (Tanvir et al., 2022).

In our research, there was no significant difference among age & gender in study groups. The patients having low socioeconomic status, oral hygiene, lack of brushing habits, having junk food provided statistically significant results having periodontitis (Table 1).

Socioeconomic status plays main role in severity and prevalence of periodontal disease. Patients from low socioeconomic status cannot afford healthy diet and due to nutritional deficiency & unawareness of dental brushing, they are high risk to develop periodontitis (Palwankar et al., 2021).

The present research shows significant difference in brushing activity (Table 1) improper brushing cause accumulation of dental plaque and calculus which invade microorganisms causing inflammation and develop periodontitis (Darby, 2022).

Major factor that has more importance now a days people are found off junk food which give over all unhealthy effects on body. We observe significant difference (Table 1) between people who take junk food. Several studies reveal that junk food has dangerous effects on teeth health because they are highly processed, sticky, and high in sugar & having more carbohydrate content that result in

increased risk of periodontitis. Diet rich in fruits & vegetables provide anti-inflammatory effects and protect teeth from diseases (Costa et al., 2022).

Saturated fatty acids lead excess production of ROS and oxidative stress and cause inflammation of periodontium (Van Ravensteijn et al., 2022). Improper brushing and fond of junk food is a combination that promote periodontitis.

CAL (clinical attachment loss) and bleeding on probing was also significant among patients with periodontitis. (Table 1). Poor oral hygiene and other factors discussed in above paragraph are important (Duangthip and Chu, 2020).

In our research, we observed a significant increase (p-value < 0.001) in level of MMP-8 in periodontitis patients as compared to normal healthy controls i.e. MMP-8 was found increased by 16.8% in mild periodontitis, 52.9% in moderate periodontitis and 66.4% in severe periodontitis patients. However, no significant difference was observed between mild PD patients and healthy control (Fig 1). MMPs level is directly related to periodontopathogen bacteria. MMPs have collagenolytic activity. Collagen is extracellular matrix protein structural component of connective tissue and increase level of MMPs promote degradation of collagen type I & III, which is involved during tissue destruction in PD (De Morais et al., 2018).

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic peptidases with various roles in tissue remodeling and degradation of tissues. In normal conditions MMP-8 is produced for tissue regeneration and in pathological condition, there is MMP-8 overproduction, enhanced activity, which is regulated by extracellular stimuli (bacterial proliferation), which induces cytokine production interleukin-1β, TGFβ TNF-α. The enhancing neutrophil activity and stress experienced by the host also contribute in overproduction of MMP8.(Kasuma et al., 2021).

Numerous studies demonstrate that, MMP-8 increases in PD patients compared to healthy controls. Furthermore, higher levels of MMP-8 were seen in accordance with the stage of PD (Mohammed et al., 2022).

It was reported in many studies that MMP-8 is considered as diagnostic biomarker of PD (Hernández-Ríos et al., 2016, Sorsa et al., 2020)

Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) is the second main parameter of our research, that might be useful predictor of developing PD having a role in maintaining health of periodontium. Our result shows, there was significant difference (p-value < 0.001) in mean TIMP-1 levels among four groups i.e. TIMP-1 was found decreased by 13.6% in mild periodontitis, 33.3% in moderate periodontitis and 56.5% in severe periodontitis patients. Similarly, there was significant difference in mean TIMP-1 levels between mild PD patients and healthy controls (Fig 2). TIMPs having their role in regulating the activity of MMPs, are composed of four types of collagenase protein, of these TIMP-1 & 2 have been well explained (Zhang et al., 2018). TIMP-1 can bind to most MMPs and is believed to be one of the most important endogenous inhibitors of MMPs (Charzewski et al., 2021).

TIMP-1 restricts the extreme expression of the MMP. Higher MMP-8 levels and low TIMP-1 levels stimulate degradation of collagen in connective tissue and alveolar bone (Khuda et al., 2021). Few studies showed that the TIMP-1 values were increased in PD patients compared with the healthy individuals (de Brouwer et al., 2022). Substantial studies revealed that TIMP-1 levels were lower in the PD patients as compared to controls (Checchi et al., 2020, Pis Pérez, 2021) . Studies reveal that TIMP-1 inhibits the activity of MMP-8, inhibiting the binding of substrate to the catalytic site of MMP. TIMP-1 is not considered as diagnostic marker for PD but may be used as treatment of PD (Balogun et al., 2021).

Zinc is third main parameter of our research, it is also very important in diagnosing & treating PD. For growth & development of periodontal tissues high Zn^{+} levels are very important (Apon and Kamble, 2019). The imbalance in zinc levels of the saliva can predispose an individual at risk of developing PD (Taru et al., 2017). Deficiency of Zn^{+} results in poorer periodontal health. Zn^{+} is a cofactor for MMP-8 hence important in regulating activity and expression (Kritikou et al., 2022)

In our results, there was significant decrease (p-value <0.001) in mean Zinc levels among four groups The mean Zn^{+} levels were significantly lower in patients with severe PD as compared to other groups.

Similarly, there was significant difference in mean Zinc levels between mild and moderate, moderate and severe PD patients & between mild PD patients and healthy controls i.e. Zn^{+} was found decreased by 68.9% in mild periodontitis, 95.9% in moderate periodontitis and 97.4% in severe periodontitis patients. (Fig 6).

Lower levels of Zn^{+} enhance the activity and expression of MMPs and high levels the of Zn^{+} suppress the MMPs activity and their destructive effects (Nosrati et al., 2019). Zn^{+} reduces the levels of TIMP 1 (Takahashi et al., 2007a). Deficiency of Zn^{+} increase the production of cytokines resulting in oxidative stress. As a result, there is reduced production of sulfhydryl groups and increased reactive oxygen species (ROS), reducing level of the zinc-superoxide-dismutase. This enzyme is present in the human periodontium, (particularly in ligaments) preventing the free radical-induced damage. Hence decreased Zn^{+} causes more oxidative stress, more periodontal damage leading to progression of PD.(Taru et al., 2017). Researches reveal that salivary Zn^{+} levels are decreased in patients with PD (Naveenaa and Malaiappan, 2022). According to current evidences, there are no significant findings in increases of Zn+ levels in PD.

In our study we have correlated MMP-8, TIMP-1 & Zn^{+} with each other. There was a negative correlation between MMP-8 and TIMP-1 among study groups. The p value shows that this correlation was statistically significant (Fig 4). Both MMP-8 & TIMP-1 levels increase in PD patients (Gul et al., 2020). Several studies show that there is inverse relation between MMP-8 and TIMP-1 showing higher MMP-8/TIMP-1 ratio (de Brouwer et al., 2022). A study showed that the N-terminal cysteine of TIMP-1 is oxidized by HOCL produced by MPO-H_2O_2-Cl system, hence controlling the interaction of TIMP-1 with MMP. The increased MPO leads to increase in MMP-8 levels and decrease in active TIMP-1 levels (Wang and Khalil, 2018).

Significantly negative correlation was found between MMP-8 and Zn^{+} (Fig 5). Structurally MMP-8 has two Zn^{+} binding sites. Site I is the coordination & site II is for catalysis (Long, 2020). Zn^{+} reduces the levels of TIMP 1 (Takahashi et al., 2019). TIMPs found in tissue are effective against MMPs by inhibiting their Zn^{+}-dependent endopeptidase activity. Specifically, TIMP-1 is considered either as a stimulator or inhibitor in the process bone resorption, depending on the quantity of TIMP-1. It may therefore generate the MMP/TIMP complex by forming a strong bond with MMPs. The complex of MMP & TIMP has been proposed as a biological marker for the possible changes in disease by researchers in several fields of study (Arpino et al., 2015).

According to our study, patients having increased MMP-8 levels in PD have low Zn^{+} levels, Zn+ inhibits the expression of MMP-8 by forming zinc hydroxide bridge which blocks the reaction site (Nosrati et al., 2019). Correlation of TIMP-1 levels with Zn^{+} levels is also done in our study (Fig 6), which shows that there is a positive relationship in TIMP-1 & Zn^{+} levels. There is no evident study showing correlation between TIMP-1 & Zn^{+} levels. In our results Patients with PD had both TIMP-1 & Zn^{+} levels reduced as compared to healthy controls. It is also reported that increased levels of Zn^{+} cause decrease in TIMP-1 levels (Takahashi et al., 2007b).

Conclusion

The salivary MMP-8 levels are increased while TIMP-1 and Zinc levels were decreased in periodontitis patients. The patients in our study with low socioeconomic status, poor brushing habits and poor oral hygiene was more prone to periodontitis. • It is hoped that the present research provides better understand to know how MMP-8, TIMP-1 and Zn+ levels are important in diagnosis and treatment of this disease. Also, it will provide a reliable data of correlation between these three markers, especially TIMP-1 & Zn+ for further research on this topic. Zinc supplementation can correct MMP-8 levels thus can prevent or treat progression of periodontitis

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