



**SALIVARY MATRIX METALLOPROTEINASE-8
LEVELS AND PERIODONTAL HEALTH STATUS
AMONG ADULTS ATTENDING THE UNIVERSITY
OF NAIROBI DENTAL HOSPITAL**

Dr. GEORGE KARIMI MWAI (BDS -NBI)

V60/80156/12

**DEPARTMENT OF PERIODONTOLOGY/COMMUNITY AND
PREVENTIVE DENTISTRY**

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE AWARD OF MASTER OF DENTAL SURGERY IN
PERIODONTOLOGY, UNIVERSITY OF NAIROBI, KENYA.**

2015

DECLARATION

I **Dr George .K. Mwai** hereby declare that this is my original work and has not been presented for the award of a degree or any other purpose in any other institution.

Signed: Date:

SUPERVISORS APPROVAL

This thesis has been submitted with the approval of my supervisors from University of Nairobi.

DR TONNIE K MULLI

BDS (Nbi), MClintDent, PhD, GCAP, AHEA (UK), CDE (Jap)

Department of Periodontology, Community and Preventive Dentistry

School of Dental Sciences, University of Nairobi

Signed.....Date.....

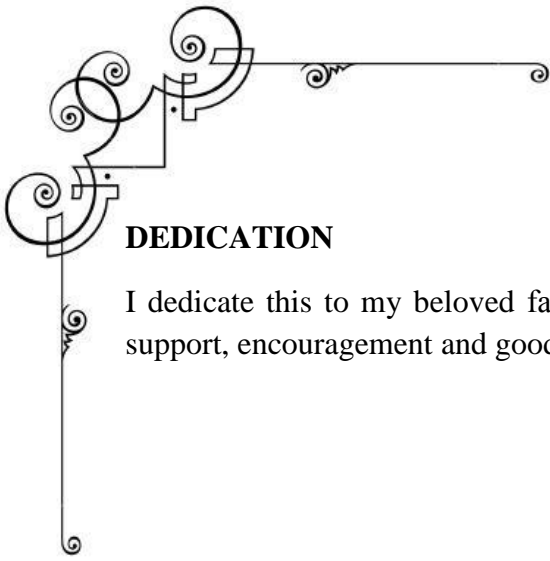
PROFESSOR FRANCIS G MACIGO

BDS,MPH,PGD-STI, FICD (Nbi)

Department of Periodontology/Community and Preventive Dentistry

School of Dental Sciences, University of Nairobi

Signed..... Date.....



DEDICATION

I dedicate this to my beloved family the Mwai's who have been a formidable pillar of support, encouragement and good cheer through this epic journey.

ACKNOWLEDGEMENTS

I thank the almighty God for giving me the opportunity and ability to carry out this research to completion. I am grateful to the University of Nairobi for the scholarship award that enabled my pursuit for this master's degree course. I am beholden and deeply grateful to my family and friends for being an unshakeable pillar of encouragement and support.

I am indebted to the Moi Teaching and Referral Hospital management for granting me study leave to pursue my postgraduate studies. I am especially grateful to my principal supervisor Doctor Tonnie Mulli for his guidance, mentorship and encouragement all through the various stages of this research. I also sincerely thank Professor Macigo and Professor Gathece for their advice and positive criticism.

I am indebted to Mr. Fred Muchache of the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) for my technical training in ELISA techniques, as well as the several hours on end spent in the laboratory. I am also thankful to Ms. Marion Makeba of UNITID for her kind assistance in timely, systematic recording, processing and storage of the saliva samples. I also thank Dr. Oyugi, Laboratory Director, UNITID for facilitating access to the laboratory and equipment. I also greatly appreciate R&D Systems Europe Limited for technical advice and permission to use their ELISA illustrations, as well as the efforts of DHL Nairobi, Kenya for the safe importation, storage and delivery of the Quantikine[®] DY908 ELISA kit and reagents.

Special thanks to Dr. Fatma Agil, and my beloved sister Esther Nyawira for kind assistance with, data entry and data validation. I extend my gratitude to Dr. Kagereki for his statistical assistance and advice in the data analysis. I am grateful to Drs. Jyoti, Alvin and Samra for their jolly participation as test run subjects and Dr. Alumera for offering office space. Finally, I especially thank all participants for their willingness to participate in this study.

TABLE OF CONTENTS

DECLARATION	ii
SUPERVISORS APPROVAL	iii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	x
LIST OF APPENDICES	xii
LIST OF ABBREVIATIONS/ACRONYMS	xiii
DEFINITIONS	xv
ABSTRACT	xvii
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW	1
1.1 Introduction	1
1.2 Literature review	4
1.2.1 Overview of periodontal disease and disease classification.....	4
1.2.2 Epidemiology of periodontal diseases.....	4
1.2.3 Pathogenesis of periodontal disease	5
1.2.4 Risk factors for periodontal disease	7
1.2.5 Host response assessment	8
1.2.6 Matrix metalloproteinases: overview	10
1.2.7 Enzyme-Linked Immunosorbent Assay (ELISA) principles	13
CHAPTER 2: STATEMENT OF THE RESEARCH PROBLEM AND JUSTIFICATION.....	16
2.1 Problem statement	16
2.2 Justification of the study	16
2.3 Objectives.....	17

2.3.1 Main objective.....	17
2.3.2 Specific objectives	17
2.4 Hypothesis.....	18
2.4.1 Null hypothesis:	18
2.4.2 Alternate hypothesis:.....	18
2.5 Study variables	19
CHAPTER 3: MATERIALS AND METHODS.....	20
3.1 Study area.....	20
3.2 Study population	20
3.3 Study design.....	20
3.4 Sample size determination	20
3.5 Sampling	22
3.6 Inclusion criteria:	22
3.7 Exclusion criteria:	22
3.8 Preliminary phase.....	22
3.9 Data collection and management	23
3.9.1 Measurement of variables	23
3.9.1.1 Sociodemographic data	23
3.9.1.2 Saliva sample collection.....	23
3.9.1.3 Clinical examination:	23
3.9.1.4 Cross infection control at sample collection	25
3.10 Laboratory procedures for quantification of Salivary MMP-8	25
3.10.1 Plate preparation	25
3.10.2 Coating of wells with capture antibody.....	26

3.10.3 Addition of recombinant MMP-8 standards and saliva samples.....	26
3.10.6 Quantification of salivary MMP-8	30
3.10.7 Calculation of salivary MM-P concentrations	30
3.10.8 Biosafety and cross infection control measures at the lab.....	31
3.10.9 Data reliability and validity.....	31
3.10.10 Minimizing bias and errors	31
3.11 Data analysis and presentation.....	32
3.12 Outcome measures	33
3.13 Constrains and limitations.....	33
3.14 Confounders	33
3.15 Ethical Considerations	33
3.16 Benefits	34
CHAPTER FOUR: RESULTS	35
4.1 Sociodemographic characteristics.....	35
4.2 Oral hygiene practices.....	36
4.3 Oral hygiene status.....	37
4.4 Gingivitis.....	38
4.5 Periodontitis	39
4.6 Salivary MMP-8.....	40
4.9 Diagnostic potential of salivary MMP-8.....	45
CHAPTER FIVE: DISCUSSION.....	47
5.1 Sociodemographic characteristics.....	47
5.2 Oral hygiene practices.....	47
5.3 Oral hygiene status.....	48
5.4 Gingivitis.....	48

5.5 Periodontitis	49
5.6 Occurrence of salivary MMP-8.....	49
5.8 Diagnostic utility of salivary MMP-8	51
5.8 Limitations	52
5.9 Conclusion	52
5.10 Recommendations	52
5.11 Conflict of interest and source of funding statement	53
REFERENCES.....	54
APPENDICES	65

LIST OF TABLES

Table 1: Salivary biomarkers of periodontal disease.....	9
Table 2: Study variables.....	19
Table 3: CDC/AAP case definitions for periodontitis	24
Table 4: Reagents used to make the double antibody ELISA sandwich.....	29
Table 5: Sociodemographic characteristics of participants.....	35
Table 6: Oral hygiene practices among participants	36
Table 7: Oral hygiene status by sociodemographic variables	37
Table 8: Gingivitis by study variables	38
Table 9: Periodontitis by sociodemographic and other variables.....	39
Table 10: Association between salivary MMP-8 levels and other variables.	40
Table 11: Results of multiple linear regression analysis to predict changes in MMP-8 levels from change in predictor variables	43
Table 12: Receiver operating characteristics (ROC) results	46

LEGEND OF FIGURES

Figure 1: Illustrations of double antibody sandwich ELISA technique	13
Figure 2: Illustrations of double antibody sandwich ELISA technique cont'd.....	14
Figure 3: Illustration to demonstrate the addition and binding of streptavidin-HRP enzyme to the biotinylated moiety of the detection antibody.....	28
Figure 4: Photograph showing ELISA microplate on a plate map.....	30
Figure 5: ELISA plate reader	30
Figure 6: Screen shot of ELISA computer software	30
Figure 7: Distribution of salivary MMP-8 levels by severity of gingivitis	41
Figure 8: Distribution of salivary MMP-8 levels by severity of periodontitis	42
Figure 9: Receiver operating characteristic (ROC) curve analysis of salivary MMP-8 on periodontitis	45

LIST OF APPENDICES

APPENDIX 1: CONSENT FORM- ENGLISH VERSION	65
APPENDIX 2: CONSENT FORM SWAHILI VERSION	67
APPENDIX 3: QUESTIONNAIRE /BIODATA FORM	69
APPENDIX 4: QUESTIONNAIRE /BIODATA FORM SCREENING SEGMENT	70
APPENDIX 5: CLINICAL EXAMINATION FORM	71
APPENDIX 6: PERIODONTAL PROBING CHART	72
APPENDIX 7: CDC / AAP CASE DEFINITIONS FOR EPIDEMIOLOGICAL STUDIES	73
APPENDIX 8: DY908 RND SYSTEMS-DUO SET ELISA DEVELOPMENT MANUAL (FRONT)	74
APPENDIX 9: DY908 RND SYSTEMS-DUO SET ELISA DEVELOPMENT SYSTEM MANUAL (BACK)	75
APPENDIX 10: LABORATORY PLATE MAPS OF MICROPLATE WELLS (PLATE 1).....	76
APPENDIX 11: LABORATORY PLATE MAPS OF MICROPLATE WELLS (PLATE 2).....	77
APPENDIX 12: LABORATORY PLATE MAPS OF MICROPLATE WELLS (PLATE 3).....	78
APPENDIX 13: LABORATORY SAMPLE ANALYSIS DATA FORM.....	79
APPENDIX 14: ETHICAL APPROVAL	80
APPENDIX 15: PERMISSION TO USE INFORMATION AND ILLUSTRATIONS FROM RND SYSTEMS LIMITED (UK).....	82

LIST OF ABBREVIATIONS/ACRONYMS

AAP/CDC	American Academy of Periodontology/Centers for Disease Control
AHEA	Associate of Higher Education Academy
BDS	Bachelor of Dental Surgery
BOP	Bleeding on probing
CAL	Clinical attachment loss
CDE	Certificate in Dental Education
ELISA	Enzyme-linked immunosorbent assay
FICD	Fellow of International College of Dentists
GI	Gingival Index
IARC	International Agency for Research on Cancer
KNH	Kenyatta National Hospital
MClinDent	Master in Clinical Dentistry
MMP-8	Matrix metalloproteinase-8
MPH	Masters in Public Health
MSc. Dent	Master of Science in Dentistry
PhD	Doctor of Philosophy
PGD-STI	Post Graduate Diploma in Sexually Transmitted Infections
ng/mL	nano grams per milliliter (nano gram= 10^{-9} g)
PPD	Periodontal probing depth

PS	Plaque score
RPM	Revolutions per minute
UK	United Kingdom
UoN	University of Nairobi
UNITID	University of Nairobi Institute of Tropical and Infectious Diseases
WHO	World Health Organization
WS	Whole saliva

DEFINITIONS

The following definitions are provided to ensure uniformity and understanding of the terms as used in the present study.

Gingivitis:

This is inflammation of the gingiva. A pattern of observable signs and symptoms that are localized to the gingiva without any loss of clinical attachment. They include bleeding on probing, bluish redness and swelling of free marginal gingiva with loss of knife edge shape.

Clinical attachment loss:

This is the vertical distance from the cemento-enamel junction to the base of the periodontal pocket. It is the apical migration of the junctional epithelium attachment to the tooth due to disease. Periodontal pocket probing depths will be measured at 6 points around each individual tooth. Pocket depths only give an indication of the amount of clinical loss of attachment and likely alveolar bone loss but are not solely reliable in measuring attachment loss from the tooth surface as the position of the free marginal gingiva may fluctuate.

Periodontitis:

A multifactorial inflammatory disease affecting the supporting tissues of the teeth. It is usually a progressively destructive process leading to pocket formation, loss of clinical attachment as well as periodontal ligament and alveolar bone destruction.

Passive saliva:

Saliva obtained by pooling at the floor of the mouth without stimulation and drooling into a collecting vessel.

Whole saliva:

A mixture of the secretions from all of the various salivary glands located in the mouth, and it also contains serum exudates from the gingiva (gingival crevicular fluid), food and cellular debris.

ABSTRACT

Background: Periodontal disease is common globally and affects about 80% of Kenyans. Late patient presentation and diagnosis coupled with lack of treatment, results in significant morbidity and tooth mortality, reduced quality of life for individuals and communities. Currently periodontal diagnosis is based on clinical findings, which utilizes techniques that are invasive and have been shown to have inherent errors and drawbacks including inability to detect current disease activity and host response. Matrix metalloproteinase 8 (MMP-8) is a key biomarker in periodontal tissue destruction and may be found in saliva. Quantification of salivary MMP-8 levels may provide a non-invasive technique for detecting the presence and activity state of periodontal disease.

Objective: To determine the relationship between the salivary MMP-8 levels and periodontal health status in adults attending The University of Nairobi Dental Hospital.

Study design: A cross-sectional study was carried out between July 2014 and April 2015.

Study participants: The study participants were adult patients aged 18 years and above visiting The University of Nairobi Dental Hospital during the period of the study.

Materials and methods: Using convenience sampling, one hundred and twenty adults were recruited out of a calculated sample size of 80 with a power of 80%. Participants who fitted the inclusion criteria had about 5mL of unstimulated whole saliva samples collected over 3 minutes, between 7:30 am and 10:00 am along with their serialized biodata. Periodontal examination to assess oral hygiene (Silness and Løe, 1964) and gingival status (Løe and Silness, 1963) were carried out. Periodontal health status was assessed using periodontal probing depth (PPD), recession and clinical attachment loss (CAL) recorded on similarly serialized forms and classified according to the CDC/AAP consensus definitions for epidemiological studies.

In the present study, target salivary MMP-8 from every participant's saliva sample was quantified using a double antibody sandwich ELISA technique utilizing specific monoclonal antibodies. Six out of the 120 were excluded as extreme outliers.

Data collected was coded and analysed using Microsoft Excel, SPSS version 20.0 for Windows and R software. Descriptive statistics were done using means and standard deviations. Chi square, t-test, Analysis of Variance (ANOVA), Pearson correlation statistics and multiple linear regression tests were also performed. Measures of prediction of salivary MMP-8 were computed using area under the curve (AUC), receiver operating characteristic (ROC), sensitivity and specificity for cut off value. Statistical significance was set at $p \leq 0.05$.

Results: Statistically significant higher levels of salivary MMP-8 were found in increasing periodontal disease severity. Out of the 120 participants recruited, 6 were dropped from the analysis as extreme outliers. The participants with no periodontitis were found to have 22.68 ng/mL, mild periodontitis 44.55 ng/mL, moderate periodontitis 46.34 ng/mL and severe periodontitis 156.62 ng/mL. A mean level of salivary MMP-8 of 40.52 ng/mL (± 66.38 SD ng/mL) with a range of 0.0 ng/mL to 295.9 ng/mL was found. A predictive value of 0.8 (AUC=0.8, $p = > 0.001$) and an optimal cut off concentration of 114.55 ng/mL was observed.

Conclusion: Within the limits of this study, a positive association was drawn between salivary MMP-8 levels and periodontal health status among adults visiting the UoN dental hospital.

Recommendation: Salivary MMP-8 should be considered as a potential diagnostic and adjunctive evaluation tool for assessing periodontal disease. However, there is further need to study this putative biomarker in better-controlled and randomized longitudinal studies in the Kenyan setting.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

This thesis consists of a study aimed at evaluating a putative salivary biomarker for the diagnosis and evaluation of periodontal diseases. This chapter describes an overview of periodontal disease, the epidemiology of periodontal disease globally and in Kenya, the effects and burden of periodontal disease and the pathogenesis of periodontal disease.

1.1 Introduction

Periodontal diseases are considered to be major contributors to the global burden of chronic disease⁽¹⁾. According to a report by the World Health Organization, advanced periodontitis leading to tooth loss affects 5–15% of most populations worldwide⁽²⁾. In Kenya, the prevalence of gingivitis is estimated to be up to 90% with periodontitis affecting about 1-10% of adults⁽³⁾. The effects of periodontal disease include, discomfort, physical, psychosocial and social disability on patients' oral health-related quality of life⁽⁴⁾. In addition, periodontal health is an integral part of general health and studies have demonstrated links between periodontal disease and systemic illnesses. Periodontal infections are implicated in a variety of other polygenic diseases for instance cardiovascular disease, diabetes mellitus, rheumatoid arthritis, obesity, osteoporosis, pulmonary disease and adverse pregnancy outcomes, for example, pre term low birth weight^(5, 6).

The classification currently used in grouping periodontal diseases and conditions clinically is based on the recommendations of the 1999 International Workshop for classification of periodontal diseases and conditions⁽⁷⁾. Conventional methods of diagnosing periodontal disease clinically include: taking medical and dental histories, assessing plaque deposits, recording clinical signs of gingival inflammation such as bleeding on probing (BOP), periodontal probing depths (PPD), gingival recession and clinical attachment loss (CAL) while assessing predisposing factors. Assessment of radiographic and microbial findings also provides additional diagnostic information on the teeth and the supporting alveolar bone⁽⁸⁾.

A joint team from the Centers for Disease Control and Prevention (CDC) and the American Academy of Periodontology (AAP) was formed in 2003 to develop and standardize case definitions for population-based studies of periodontitis. Consensus was sought due to lack of universal uniformity in the definition of periodontitis in epidemiologic studies leading to substantial inconsistencies in prevalence rates globally.

Conventional diagnostic techniques lack the ability to distinguish current or effects of previous disease and are also unable to identify those patients at greatest risk for further periodontal disease progression⁽⁹⁾. A recent publication by the AAP in July 2015, describes challenges with the potential of errors in assessment of clinical attachment loss especially when the gingival margin is coronal to the cemento-enamel junction as well as the time consuming nature⁽¹⁰⁾. Studies have also shown that not all patients who experience progressive periodontal destruction present with classical clinical features measured using conventional methods⁽¹¹⁾. Evidence also suggests that periodontal disease may have dynamic periods of disease exacerbation and remission within the individual which may not be clinically detectable⁽¹²⁾. As for microbial analysis, it is established that oral micro-organisms are necessary but not sufficient for the causation of periodontitis, as this is mostly influenced by the host response⁽¹³⁾. This is further confirmed by studies establishing individuals who are periodontally healthy being found to harbor periodontal pathogens as part of their commensal microbes⁽¹⁴⁾. The conventional diagnostic techniques have been found to be labour intensive, cumbersome and poorly utilized in screening programs⁽¹⁵⁾. It also requires a substantial amount of irreversible periodontal damage before clinical parameters can be measurable.

Technological advances are trending towards point of care biomarker testing with miniature portable devices. An example is the ongoing development of a point of care device that will provide a simple, rapid, and affordable method for counting CD4⁺ lymphocytes using microchips. It has been tested in the United States and Botswana and showed close agreement to standard laboratory analysis. It is predicted to facilitate large scale HIV monitoring in resource-poor settings⁽¹⁶⁾. Saliva is easily accessible, obtained via non invasive methods and contains factors involved in the host response. Saliva may be a diagnostic medium that is able to aid clinicians in risk determination, diagnosis and treatment planning for periodontal diseases^(17, 18).

Studies have shown salivary matrix metalloproteinase 8 (MMP-8) to be most promising as an indicator for periodontal and peri-implant tissue destruction⁽¹⁹⁾. There are no Kenyan studies describing the salivary MMP-8 profile in periodontal disease. Successful application of biologic assays requires the determination of the ability of the potential biomarker to indicate the severity and progression of periodontal disease.

The application of simple non invasive diagnostic tests will facilitate the identification of patients at risk of periodontitis, predict the progression of periodontitis and allow monitoring of the response to therapy in settings other than the dental practice, such as health care centers or at home. This will also allow individuals to be directed for more personalized treatments. Periodontal oral diagnostic devices may enable large populations to be screened accurately, in particular, underserved communities and resource-limited areas may be accessed more efficiently than by current cumbersome and poorly utilized screening programs⁽¹⁵⁾. This will by and large allow timely intervention, facilitate well targeted periodontal therapy, hence mitigate the effects of progressive periodontal destruction⁽²⁰⁾.

From the aforementioned, the aim of the current study was to determine the levels of salivary MMP-8 in adults attending the University of Nairobi Dental Hospital and the association of salivary MMP-8 concentration with varying severities of periodontal disease. This evaluated the diagnostic potential of salivary MMP-8 in the Kenyan setting. This will also provide a platform for further proteomic studies.

1.2 Literature review

Periodontitis is a progressively destructive process initiated by microbial plaque biofilms. It is characterized by initial inflammation of the gingiva, progressive destruction of tooth supporting structures causing clinical attachment loss, alveolar bone loss and pocket formation if the disease goes on unabated.

1.2.1 Overview of periodontal disease and disease classification

Historically, there have been various ways of classifying and defining various forms of periodontal disease. Most forms have been characterized by gingival inflammation, periodontal pocket formation, clinical attachment loss and alveolar bone loss⁽²¹⁾. The overall eventual result of periodontal disease is compromised periodontium with the likelihood of impaired masticatory function and tooth loss.

Currently, periodontal diseases are broadly classified clinically into several groups based on recommendations given at the 1999 International Workshop for the Classification of Periodontal diseases and conditions^(7, 22): Gingival diseases, chronic periodontitis, aggressive periodontitis, periodontitis as a manifestation of systemic disease, necrotizing periodontal diseases, abscesses of the periodontium, and periodontitis as a manifestation of systemic disease, developmental or acquired mucogingival deformities. Previous application of cross sectional studies in the definition and classification of periodontal diseases led to inconsistencies in results reported. In view of the drawbacks, a joint team from the Centers for Disease Control and Prevention (CDC) and the American Academy of Periodontology (AAP) formulated consensus case definitions for population based studies of periodontitis based on longitudinal prospective studies⁽²³⁾.

1.2.2 Epidemiology of periodontal diseases

Oral biofilm associated diseases are the major cause for tooth loss globally and pose a significant cost burden on societies and national expenditure. Periodontal disease is common among different populations and regions of the world at varying prevalence, extent and severity. Severe periodontal disease affects 5–15% of most populations globally⁽²⁾.

In Africa, gingivitis affects 50% of the population while 35% are affected by varying degrees of periodontitis⁽²⁴⁾. In Kenya, gingivitis affects up to 90% of the population while 1-10% suffer from chronic periodontitis⁽³⁾.

The CDC/AAP consensus periodontal case definition⁽²⁵⁾ was used in classifying periodontal disease in this study as it considers both the probing depths and clinical attachment loss, and has stringent criteria for defining severe periodontitis to ensure that participants identified by the provided criteria actually have the disease. Furthermore, it is well accepted and applied in population-based surveillance of periodontal disease hence applicable for comparisons with levels of the putative salivary biomarker MMP-8. Categories in this classification include “severe”, “moderate” periodontitis and “no or mild” periodontitis. A recent study estimating the prevalence, severity, and extent of periodontitis in the United States adult population applying the CDC/AAP criteria established that over 50% of the population had periodontitis. In terms of severity: 8.7% had mild periodontitis, 30.0% had moderate periodontitis and 8.5% with severe periodontitis. Periodontitis was also highest in males with less than a high school education⁽²⁶⁾. A study done in Germany reported a prevalence rate of 90% of periodontitis⁽²⁷⁾ while in Australia, a 61% prevalence of moderate and severe periodontitis has been reported⁽²⁸⁾. A study carried out on periodontal diseases in Kenyans involving 1,131 participants between the ages of 15-65 years established that clinical attachment loss affected 75-95% of individuals, the prevalence increasing with age⁽²⁴⁾. The authors recommended specific biologic characterization of the features of periodontal breakdown in susceptible patients.

1.2.3 Pathogenesis of periodontal disease

Our understanding of periodontal disease pathogenesis has evolved over the decades. There have been paradigm shifts in our way of thinking, such as from the initiating role of plaque bacteria in gingivitis by Loe et al 1965⁽²⁹⁾, to histological studies of inflammation in the periodontium by Page and Schroeder 1976⁽³⁰⁾, the recognition of variations in periodontal disease susceptibility between individuals and the landmark importance of the host response by Page et al in 1997⁽³¹⁾.

Dental plaque is important in the pathogenesis of periodontal disease. Microbes in dental plaque initiate an immune-inflammatory response in the periodontal connective tissue. There is a complex interaction between periodontal pathogens and the host. A combination of the up-regulation of immune inflammatory cytokines, eicosanoids and tissue destructive enzymes for instance matrix metalloproteinases and lower levels of protective anti inflammatory cytokines⁽³²⁾. An unfavourable imbalance between host protective and destructive mechanisms occurs in disease resulting in a net effect of irreversible periodontal tissue destruction. A paradox in that, it is the host defensive processes that cause majority of the periodontal tissue damage. In health, balanced levels of these factors are released for tissue turnover mechanisms. The role of autoimmunity in periodontal disease has also been explored whereby findings suggest that autoimmune reactions to pathologically modified extracellular matrix components, play a role in periodontal disease pathogenesis⁽³³⁾.

Periodontal diseases involve the interplay of diverse aetiologic factors including: host social and behavioral factors, genetic or epigenetic traits and the host's immune and inflammatory response⁽³⁴⁾. Genetic polymorphisms influence the progression and severity of periodontitis. Evidence for the role of genetic variations in periodontal disease has been shown in studies of identical twins, suggesting a 50% of susceptibility to periodontal disease attributable to host factors⁽³⁵⁾. The presence of various genetic polymorphisms including those in cytokine and receptor molecule genes has been related to periodontal disease susceptibility.

A recent landmark study in Kenya by Wagaiyu and colleagues on IL-1A and IL-1B isoforms, associated genetic polymorphisms in specific loci with varied periodontitis susceptibility between individuals, with IL-1A-889 locus implicated in periodontitis occurrence⁽³⁶⁾. Carriage of these genetic polymorphisms varies among populations. A meta analysis in 2008 found statistically significant associations between cytokine gene polymorphisms and periodontal disease with statistically significant differences noted between Caucasians and Asians⁽³⁷⁾. These inflammatory cytokines have been shown to play a key role in regulating MMP-8 expression, secretion and activity in the periodontium^(38, 39).

A Turkish study by Emingil in 2014 established that gene polymorphisms particularly in MMP-8 genotypes may also be associated with severe forms of periodontal disease⁽⁴⁰⁾. This indicated that inherent differences in MMP-8 expression also exist between individuals, races and regions. Furthermore, epigenetic mechanisms have been recently shown to influence chronic inflammation, including gingivitis and periodontitis⁽⁴¹⁾.

1.2.4 Risk factors for periodontal disease

Risk factors are aspects of personal behavior or lifestyle, environmental exposure, or host characteristics, which on the basis of longitudinal epidemiological evidence are known to increase the probability of developing a measurable deterioration of periodontal supporting tissues⁽⁴²⁾. Periodontitis is a multifactorial disease initiated by plaque microbes, however the manifestation and progression of disease is influenced by a wide spectrum of determinants and factors⁽⁴²⁾.

The progression of periodontal disease is also influenced by tooth level factors, socioeconomic, behavioral and environmental risk factors, which vary among populations^(43, 44). Studies have shown a relationship between systemic factors for example poor glycemic control and periodontal disease parameters due to the unfavourable effect on the gingival response to aetiologic factors⁽⁴⁵⁾. A Thai study involving 2,005 adults established that age, gender, education level, oral hygiene status, smoking history and uncontrolled diabetes are significantly associated with periodontal disease severity⁽⁴⁶⁾. A study done in neighbouring Tanzania by Mumghamba et al in 1995 found similar risk factors to be significant also including rural residence, plaque and calculus deposits⁽⁴⁷⁾.

Host genetic variability is a proven risk factor. The specific role of implicated genes still remains unclear. However, some association studies have identified a number of host gene polymorphisms that may have a key role in 38% to 82% of all cases of periodontitis^(48, 49). Racial differences, may also predispose individuals to periodontal disease⁽⁵⁰⁾. It is thus evident that periodontal disease susceptibility, the development of periodontal disease, and presentation vary greatly between individuals and populations due to varied inherent individual factors as well as exposure to a vast array of risk factors⁽⁵¹⁾.

In this current study, patients exposed to risk factors such as cigarette smoking, uncontrolled systemic disease, pregnancy and lactation were excluded.

1.2.5 Host response assessment

Diagnostic methods should provide accurate information to identify the presence, severity, as well as the prognosis of the disease as these have a pivotal role in targeted, timely periodontal disease management.

Host response factors may be found in patients' serum, saliva and gingival crevicular fluid. Examples include: neutrophil elastase, aspartate aminotransferase, matrix metalloproteinases, glucuronidases and alkaline phosphatase, prostaglandin E₂ and interleukin-I. factors that occur as a consequence of tissue damage for example collagen fragments, analysis of autoantibodies against native and modified host periodontal tissues⁽³³⁾. Other techniques employed to assess the inflammatory process include use of subgingival temperature probes, optical spectroscopy to determine gingival tissue oxygenation through total tissue hemoglobin and tissue edema in periodontal tissues *in vivo*⁽⁵²⁾. There have been advances in the detection of host response biomarkers for example: ELISA for various proteins, genomic analyses as well as prototypes for point of care devices for local and systemic diseases as well as pharmaceutical studies⁽⁵³⁾.

A study by Mantyla and colleagues in 2003 established that a gingival crevicular fluid MMP-8 may be used for a rapid chair-side diagnosis and monitoring of periodontitis before and after interventional therapy. The technique involved use of a site specific test stick employing immunofluorometric analysis⁽⁵⁴⁾. The gingival crevicular fluid contribution to whole saliva provides saliva with agents that are derived from the host's local circulation. This makes it feasible to utilize whole saliva to evaluate periodontal disease biomarkers.

The host inflammatory agents associated with periodontal disease can be detected in saliva via non invasive proteomic analysis techniques ^(20, 55). Table 1 summarizes several salivary biomarkers of periodontal disease. Saliva has abundant advantages compared to other body fluids.

Saliva is a convenient and simple point of care diagnostic tool as it is easily accessible being obtained via non-invasive, stress-free and inexpensive methods, which offers distinctive advantages over patient blood for analysis⁽⁵⁶⁾.

Unstimulated whole saliva is a passive pooling of secretions from all non-salivary and salivary gland origin at rest whereas stimulated saliva is obtained as a result of gustatory stimuli for instance using citric acid, paraffin wax, rubber⁽²⁰⁾. Whole saliva contains significant contributions from gingival crevicular fluid (GCF)⁽⁵⁷⁾. For purposes of this study, unstimulated whole saliva from every participant will be collected and analyzed.

TABLE 1: SALIVARY BIOMARKERS OF PERIODONTAL DISEASE. ADAPTED FROM ZHANG ET AL 2009⁽⁹⁾ AND PRIYANKA ET AL 2012⁽⁵⁸⁾

SALIVARY BIOMARKERS				
DENTAL BIOFILM	INFLAMMATORY BIOMARKERS	GENETIC BIOMARKERS	COLLAGEN BREAKDOWN	BONE REMODELLING
Immunoglobulins (IgA,IgM,IgG)	β -glucuronidase, β -glucosidase	Cathepsin C gene mutations	α 2-macroglobulin	Alkaline phosphatase, Acid phosphatase
Mucins	C reactive protein	Tumor Necrosis Factor polymorphisms	MMP-8	Osteoprotegerin
Lysozyme	Interleukin-1	Interleukin-1 polymorphisms(IL-1 β)	MMP-9	Osteocalcin, Osteopontin
Lactoferrin	Interleukin-6	Collagen synthesis gene mutation	Aspartate aminotransferase	Receptor Activator on Nuclear factor Kappa Ligand (RANKL)
Histatin	Macrophage Inflammatory Protein 1- α	Peroxisome proliferator-activated receptors (PPARs)	Alanine aminotransferase	C-terminal type I Collagen telopeptide

There are several advantages of using saliva as a diagnostic body fluid compared to blood or GCF for instance: saliva sampling is non-invasive, rapid and the collection process is also relatively simple, painless, generally tolerable to patients⁽⁵⁹⁾. It is also ideal for population based screening activities.

The method of collection is relatively simple and does not require a skilled work force, hence sample collection can be carried out by patients for self monitoring at home or care givers in clinical settings including in remote areas. It is thus ideal for population based screening activities. Saliva can be used in clinically challenging situations, for example obtaining samples from children, physically and mentally challenged individuals or anxious patients, in whom blood sampling could be a difficult procedure to perform.

Saliva also has minimal risk of contracting infections compared to blood. On the other hand, GCF collection is technique sensitive in terms of the active sites selection and access to the gingival sulci. Furthermore, GCF sample findings may not be representative of the entire mouth as disease states may vary between individual teeth and sites.

The use of non invasive host biomarkers has been proposed for the assessment of periodontal diseases including in larger epidemiological surveys as they would be simple, efficient and cost effective⁽⁶⁰⁾. Management of periodontal disease would also be more targeted, based on the individual's periodontal disease activity. The development of techniques with universally standardized methodology and establishing regional reference values in view of factors influencing disease is key in making salivary diagnostics an adjunctive gold standard⁽⁶¹⁾. Furthermore, saliva biomarker analysis will also increase the understanding of the pathogenesis of periodontal disease in the Kenyan setting.

Among all the aforementioned salivary biomarkers, matrix metalloproteinases particularly MMP-8 has shown great potential as an indicator for periodontal disease activity⁽⁶²⁾ and was hence of the focus of this study.

1.2.6 Matrix metalloproteinases: overview

Matrix metalloproteinases were discovered in a landmark study by Jerome Gross and Charles Lapière in 1962⁽⁶³⁾. Degradation of collagen and other extracellular matrix elements is a key component of normal tissue turnover and remodeling. Several enzymes are involved in the destruction of connective tissue components; however, collagen breakdown is primarily mediated by proteolytic enzymes called collagenases.

Matrix metalloproteinases (MMPs) are a family of over twenty zinc and calcium-dependent, proteolytic enzymes that are responsible for the turnover and degradation of the extracellular matrix in both physiological and pathological conditions. They play critical roles in reproduction, development, wound healing and repair, regeneration, tissue remodeling and cell movement⁽⁶⁴⁾. They also play a role in a number of pathological inflammatory processes such as osteoarthritis, cardiovascular diseases, rheumatoid arthritis, fibrosis and cancer.

MMPs also influence the activity of various cytokines, indicating a role in local tissue activation and inactivation of the immune inflammatory system. The MMPs can be grouped into collagenases, gelatinases, stromelysins, membrane type MMPs and matrilysins based on their domain structure. Two distinct types of interstitial collagenases have been characterized in vertebrate tissues: fibroblast collagenase (MMP-1) and the neutrophil collagenase (MMP-8)⁽⁶⁵⁾.

Matrix metalloproteinase-8 is also known as neutrophil collagenase as it is primarily synthesized by neutrophils. Other host cells, such as fibroblasts, endothelial cells and chondrocytes have been found to secrete the enzyme. The MMP-8 structure consists of several domains. The N-terminal pro-domain contains the cysteine switch specifically conserved in MMPs, it maintains the latent state. Enzyme activation occurs locally in the extracellular space by the removal of the pro-domain⁽⁶⁶⁾. Neutrophil pro-MMP-8 has a molecular weight of 75 kD (kilo Daltons); whereas other cells may produce smaller 50-55 kD forms of pro-MMP-8. The differences originate by variations in enzyme structure via glycosylation, these succinct structural differences allow specific proteomic detection and quantification⁽⁶⁷⁾.

Several studies including a recent study by Gursoy and colleagues in 2010 showed MMP-8 to be the main enzyme that initiates type I collagen degradation associated with periodontal tissue destruction⁽⁶⁸⁻⁷⁰⁾. Type I collagen is the main component of periodontal connective tissue. During the destructive phases of periodontal disease, various gene regulated cytokines, such as Interleukin-1, Interleukin-6, Interleukin-8 and Tumour Necrosis Factor- α , stimulate the synthesis and release of MMP-8 from neutrophils into the periodontal connective tissues.

Neutrophils have a high capacity to infiltrate tissues, studies have shown that most of the matrix metalloproteinase found in whole saliva originates from neutrophils (PMNs)⁽⁶¹⁾. The enzymes permeate into the gingival sulcus or periodontal pocket to the gingival crevicular fluid, where they contribute to overall saliva composition. MMP-8 is not only up-regulated in affected periodontal connective tissues, but also in serum and plasma⁽⁷¹⁾.

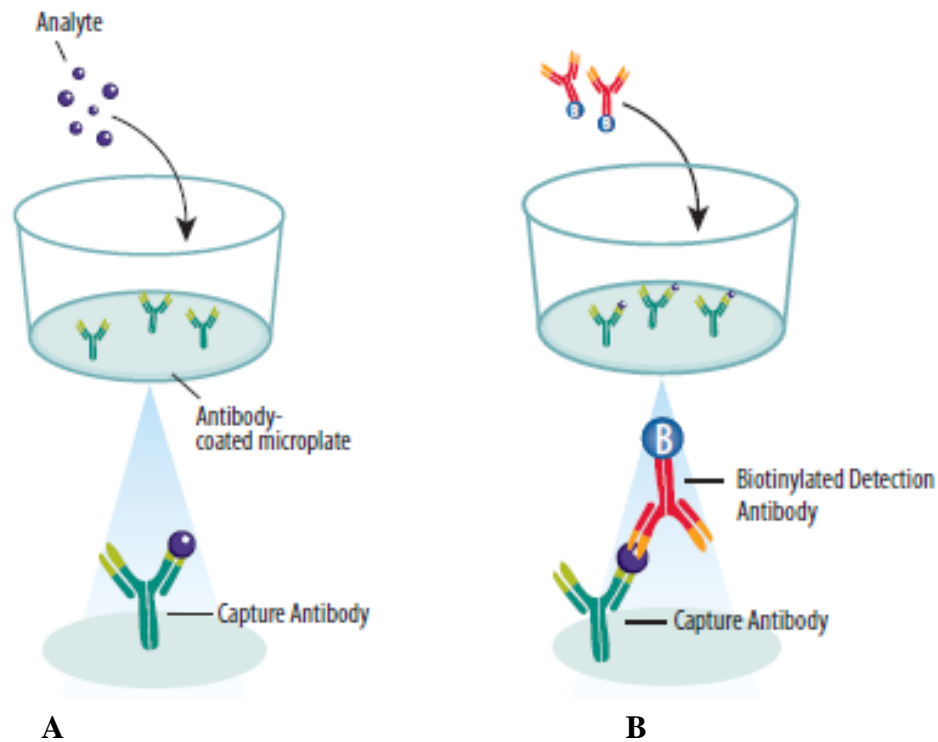
The MMP-8 gene localizes to chromosome 11q22.3. A study by Chou and colleagues in 2011 demonstrated that the MMP-8 gene has polymorphisms in -799 C >T alleles at the promoter region between individuals. The T allele was shown to increase MMP-8 expression and activity ⁽⁷²⁾. Thus various sociodemographic, racial and genetic variations may influence the expression of MMP-8 among individuals.

The regulation of MMP secretion and activity is carried out at various levels. Quantity or expression is regulated at the level of gene transcription, whereas enzyme activity is through regulation by endogenous MMP-specific inhibitors for example the tissue inhibitors of metalloproteinases (TIMPs) and general circulatory inhibitors, such as α 2 macroglobulin⁽⁶²⁾. In periodontal disease, an unbalanced ratio exists between matrix metalloproteinases and endogenous inhibitors of metalloproteinases favoring periodontal tissue destruction. Salivary MMP-8 is thus a promising candidate for non invasive diagnosis and possibly more importantly, assessing the severity, activity and progression of periodontal disease^(57, 73). In conclusion, it is possible to detect and quantify these biomolecules in saliva using molecular techniques such as ELISA. Benchmarking of salivary MMP-8 with clinical parameters may prove the putative biomarker to be a valuable adjunct or substitute to traditional periodontal diagnostic methods.

1.2.7 Enzyme-Linked Immunosorbent Assay (ELISA) principles

ELISA is a biochemical technique used to detect the presence of molecules. In ELISA, an unknown amount of antigen to be detected is affixed to an inert solid surface (usually a polystyrene microtiter plate) using an antibody (capture antibody). A second antibody specific to the same unknown molecules/antigens (detection antibody) is then washed over the surface so that it can specifically bind to the affixed antigen. This forms a double antibody sandwich (figure B). The detection antibody is then linked to an enzyme (such as peroxidase). Substrates: H_2O_2 and Tetramethylbenzidine (TMB) are then added such that the streptavidin enzyme can convert the assemble to a detectable, quantifiable colour signal⁽⁷⁴⁾.

FIGURE 1: ILLUSTRATIONS OF DOUBLE ANTIBODY SANDWICH ELISA TECHNIQUE
With permission from RnD Systems Europe Limited (UK)

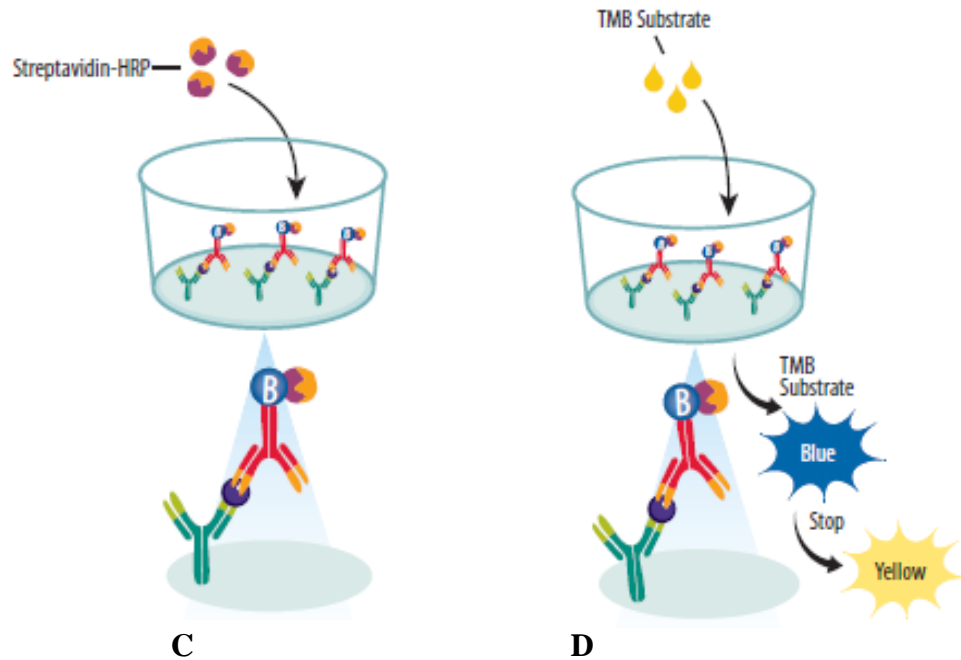


A: Polystyrene microplate well, coated with capture antibodies to immobilize/affix antigen (MMP-8) in analyte (saliva sample) being added.

B: Detection antibody added- Biotinylated goat antihuman MMP-8 (to bind to captured MMP-8 and form the sandwich)

FIGURE 2: ILLUSTRATIONS OF DOUBLE ANTIBODY SANDWICH ELISA TECHNIQUE CONT'D

With permission from RnD Systems Europe Limited (UK)



C: Addition of Streptavidin-Horse radish peroxidase (HRP) enzyme to link with biotinylated moiety on detection antibody.

D: Addition of substrates: H_2O_2 and Tetramethylbenzidine (TMB) - visualizing agents that turn blue on breakdown by streptavidin and turn yellow addition of an acid solution (2N Sulfuric acid). The acid stops the reaction after the prescribed incubation period with a resultant colour change to shades of yellow. The shades of yellow are then read in a spectrophotometer to indirectly detect and quantify the amount of antigen (salivary MMP-8).

NOTE: Incubation of reagents is done between steps to allow the reactions to proceed to completion and thereafter flushing of wells with a mild detergent to clear unbound substances

Other techniques utilized in identifying and quantifying human MMP-8 are Western blot, Immunofluorometric method (IFMA) as well as Gelatin and Casein zymography. These require complex sample purification stages, electrophoresis, are time consuming and not as sensitive ⁽⁷⁵⁾.

A recent study by Leppilahti established that MMP-8 concentrations can be specifically quantified with increasing levels of periodontal inflammatory burden by analyzing an oral rinse using ELISA ⁽⁷⁵⁾. Thus, ELISA is a reliable convenient diagnostic tool with highly specific detection and quantification of target biomolecules. Quantification allowed for associations to be made between levels of salivary MMP-8 and clinical parameters including periodontitis severity. ELISA kits are commercially available, and provided with well developed pre optimized components for simplified analysis of a large number of samples.

In conclusion, owing to these advantages, a double antibody sandwich ELISA technique was employed in this study to specifically detect and quantify the total human salivary MMP-8 in participants' saliva samples.

CHAPTER 2: STATEMENT OF THE RESEARCH PROBLEM AND JUSTIFICATION

2.1 Problem statement

Periodontal disease has a high prevalence in Kenya, estimated to affect about 80% of the adult population⁽²⁴⁾. Periodontal disease is also prevalent worldwide and a leading cause of tooth loss in adults. Periodontitis is a multifactorial disease initiated by plaque microbes however the host immuno inflammatory response is critical in instigating periodontal tissue damage. Uncontrolled periodontal disease leads to progressive loss of clinical attachment, loss of supporting tissues and eventual tooth loss.

Current clinical and epidemiological periodontal diagnostic methods have been shown to have inherent errors failing to provide precise diagnosis of periodontal disease status activity, and prediction of outcomes with or without appropriate therapy^(7, 76, 77). The techniques are also labour intensive, invasive, challenging, time consuming and require skilled and trained personnel. Furthermore, a significant level of periodontal damage has to occur for clinical parameters to be measurable. Research is thus needed to develop diagnostic strategies that utilize the current global understanding of periodontal tissue metabolism in health and disease. This will result in more accurate, efficient, less invasive, less tedious methods in large scale epidemiologic studies as well as in the clinical settings.

2.2 Justification of the study

Salivary MMP-8 seems promising as a biomarker for periodontitis in Caucasian studies, however it has been well established that inheritable elements of susceptibility, epigenetic factors, racial, ethnic, socioeconomic, environmental and demographic factors alter the expression of the host response and hence expression of specific biomarkers^(50, 78). Genetic, racial, socioeconomic and behavioral variability in periodontal disease pathogenesis and presentation has been established⁽⁷⁶⁾. This includes genetic polymorphisms in MMP-8 enzyme expression in individuals⁽⁷²⁾. Notably, some of the genetic variations may also be protective and beneficial to the host⁽⁷⁹⁾. Hence, the need to

determine the levels and optimal cut-off concentrations of biomarkers between various populations.

No data exists on the putative salivary biomarker MMP-8 in the Kenyan population and in particular, how the levels relate to periodontal health. The results of this study will give further insight into the periodontopathic process of periodontal disease which may help clinicians to better understand and manage periodontitis as well as provide a platform for further molecular studies in the pathogenesis of periodontal disease.

With the aforementioned, it would be useful to evaluate the expression of salivary MMP-8 in an African context especially in Kenya with an aim to determine its diagnostic utility as a putative biomarker for periodontitis. This will also aid in development and application of rapid point of care devices in our settings to allow early identification and treatment. The use of such biomarkers would be more useful in such a population as the burden of this irreversible disease far outstrips the physical and human resources available to detect and monitor it.

2.3 Objectives

2.3.1 Main objective

To determine salivary MMP-8 levels and periodontal health status among adults attending the University of Nairobi dental hospital.

2.3.2 Specific objectives

- a.** To establish the levels of severity of periodontitis among adult patients attending the University of Nairobi dental hospital.
- b.** To determine the salivary MMP-8 levels among the adult patients attending the University of Nairobi dental hospital.
- c.** To determine the association between levels of salivary MMP-8 and periodontal disease severity among the adult patients attending the University of Nairobi dental hospital.

2.4 Hypothesis

2.4.1 Null hypothesis:

There is no association between periodontal health status and salivary MMP-8 levels among adult patients attending the University of Nairobi dental hospital.

2.4.2 Alternate hypothesis:

There is an association between periodontal health status and salivary MMP-8 levels among adult patients attending the University of Nairobi dental hospital.

2.5 Study variables

TABLE 2: STUDY VARIABLES

VARIABLES	MEASUREMENT
<i>Sociodemographic variables</i>	
Age	Number of years
Gender	Male or female
Residence	Where the participants currently reside (Nairobi or outside Nairobi county)
Education	Level of education attained: Primary, Secondary, Tertiary
<i>Independent (exposure) variables</i>	
Gingival inflammatory status	Presence and severity of gingival inflammation – (Gingival Index by Loe and Silness, 1963)
Severity of periodontitis	No, mild ,moderate and severe periodontitis(CDC/AAP)
<i>Dependent (Outcome) variables</i>	
Salivary MMP-8	Salivary MMP-8 ELISA concentration nano grams per milliliter (ng/mL) in the 100 μ L of supernatant from participants' centrifuged whole passive saliva
<i>Confounder variables</i>	
Oral hygiene status	Presence and amount of plaque deposits (Plaque scores by Silnes and Loe, 1964)
Cigarette smoking	Presence or absence of a cigarette smoking habit

CHAPTER 3: MATERIALS AND METHODS

3.1 Study area

The study was carried out at The University of Nairobi Dental Hospital, situated a few kilometers from the central business district of Nairobi, located opposite the Lee Funeral Home and Nairobi Hospital, off Argwings Kodhek road. It serves as the national dental referral hospital in Kenya and is a training centre for both undergraduate and postgraduate students. An average of 4,000 patients visit the hospital annually, with an estimate of 25 patients daily. It serves both rural and urban populations with a wide socio economic spectrum.

3.2 Study population

The study population consisted of adult patients visiting The University of Nairobi Dental hospital with and without periodontal diseases of various severities.

3.3 Study design

This was a descriptive cross sectional study

3.4 Sample size determination

The difference in the mean (μ) salivary MMP-8 level was evaluated in four groups of participants:

1. No periodontal disease (μ_1)
2. Mild periodontal disease (μ_2)
3. Moderate periodontal disease (μ_3)
4. Severe periodontal disease (μ_4) (as described in appendix 6)

Hence this required an **analysis of variance (ANOVA) model**.

Given the null hypothesis stated that $\mu_1=\mu_2=\mu_3=\mu_4$, the alternative hypothesis stated that $\mu_1 \neq \mu_2 \neq \mu_3 \neq \mu_4$, and that the population variance was unknown. Findings aimed to reject the null hypothesis at a significance level of 0.05, which gave a corresponding Z score, referred to as $Z_{\alpha/2}$. Therefore, in the power analysis as outlined by Cohen in 1988⁽⁸⁰⁾ the power function was:

$$f = \sqrt{\frac{\sum_{i=1}^k p_i * (\mu_i - \mu)^2}{\sigma^2}}$$

where $p_i = n_i / N$,
 n_i = number of observations in group i
 N = total number of observations
 μ_i = mean of group i
 μ = grand mean
 σ^2 = error variance within groups

Where:

f- is the effect size

k - is the number of groups

n- is the sample size in each group.

sig level- is the significance level (Type I error probability)

Power-is the power of test (1 minus Type II error probability)

Cohen suggests that f values of 0.1, 0.25, and 0.4 represent small, medium, and large effect sizes respectively. Statistical significance indicates whether there is a difference between groups, whereas effect size defines the magnitude or size of the change/difference. Conventionally, a large effect size is desirable. Power is probability that a statistical test will indicate a significant difference when a difference actually exists⁽⁸¹⁾. Thus applying the $f = 0.4$, $k = 4$, significance level of 0.05 and power of 80% (0.8) in R software (pwr package) developed by Stéphane Champely (August 2013) which functions along the lines of Cohen methods; resulted in a sample size of 16 participants per group as shown in the output below:

f (effect size) = 0.4, k = 4 (groups), sig.level = 0.05, power = 0.8. Hence, $n = 15.89713$

Thus with approximately 20 participants per group, the sample size calculated was 80 participants. For the purposes of the study, 120 adults were recruited.

3.5 Sampling

Convenience sampling method was used to select the study participants whereby morning patients presenting at the oral diagnosis clinic of the UoN dental hospital during the period of the study were recruited.

3.6 Inclusion criteria:

All persons, 18 years and above, who gave voluntary informed consent to participate in the study.

3.7 Exclusion criteria:

Non-consenting patients.

Persons who were below the age of 18 years.

Persons who had a periodontal treatment procedure within the past 6 months.

Persons who had consumed any antibiotic within the past 6 months.

Persons who had concurrent systemic illness for example rheumatoid arthritis.

Female patients who were pregnant or lactating.

Persons who were smokers or had smoked cigarettes in the last 3 years

Edentulous patients and patients who had less than 20 teeth.

3.8 Preliminary phase

A preliminary visit was also made to the University of Nairobi Institute of Infectious and Tropical Diseases (UNITID) laboratory situated at Kenyatta National Hospital (KNH). This was to work out feasibility, logistics and procedures concerning human salivary MMP-8 enzyme analysis using ELISA. A pilot run was done at the University of Nairobi dental hospital oral diagnosis clinic, whereby biodata, clinical examination forms, saliva collection protocols were carried out using five test subjects.

The five test samples were analysed using the DY908 ELISA kit at the laboratory to test if the protocols applied could achieve the objectives. Necessary corrections and adjustments were then done.

3.9 Data collection and management

Data was collected using various tools, equipment and techniques as follows:

3.9.1 Measurement of variables

3.9.1.1 Sociodemographic data

Data concerning sociodemographic variables was collected from participants using interviewer administered and serialized questionnaires.

3.9.1.2 Saliva sample collection

Prior to saliva sample collection, the participants were allowed to rest seated comfortably for a few minutes and then asked to thoroughly rinse their mouth with water into the spittoon. They were then asked to slightly lean forward and not to swallow or speak. After about 5 minutes, the saliva had pooled in the anterior floor of the mouth. A minimum of 3mL whole saliva sample collection was then done by passively drooling into 50mL pre weighed, airtight, serialized, centrifuge compatible polystyrene tubes⁽⁸²⁾. Absorbent paper towels were provided for any untoward spillages. Saliva collection was done between 8:00 a.m and 11:00 a.m.

Samples in tubes were placed in a sealable polythene bag, covered with ice cubes and gel ice packs inside a cooler box. These were then transported in the shortest time possible (maximum 2 hours) to the UNITID laboratory within KNH for processing.

3.9.1.3 Clinical examination:

Clinical examination was then done on a dental chair by the investigator using sterile dental mirrors and Marquis periodontal probes in dental trays. Plaque scores were taken using Silness-Loe index (1964) and gingival index using the Loe and Silness index (1963) as shown in Appendix 5 on Ramfjord's index teeth which have been established to be of representative and epidemiological significance including the East African region⁽⁸³⁾. Full mouth periodontal examination was done and probing depths, recession and clinical attachment loss in millimeters was measured at six sites per tooth (mesiobuccal, mid-buccal, distobuccal, mesiolingual, midlingual and distolingual) using a

sterile Marquis periodontal probe at all teeth excluding third molars.

The 6 point periodontal measurements have been shown to provide high sensitivity for prevalence evaluation and low relative bias for disease severity and extent estimates⁽⁸⁴⁾. Findings were recorded in a periodontal probing chart to the nearest millimeter (Appendix 6). Periodontitis category was then determined using the CDC/AAP consensus definitions, However, since it classifies individuals with mild periodontitis and no periodontitis together, separate categories for mild and no diseases were set as shown in table 3.

TABLE 3: CDC/AAP CASE DEFINITIONS FOR PERIODONTITIS - ADAPTED FROM PAGE ET AL 2007⁽²³⁾

Disease category	Clinical attachment loss	Periodontal pocket depths
Severe periodontitis	More than 2 interproximal sites with CAL of more or equal to 6 mm (not on the same tooth)	AND 2 or more interproximal sites with PPD of more than or equal to 5 mm
Moderate periodontitis	More than 2 interproximal sites with CAL of more or equal to 4 mm (not on the same tooth)	OR 2 or more interproximal sites with PPD of more than or equal to 5 mm
Mild periodontitis	More than 2 interproximal sites with CAL of more or equal to 2 mm (not on the same tooth)	OR 2 or more interproximal sites with PPD of more than or equal to 4 mm
No periodontitis	No evidence of mild, moderate or severe periodontitis	

**Third molars excluded. *PPD- periodontal probing depth, *CAL-clinical attachment loss.*

3.9.1.4 Cross infection control at sample collection

All instruments and gauze to be used in saliva collection and periodontal examination had been sterilized in a calibrated and serviced autoclave at the University to prevent cross infection.

Hand washing as well as use of personal protective equipments for example masks and clean disposable examination gloves, cups and paper towels were used for examination of every participant. Any inadvertent spillage on surfaces was cleaned and disinfected using hypochlorite swabs. Saliva samples were collected in airtight plastic saliva collection tubes and transported in a spill resistant cooler box packed with ice cubes and gel ice sachets to the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) laboratory for processing.

3.10 Laboratory procedures for quantification of Salivary MMP-8

Enzyme linked immunosorbent assay (ELISA) technique was employed in this study. For ELISA, it is required to have a known concentration of the target biomolecule (MMP-8), usually a recombinant (standard) which will be serially diluted and analysed obtain optical readings along with the samples. The known reducing concentration of the standards is employed to generate a standard logarithmic curve of best fit to allow translation of sample optical densities to corresponding target biomolecule concentrations.

A commercially available ELISA kit, Quantikine® Colorimetric double antibody sandwich ELISA kit DY908 by R&D Systems (United Kingdom), was used to detect and quantify concentrations of MMP-8 in saliva samples from the participants. The ELISA kit is all inclusive supplied with optimized reagents ready for use or by simple reconstitution as per the manuals provided. R&D Systems is a well referenced ELISA manufacturer in various studies on salivary MMP-8.

A pilot run was carried out using samples from test subjects, the MMP-8 standards as well as negative controls (blanks) to optimize and streamline the analysis protocols. The study samples assay was carried out as described in the steps below.

3.10.1 Plate preparation

The 96-well microplates were mapped out in rows and columns in duplicate on lab data sheets to indicate the positions of standards and coded samples in duplicate wells (Appendix 10-12). The reagents supplied were reconstituted using dilution formulas as per the manufacturer's instructions (Appendix 8 and 9).

3.10.2 Coating of wells with capture antibody

The capture antibody (mouse anti-human MMP-8) in powder form was reconstituted by first adding 1mL of phosphate buffered saline to the vial and shaking to make 360µg/ml stock solution. This was then diluted to make 30mL (30,000µL) at a working concentration of 2µg/mL of capture antibody for the assay. 100µL of the prepared capture antibody at the working concentration was then placed to each well of the inert (polystyrene) microplates using micropipettes and allowed incubate overnight. This step allowed the monoclonal capture antibody specific for human MMP-8 to be immobilized onto the microplate wells.

3.10.3 Addition of recombinant MMP-8 standards and saliva samples

Following overnight incubation, any unbound capture antibody was flushed out of the wells using the wash buffer concentrate (a mild detergent) employing a pre programmed autowasher machine (ASYS[®] by Atlantis). The machine accurately and automatically washes each plate 2 times for 3 cycles (6 washes) by repeatedly filling each well with 400µL of wash buffer and aspirating in sequence. Any remaining wash buffer after aspiration, was removed from the microplates by inverting them against disposable absorbent paper towels.

Unbound sites in the capture antibody layer were then blocked by adding 300µL of reconstituted reagent diluent 1% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (pH 7.2 to 7.4, 0.2µm filtered) in every microplate well and incubating at room temperature for a minimum of 1 hour. After the incubation period had lapsed, flushing of wells was done to remove any residual reagents.

The reconstituted standards (recombinant human MMP-8) were then placed in duplicate wells and serially diluted in duplicate columns as per the pre set plate maps (appendix 10

to 12) to result in 100µL of known reducing concentrations of recombinant MMP-8 per well. The last 2 wells in the standards columns had reagent diluent placed as a blank reference (figure 3). Saliva supernatants previously stored at -80⁰ were left to thaw at room temperature and similarly, 100µL of supernatant pipetted into each well in duplicate as per the serial numbers on the plate maps.

Incubation was then allowed for a minimum of 2 hours at room temperature to allow the recombinant MMP-8 standard as well as any human MMP-8 present in the test samples (antigen) to be bound to the capture antibody coating the wells.

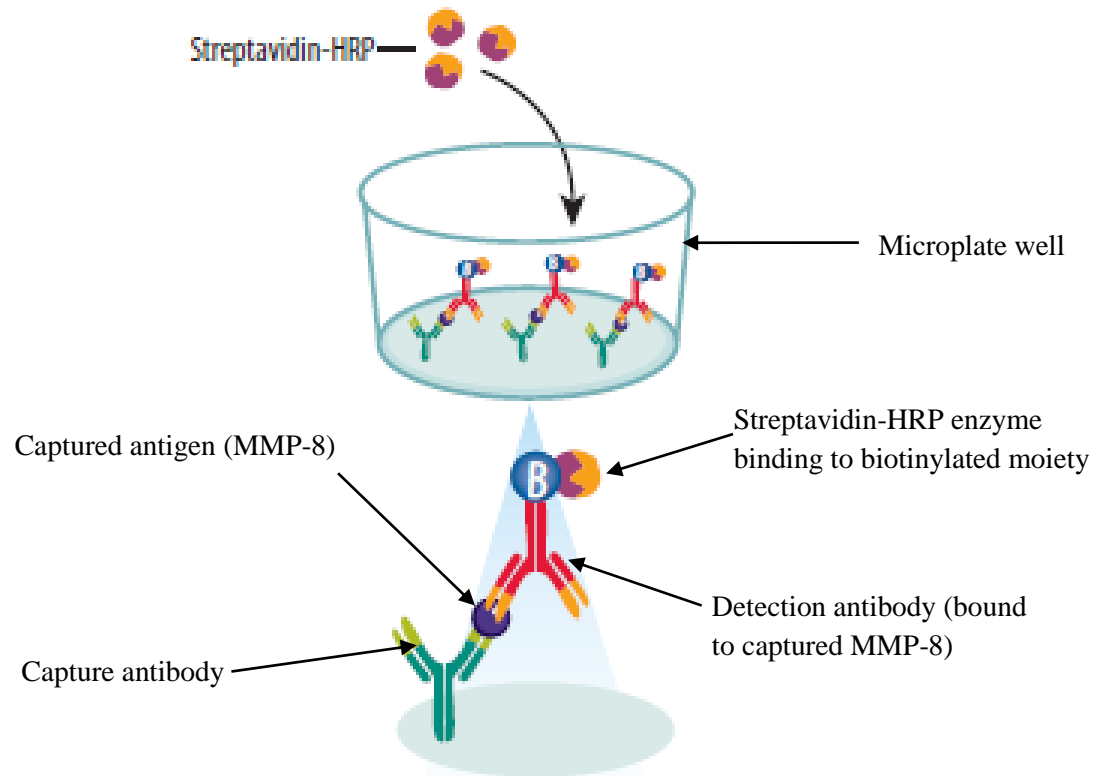
3.10.4 Addition of detection antibody and enzyme linkage

Aspiration and washing of wells was done using the ELISA plate autowasher machine to remove any unbound analytes. A second monoclonal antibody specific for the captured MMP-8 (Biotynylated goat antihuman MMP-8) was then prepared as per manufacturer's instructions to the required working 200ng/mL concentration and then added to the wells. This was then incubated for the recommended 2 hours at room temperature allowing binding of the capture antibody via the already bound antigens (MMP-8) thus making the sandwich (figure 2). After the incubation period had elapsed, aspiration/wash cycles of the wells was done using the autowasher to clear any unbound reagents.

100µL of an enzyme, streptavidin-horse radish peroxidase (HRP) was then added per well to specifically bind to the biotinylated moiety of the detection antibody. This is illustrated in figure 2. The prepared Streptavidin-HRP enzyme was used within 1 hour of reconstitution and placed away from direct light to ensure stability.

FIGURE 3: ILLUSTRATION TO DEMONSTRATE THE ADDITION AND BINDING OF STREPTAVIDIN-HRP ENZYME TO THE BIOTINYLATED MOIETY OF THE DETECTION ANTIBODY.

With permission from RnD Systems Europe Limited (UK)



3.10.5 Addition of substrate and reactions

Aspiration/wash sequence of wells was done using the autowasher to remove any excess unbound Streptavidin-HRP. The substrate for the detection antibody-bound enzyme was a 1:1 ratio of 15mL of colour reagent A (H_2O_2) and 15mL of reagent B (Tetramethylbenzidine TMB). 100 μ L of substrate was added to each well. The plates were covered with plate sealers and incubated for 20 minutes at room temperature away from direct sunlight.

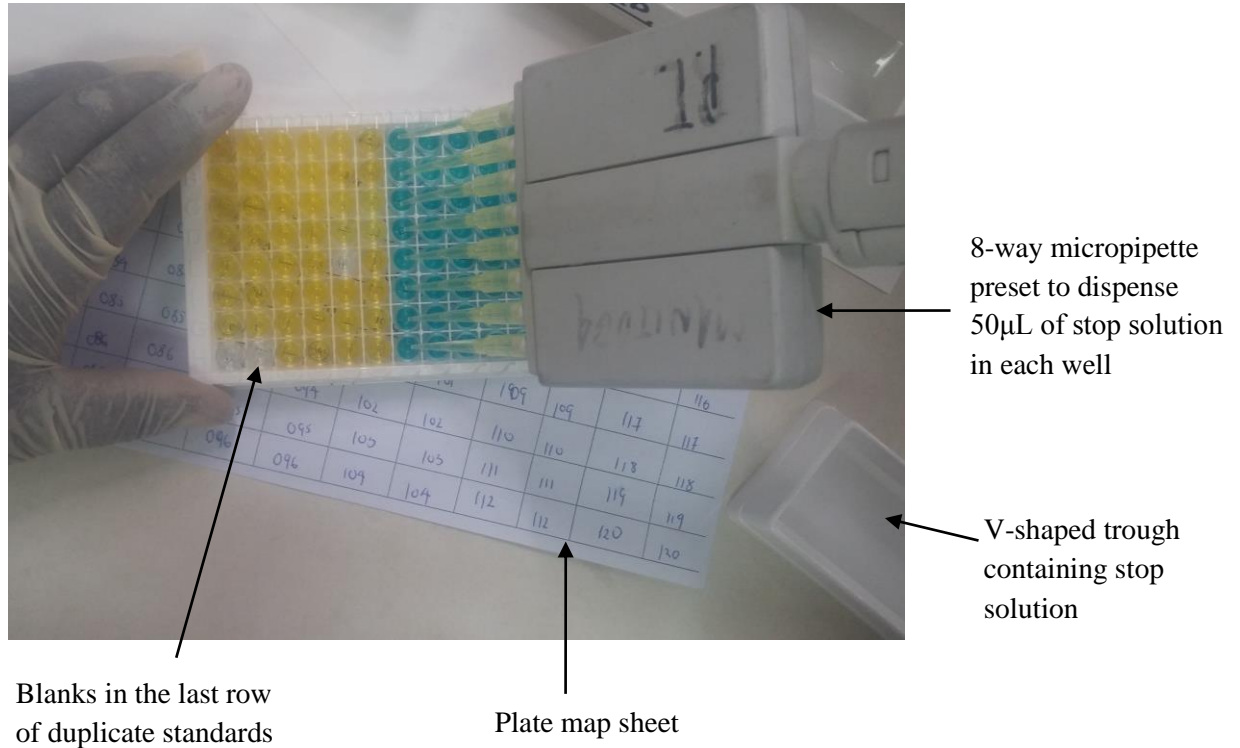
A varying pale blue colour appeared in the wells after the minimum incubation period had elapsed. A 50 μ L of Stop solution (2N H_2SO_4 acid solution) was then added to each well to halt further reactions. Thereafter, the intensity of the yellow color that developed (figure 2) was then measured using spectrophotometry⁽⁸⁵⁾

Table 4 gives a summary of the reagents used in the salivary MMP-8 ELISA. Figure 4 is a photograph showing the microplate set up at the stage of stopping reactions.

TABLE 4: REAGENTS USED TO MAKE THE DOUBLE ANTIBODY ELISA SANDWICH

Reagent	Volume per well and concentration
Capture antibody	Mouse antihuman MMP-8 (100µL at 2.0 µg/mL)
Standard	Recombinant human MMP-8 (100µL of 120ng/mL to be serially diluted)
Saliva sample	Salivary MMP-8 (100µL-at unknown concentration)
Detection antibody	Biotynylated goat antihuman MMP-8 (100µL at 200ng/mL)
Blocking agent	Reagent diluent 1% Bovine Serum Albumin in PBS (pH 7.2 to 7.4 0.2µm filtered)
Enzyme to bind to biotinylated moiety of detection antibody and act on substrate.	Streptavidin-Horse Radish Peroxidase 100µL at 1:200 (in PBS)
Substrate solution	1:1 ratio of 15mL of Colour reagent A -Hydrogen peroxide (H ₂ O ₂) and 15mL of B (Tetramethylbenzidine TMB)
Stop solution	50µL of 2N Sulphuric acid (H ₂ SO ₄)

FIGURE 4: PHOTOGRAPH SHOWING ELISA MICROPLATE ON A PLATE MAP



3.10.6 Quantification of salivary MMP-8

A serviced and calibrated microplate reader Emax spectrophotometer -Molecular devices x[®] as shown in figure 5, set at 450nm filter was used to measure the optical density of each well as per the pre set plate map. A linked computer software (SOFTmax[®] PRO 4.3.1 Life Sciences edition by Carey Farquhar, University of Washington) as shown in figure 6, was used to automatically display the resultant optical densities from every well onto a Microsoft excel sheet.

FIGURE 5: ELISA PLATE READER

FIGURE 6: SCREEN SHOT OF ELISA COMPUTER SOFTWARE



Figure 5

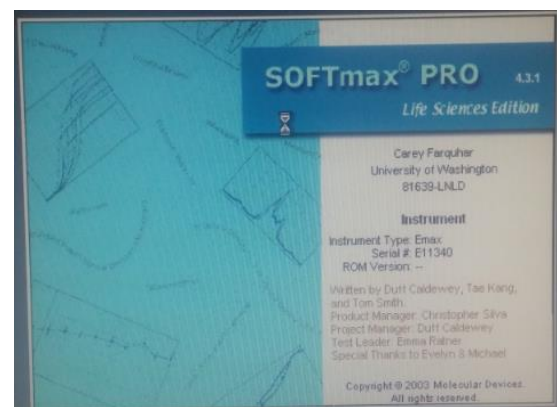


Figure 6

Average optical readings for each duplicate standard and saliva samples were recorded and subtraction of the average zero/blank optical density was done to correct the results. The mean absorbance for each MMP-8 standard on every microplate was then plotted on y axis, against the MMP-8 concentration in (ng/mL) on the x axis. Thereafter, using computer software, a *4-parameter logistic* (4PL), non linear regression line of best fit curve was plotted. Non linear regression line of best fit is ideal for ELISA analysis. Each curve provided a trend line formula that enabled conversion of the samples optical densities to the corresponding salivary MMP-8 concentrations in ng/mL.

3.10.8 Biosafety and cross infection control measures at the lab

Saliva sample handling including collection, transportation to the lab, processing and disposal following analysis was done in consultation with a qualified supervisor and laboratory technologist. This was to ensure coherence to international biosafety protocols, prevent contamination and ensure sample viability. Personal protective equipment for eyes, face, hands and clothing were used as per lab biosafety protocols during the constitution and handling of reagents as well as ELISA analysis of samples.

3.10.9 Data reliability and validity

To determine intra examiner consistency, readings from every tenth participant were reexamined on the same day of data collection to evaluate variability. The saliva collection protocol was also assessed on the pilot run. Both data collection procedures and saliva collection processes were correct. Data processing included data cleaning and validation.

3.10.10 Minimizing bias and errors

The principal investigator (G.K.M) was calibrated by his supervisor, a specialist periodontist (T.K.M) prior to data collection on participant examination as well as saliva sample collection protocols. All clinical measurements were carried out by the principal investigator. In view of the epidemiologic inconsistencies, this study adopted the CDC/AAP consensus definitions of periodontal status⁽²³⁾.

A test run was carried out to streamline sample collection, transport, sample storage as well as to optimize the ELISA assay technique. Saliva sample collection was standardized for all patients. The transport, handling and storage of samples was done in consultation with a laboratory technologist (F.M) who was qualified with ELISA analysis techniques. The principal investigator was trained on lab protocols in ELISA. Measures were taken to ensure and document the laboratory equipment used were calibrated and serviced. All samples were analyzed within 3 months of collection and care was taken to prevent any repeated thawing and freezing of samples. In the ELISA technique, MMP-8 standards and samples were run in duplicate to evaluate reproducibility of the laboratory techniques. The technologist was blinded on the participants' clinical findings and periodontal diagnoses as the clinical examination form was not submitted to the lab.

3.11 Data analysis and presentation

Following data entry, data cleaning and validation was carried out. The data was coded and processed with Statistical Packages for Social Sciences (SPSS) 20.0 for Windows (SPSS inc. Chicago, Illinois, USA), Microsoft- Excel as well as R software (ver. 3.1.2) [R Core Team, 2013] . Descriptive statistics applied were measures of central tendency and dispersion for continuous variables for example age, gender, plaque scores, gingival status and periodontal health status. Grubb's test and visual examination of histograms and box plots was carried out to identify the six extreme outliers. Comparison of means and proportions was done using t-test and chi square test. Where appropriate, ANOVA, Pearson's and Spearman's rank correlation tests were used to determine the associations between key variables including periodontitis severity, oral hygiene practices, gingival status, sociodemographic and other variables when compared with the salivary MMP-8 levels. Confidence level was set at 95% (α level <0.05) to assess strength of association. Presentation of findings was done in form of tables, graphs and box plots.

3.12 Outcome measures

- 1.Oral hygiene practices of adults visiting UoN dental hospital.
- 2.Oral hygiene status of adults visiting UoN dental hospital.
- 3.Gingival health status of adults visiting UoN dental hospital.
- 4.Periodontal health status of adults visiting UoN dental hospital.
- 5.Salivary MMP-8 levels among adults visiting UoN dental hospital.

3.13 Constrains and limitations

The study population was hospital based and convenience sampling was used to select the study sample. Ramfjord's index teeth were used in calculation of gingival scores and plaque scores, as much as they have been shown to have epidemiological validity in the East African population⁽⁸³⁾, they do not give the true picture of the condition of the entire periodontium. The study also required costly items with the cost being borne by the researcher. Time limitations due to compact post graduate academic and clinical schedules.

3.14 Confounders

Non disclosure or inaccurate information provided by participants concerning exclusion criteria for instance: Smoking history, recent dental procedures, medications or systemic illness.

3.15 Ethical Considerations

Approval to conduct the study was sought and obtained from the Kenyatta National Hospital (KNH) and University of Nairobi (UoN) research ethics and standards committee. Permission to conduct the study was sought and granted by the University of Nairobi School of Dental Sciences clinics and UNITID laboratories.

The purpose of the study was explained to the participants in a language they understood best, any queries were answered appropriately and written informed voluntary consent

was obtained from every participant before commencement of the study as shown in Appendix 1 and 2.

Data confidentiality and anonymity of patients' identity was ensured. All persons who met the inclusion criteria had an equal chance of being included in the study. There were no risks to participants during clinical examination and saliva sample collection as these were carried out within the setting of a dental clinic with all cross infection measures being strictly followed. There was also a provision for participants to withdraw from the study at will without any dire consequences. There were no anticipated financial benefits either to the investigator or the participants from this study.

3.16 Benefits

This study provides new knowledge on levels of salivary MMP-8 in Kenya, in view of genetic, regional and racial differences in periodontitis. Associations of MMP-8 with varying periodontitis severity as well as sociodemographic variables, provides indicators its utility as a biomarker in our settings. The results of this study will be published with the aim of further expanding clinicians' current knowledge of the host response to periodontal disease, and assessment of periodontal disease activity in the Kenyan population. This also forms a vital platform for further salivary proteomic studies.

Direct benefit to participants included receiving free information and consultation regarding their various dental conditions found on examination and management accordingly. Comparisons between the participants with periodontal disease in various severities and those without periodontal disease assess the diagnostic potential of MMP-8 in the Kenyan setting and provide a platform for large scale studies, prospective longitudinal studies and further proteomic analysis of salivary biomarkers in the African setting.

This study is also in partial fulfilment of the requirements for the award of Masters in Dental Surgery in Periodontology, at the University of Nairobi, Kenya.

CHAPTER FOUR: RESULTS

4.1 Sociodemographic characteristics

A total of one hundred and twenty patients were recruited into the study, however six participants were identified using Grubb's test as extreme outliers and excluded. Of the 114 evaluated, 64(56.1%) were female and 50 (43.9%) were male. The age ranged from 18 - 77 years with a mean of 36 years (± 13.87 SD). The females were slightly older with a mean of 36.6 years (± 13.1 SD) than males with 36.2 years (± 14.91 SD). However, the difference was not statistically significant ($t= 0.168$ $p=0.87$). Table 5 shows sociodemographic characteristics of participants. Males had a statistically significant higher level of education ($X^2=5.827$ $p=0.05$) and more were in formal employment ($X^2=6.597$ $p=0.04$) compared to their female counterparts.

TABLE 5: SOCIODEMOGRAPHIC CHARACTERISTICS OF PARTICIPANTS

Variable	Gender		X^2	p -value	
	Male <i>n</i> (%)	Female <i>n</i> (%)			
Age	18-30 years	22 (44.0)	28 (56.0)	1.016	0.60
	31-45 years	16 (50.0)	16(50.0)		
	Above 46 years	12 (37.5)	20 (62.5)		
Level of education	Primary	1(11.1)	8(88.9)	5.827	0.05
	Secondary	12(37.5)	20(62.5)		
	Tertiary	37(50.7)	36(49.3)		
Occupation	Formal	20 (62.5)	12 (37.5)	6.597	0.04
	Informal	13(33.3)	26(66.7)		
	Unemployed	17(39.5)	26 (60.5)		
Residence	Nairobi	44(45.4)	53(54.6)	0.60	0.31
	Out of Nairobi	6(35.3)	11(64.7)		

4.2 Oral hygiene practices

All participants brushed their teeth. Majority of the participants 68(59.6%) reported brushing their teeth twice daily with the remaining 46 (40.4%) brushing their teeth once daily. There was no statistically significant association between toothbrushing frequency and sociodemographic variables as summarized in table 6.

TABLE 6: ORAL HYGIENE PRACTICES AMONG PARTICIPANTS

Variable		Brushing frequency		X^2	<i>p</i> -value
		Once daily	Twice daily		
		<i>n</i> (%)	<i>n</i> (%)		
Gender	Male	20(40.0)	30(60.0)	1.00	
	Female	26(40.6)	38(59.4)		
Level of education	Primary	5(55.6)	4(44.4)	2.11	0.348
	Secondary	15(46.9)	17(53.1)		
	Tertiary	26(35.6)	47(64.4)		
Last dental visit	Never been	6(60.0)	4(40.0)	3.43	0.331
	< 3 months	0(0.0)	1(100)		
	3-6months	4(57.1)	3(42.9)		
	>6 months	36 (37.5)	60(62.5)		

4.3 Oral hygiene status

All participants had plaque deposits. The mean plaque score was 0.94(\pm 0.49SD). A statistically significant association was found between plaque scores and increasing age ($F=5.09$; $p=0.008$) as well as level of education ($F=3.39$; $p=0.037$). There was no statistically significant association between mean plaque scores and other sociodemographic variables as summarized in table 7.

TABLE 7: ORAL HYGIENE STATUS BY SOCIODEMOGRAPHIC VARIABLES

Variable		Mean plaque score	Test	<i>p</i>-value
Gender	Male	0.92 (\pm 0.45 SD)	$t=0.405$	0.686
	Female	0.96 (\pm 0.51 SD)		
Age groups	18-30 years	0.90 (\pm 0.49 SD)	$F=5.09$	0.008
	31-45 years	0.79 (\pm 0.30 SD)		
	46 years and above	1.16 (\pm 0.49 SD)		
Frequency of brushing	Once a day	1.0(\pm 0.50 SD)	$t= 1.307$	0.194
	Twice daily	0.89 (\pm 0.48 SD)		
Level of education	Primary	1.19(\pm 0.53 SD)	$F= 3.39$	0.037
	Secondary	1.07(\pm 0.52 SD)		
	Tertiary	0.86(\pm 0.45 SD)		
Last dental visit	Never been	0.88(\pm 0.39 SD)	$F= 1.341$	0.265
	< 3 months	0.70(\pm 0.00 SD)		
	3-6months	1.29(\pm 0.70 SD)		
	>6 months	0.93 (\pm 0.48 SD)		

4.4 Gingivitis

All participants had gingival inflammation. The mean gingival index was 1.1 (± 0.33 SD) with a range of 0.3-2.4. Out of the participants, 48 (42.1%) had mild gingivitis, 63(55.3%) had moderate gingivitis while 3(2.6%) had severe gingivitis. Higher levels of gingival inflammation were noted in older age groups ($F=11.65$; $p<0.001$). Higher levels of education were associated with statistically significant lower gingival indices ($F=4.215$; $p=0.017$). Predictably, lower gingival indices were noted in participants who reported brushing twice a day than those who brushed once daily. However the difference was not statistically significant ($F=1.114$; $p=0.293$). Table 8 summarizes the distribution of gingival inflammation by sociodemographic and other variables.

TABLE 8: GINGIVITIS BY STUDY VARIABLES

VARIABLES		Gingival index	Test	p-value
Gender:	Male	1.148(± 0.27)	$t=0.444$	0.658
	Female	1.120(± 0.37)		
Age groups (yrs):	18-30	1.074 (± 0.33 SD)	$F=10.379$	<0.001
	31-45	1.019(± 0.17 SD)		
	46 and above	1.338(± 0.37 SD)		
Level of education:	Primary	1.322(± 0.29 SD)	$F=3.914$	0.023
	Secondary	1.216(± 0.33 SD)		
	Tertiary	1.073(± 0.32 SD)		
Toothbrushing frequency:	Once daily	1.161 (± 0.33 SD)	$t=0.756$	0.451
	Twice daily	1.113 (± 0.33 SD)		
Plaque scores			$r^2=0.84$	<0.001

Further analysis using Pearson correlation revealed a strong positive correlation between plaque score and gingival index ($r^2=0.85$, $p<0.001$).

4.5 Periodontitis

Out of the 114 participants, 69 had no periodontitis, whereas 45 had periodontitis. Out of those with periodontitis, 9(7.9 %) had severe periodontitis, 23(20.2%) had moderate periodontitis, 13(11.4 %) had mild periodontitis, majority 69(60.5%) had no periodontitis. Mean periodontal probing depth was 1.8 mm ($\pm 0.47SD$), whereas mean clinical attachment loss was 0.2 mm ($\pm 0.06SD$). Associations between periodontitis and sociodemographic variables are shown in table 9. A statistically significant association was found between periodontal status and increasing age ($X^2=17.99, p=0.007$).

TABLE 9: PERIODONTITIS BY SOCIODEMOGRAPHIC AND OTHER VARIABLES

VARIABLE/ CHARACTERISTIC	PERIODONTITIS <i>n</i> (%)				Test	<i>p</i> - value	
	No	Mild	Moderate	Severe			
Gender	Male	28(40.6)	7(53.8)	11(47.8)	4(44.4)	$X^2=0.976$	0.807
	Female	41(59.4)	6(46.2)	12(52.2)	5(55.6)		
Age groups	18-30 years	36(52.2)	6(46.2)	6(26.1)	2(22.2)	$X^2=17.99$	0.007
	31-45 years	23(33.3)	2(15.4)	5(21.7)	2(22.2)		
	46 years and above	10(14.5)	5(38.5)	12(52.2)	5(55.6)		
Frequency of brushing	Once daily	28 (40.6)	5(38.5)	9(39.1)	4(44.4)	$X^2=0.098$	0.992
	Twice daily	41(59.4)	8(61.5)	14(60.9)	5(55.6)		
Level of education	Primary	5(7.2)	1(7.7)	1(4.3)	2(22.2)	$X^2=10.039$	0.123
	Secondary	15(21.7)	5(38.5)	11(47.8)	1(11.1)		
	Tertiary	49(71.0)	7(53.8)	11(47.8)	6(66.7)		

ANOVA analysis revealed statistically significant associations between plaque index and periodontitis severity ($F=12.88, p<0.001$).

4.6 Salivary MMP-8

Of the 120 samples collected, 6 had MMP-8 levels that were extremely high and deemed as outliers using Grubbs test (R-software). Including them in the statistical analysis skewed the data unfavourably and they were thus omitted in the final data analysis. It is envisaged that this did not mask the general trend of the MMP-8 expression in the different periodontal status groups. Further, since the number recruited far exceeded the calculated samples size of 80, removing these outliers was deemed safe in retaining the general characteristics of the parameters under study.

Out of the 114 samples analysed, salivary MMP-8 was present in majority of participants 91(79.8%) and not detectable in 23(20.2%). The MMP-8 values ranged from undetectable values to 295.9 ng/mL with a mean of 40.52 ng/mL (± 66.37 SD). Increase in age was correlated with a statistically significant increase in MMP-8 levels ($r= 0.316$, $p=0.001$). Correlations between MMP-8 levels and sociodemographic variables are summarized in table 10.

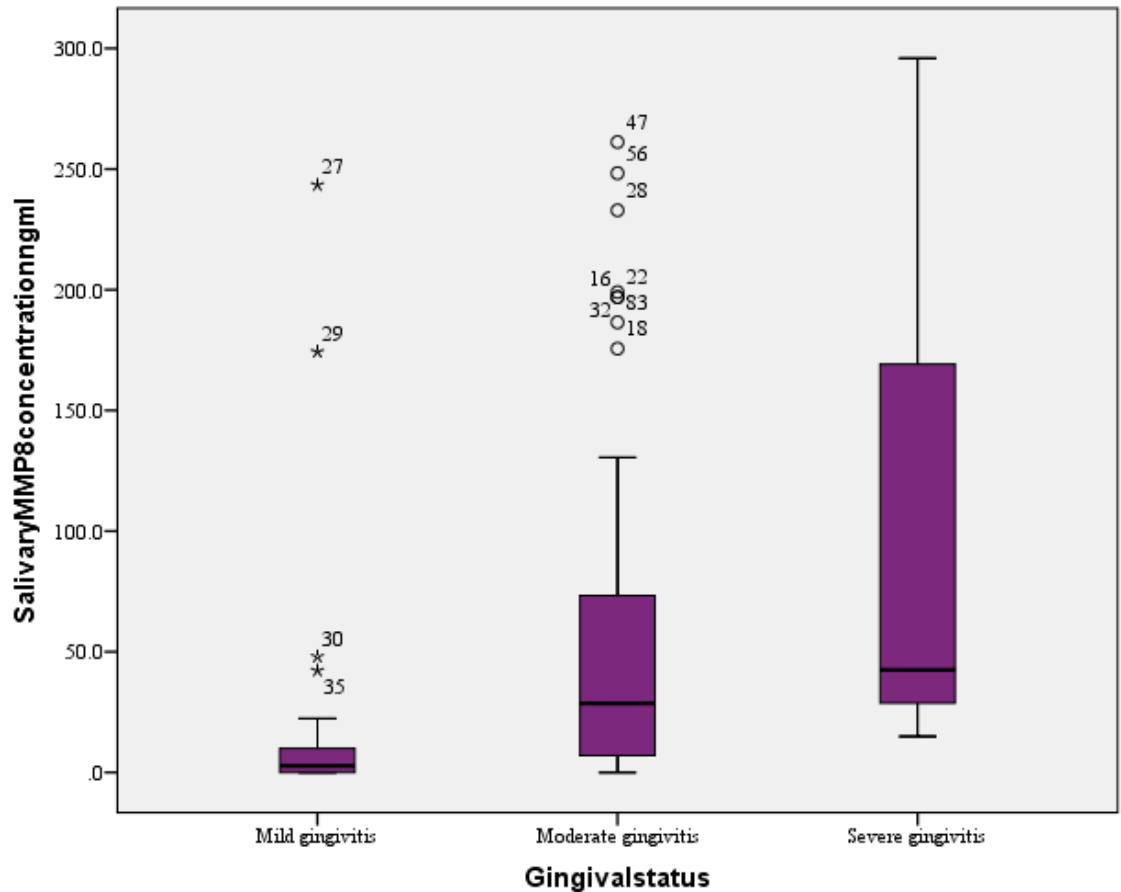
TABLE 10: ASSOCIATION BETWEEN SALIVARY MMP-8 LEVELS AND OTHER VARIABLES.

Variable/ Characteristic	Mean values	Test	<i>p</i>-value
Age (Years)	36.45 (± 13.87 SD) years	$r= 0.316$	0.001
Gender			
Male	36.61(61.34SD) ng/mL	$t=-0.555$	0.580
Female	43.58(70.39SD) ng/mL		
Plaque scores	0.94 (± 0.49 SD)	$r=0.389$	<0.001
Gingival indices	1.1(± 0.33 SD)	$r= 0.425$	<0.001
Periodontal probing depths (PPD)	1.8(± 0.47 SD) mm	$r= -0.146$	0.122
Clinical attachment loss (CAL)	0.2 (± 0.59 SD) mm	$r= -0.129$	0.171

4.7 Association between salivary MMP-8 levels and gingivitis severity

The mean salivary MMP-8 levels tended to be higher with increasing gingivitis severity. Participants with mild gingivitis had 14.84 ng/mL (± 42.68 SD), moderate gingivitis had 56.40 ng/mL (± 69.43 SD) while those with severe gingivitis had 117.76 ng/mL (± 154.88 SD). ANOVA analysis revealed a statistically significant association between the groups based on salivary MMP-8 levels ($F=8.398$, $p<0.001$). The box and whisker plots (figure 6) illustrate the distribution of MMP-8. Notably, some participants had higher levels of MMP-8 with less severity of gingivitis and vice versa.

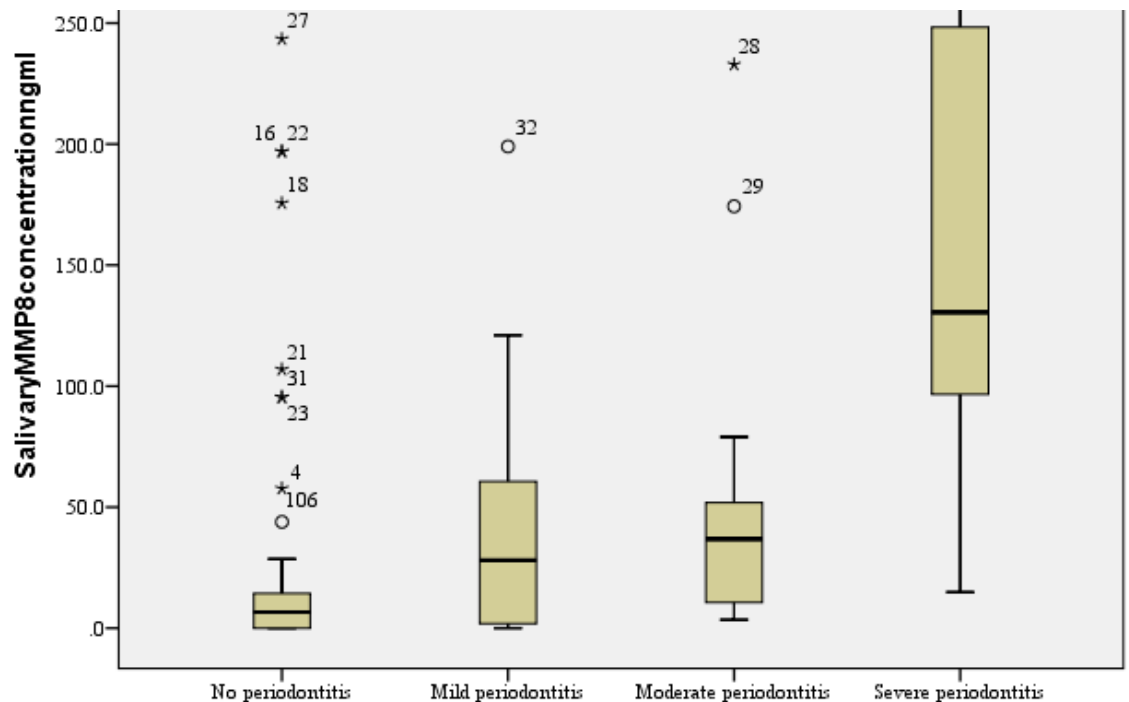
FIGURE 7: DISTRIBUTION OF SALIVARY MMP-8 LEVELS BY SEVERITY OF GINGIVITIS.



4.8 Association between salivary MMP-8 levels and severity of periodontitis

The mean salivary MMP-8 levels also tended to be higher with increasing level of periodontitis severity. The group with no periodontitis (69) had 22.67 ng/mL (± 50.32 SD), those with mild periodontitis(13) had 44.55 ng/mL (± 59.11 SD), the moderate periodontitis group(23) had 46.34 ng/mL (± 54.44 SD) whereas severe periodontitis group(9) had 156.62 ng/mL (± 96.36 SD). The salivary MMP-8 concentration was generally higher in the 45 participants with periodontitis (67.92 ng/mL ± 78.38 SD) compared to those with no periodontitis (22.67 ng/mL ± 50.32 SD). One way ANOVA analysis revealed a statistically significant difference between the four groups of periodontitis severity ($F=14.96$, $p<0.001$). The null hypothesis was thus rejected. The box and whisker plot (Figure 8) illustrates the distribution of salivary MMP-8 among the groups of individuals. Notably, some individuals within the groups of no and mild

FIGURE 8: DISTRIBUTION OF SALIVARY MMP-8 LEVELS BY SEVERITY OF PERIODONTITIS



periodontitis severity had relatively high concentrations of MMP-8.

A multiple linear regression analysis was used to evaluate association between: gender, increase in age, gingival index, mean probing depths, mean clinical attachment levels and salivary MMP-8 levels after controlling for plaque scores and toothbrushing frequency for participants. The regression analysis yielded a coefficient of determination R squared (R^2) of 0.24. Therefore, 24.0% of the variation in salivary MMP-8 levels was accounted for by the independent/predictor variables aforementioned.

However, individually, the change in gingival scores ($B=70.47$, $p<0.001$) and age ($B=0.94$, $p=0.03$) were statistically significant predictors of changes in salivary MMP-8 levels. Table 11 summarizes the regression results.

TABLE 11: RESULTS OF MULTIPLE LINEAR REGRESSION ANALYSIS TO PREDICT CHANGES IN MMP-8 LEVELS FROM CHANGE IN PREDICTOR VARIABLES

<i>Variable</i>	<i>B</i>	<i>t value</i>	<i>95% Confidence interval</i>		<i>p-value</i>
			Upper	Lower	
Age (Years)	0.940	2.18	0.087	1.793	0.031*
Gingival index	70.47	3.88	34.44	106.49	0.000*
Mean periodontal probing depth	-14.34	-0.95	-44.17	15.48	0.342

Mean clinical attachment loss	-5.37	-0.45	-29.00	18.27	0.654
Gender	9.84	0.88	-12.46	32.14	0.384

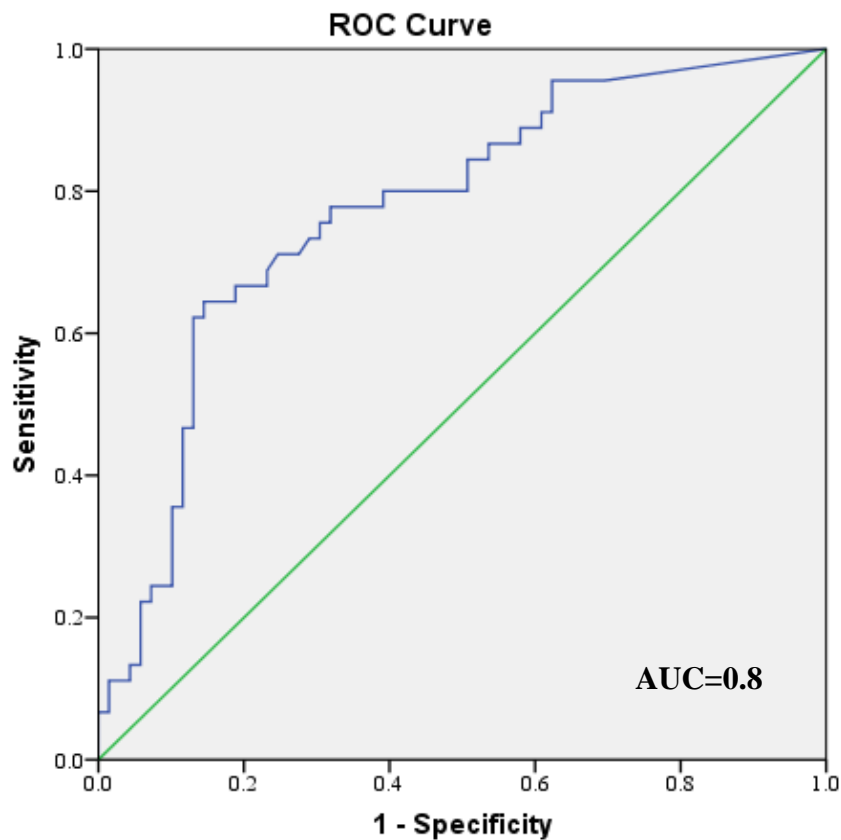
*There was a statistically significant association between increase in age, in gingival inflammation and salivary MMP-8 levels

4.9 Diagnostic potential of salivary MMP-8

Receiver operating characteristic (ROC) curves are an effective and widely accepted method of evaluating the performance of diagnostic tests. They are curves plotting sensitivity (true positive) against specificity (false positives rate or 1-specificity) and may vary between studies.

Analysis was performed to determine the diagnostic accuracy of salivary MMP-8 as a specific and sensitive diagnostic test. The periodontitis diagnosis of participants was first recoded to dichotomous state values of periodontitis and no periodontitis thereafter a ROC curve was then plotted as shown in figure 9.

FIGURE 9: RECEIVER OPERATING CHARACTERISTIC (ROC) ANALYSIS OF SALIVARY MMP-8 ON PERIODONTITIS



Zweig and Campbell 1993 demonstrated that the closer the ROC curve is to the upper left corner, the higher the overall accuracy of the test⁽⁸⁶⁾. For comparative analysis, the higher

the AUC value, the better the overall performance of the diagnostic test to correctly distinguish diseased and non-diseased patients.

The area under the curve (AUC) value was **0.8**. This indicated that the measure of salivary MMP-8 can distinguish between individuals with periodontitis and no periodontitis with an accuracy of about 80.0%. Table 12 summarizes the results of the ROC analysis.

TABLE 12: RECEIVER OPERATING CHARACTERISTICS (ROC) RESULTS

Area under the curve	Standard error	<i>95% Confidence interval</i>		<i>p-value</i>
		Lower	Upper	
0.774	0.045	0.686	0.863	>0.001

Coordinates of the curve were then assessed to get a trade-off of sensitivity (at 0.133) and specificity (at 0.05). This estimated an approximate cut-off value of **114.55 ng/mL** of MMP-8 above which indicates a likelihood of periodontitis in participants.

CHAPTER FIVE: DISCUSSION

5.1 Sociodemographic characteristics

The age of participants ranged from 18 -77 years with a mean of 37 years and a broad standard deviation of 14.2 years. This implied that diverse and varied ages of adults sought dental treatment for various illnesses and conditions at the study area. In the current study, more participants were of female gender than males. The higher ratio of adult females to males at the UoN dental hospital indicated that females have better health seeking behaviour and was in agreement with recent findings by Maubi et al 2013⁽⁸⁷⁾.

Majority of participants had achieved tertiary education (college, undergraduate or postgraduate education). This high percentage of participants with higher education level is possibly due to the fact that this study was carried out in an urban setting whereby the urban population is probably more educated. There was a statistically significant disparity regarding level of education and gender with more males having achieved tertiary education as well as more in formal employment. This reflects the existent socioeconomic gender imbalances. Most participants were found to reside in Nairobi. This is possibly because the study was carried out at The University of Nairobi Dental Hospital, situated a few kilometers from the central business district of Nairobi. Hence most participants were likely to have been drawn from the city and its close environs.

5.2 Oral hygiene practices

Globally, toothbrushing has been found to be one of the most effective ways to control dental plaque and maintain good oral hygiene. It is recommended that one brushes at least twice a day. In this study, all participants reported to practice tooth brushing. Majority reported to brush their teeth twice a day. Similar findings were found in a study done in Germany in an urban set dental hospital which found a majority of adult participants (79.6%) also brushing twice daily⁽⁸⁸⁾.

In this study more females reported to brush twice daily than males, however the difference was not statistically significant. This finding was similar to a study by Davidson et al in 2007 which found higher frequencies of daily toothbrushing among females⁽⁸⁹⁾.

The relatively high number of patients brushing only once daily emphasizes the need for ongoing oral hygiene education in the population.

5.3 Oral hygiene status

In this study, lower plaque scores were observed among participants who brushed twice daily. A higher level of education was associated with lower plaque scores. This may be attributed to the participants being more enlightened on oral hygiene practices. More positive dental attitudes and knowledge about oral diseases have been shown among urban populations compared with the rural populations⁽⁹⁰⁾. Predictably, plaque scores correlated positively with increasing severity of gingival disease. The microbial plaque biofilm has been evidenced to be key in initiating and propagating gingival inflammation.

A higher mean clinical attachment loss was found among individuals who brushed only once daily. However the relationship was not statistically significant ($t=0.465$, $p=0.643$). A study among Chinese adults by Corbet, Wong and colleagues in 2001 similarly found that less frequent toothbrushing was associated with increased clinical attachment loss⁽⁹¹⁾. Unabated periodontal inflammation results in alveolar bone loss, connective tissue destruction and junctional epithelial migration apically noted clinically as attachment loss.

5.4 Gingivitis

Gingival bleeding is commonly used to assess levels of gingival inflammation. Positive correlations were found between gingivitis and plaque confirming known concepts in pathogenesis of gingivitis. There was no statistically significant gender difference in gingivitis levels ($F = 1.011$ $p=0.317$). Positive correlations were observed between increasing age and gingivitis levels possibly due to a prolonged exposure to risk factors and the likelihood of reduced manual dexterity in controlling dental plaque. Participants with higher levels of education were found to have statistically significant lower mean

gingival scores; this is likely due to participants being better enlightened on oral hygiene practices. A recent study by Peeran et al 2015 demonstrated that education level plays a role in inequities in oral hygiene practices⁽⁹²⁾. This reinforces the importance of oral health education within teaching institutions in reducing the prevalence of periodontal diseases.

5.5 Periodontitis

Periodontitis severity was classified using consensus CDC/AAP definitions⁽²⁵⁾. A relatively higher number of individuals in the study had moderate or severe periodontitis. This could be explained by the fact that the participants were drawn from a population of patients seeking treatment at a dental hospital. Associations with tooth brushing practices, level of education and gender disparities were not statistically significant. In this study, no statistically significant association was between periodontitis prevalence in participants residing within Nairobi and those out of Nairobi. However a study by Amenaghawon et al in 2013 described differences in the prevalence of periodontal disease between urban and rural areas⁽⁹³⁾. Association of periodontitis with increasing age was statistically significant, plausible explanations for increased periodontal disease severity with increasing age is prolonged exposure to risk factors over the years such as periodontopathic bacteria, decreased manual dexterity in plaque control and undiagnosed concurrent systemic diseases⁽⁹⁴⁾.

5.6 Occurrence of salivary MMP-8

MMP-8 is a major factor secreted by neutrophils during inflammatory injury. In this study, a statistically significant, positive correlation was found between increasing age and the salivary MMP-8 levels. These findings were in agreement with a recent study on age-related changes in salivary biomarkers where authors used a similar saliva collection and ELISA analysis technique⁽⁹⁵⁾. A plausible explanation for increased periodontal disease severity with increasing age is prolonged exposure to risk factors over a longer duration and possible influence of undiagnosed concurrent systemic diseases predisposing periodontal breakdown⁽⁹⁴⁾.

Salivary concentrations of MMP-8 were compared between male and female participants. Within the limits of this study, there was no statistically significant difference in salivary MMP-8 levels between the sexes. Statistically significant positive correlations were found between plaque indices, gingival indices and salivary MMP-8 levels this was in agreement with a study by Gupta 2014 which established similar relationships in the periodontitis groups studied⁽⁹⁶⁾.

An interesting aspect of the MMP-8 occurrence was the wide range of concentrations between individuals and within groups. Of note were participants in the gingivitis and even the healthy groups with relatively high levels of MMP-8, it is plausible that this was an indicator characterizing periodontal disease susceptibility. Similar findings were noted in a recent proteomic study by Ebersole and colleagues 2015⁽⁹⁷⁾.

5.7 Associations of MMP-8 and periodontitis

Salivary MMP-8 levels tended to increase with disease severity. The higher MMP-8 concentration in the participants diagnosed with periodontitis compared to those with no periodontitis was similar to findings from a cross-sectional study by Miller in 2006 that reported higher average salivary MMP-8 concentration in periodontally diseased patients ($623.8\text{ng/ml} \pm 204.0\text{SD}$, $p < 0.05$) than that of healthy patients ($64.6\text{ ng/ml} \pm 16.4\text{SD}$)⁽⁹⁸⁾. Genetic and racial differences in the Kenyan population may be responsible for the varied mean concentrations of salivary MMP-8 between the aforementioned studies.

Varied levels of MMP-8 were also found within the groups of periodontitis severity as illustrated in figure 9. Furthermore, individuals in the mild and moderate periodontitis had closely approximated means of salivary MMP-8 of 44.55ng/mL and 46.34ng/mL respectively. A plausible explanation for the varied levels and close figures is the existence of different disease activity states within individuals in the study. Studies have demonstrated that cyclic periods of “quiescent or linear” and ‘burst’ episodic periods and subsequent repair occur in periodontitis⁽⁹⁹⁾. A study done by Herr in 2007 in the United States similarly demonstrated that, salivary MMP-8 concentrations exhibit variability, even among periodontally diseased groups. This further confirmed the dynamism in periodontitis disease activity⁽⁷³⁾.

Another plausible explanation may be that some individuals innately produce higher amounts of MMP-8 that predispose them to more severe periodontitis, while those who produce less may be less susceptible. Heterogeneity in the host immuno-inflammatory response to the subgingival biofilm has been purported to explain the varied susceptibility to periodontal disease by its established influence on the progress of inflammation in the periodontium^(100, 101). A study by Chou in 2011 among Taiwanese adults associated susceptibility to chronic and aggressive forms of periodontitis to genetic polymorphisms in genomic DNA for MMP-8-799 C and T alleles. The T allele being related to increased expression of MMP-8⁽⁷²⁾. The role of cytokine and receptor gene polymorphisms in altered immuno inflammatory pathways leading to susceptibility to periodontal tissue destruction has also been evidenced in other studies⁽¹⁰²⁾.

5.8 Diagnostic utility of salivary MMP-8

Studies have indicated the potential utility of using saliva as a diagnostic fluid as well as in monitoring therapeutic interventions⁽⁷¹⁾. Salivary MMP-8 demonstrated diagnostic utility in differentiating periodontitis and gingivitis severity as well as healthy status. After controlling for plaque scores, twenty four percent of the variation in salivary MMP-8 levels was accounted for by gender, increase in age, gingival index, mean probing depths and mean clinical attachment levels. This highlighted the influential role of other factors, possibly genetic polymorphisms in inducing variations in MMP-8 concentrations. Individually, the change in gingival scores was observed to be a significant predictor of salivary MMP-8 levels, which thus reinforced the concept that salivary MMP-8 levels do indicate individual disease activity.

Receiver operating characteristic (ROC) curve values may vary between studies and are an established technique in comparing diagnostic tests. The ROC curve demonstrated that salivary MMP-8 could distinguish between individuals with and without periodontitis with an area under the curve (AUC) value of **0.8**. This was in agreement with a recent study on salivary biomarkers by Ebersole and colleagues who established an AUC value of 0.7 using salivary MMP-8⁽⁹⁷⁾. Similar findings were also observed in a study by Ramseier, Kenney and colleagues where an AUC of 0.9 was derived ⁽¹⁰³⁾. The more robust AUC value in the Ramseier study was possibly due to the analysis of a

combination of biomarkers (MMP-8, MMP-9 and osteoprotegerin) as well as genomic identification of red complex periodontal pathogens.

Further analysis to establish cut off concentrations of salivary MMP-8 established an approximate threshold /optimal concentration of **114.55 ng/mL** above which would indicate a high likelihood of periodontitis activity in individuals. This was slightly lower but comparable to findings in a recent proteomic study by Ebersole and colleagues August 2015 who found an optimal cut off value of 165.92 ng/mL⁽⁹⁷⁾. Despite the heterogeneity, studies have shown that salivary levels of MMP-8 alone as well as in combination with other biomarkers such as IL-1 β , IL-6, MIP- α have valuable diagnostic potential^(97, 104).

5.8 Limitations

This was a cross sectional study and as such could not monitor the true relationship between salivary MMP-8 levels and changes in disease severity. Secondly, the study was carried out in a hospital setting and was not randomized potentially introducing selection bias. The study was also hospital based posing a challenge in making inferences to the general population. The study also required reagents and materials that were costly with the investigator bearing the costs. This and the prevailing time constraints made it impractical to carry out a randomized study as well as validating the lab analysis data by running repeat measures for the six samples identified as outliers.

5.9 Conclusion

Salivary MMP-8 levels were higher in individuals with periodontitis compared to individuals with no periodontitis. There was a positive correlation between the increased salivary MMP-8 levels and the increasing periodontitis and gingivitis severity assessed with the clinical parameters. A rapid, convenient and reliable method of correctly diagnosing and monitoring periodontal disease activity will aid population prevalence studies as well as decision making in treatment.

5.10 Recommendations

Due to the drawbacks of current disease classifications, the use of putative salivary biomarkers should be developed as an adjunct in identifying individuals' disease activity and susceptibility to periodontal destruction. In order to conclusively associate salivary MMP-8 and periodontal disease as well as evaluate outcomes of non surgical and surgical periodontal interventions in the population, there is need for larger longitudinal cohort studies and randomized controlled studies. The exploration of combinations of multiple putative biomarkers will further describe periodontal disease activity on our setup. The use of chairside diagnostic devices will further increase efficiency.

5.11 Conflict of interest and source of funding statement

The cost of the study was met solely by the principal investigator for scientific and academic purposes. There was no conflict of interest related to this study.

REFERENCES

1. Petersen PE, Ogawa H. The global burden of periodontal disease: towards integration with chronic disease prevention and control. *Periodontology* 2000. 2012;60(1):15-39.
2. Papapanou PN. Epidemiology of periodontal diseases: an update. *Journal of the International Academy of Periodontology*. 1999;1(4):110-6.
3. Kaimenyi JT. Oral health in Kenya. *International Dental Journal* (2004) 54, 378–382.
4. O'Dowd LK, Durham J, McCracken GI, Preshaw PM. Patients' experiences of the impact of periodontal disease. *Journal of clinical periodontology*. 2010;37(4):334-9.
5. Slots J. Periodontology: past, present, perspectives. *Periodontology* 2000. 2013;62(1):7-19.
6. Kuo L-C, Polson AM, Kang T. Associations between periodontal diseases and systemic diseases: A review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. *Public Health*. 2008;122(4):417-33.
7. Armitage GC. Periodontal diagnoses and classification of periodontal diseases. *Periodontology* 2000. 2004;34(1):9-21.
8. Teles R, Teles F, Frias-Lopez J, Paster B, Haffajee A. Lessons learned and unlearned in periodontal microbiology. *Periodontology* 2000. 2013;62(1):95-162.
9. Zhang L, Henson BS, Camargo PM, Wong DT. The clinical value of salivary biomarkers for periodontal disease. *Periodontol* 2000. 2009;51:25-37.
10. American Academy of Periodontology Task Force Report on the Update to the 1999 Classification of Periodontal Diseases and Conditions. *Journal of periodontology*. 2015;86(7):835-8.

11. Page RC, Sturdivant EC. Noninflammatory destructive periodontal disease (NDPD). *Periodontology 2000*. 2002;30(1):24-39.
12. Jeffcoat MK, Reddy MS. Progression of probing attachment loss in adult periodontitis. *Journal of periodontology*. 1991;62(3):185-9.
13. Kinane DF, Podmore M, Ebersole J. Etiopathogenesis of periodontitis in children and adolescents. *Periodontology 2000*. 2001;26(1):54-91.
14. Dahlén G, Manji F, Baelum V, Fejerskov O. Putative periodontopathogens in “diseased” and “non-diseased” persons exhibiting poor oral hygiene. *Journal of clinical periodontology*. 1992;19(1):35-42.
15. Yager P, Edwards T, Fu E, Helton K, Nelson K, Tam MR, et al. Microfluidic diagnostic technologies for global public health. *Nature*. 2006;442(7101):412-8.
16. Rodriguez WR, Christodoulides N, Floriano PN, Graham S, Mohanty S, Dixon M, et al. A microchip CD4 counting method for HIV monitoring in resource-poor settings. *PLoS medicine*. 2005;2(7):e182.
17. Fuentes L, Yakob M, Wong DT. Emerging horizons of salivary diagnostics for periodontal disease. *British dental journal*. 2014;217(10):567-73.
18. Wong DT. Salivaomics. *Journal of the American Dental Association (1939)*. 2012;143(10 Suppl):19S-24S.
19. Sorsa T, Tervahartiala T, Leppilahti J, Hernandez M, Gamonal J, Tuomainen AM, et al. Collagenase-2 (MMP-8) as a point-of-care biomarker in periodontitis and cardiovascular diseases. Therapeutic response to non-antimicrobial properties of tetracyclines. *Pharmacological Research*. 2011;63(2):108-13.
20. Sahingur SE, Cohen RE. Analysis of host responses and risk for disease progression. *Periodontology 2000*. 2004;34(1):57-83.
21. Listgarten MA. Nature of periodontal diseases: Pathogenic mechanisms. *Journal of Periodontal Research*. 1987;22(3):172-8.

22. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Annals of periodontology / the American Academy of Periodontology*. 1999;4(1):1-6.
23. Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. *Journal of periodontology*. 2007;78(7 Suppl):1387-99.
24. Baelum V, Fejerskov O, Manji F. Periodontal diseases in adult Kenyans. *Journal of clinical periodontology*. 1988;15(7):445-52.
25. Page RC, Eke PI. Case Definitions for Use in Population-Based Surveillance of Periodontitis. *J Periodontol*. 2007;Volume 78(Number 7 (Supl.)):1387-99.
26. Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ. Prevalence of Periodontitis in Adults in the United States: 2009 and 2010. *Journal of Dental Research*. 2012;91(10):914-20.
27. Holtfreter B, Schwahn C, Biffar R, Kocher T. Epidemiology of periodontal diseases in the study of health in Pomerania. *J Clin Periodontol*. 2009;36:114-23.
28. Corbet EF, Leung WK. Epidemiology of periodontitis in the Asia and Oceania regions. *Periodontology 2000*. 2011;56:25-64.
29. Loe H, Theilade E, Jensen SB. EXPERIMENTAL GINGIVITIS IN MAN. *Journal of periodontology*. 1965;36:177-87.
30. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest*. 1976;34(3):235-49.
31. Page RC, Offenbacher S, Schroeder HE, Seymour GJ, Kornman KS. Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontology 2000*. 1997;14(1):216-48.
32. Kinane DF, Preshaw PM, Loos BG, on Behalf of Working Group 2 of the Seventh European Workshop on P. Host-response: understanding the cellular and molecular mechanisms of host-microbial interactions – Consensus of the Seventh

European Workshop on Periodontology. *Journal of clinical periodontology*. 2011;38:44-8.

33. Hendler A, Mulli TK, Hughes FJ, Perrett D, Bombardieri M, Hourri-Haddad Y, et al. Involvement of Autoimmunity in the Pathogenesis of Aggressive Periodontitis. *Journal of Dental Research*. 2010;89(12):1389-94.

34. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000*. 1994;5:78-111.

35. Michalowicz BS, Aeppli D, Virag JG, Klump DG, Hinrichs JE, Segal NL, et al. Periodontal findings in adult twins. *Journal of periodontology*. 1991;62(5):293-9.

36. Wagaiyu EG. A Comparative Study To Assess Interleukin-1 Polymorphisms And Their Relationship With Chronic Periodontitis In Two Kenyan Coastal Communities: University of Nairobi; 2013.

37. Nikolopoulos GK, Dimou NL, Hamdrakas SJ, Bagos PG. Cytokine gene polymorphisms in periodontal disease: a meta-analysis of 53 studies including 4178 cases and 4590 controls. *Journal of clinical periodontology*. 2008;35(9):754-67.

38. Beklen A, Ainola M, Hukkanen M, Gurgan C, Sorsa T, Kontinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. *J Dent Res*. 2007;86(4):347-51.

39. Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *The FASEB Journal*. 1991;5(8):2145-54.

40. Emingil G, Han B, Gurkan A, Berdeli A, Tervahartiala T, Salo T, et al. Matrix metalloproteinase (MMP)-8 and tissue inhibitor of MMP-1 (TIMP-1) gene polymorphisms in generalized aggressive periodontitis: gingival crevicular fluid MMP-8 and TIMP-1 levels and outcome of periodontal therapy. *Journal of periodontology*. 2014;85(8):1070-80.

41. Larsson L, Castilho RM, Giannobile WV. Epigenetics and Its Role in Periodontal Diseases: A State-of-the-Art Review. *Journal of periodontology*. 2015;86(4):556-68.

42. Nunn ME. Understanding the etiology of periodontitis: an overview of periodontal risk factors. *Periodontology* 2000. 2003;32(1):11-23.
43. Offenbacher S, Barros SP, Beck JD. Rethinking periodontal inflammation. *Journal of periodontology*. 2008;79(8 Suppl):1577-84.
44. Buchwald S, Kocher T, Biffar R, Harb A, Holtfreter B, Meisel P. Tooth loss and periodontitis by socio-economic status and inflammation in a longitudinal population-based study. *Journal of clinical periodontology*. 2013;40(3):203-11.
45. Cutler CW, Machen RL, Jotwani R, Iacopino AM. Heightened gingival inflammation and attachment loss in type 2 diabetics with hyperlipidemia. *Journal of periodontology*. 1999;70(11):1313-21.
46. Torrungruang K, Tamsailom S, Rojanasomsith K, Sutdhibhisal S, Nisapakultorn K, Vanichjakvong O, et al. Risk Indicators of Periodontal Disease in Older Thai Adults. *Journal of periodontology*. 2005;76(4):558-65.
47. Mumghamba EGS, Markkanen HA, Honkala E. Risk factors for periodontal diseases in Ilala, Tanzania. *Journal of clinical periodontology*. 1995;22(5):347-54.
48. Clarke NG, Hirsch RS. Personal risk factors for generalized periodontitis. *Journal of clinical periodontology*. 1995;22(2):136-45.
49. Michalowicz BS. Genetic and heritable risk factors in periodontal disease. *Journal of periodontology*. 1994;65(5 Suppl):479-88.
50. Levine ME, Kim JK, Crimmins EM. The role of physiological markers of health in the association between demographic factors and periodontal disease. *Journal of Periodontal Research*. 2012:n/a-n/a.
51. Tarannum F, Faizuddin M. Effect of gene polymorphisms on periodontal diseases. *Indian Journal of Human Genetics*. 2012;18(1):9-19.

52. Liu KZ, Xiang XM, Man A, Sowa MG, Cholakis A, Ghiabi E, et al. In vivo determination of multiple indices of periodontal inflammation by optical spectroscopy. *Journal of Periodontal Research*. 2009;44(1):117-24.
53. Giannobile WV, McDevitt JT, Niedbala RS, Malamud D. Translational and clinical applications of salivary diagnostics. *Adv Dent Res*. 2011;23(4):375-80.
54. Mäntylä P, Stenman M, Kinane DF, Tikanoja S, Luoto H, Salo T, et al. Gingival crevicular fluid collagenase-2 (MMP-8) test stick for chair-side monitoring of periodontitis. *Journal of Periodontal Research*. 2003;38(4):436-9.
55. Nomura Y, Shimada Y, Hanada N, Numabe Y, Kamoi K, Sato T, et al. Salivary biomarkers for predicting the progression of chronic periodontitis. *Archives of Oral Biology*. 2012;57(4):413-20.
56. Mandel ID, Wotman S. The salivary secretions in health and disease. *Oral sciences reviews*. 1976(8):25-47.
57. Giannobile WV, Beikler T, Kinney JS, Ramseier CA, Morelli T, Wong DT. Saliva as a diagnostic tool for periodontal disease: current state and future directions. *Periodontology 2000*. 2009;50(1):52-64.
58. Pradeep A. RECENT APPROACHES IN SALIVA AS A CREDIBLE PERIODONTAL DIAGNOSTIC AND PROGNOSTIC MARKER.
59. Koka S, Beebe TJ, Merry SP, DeJesus RS, Berlanga LD, Weaver AL, et al. The Preferences of Adult Outpatients in Medical or Dental Care Settings for Giving Saliva, Urine or Blood for Clinical Testing. *The Journal of the American Dental Association*. 2008;139(6):735-40.
60. Rathnayake N, Åkerman S, Klinge B, Lundegren N, Jansson H, Tryselius Y, et al. Salivary biomarkers of oral health – a cross-sectional study. *Journal of clinical periodontology*. 2013;40(2):140-7.
61. Sindhu S, Jagannathan N. Saliva: A Cutting Edge in Diagnostic Procedures. *Journal of Oral Diseases*. 2014;2014:8.

62. Sorsa T, Tjaderhane L, Konttinen YT, Lauhio A, Salo T, Lee HM, et al. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Annals of medicine*. 2006;38(5):306-21.
63. Gross J, Lapiere CM. COLLAGENOLYTIC ACTIVITY IN AMPHIBIAN TISSUES: A TISSUE CULTURE ASSAY. *Proceedings of the National Academy of Sciences of the United States of America*. 1962;48(6):1014-22.
64. Bartlett JD, Smith CE. Modulation of cell-cell junctional complexes by matrix metalloproteinases. *J Dent Res*. 2013;92(1):10-7.
65. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, et al. Matrix metalloproteinases: a review. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists*. 1993;4(2):197-250.
66. Sorsa T, Mäntylä P, Tervahartiala T, Pussinen PJ, Gamonal J, Hernandez M. MMP activation in diagnostics of periodontitis and systemic inflammation. *Journal of clinical periodontology*. 2011;38(9):817-9.
67. Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, et al. Matrix Metalloproteinases: A Review. *Critical Reviews in Oral Biology & Medicine*. 1993;4(2):197-250.
68. Gursoy UK, Könönen E, Huumonen S, Tervahartiala T, Pussinen PJ, Suominen AL, et al. Salivary type I collagen degradation end-products and related matrix metalloproteinases in periodontitis. *Journal of clinical periodontology*. 2013;40(1):18-25.
69. Sorsa T, Tjäderhane L, Salo T. Matrix metalloproteinases (MMPs) in oral diseases. *Oral diseases*. 2004;10(6):311-8.
70. Gursoy UK, KnnE, Pradhan-Palikhe P, Tervahartiala T, Pussinen PJ, Suominen-, Taipale L ST. Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *J Clin Periodontol* 2010; 37: 487–493 doi: 101111/j1600-051X201001563x.

71. Sexton WM, Lin Y, Kryscio RJ, Dawson DR, 3rd, Ebersole JL, Miller CS. Salivary biomarkers of periodontal disease in response to treatment. *Journal of clinical periodontology*. 2011;38(5):434-41.
72. Chou YH, Ho YP, Lin YC, Hu KF, Yang YH, Ho KY, et al. MMP-8 -799 C>T genetic polymorphism is associated with the susceptibility to chronic and aggressive periodontitis in Taiwanese. *Journal of clinical periodontology*. 2011;38(12):1078-84.
73. Herr AE, Hatch AV, Throckmorton DJ, Tran HM, Brennan JS, Giannobile WV, et al. Microfluidic immunoassays as rapid saliva-based clinical diagnostics. *Proceedings of the National Academy of Sciences*. 2007;104(13):5268-73.
74. Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical chemistry*. 2005;51(12):2415-8.
75. Leppilähti JM, Ahonen MM, Hernández M, Munjal S, Netuschil L, Uitto VJ, et al. Oral rinse MMP-8 point-of-care immuno test identifies patients with strong periodontal inflammatory burden. *Oral diseases*. 2011;17(1):115-22.
76. Brinkmann O, Spielmann N, Wong DT. Salivary diagnostics: moving to the next level. *Dentistry today*. 2012;31(6):54, 6-7; quiz 8-9.
77. Pihlstrom BL. Measurement of attachment level in clinical trials: probing methods. *Journal of periodontology*. 1992;63(12 Suppl):1072-7.
78. Wahaidi VY, Dowsett SA, Eckert GJ, Kowolik MJ. Neutrophil Response to Dental Plaque by Gender and Race. *Journal of Dental Research*. 2009;88(8):709-14.
79. Mout R, Willemze R, Landegent JE. Repeat polymorphisms in the interleukin-4 gene (IL4). *Nucleic Acids Research*. 1991;19(13):3763.
80. Cohen J. *Statistical Power Analysis for the Behavioral Sciences*: L. Erlbaum Associates; 1988.
81. Case LD, Ambrosius WT. Power and sample size. *Methods in molecular biology* (Clifton, NJ). 2007;404:377-408.

82. Uitto V-J, Overall CM, McCulloch C. Proteolytic host cell enzymes in gingival crevice fluid. *Periodontology* 2000. 2003;31(1):77-104.
83. Mumghamba EGS, Pitiphat W, Matee MIN, Simon E, Merchant AT. The usefulness of using Ramfjord teeth in predicting periodontal status of a Tanzanian adult population. *Journal of clinical periodontology*. 2004;31(1):16-8.
84. Tran DT, Gay I, Du XL, Fu Y, Bebermeyer RD, Neumann AS, et al. Assessing periodontitis in populations: a systematic review of the validity of partial-mouth examination protocols. *Journal of clinical periodontology*. 2013;40(12):1064-71.
85. Van Weemen BK, Schuurs AH. Immunoassay using antigen-enzyme conjugates. *FEBS letters*. 1971;15(3):232-6.
86. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clinical chemistry*. 1993;39(4):561-77.
87. Maubi GK. Oral treatment seeking behaviour among patients visiting University of Nairobi dental hospital: University of Nairobi; 2013.
88. Ganss C, Schlueter N, Preiss S, Klimek J. Tooth brushing habits in uninstructed adults--frequency, technique, duration and force. *Clinical oral investigations*. 2009;13(2):203-8.
89. Davidson P, Rams T, Andfrsen R. Socio-behavioral determinants of oral hygiene practices among USA ethnic and age groups. *Advances in dental research*. 1997;11(2):245-53.
90. Ogunbodede EO, Kida IA, Madjapa HS, Amedari M, Ehizele A, Mutave R, et al. Oral Health Inequalities between Rural and Urban Populations of the African and Middle East Region. *Advances in Dental Research*. 2015;27(1):18-25.
91. Corbet EF, Wong MC, Lin HC. Periodontal conditions in adult Southern Chinese. *J Dent Res*. 2001;80(5):1480-5.

92. Peeran SA, Peeran SW, Al Sanabani F, Almakramani B, Elham EI, Naveen Kumar PG. "Education level" responsible for inequities in oral practices among 15–34-year-old individuals in Jizan, Saudi Arabia. *Journal of International Society of Preventive & Community Dentistry*. 2015;5(2):120-4.
93. Azodo C, Amenaghawon O. Oral hygiene status and practices among rural dwellers 2013 January 1, 2013. 42-5 p.
94. Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, et al. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *Journal of periodontology*. 1994;65(3):260-7.
95. Nassar M, Hiraishi N, Islam MS, Otsuki M, Tagami J. Age-related changes in salivary biomarkers. *Journal of Dental Sciences*. 9(1):85-90.
96. Gupta N, Gupta ND, Gupta A, Khan S, Bansal N. Role of salivary matrix metalloproteinase-8 (MMP-8) in chronic periodontitis diagnosis. *Frontiers of medicine*. 2015;9(1):72-6.
97. Ebersole JL, Nagarajan R, Akers D, Miller CS. Targeted salivary biomarkers for discrimination of periodontal health and disease(s). *Frontiers in Cellular and Infection Microbiology*. 2015;5:62.
98. Miller CS, King CP, Jr., Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing periodontal disease: a cross-sectional study. *Journal of the American Dental Association (1939)*. 2006;137(3):322-9.
99. Borrell LN, Papapanou PN. Analytical epidemiology of periodontitis. *J Clin Periodontol*. 2005;32 (Suppl. 6):132-58.
100. Van Dyke TE, Dave S. Risk Factors for Periodontitis. *Journal of the International Academy of Periodontology*. 2005;7(1):3-7.
101. Preshaw PM, Taylor JJ. How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis? *Journal of clinical periodontology*. 2011;38:60-84.

102. Yoshie H, Kobayashi T, Tai H, Galicia JC. The role of genetic polymorphisms in periodontitis. *Periodontology 2000*. 2007;43(1):102-32.
103. Ramseier CA, Kinney JS, Herr AE, Braun T, Sugai JV, Shelburne CA, et al. Identification of pathogen and host-response markers correlated with periodontal disease. *Journal of periodontology*. 2009;80(3):436-46.
104. Sorsa T, Mantyla P, Ronka H, Kallio P, Kallis GB, Lundqvist C, et al. Scientific basis of a matrix metalloproteinase-8 specific chair-side test for monitoring periodontal and peri-implant health and disease. *Ann N Y Acad Sci*. 1999;878:130-40.

APPENDICES

APPENDIX 1: CONSENT FORM- ENGLISH VERSION

This research study entitled “**Salivary MMP-8 levels and periodontal health status among adults attending the University of Nairobi dental hospital.**” is being carried out by Dr. George K. Mwai, a postgraduate student in the department of Periodontology, Community and Preventive Dentistry, University of Nairobi, School of Dental Sciences, towards his attainment of a Master of Dental Surgery in Periodontology.

Part of this research will entail collection of a small sample of saliva following rinsing the mouth with clean water. Clinical examination will be done using standard calibrated dental instruments on all participants’ teeth and gums and also include a collection of a small sample of saliva after rinsing with. There is no health risk posed to participants during the examination.

All information gathered during this study will be treated with utmost confidentiality and will only be used for the purposes of this research. Participation is voluntary you may opt out of the study at your own free will without any threats or dire consequence. No monetary compensation or reward of any form will be offered to participants. The findings of this research may be of great use in understanding, in evaluation, treatment and follow up of periodontal diseases in our setting using current emerging technology and globally accepted methodology.

Having read and understood the above information, and with any concerns I may have had having been answered satisfactorily by the principal investigator Dr. Mwai, I do hereby voluntarily consent to participate in this research.

Signed by participant.....

Date.....

Declaration by the principal investigator

I have explained to the participant the purpose and expected benefits of this study and have also answered his/her questions and concerns regarding this research on the date on this consent form.

Signed by investigator..... Date.....

For clarifications and enquiries on the consent please contact

The KNH/UoN Ethics and Research Standards Committee Secretariat

Email: uonknh_erc@uonbi.ac.ke

Telephone Number +254-20 2726300 Ext 44355

APPENDIX 2: CONSENT FORM SWAHILI VERSION

FOMU YA KUTAFUTA IDHINI KUTOKA KWA WAHUSIKA KATIKA UTAFITI:

Utafiti huu unaitwa “**Uhusiano kati ya salivary MMP-8 na afya ya ufizi kati ya watu wazima katika Hospitali ya Matibabu ya Meno katika Chuo Kikuu cha Nairobi.**” Unafanywa na daktari Mwai K. George ambaye ni mwanafunzi katika chuo kikuu cha Nairobi.

Katika utafiti huu hali ya afya ya ufizi na uchafu ulio katika meno za wahusika utachunguzwa kwa kutumia vifaa safi. Pia sampuli kidogo ya mate itachunguzwa baada ya kuosha mdomo na maji safi. Hakuna madhara ya aina yoyote kwa wahusika katika utafiti huu.

Habari zote zitakazotolewa na wahusika zitatumwa kwa utafiti huu peke yake. Pia zitawekwa kwa njia ya kibinafsi na kisiri katika rekodi ambazo mchunguzi mkuu ataziweka vyema. Wahusika watahiriki kwa utafiti huu kwa hiari yao bila kushurutishwa au kulazimishwa na ye yote. Wahusika pia wanaweza kujiondoa kutoka kwa utafiti huu wakati wo wote bila vitisho au madhara yo yote. Hakuna malipo ya kifedha au aina nyingine ambayo washirika watapewa kwa kushiriki katika utafiti huu. Matokeo ya utafiti huu yataweza kusaidia madaktari na wanasayansi kuelewa magonjwa ya ufizi na jinsi ya kuyatibu vyema zaidi kwa kutumia mbinu za kisasa zinazokubaliwa kote duniani.

Baada ya kusoma na kuelewa maelezo haya, na baada ya maswali yoye niliyokuwa nayo kuhusu utafiti huu kujibiwa na Daktari Mwai, ninakubali kuhusishwa katika utafiti huu kwa kutia sahihi hapa chini.

Sahihi ya mshiriki..... Tarehe.....

Maelezo ya mchunguzi mkuu:

Nimemweleza mshiriki kuhusu maudhui na manufaa ya uchunguzi huu na nimejibu maswali alizokuwa nazo siku ambayo imetiwa sahihi hapa chini.

Sahihi ya mchunguzi mkuu.....Tarehe.....

Kwa maelezo zaidi wasiliana na:

The KNH/UoN Ethics and Research Standards Committee Secretariat

Email: uonknh_erc@uonbi.ac.ke

Nambari ya simu +254-20 2726300 Ext 44355

APPENDIX 3: QUESTIONNAIRE /BIODATA FORM

SALIVARY MMP-8 LEVELS AND PERIODONTAL HEALTH STATUS AMONG ADULTS ATTENDING THE UNIVERSITY OF NAIROBI DENTAL HOSPITAL.

Date..... Saliva sample serial/code number.....

Age (Years)..... File number.....

Gender: Male Female

Residence..... Occupation.....,

Highest level of education:

Primary Secondary Tertiary

Tooth brushing habits: Once daily Twice daily Thrice daily

Other

Inter dental cleaning: Dental floss: No Yes If yes, state frequency?

Interdental brushes: No Yes if yes, state frequency?.....

Last dental visit:

Never been to a dentist Less than 3 months ago 3-6 months ago

>6 months ago Specify procedure(s) done.....

Modifying factors:

Tobacco cigarette smoking: Never smoked Former smoker >3 yrs ago

If former smoker, specify years smoked and sticks smoked per day.....

Diabetes: No Yes If yes, state duration and control.....

Immunologic disorder: No Yes If yes, specify

Other illness.....

APPENDIX 4: QUESTIONNAIRE /BIODATA FORM SCREENING SEGMENT

Screening (TICK)

Persons below the age of 18 years.

Periodontal procedure within the past 6 months.

Antibiotic therapy within the past 6 months.

Concurrent systemic illness for example rheumatoid arthritis.

Pregnancy or lactation.

Current smoker or history of smoking in the last 3 years

Edentulous patients and patients who have less than 20 teeth.

APPENDIX 5: CLINICAL EXAMINATION FORM

PLAQUE SCORE: Silness and Loe Index 1964

Tooth												
Surface	F	L	F	L	F	L	F	L	F	L	F	L
Score												

Total..... Mean.....

GINGIVAL INDEX: Silness-Loe index (1963)

Tooth												
Surface	F	L	F	L	F	L	F	L	F	L	F	L
Score												

Total..... Mean..... Gingival health status:

Plaque score: Silness and Loe Plaque index 1964

Scores	Criteria
0	No plaque
1	A film of plaque adhering to the tooth and the free gingival margin. The plaque may be seen in situ only after passing the probe on the tooth surface.
2	Moderate accumulation of soft deposits within the gingival sulcus, or the tooth surface and gingival margin which can be seen with the naked eye.
3	Abundance of soft matter in the gingival sulcus and/or on the tooth and gingival margin

Gingival index: Silness and Loe Index (1963)

- 0- Normal, absence of oedema and no bleeding on probing
- 1- Presence of oedema with absence of bleeding
- 2- Oedema present, glazing and bleeding on probing
- 3- Oedema, ulcerations with spontaneous bleeding.

Gingivitis severity

- No gingivitis
- 0.1– 1 Mild gingivitis
- 1.1-2 Moderate gingivitis
- 2.1-3 Severe gingivitis

APPENDIX 6: PERIODONTAL PROBING CHART

Maxillary arch Serial Number.....

Tooth	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27
Palatal (mm)															
Recession(mm)															
CAL(mm)															
Facial (mm)															
Recession(mm)															
CAL(mm)															

Mandibular arch:

Tooth	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37
Lingual (mm)															
Recession(mm)															
CAL(mm)															
Facial															
Recession(mm)															
CAL(mm)															

Number and measure of interproximal sites with CAL..... PPD.....

CDC/AAP consensus periodontitis classification.....

Disease Category	Clinical Attachment Loss (CAL)	Periodontal Pocket Depths (PPD)
Severe periodontitis	More than 2 interproximal sites with CAL of more or equal to 6 mm (not on the same tooth)	AND 2 or more interproximal sites with PPD of more than or equal to 5 mm
Moderate periodontitis	More than 2 interproximal sites with CAL of more or equal to 4 mm (not on the same tooth)	OR 2 or more interproximal sites with PPD of more than or equal to 5 mm
Mild periodontitis	More than 2 interproximal sites with CAL of more or equal to 2 mm (not on the same tooth)	OR 2 or more interproximal sites with PPD of more than or equal to 4 mm
No periodontitis	No evidence of mild, moderate or severe periodontitis	

APPENDIX 7: CDC / AAP CASE DEFINITIONS FOR EPIDEMIOLOGICAL STUDIES

The periodontitis severity was based on the criteria defined by the Centers for Disease Control/American Academy of Periodontology.

Severe periodontitis: 2 or more interproximal sites with **6 mm** or more of clinical attachment loss or 5mm or more of periodontal pocket depth **and** more than 1 interproximal site with PPD of more than or equal to **5 mm**.

Moderate periodontitis: 2 or more interproximal sites with **4mm** or more of clinical attachment loss or 5 mm or more of periodontal pocket depth or 2 or more interproximal sites with PPD of more than or equal to **5 mm**.

Mild periodontitis - More than 2 interproximal sites with clinical attachment loss of more or equal to **2 mm** (not on the same tooth) or 2 or more interproximal sites with PPD of more than or equal to **4 mm**.

No periodontitis: The absence of any periodontal disease (including no loss of clinical attachment).

APPENDIX 8: DY908 RND SYSTEMS-DUO SET ELISA DEVELOPMENT MANUAL (FRONT)

Tools for Cell Biology Research™

DuoSet® ELISA DEVELOPMENT SYSTEM



human Total MMP-8 Catalog Number: DY908

SPECIFICITY

The following factors prepared at 50 ng/mL were assayed and exhibited no cross-reactivity or interference.

Recombinant human factor:	MMP-7	MMP-13
MMP-1	MMP-9	TIMP-1
MMP-2	MMP-10	TIMP-2
MMP-3	MMP-12	

CALIBRATION

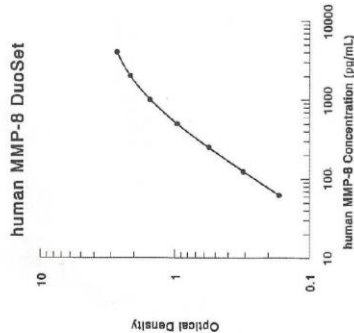
This DuoSet is calibrated against a highly purified NSO-expressed recombinant human MMP-8 produced at R&D Systems.

TYPICAL DATA

This standard curve is only for demonstration purposes.

A standard curve should be generated for each set of samples assayed.

The graph below represents typical data generated when using this human MMP-8 DuoSet. The standard curve was calculated using a computer-generated 4-PL curve-fit.



FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 | (612) 379-2956 | FAX: (612) 656-4400

UK & Europe | R&D Systems Europe, Ltd.
19 Baron Lane, Abingdon Science Park, Abingdon, OX14 3NB, UK
TEL: +44 (0)1235 529449 | FAX: +44 (0)1235 539420

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road
Shanghai P.R.C 200050
TEL: +86 (21) 52380373 | FAX: +86 (21) 52371001

751017.6

11/11



www.RnDSystems.com

This DuoSet ELISA Development kit contains the basic components required for the development of sandwich ELISAs to measure natural and recombinant human active and pro-Matrix Metalloproteinase 8 (Total MMP-8). DuoSets are designed for the analysis of cell culture supernates. Other sample types, such as serum and plasma, need to be validated prior to use in this DuoSet. Each kit contains sufficient materials to run ELISAs on approximately fifteen 96-well plates, provided that the following conditions are met:¹

- The assay is run as summarized in the General ELISA protocol.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

This package insert must be read in its entirety before using this product.

¹If assaying sample types other than cell culture supernates, each laboratory should develop and validate its own diluent. We suggest using a 1:100 dilution of sample in assay buffer serum. The diluent must not be used to dilute the Detection Antibody or the Streptavidin-HRP.

²Individual results may vary due to differences in technique, plasticware and water sources.

³Provided this is within the expiration date of the kit.

⁴Allow all components to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions should be prepared and used immediately.

⁵All buffers containing BSA must be stored at 2-8°C.

⁶Costar EIA Plate (Costar Catalog # 2592 or R&D Systems Catalog # DY908) is suggested. R&D Systems ELISA Plate Sealers (Catalog # DY952) are also available.

APPENDIX 9: DY908 RND SYSTEMS-DUO SET ELISA DEVELOPMENT SYSTEM MANUAL (BACK)

ALERO and 27/9/2015

MATERIALS PROVIDED

Bring all reagents to room temperature before use.

Capture Antibody (Part 841031, 1 vial) - 360 µg/mL of mouse anti-human MMP-8 when reconstituted with 1.0 mL of PBS. After reconstitution, store at 2-8° C for up to 60 days or aliquot and store at -20° C to -70° C in a manual defrost freezer for up to 6 months.³ Dilute to a working concentration of 2.0 µg/mL in PBS,⁴ without carrier protein.

Detection Antibody (Part 841032, 1 vial) - 36 µg/mL of biotinylated goat anti-human MMP-8 when reconstituted with 1.0 mL of Reagent Diluent (see Solutions Required section). After reconstitution, store at 2-8° C for up to 60 days or aliquot and store at -20° C to -70° C in a manual defrost freezer for up to 6 months.³ Dilute to a working concentration of 200 ng/mL in Reagent Diluent with 2% heat inactivated normal goat serum (NGS). Prepare 1-2 hours prior to use.

Standard (Part 841033, 3 vials) - Each vial contains 120 ng/mL of recombinant human MMP-8 when reconstituted with 0.5 mL of deionized or distilled water. Store reconstituted standard at 2-8° C or aliquot and store at -70° C for up to 2 months.³ A seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 4000 pg/mL is recommended.

Streptavidin-HRP (Part 890803, 1 vial) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2-8° C for up to 6 months after initial use.³ **DO NOT FREEZE.** Dilute to the working concentration specified on the vial label using Reagent Diluent (see Solutions Required section).

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 0.2 µm filtered. (R&D Systems Catalog # W1120).

Wash Buffer - 0.05% Tween[®] 20 in PBS, pH 7.2-7.4 (R&D Systems Catalog # W1120).

Reagent Diluent¹ - 1% BSA¹ in PBS, pH 7.2-7.4, 0.2 µm filtered. (R&D Systems Catalog # DY995).

Quality of BSA is critical (see Technical Hints).

Substrate Solution - 1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent E (Tetramethylbenzidine) (R&D Systems Catalog # D1999).

Stop Solution - 2 N H₂SO₄ (R&D Systems Catalog # D1994).

Normal Goat Serum - (R&D Systems Catalog # D1005).

¹Tween is a registered trademark of C.I. Americas.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate² with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µL of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Add 100 µL of the Detection Antibody, diluted in Reagent Diluent with NGS, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature.
6. Repeat the aspiration/wash as in step 2.
7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature.
8. Avoid placing the plate in direct light.
9. Determine the optical density of each well on the plate to ensure thorough mixing. Turn immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

TECHNICAL HINTS AND LIMITATIONS

- The use of high quality Bovine Serum Albumin (BSA) for the Reagent Diluent is crucial for the optimum performance of the DuoSet ELISA Development kit. Impurities such as proteases, binding proteins, soluble receptors or other interfering substances can be found to varying degrees in virtually all BSA preparations and can inhibit or interfere with the detection of certain analytes. If the standard curve appears suppressed, consider evaluating a different preparation of BSA.
- We recommend the use of R&D Systems' Reagent Diluent (Catalog # D1995) or the use of Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045). Prepare your own Reagent Diluent.
- This DuoSet should not be used beyond the expiration date on the label.
- It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. The diluent suggested in this protocol should be suitable for most cell culture supernatant samples. Validate diluents for specific sample types prior to use.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing a large quantity of protein should be made under sterile conditions and stored at 2-8° C or be prepared fresh daily.

PRECAUTION


The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

APPENDIX 10: LABORATORY PLATE MAPS OF MICROPLATE WELLS (PLATE 1)

PLATE 1

UNIVERSITY OF NAIROBI INSTITUTE OF TROPICAL AND INFECTIOUS DISEASES

Dr. George Mwangi MDI III - Serology M.M.P.8 thesis

UNIVERSITY OF NAIROBI

 Serology Laboratory
 ELISA Worksheet -

Date 13.4.2015 Lot# _____ Exp. Date _____

Run _____ Coated plate: _____ Diluent: _____

Date _____ Positive control _____ Negative control _____

Technologist AREA MUIE

	1	2	3	4	5	6	7	8	9	10	11	12
A	STANDARD	STANDARD	SAMPLE 001	SAMPLE 001	009	009	017	017	023	023	033	033
B			002	002	010	010	018	018	026	026	034	034
C			003	003	011	011	019	019	027	027	035	035
D			004	004	012	012	020	020	028	028	036	036
E			005	005	013	013	021	021	029	029	037	037
F			006	006	014	014	022	022	030	030	038	038
G			007	007	015	015	023	023	031	031	039	039
H	BLANK	BLANK	008	008	016	016	024	024	032	032	040	040

APPENDIX 11: LABORATORY PLATE MAPS OF MICROPLATE WELLS (PLATE 2)

PLATE 2

*Dr George Muriu - MD III
Salivary MMP-8 Inhi*



UNIVERSITY OF NAIROBI INSTITUTE OF TROPICAL AND INFECTIOUS DISEASES

Serology Laboratory
ELISA Worksheet -
Lot# Exp. Date

Date — 13.4.2015

Exp. Date

Lot#

Diluent:

Coated plate:

Positive control

Negative control

Run

Date

Technologist *FOO MORE*

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard	Standard	0A1	0A1	0A9	0A9	0A7	0A7	0B5	0B5	0B3	0B3
B			0A2	0A2	0B0	0B0	0B8	0B8	0B6	0B6	0B4	0B4
C			0A3	0A3	0B1	0B1	0B9	0B9	0B7	0B7	0B5	0B5
D			0A4	0A4	0B2	0B2	0B0	0B0	0B8	0B8	0B6	0B6
E			0A5	0A5	0B3	0B3	0B1	0B1	0B9	0B9	0B7	0B7
F			0A6	0A6	0B4	0B4	0B2	0B2	0B0	0B0	0B8	0B8
G			0A7	0A7	0B5	0B5	0B3	0B3	0B1	0B1	0B9	0B9
H	BLANK	BLANK	0A8	0A8	0B6	0B6	0B4	0B4	0B2	0B2	0B0	0B0

APPENDIX 12: LABORATORY PLATE MAPS OF MICROPLATE WELLS (PLATE 3)

09 January M.P. 8 Thesis



UNIVERSITY OF NAIROBI INSTITUTE OF TROPICAL AND INFECTIOUS DISEASES

Date 13 April 2015

Serology Laboratory

ELISA Worksheet -

Lot# _____ Exp. Date _____

Run _____ Coated plate: _____

Diluent: _____

Positive control _____

Negative control _____

Date _____

Technologist FAE-MARE

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard	Standard	081	081	089	089	094	094	105	105	113	113
B			082	082	090	090	098	098	106	106	114	114
C			083	083	091	091	099	099	107	107	115	115
D			084	084	092	092	100	100	108	108	116	116
E			085	085	093	093	101	101	109	109	117	117
F			086	086	094	094	102	102	110	110	118	118
G			087	087	095	095	103	103	111	111	119	119
H	BLANK	BLANK	088	088	096	096	104	104	112	112	120	120

APPENDIX 13: LABORATORY SAMPLE ANALYSIS DATA FORM

Saliva sample serial No: Date and time of collection.....

Date and time sample received, centrifuged and supernatant stored.....

ELISA analysis:

Salivary MMP-8 level: (ng/mL)

Laboratory technologist: Signed.....

APPENDIX 14: ETHICAL APPROVAL



UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355



KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: www.uonbi.ac.ke



KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/228

Link: www.uonbi.ac.ke/activities/KNHUoN

9th July 2014

Dr. George K. Mwai
Dept. of Periodontology/Community and Preventive Dentistry
School of Dental Sciences
University of Nairobi

Dear Dr. Mwai

**RESEARCH PROPOSAL: SALIVARY MMP-8 LEVELS AND PERIODONTAL HEALTH STATUS, AMONG
ADULTS ATTENDING THE UNIVERSITY OF NAIROBI DENTAL HOSPITAL (P179/04/2014)**

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 9th July 2014 to 8th July 2015.

This approval is subject to compliance with the following requirements:

- a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
- b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
- c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
- d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
- e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
- f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
- g) Submission of an *executive summary* report within 90 days upon completion of the study. This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website www.uonbi.ac.ke/activities/KNHUoN.

Protect to Discover

Yours sincerely



PROF. M. L. CHINDIA
SECRETARY, KNH/UON-ERC

- c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Chairperson, KNH/UoN-ERC
The Assistant Director, Health Information, KNH
The Director, School of Dental Sciences, UoN
The Chairman, Dept. of Peridontology/Community and Preventive Dentistry, UoN
Supervisors: Dr. Tonnie K. Mulli, Prof. Francis G. Macigo

APPENDIX 15: PERMISSION TO USE INFORMATION AND ILLUSTRATIONS FROM RND SYSTEMS LIMITED (UK)

● **Brad Mabbutt** <Brad.Mabbutt@bio-techne.com>
To kanjimwai16@yahoo.com

Oct 26 at 2:45 PM

Dr George Mwai,

Thank you for your email. Please be aware that Andrew Haynes is no longer working at Bio-Techne, so I am looking after your enquiry.

I am very pleased to learn that your research was successful and you are very happy with the human MMP-8 DuoSet Elisa development kit. I can confirm permission for you to use information and illustrations given in the kit insert in your publication. If you are able to send details of the publication, once published that would be great.

For future enquires regarding R&D Systems products (or products from other Bio-Techne brands) please send your email correspondence to info@bio-techne.com

Thank you for choosing R&D Systems products and I look forward to hearing from you again soon.

Regards

Bradley Mabbutt
Sales Executive - Export

Bio-Techne
19 Barton Lane, Abingdon Science Park
Abingdon, OX14 3NB, UK

Office +44 (0)1235 529449
Direct +44 (0) 1235 551142
Mobile +44 (0)7827 302931

brad.mabbutt@bio-techne.com
bio-techne.com