FORMAT OF RESEARCH PLAN

1. **Title of the proposed research project: EVALUATION OFTHE RELATIONSHIP OF S100A8 AND MMP-9 LEVELS IN GINGIVAL CREVICULAR FLUID WITH CLINICAL PARAMETERS OF PERIODONTAL DISEASE AND HBA1c LEVELS BEFORE AND AFTER NON-SURGICAL PERIODONTAL TREATMENT IN CONTROLLED AND UNCONTROLLED TYPE 2 DIABETES MELLITUS SUBJECTS WITH PERIODONTITIS AND IN NON-DIABETIC PERIODONTITIS SUBJECTS-A LONGITUDINAL STUDY**

    1. **Summary (up to 250 words):**

Background: This study aims to compare the total amount of S100A8 AND MMP-9 levels in gingival crevicular fluid (GCF) and change in the HBA1c levels before and after non-surgical periodontal treatment in controlled and uncontrolled type 2 diabetes mellitus subjects with periodontitis and in non-diabetic periodontitis subjects and to evaluate diagnostic and prognostic ability of GCF MMP-9 and S100A8 for periodontitis with diabetes mellitus in Indian population.

Novelty: Liquid biopsy using Gingival crevicular fluid (GCF) offers a non-invasive opportunity to weigh risks, predict disease initiation, refine diagnosis and stratify treatment modalities. In search for functional and regulatory periodontitis biomarkers especially in patients with diabetes mellitus who are at increased risk of periodontal destruction, this study for the first time evaluates the of diagnostic and prognostic ability of S100A8 AND MMP-9 levels in Gingival crevicular fluid in periodontis affected patients with Diabetes mellitus.

Objectives:

- To estimate S100A8 and MMP-9 GCF levels in subjects with periodontitis, periodontitis with controlled type 2 diabetes mellitus and periodontitis with uncontrolled type 2 diabetes mellitus before and after non-surgical periodontal treatment.

    • To compare the changes in the HbA1c levels before and after treatment in subjects with periodontitis, periodontitis with controlled type 2 diabetes mellitus and uncontrolled type 2 diabetes mellitus and correlate it with changes in the clinical and biochemical parameters in the 3 groups mentioned above.

    • To find out the relationship of S100A8 and MMP-9 GCF levels with clinical parameters of periodontal disease and HBA1c levels in subjects with periodontitis, periodontitis with controlled type 2 diabetes mellitus and periodontitis with uncontrolled type 2 diabetes mellitus before and after treatment.

    • To explore the possibility of using S100A8 and MMP-9 as an inflammatory biomarker in periodontal disease.

Methods: Total 39 participants (convenient sample) which includes 13 non diabetic (DM) periodontitis and 13 subjects with periodontitis well controlled DM, and 13 uncontrolled DM with periodontitis subjects are recruited. Periodontitis will be classified as stage III-IV of new classification of periodontitis proposed in 2018. Enzyme linked immunosorbent assay kit is used to quantify GCF MMP-9 and S100A8. Biochemical levels (MMP-9 and S100A8 levels) in GCF, periodontal parameters, and HbA1c are measured at baseline for all the groups and at one and three months after the initial non-surgical periodontal therapy (NSPT) and data will be analyzed with appropriate statistical analysis to determine the diagnostic and prognostic ability of biochemical markers for periodontitis with diabetes mellitus in Indian population.

Expected outcome: This study aims to evaluate for the first time, the Diagnostic and Prognostic ability of GCF MMP-9 and S100A8 for periodontitis in the presence of Diabetes mellitus in Indian population and to validate for clinical application to advance precision oral medicine. The result of this study may help in using GCF MMP-9 and S100A8 levels as a diagnostic and prognostic marker in periodontitis patients with Diabetes mellitus. This

may help in developing a chair side kit for early detection.

2. **Keywords:** Periodontal disease, Gingival crevicular fluid, Biomarkers, Diabetes mellitus,
3. **Abbreviations:** Periodontal disease (PD), Gingival crevicular fluid (GCF), Bleeding on probing (BOP), Pocket probing depth (PPD),Matrix metalloproteinase(MMP-9).Non surgical periodontal treatment(NSPT)

4. **Background (up to 500 words):** Periodontal diseases (PD), including periodontitis, are chronic inflammatory pathologies caused by bacteria in the subgingival biofilm which affect the periodontal tissues. The chronic immune-inflammatory response to microbial biofilms at the tooth or dental implant surface is associated with systemic conditions such as cardiovascular disease, diabetes or gastrointestinal diseases. Current scientific evidence points to a two-way relationship between DM and periodontal disease, whereby DM is associated with an increase in the incidence and progression of periodontitis, while periodontal infection is associated with worsening glycemic control in diabetic patients.[1]This two-way relationship points to a need to promote oral health in DM patients, and to implement a joint management protocol between endocrinologist and dentist that aims to create adequate conditions for early diagnosis and the effective treatment of both diseases. Currently available conventional clinical diagnosis of periodontitis such as bleeding on probing (BOP), measurement of pocket depth (PD), clinical attachment loss (CAL) and alveolar bone loss using radiograph in clinical practice do not allow the detection of periodontitis risks in advance of the onset.[2] Early detection of periodontitis, especially in DM patients is necessary for public health in a preventive dimension in advance of clinical sign and symptoms, because it could reduce the periodontal burden such as tooth mobility, tooth loss, mastication deficiencies and digestive problems.

Biofluids like blood, urine, tears have been used as sources of biomarkers for certain disease.[3] Oral fluid-based biomarkers have demonstrated easy accessibility and potential as diagnostics for oral and systemic diseases. Gingival crevicular fluid (GCF) is essentially a serum exudate that accumulates in the gingival sulcus or pocket that is generally rich in biological markers. This site-specific fluid is easily obtainable and generally predictive of periodontal pathogenesis.[4]Over the past 2 decades studies have analyzed biomarkers in the GCF and their predictive ability in the periodontitis identification and progression.

S100A8 is a subgroup of molecules within the broader family of S100 calcium-binding protein and it has ability to bind with zinc. These proteins are mostly expressed on neutrophils and monocytes or macrophages and previously it has been reported that increased concentration of S100A8 in saliva were associated with periodontitis patients in Korean population.[5]
Matrix metalloproteinases (MMPs) are the major group of enzymes, which are responsible for degradation of extracellular matrix. MMP-9 [Gelatinase B] is mainly secreted by polymorphonuclear leukocytes (PMNLs) and they degrade type IV collagen present in gingival tissues.[6] MMP-9 are increased in gingival crevicular fluid (GCF) of sites with active periodontal disease and are present in the granules of PMNLs.[7] Previous salivary studies have showed that S100A8 and MMP-9 are candidate biomarker for periodontitis among Koreans.[8,9,10] These biomarkers offer a potential therapeutic target.

This study aims to evaluate for the first time, the Diagnostic and Prognostic ability of GCF MMP-9 and S100A8 for periodontitis in the presence of Diabetes mellitus in Indian

population and to validate for clinical application to advance precision oral medicine

5.Literature review (up to 1000 words):

- A longitudinal study was conducted in 110 subjects to compare the levels of calprotectin (S100A8/A9), in GCF and serum among patients with Diabetes Mellitus-periodontitis (DM-P), Chronic Periodontitis(CP) and healthy controls and also to observe the effect of initial periodontal therapy on its levels in GCF and serum in DM-P group. The study concluded that levels of calprotectin in serum and GCF in the DM-P patients are significantly higher than those in CP patients and healthy controls, which significantly reduced 3 months after initial periodontal therapy. Furthermore, it suggests diabetic patients might exhibit more pronounced inflammation periodontally and systemically.[1]

- This cross-sectional study on 326 Korean participants aims to evaluate the association of salivary S100A8 and A9 proteins with periodontitis and its screening ability for periodontitis. The results of the study concluded that salivary S100A8 and S100A9 could be practical markers for periodontitis. Its screening ability for periodontitis could be beneficial in clinics and at home.[5]

- Rai et al conducted a study to determine the levels of GCF MMP-8 and MMP-9 in patients with periodontitis and healthy controls. Significantly higher crevicular MMP-8 and -9 were observed in cases of periodontitis compared to healthy adults and concluded that these molecules may serve as biomarkers of periodontal disease and aid in early detection of periodontitis.[6]

- A cross-sectional study was conducted to compare the levels of MMP-8, Tissue Inhibitor of MMPs (TIMP-1 and TIMP-2), Myeloperoxidase (MPO), and MMP-9 in GCF of CP patients and controls at baseline and 3 months after non-surgical therapy. Higher levels of these molecules were found in GCF of patients compared with controls, and these markers decreased 3 months after periodontal therapy.[7]

- A cross sectional observational study aims to evaluate the association of salivary matrix metalloproteinase-9 (MMP-9) and interleukin-8 (IL-8) with periodontitis and its screening ability on periodontitis. The data showed that salivary MMP-9 and IL-8 could be potential markers for periodontitis. The screening model for periodontitis could be useful in clinics and home. A future prospective study is indicated for predicting the occurrence of periodontitis.[8]

- A prospective study was conducted among 149 Korean participants to evaluate the prognostic ability of salivary MMP-9 and S100A8 for periodontitis through non-surgical periodontitis treatment trial. Study concluded that algorithm using salivary MMP-9 and S100A8 showed high diagnostic power for periodontitis. Both showed prognostic ability for periodontitis, but S100A8 was better.[9]

- In this cross-sectional study, Saliva samples were collected from 207 participants including 36 pairs matched for age, sex, and smoking. Shotgun proteomics was applied to detect proteins from saliva samples. Enzyme-linked immunosorbent assay (ELISA) was used to verify the candidate protein markers among another matched participants (n = 80). Shotgun proteomics indicated that salivary S100A8 and S100A9 were candidate biomarkers for periodontitis. ELISA confirmed that both salivary S100A8 and S100A9 were higher in those with periodontitis compared to those without periodontitis. The results showed that S100A8 and S100A9 in saliva could be candidate biomarkers for periodontitis. The rapid-test-kit using salivary S100A8 and S100A9 will be a practical tool for reducing the risk of periodontitis and promotion of periodontal health.[10]

6.Novelty/Innovation (up to 250 words): Traditional methods of clinical diagnosis lacks the

ability of detection of periodontitis risks in advance of the onset. Liquid biopsy using Gingival crevicular fluid (GCF) offers a non-invasive opportunity to weigh risks, predict disease initiation, refine diagnosis and stratify treatment modalities. In search for functional and regulatory periodontitis biomarkers especially in patients with diabetes mellitus who are at increased risk of periodontal destruction, this study for the first time evaluates the of diagnostic and prognostic ability of S100A8 and MMP-9 levels in Gingival crevicular fluid in periodontis affected patients with Diabetes mellitus. This study explores the possibility of using S100A8 and MMP-9 as an inflammatory biomarker in periodontal disease in diabetic patients and may help in developing chairside kits which may have diagnostic and prognostic potential.

7. Study Objectives:
- To estimate S100A8 and MMP-9 GCF levels in subjects with periodontitis, periodontitis with controlled type 2 diabetes mellitus and periodontitis with uncontrolled type 2 diabetes mellitus before and after non-surgical periodontal treatment.
- To compare the changes in the HbA1c levels before and after treatment in subjects with periodontitis, periodontitis with controlled type 2 diabetes mellitus and uncontrolled type 2 diabetes mellitus and correlate it with changes in the clinical and biochemical parameters in the 3 study groups.
- To find out the relationship of S100A8 and MMP-9 GCF levels with clinical parameters of periodontal disease and HbA1c levels in subjects with periodontitis, periodontitis with controlled type 2 diabetes mellitus and periodontitis with uncontrolled type 2 diabetes mellitus before and after treatment.
- To explore the possibility of using S100A8 and MMP-9 as an inflammatory biomarker in periodontal disease.
- To explore the impact of glycemic control (HbA1c on the clinical parameters of periodontal disease and biochemical parameters in GCF after periodontal treatment.

8. Methodology (up to 2000 words): Include the following subheads

i. Study Design: Longitudinal Study

SOURCES OF THE DATA: The 39 participants with periodontitis recruited in this study will be selected from the outpatient section, Department of Periodontology, Government Dental College and Research Institute Bengaluru. Ethical clearance has been obtained from institutional ethical committee

The consecutive subjects fulfilling the inclusion and exclusion criteria will be enrolled for the study (convenient sample). Informed written consent will be taken from the subjects willing voluntarily to participate in the study. The subjects enrolled will be categorized into three groups.
GROUP I: Subjects with diagnosis of Stage III or IV periodontitis[11] followed for 3 months after non-surgical periodontal treatment. (Non diabetic)

GROUP II: Subjects with diagnosis of Stage III or IV periodontitis[11]
and well controlled Type 2 diabetes mellitus. [12,13], followed for 3 months after non-surgical periodontal treatment.

GROUP III: Subjects with diagnosis of Stage III or IV periodontitis[11] and uncontrolled Type 2 diabetes mellitus,[12,13] followed for 3 months after non-surgical periodontal treatment with

pharmacotherapy.

PLACE OF STUDY: Government Dental College and Research Institute, Bangalore.

INCLUSION CRITERIA: COMMON CRITERIA:
1. Male and Female Subjects in the age group of 25-65 years.
2. Subjects should have at least 20 natural teeth.
3. Subjects in good systemic health other than the systemic condition included in the study.

EXCLUSION CRITERIA:
1. Subjects having heart disease & hypertension.
2. Subjects having the habit of smoking.
3. Subjects having aggressive form of periodontitis.
4. Subjects having other complications of type 2 diabetes other than periodontitis.
5. Subjects with vitamin D deficiency.
6. Pregnant and lactating women.
7. Any other systemic disease which can alter the course of periodontal disease.
8. Subjects who have received any anti-inflammatory drugs and antibiotic in the previous one month.
 5. Subjects who have received periodontal therapy within 6 months before the study

Inclusion criteria for subjects included in this study:
GROUP 1
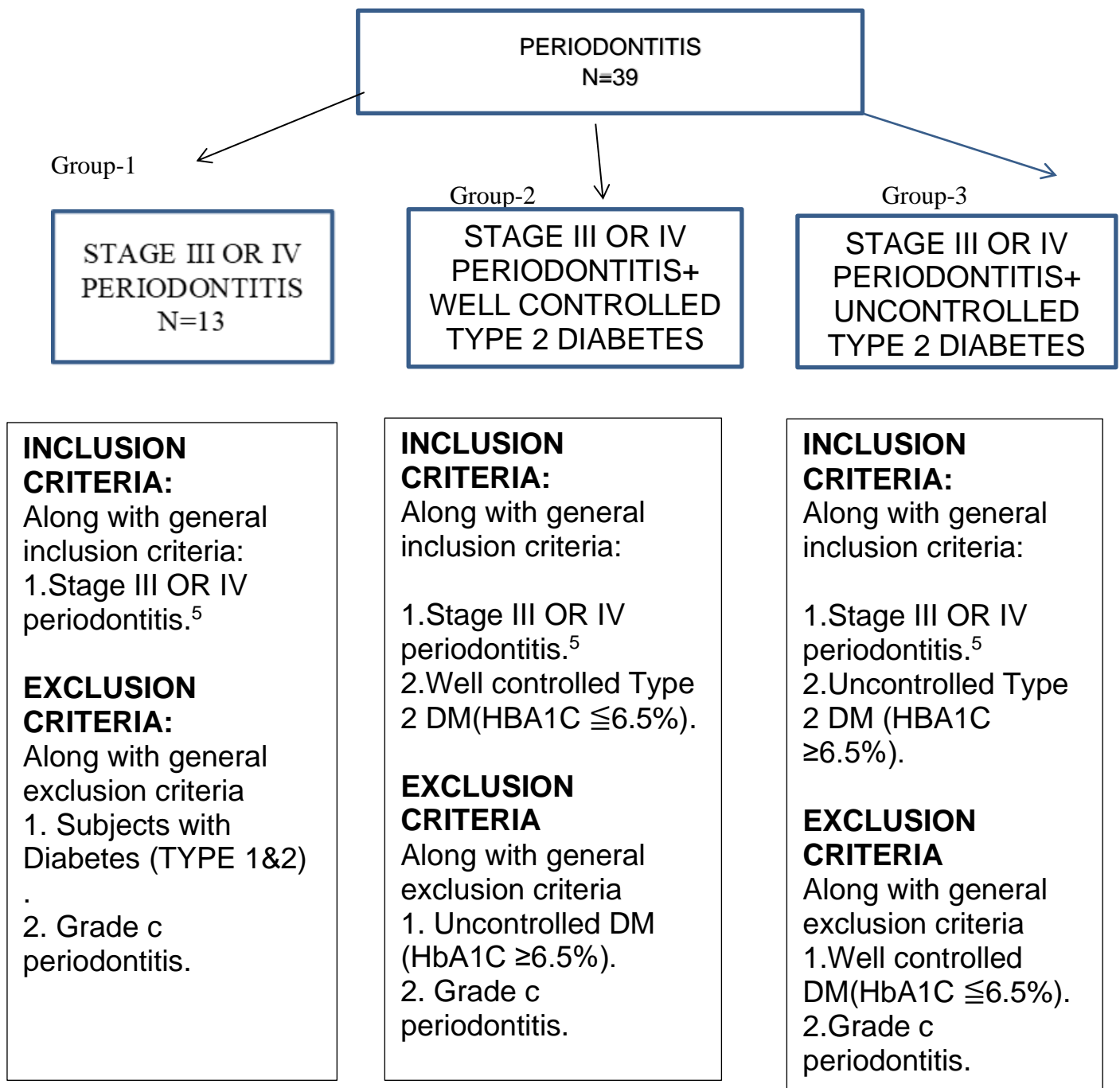Stage III or IV periodontitis subjects.[11]

GROUP 2
Stage III or IV periodontitis subjects (AAP & EFP 2017 CLASSIFICATION)[11] having Type 2 Diabetes Mellitus with HBA1c levels maintained less than or equal to 6.5% considered as controlled group.[12]

GROUP 3
Stage III or IV periodontitis subjects .[11]Having Type 2 Diabetes Mellitus with HbA1c levels more than 6.5% considered as Uncontrolled group.[12]

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                    │      PERIODONTITIS          │
                    │          N=39               │
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Group-1   Group-2   Group-3

STAGE III OR IV PERIODONTITIS N=13	STAGE III OR IV PERIODONTITIS+ WELL CONTROLLED TYPE 2 DIABETES	STAGE III OR IV PERIODONTITIS+ UNCONTROLLED TYPE 2 DIABETES
INCLUSION CRITERIA: Along with general inclusion criteria: 1.Stage III OR IV periodontitis.[5] **EXCLUSION CRITERIA:** Along with general exclusion criteria 1. Subjects with Diabetes (TYPE 1&2) . 2. Grade c periodontitis.	**INCLUSION CRITERIA:** Along with general inclusion criteria: 1.Stage III OR IV periodontitis.[5] 2.Well controlled Type 2 DM(HBA1C ≦6.5%). **EXCLUSION CRITERIA** Along with general exclusion criteria 1. Uncontrolled DM (HbA1C ≥6.5%). 2. Grade c periodontitis.	**INCLUSION CRITERIA:** Along with general inclusion criteria: 1.Stage III OR IV periodontitis.[5] 2.Uncontrolled Type 2 DM (HBA1C ≥6.5%). **EXCLUSION CRITERIA** Along with general exclusion criteria 1.Well controlled DM(HbA1C ≦6.5%). 2.Grade c periodontitis.

METHODS OF COLLECTION OF DATA
METHODOLOGY:
- After obtaining approval and clearance from the institutional ethical committee and review board, the patients fulfilling the inclusion criteria will be enrolled for the study after obtaining

informed consent. Consecutive Subjects fulfilling the criteria will be enrolled and categorized into 3 groups based on the inclusion and exclusion criteria.

- Fasting blood glucose (FBG), HbA1c levels will be monitored before treatment (baseline). Gingival index (Loe & Silness),[14] plaque index (Sillness & Loe), bleeding on probing, probing pocket depth and clinical attachment level also will be noted.
- It will be made clear to the potential subjects that participation will be voluntary and written informed consent will be obtained from those who agree to participate.

Group I: 39 GCF samples (13 GCF samples at baseline followed by 13 GCF samples 1 month after non-surgical periodontal therapy) from 13 subjects.

Group II: 39 GCF samples (13 GCF samples at baseline followed by 13 GCF samples 1 month and 3 months after scaling and root planning) from 13 subjects with periodontitis and type 2 controlled diabetes mellitus.

Group III: 39 GCF samples (13 GCF samples at baseline followed by 13 GCF samples 1 month and 3 months after scaling and root planning) from 13 subjects with periodontitis and type 2 uncontrolled diabetes mellitus along with antimicrobial medication.

Gingival index, bleeding on probing, probing pocket depth and clinical attachment level will be measured one day before GCF collection to avoid contamination of the sample with blood. The radiographs will be done to confirm site assessment. The clinical measurements will be carried out by the same examiner, using UNC-15 periodontal probe which are colour coded at every millimeter demarcation. The site showing probing pocket depth of ≥5mm with clinical attachment level of ≥3mm (in case of Periodontitis) will be selected for GCF sample collection.

GCF collection: GCF samples will be collected in Group I, Group II and Group III with micropipettes/perio-paper. GCF samples will be stored at −70ºC till the assay procedure.

After collection of GCF, non-surgical periodontal therapy followed by oral hygiene instructions and motivation will be given for all the three groups. Subjects with uncontrolled diabetes mellitus will be given treatment after obtaining the consent from the physician along with antibiotic coverage.

➢ All the subjects will be given initial periodontal therapy, including oral hygiene instruction, supragingival scaling, subgingival scaling, and root planing. Antimicrobial agents will be prescribed to uncontrolled Type 2 DM group as needed.

➢ The subjects will be recalled at 1 month and 3 months after non-surgical periodontal treatment (NSPT).

➢ S100A8 and MMP 9 levels in GCF will be measured and recorded 1 month and 3 months after NSPT and compared with their baseline levels.

➢ Clinical parameters will be measured and recorded at 1 month and 3 months after NSPT and compared with baseline.

➢ HBA1c levels will be measured at 1 month and 3 months after NSPT and compared to the baseline.

➢ DM-P patients will have to maintain their original diet, exercise, and the dose and kinds of anti-diabetic drugs during the course of the study.

➢ Estimation of S100A8 & MMP-9 will be done by using Enzyme Linked Immunosorbent Assay (ELISA) Kit obtained from commercially available ELISA kit manufacturers. ELISA procedures will be performed based on the instructions provided by the ELISA kit manufacturers.

ii.Sample Size:

39 subjects will be included in the study.Subjects will be categorized into 3 groups
with 13 subjects in each group. From each subject, GCF samples will be taken before and after
treatment resulting in a total of 78 GCF samples.

SAMPLE SIZE CALCULATION: Based on the published literature,[1]
F tests - ANOVA: Fixed effects, omnibus, one-way
Analysis: A priori: Compute required sample size
Input: Effect size f = 0.55
α err prob = 0.05
Power (1-β err prob) = 0.80
Number of groups = 3
Output: Non centrality parameter λ = 10.8900000
Critical F = 3.2849177
Numerator df = 2, df = Degrees of freedom
Denominator df = 33
Total sample size = 36
 Actual power + 0.8125474
Considering the effect size of 0.55 (medium: ratio of mean difference and pooled Standard
   deviation), the power of the study (1-β) =80%, type 1 error 5%, sample size was calculated
   using G*power software ver. 3.1.9.2.
Total calculated sample size was 36, considering 10% dropout, 3 patients will be added. Hence,
   total sample size 39, 13 in each group.

iii, Project Implementation Plan: Consecutive patients will be screened for recruitment
from the outpatient section, Department of Periodontology, Government Dental College and
Research Institute Bengaluru based on the inclusion and exclusion criteria, and 39
participants will be recruited. Ethical clearance has already been obtained from the
institution. Informed written consent will be taken from the subjects willing voluntarily to
participate in the study. The subjects enrolled will be categorized into three groups. Subjects
in the group 3 may be consulted with their physician and opinion may be sought for the
fitness if required for participation in the study. Laboratory investigation for FBG and HbA1c
levels will be analyzed at the laboratory attached to the institution and non-surgical
periodontal treatment will be carried out in the department by the co-investigator under the
supervision of principal investigator. A blinded calibrated examiner will carry out the
intraoral clinical examination and record the clinical parameters. Samples of GCF collected at
3 time points from the participants will be transferred to tubes containing Phosphate buffered
saline and stored at -70 ɔ C (sanctioned in another project) till analyzed using ELISA Kits
(ELISA READER sanctioned in another ICMR PROJECT). Data thus collected will be
statically analyzed and interpreted

iv. Ethics Review: This is longitudinal study on human subjects and involves collection of
data on periodontal clinical parameters. Collection of oral biological fluids. This study will be
carried out in accordance to the principles of Declaration of Helsinki 2008 and as revised in
2013.Institutional ethical clearance has already been obtained .Each potential participants is
given the information regarding the purpose of the research, how the participants are
recruited (Participant selection),option of voluntary participation ,information on Procedures
and Protocol, side effects, risks, benefits, assurance of maintaining the confidentiality

,sharing the results, Right to Refuse or Withdraw, contact details. Informed written consent will be obtained from all the participants.

v. Data collection & statistical analysis plan: The data will be collected & entered into excel spread sheet. Data will be analyzed using the Statistical Package for Social Sciences (SPSS) version 24 (SPSS Inc., Chicago, IL, USA). Descriptive and inferential analysis will be done. Discrete data will be represented with frequency & proportion and continuous data will be represented by mean (SD). Normality of data will be determined and parametric/non-parametric test will be applied accordingly based on the type and pattern of data. Pre and post treatment mean difference of variable will be measured with paired t test\ Wilcoxon sum rank test. Chi Square Test will be used to find out difference between proportions. The mean difference between the group variables will be determined by One Way ANOVA/ Kruskal Wallis Test and multiple comparison will be done by Bonferroni post hoc test. The receiver operating characteristic (ROC) curve will be applied for estimating sensitivity, specificity and c-statistics (area under the curve: AUC) as diagnostic ability of S100A8, MMP-9 and the algorithm using both S100A8 and MMP-9 for periodontitis. The cut-off will be decided to estimate the highest value for the sum of sensitivity and specificity. Statistical significance will be considered at p <0.05 (confidence interval of 95%).

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9.Expected Outcomes (up to 100 words): This study aims to evaluate for the first time, the Diagnostic and Prognostic ability of GCF MMP-9 and S100A8 for periodontitis in the presence of Diabetes mellitus in Indian population and to validate for clinical application to advance precision oral medicine. The result of this study may help in using GCF MMP-9 and S100A8 levels as a diagnostic and prognostic marker in periodontitis patients with Diabetes mellitus. This may help in developing a chair side kit for early detection.

10.Limitations of this study (up to 100 words).

 A group of type 2 diabetes patients without periodontitis for the observation and a control group that does not receive any therapies for the longitudinal observation should have been designed and recruited. However due to ethical issues has not been included.Since we are evaluating the diagnostic and prognostic ability of the biochemical markers for periodontitis, large multicentric trials may be required to validate the results of the study. Further, the biochemical markers that are being evaluated are not specific to periodontitis / Diabetes mellitus alone and are influenced by systemic inflammation other than under observation, hence may influence the results of this study, Hence careful screening of the study participants have to be done

11Future plans based on expected outcomes if any (up to 100 words). If the results prove that the investigational biomolecules have diagnostic and prognostic potential, future multicentric study with large samples size may be undertaken to validate the results of the study. Chair side kits may be developed if the study proves that if the biomolecules can be developed as diagnostic and prognostic markers for periodontitis. Advances in biotechnology have led to innovations in lab-on chip and biosensors to interface with oral -based biomarker assessment. The development of investigational biomolecules as biomarker and its validation for clinical application may advance the precision periodontal medicine through improved diagnosis, prognosis, and periodontal disease phenotype stratification,

12Timelines: 18 months for screening, recruitment, collection of data on clinical parameters at baseline, collection of samples at baseline, to provide non-surgical periodontal treatment and to collect data on the clinical parameters and collection of oral fluid at different time points and running the ELISA. Next 6 months for data analysis and reporting.

13.Institutional Support: Institution has provided necessary infrastructure required to carryout the previous projects funded by ICMR. There is state of art laboratory attached to the institution required for the hematological assessment required for study. Institution is also

attached to the medical college where participants can be referred for evaluation by the physician. ELISA reader, deep freezer for refrigeration of the sample is available funded from the previous projects.