ASSOCIATION OF SALIVARY RECEPTOR ACTIVATOR OF NUCLEAR FACTOR LIGAND AND OSTEOPROTEGERIN LEVELS WITH PERIODONTAL CLINICAL STATUS

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DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my wife Emily whose unyielding love, support and encouragement enriched my soul and inspired me to pursue and complete the research.

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LIST OF ACRONYMS

AA	Amino acid
AAP	American Academy of Periodontology
AG	Aggressive periodontitis
AHEA	Associate of Higher Education Academy
BDS	Bachelor of Dental Surgery
CAL	Clinical attachment loss
CDC	Centre for Disease Control
CDE	Certificate in Dental Education
CP	Chronic periodontitis
ELISA	Enzyme-linked immunosorbent assay
GCAP	Graduate Certificate of Academic Practice.
GCF	Gingivocrevicular fluid
HRP	Horseradish peroxidase
KAVI	Kenya Aids Vaccine Initiative
kDa	Kilo Dalton
MClin.Dent	Master in Clinical Dentistry
MDS	Master of Dental Surgery
MSc.Dent	Master of Science in Dentistry
NOHP	National Oral Health Policy
Nrb	Nairobi

OPG	Osteoprotegerin
PCR	Polymerase chain reaction
PDL	Periodontal ligament
PBS	Phosphate buffer saline
PhD	Doctor of Philosophy
RANKL	Receptor activator of Nuclear Factor ligand
SP	Strategic plan
SPSS	Statistical Package for Social Sciences
TNF	Tumour Necrosis Factor
UoN	University of Nairobi
WHO	World Health Organization
UWC	University of the Western Cape.

DEFINITION OF TERMS

Biofilms – Microbial communities that adhere to solid surfaces where there is sufficient moisture (including plants and animal tissues).

Dental plaque – Soft deposits that form the biofilm adhering to the tooth surface or other hard tissues in the oral cavity (including removable and fixed prostheses).

Gingivitis – Refers to gingival inflammation without loss of connective tissue attachment.

Junctional epithelium – Refers to a single or multiple layers of non-keratinizing epithelial cells that surround the tooth in a collar like fashion.

Ligand - Is a signal triggering molecule that binds to a site on a target protein.

Periodontitis – This is an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms resulting in progressive destruction of the PDL and alveolar bone with pocket formation, recession or both (AAP 1999).

Receptor – A protein molecule usually found inside or on the surface of a cell, that receives chemical signals from outside the cell. It binds a ligand.

Clinical attachment loss – The sum of probing pocket depth and the gingival recession will give the clinical attachment loss

Probing pocket depth – The distance between the gingival margin and the bottom of the pocket to the nearest whole millimeter.

Gingival recession - the distance between the cemento-enamel junction (CEJ) and the gingival margin

Analyte – The substance whose chemical constituents are being identified and measured

Stock solution – A concentrated solution that will be diluted to some lower concentration for actual use.

ABSTRACT

Introduction

Periodontal diseases are some of the commonest oral health problems. They affect every race worldwide and their prevalence varies from region to region. An important aspect of tissue breakdown in these diseases is alveolar bone loss. However, the mechanisms that lead to the bone loss are not well understood. Increasing evidence has shown that resorption of bone is regulated by two biomarkers - receptor activator of nuclear factor ligand (RANKL) and osteoprotegerin (OPG).

Aim: To determine the association of salivary levels of the bone remodelling regulators RANKL and OPG with periodontal clinical status.

Materials and Methods: This was a descriptive cross sectional study and was carried out at the University of Nairobi Dental Hospital.158 participants were selected from a pool of adult patients who visited the dental hospital during the period of study via systematic random sampling method. Saliva was collected from each participant followed by periodontal clinical examination.

Saliva collection: About 5 mL of saliva was collected from each participant (unstimulated whole saliva).Collection was done between 7 and 9 am through expectoration and transferred to the lab for immediate centrifugation. The supernatant was collected and aliquoted in 500 µL using micropipettes into a clean microcap tubes prior to freezing at -70°C until processing.

Clinical evaluation: Periodontal examination of all teeth was done at six points and included pocket depths, gingival recession and clinical attachment loss. Other clinical parameters included plaque score and gingival index.

Biomarker analysis: Concentrations of RANKL and OPG was determined using human RANKL and OPG enzyme linked immunosorbent assay (ELISA) kits as per the manufacturer's instructions. The results were expressed as picograms per milliliter (pg/mL). The laboratory stage of the study was performed at the Kenya AIDS Vaccine

Initiative (KAVI) - Institute of Clinical Research, College of Health Sciences, University of Nairobi.

Data analysis: Coding, entry and analysis was done by Statistical Packages for Social Sciences (SPSS) 20 for windows. Categorical data were presented in frequencies and percentages. Continuous data was presented in mean, median, range and standard deviation. Association between a categorical variable (e.g. gingival index, CAL) and a continuous variable (e.g. RANKL, OPG level) was tested through t-test and ANOVA. The independence of the association was analyzed by ordinal hierarchical multiple linear progression, adjusting for age and smoking habit.

Results: A total of 158 participants were included in the study with 92 (58.2%) being females while 66 (41.8%) being males and age range of 18-75 years. Of the 158, 9 were extreme outliers hence excluded at the analysis stage. RANKL had a mean salivary level of 14.65 pg/mL (± 18.72SD) and strong level of association with the severity of periodontal disease ($F = 64.82, p < 0.001$) where higher levels were detected in severe grades of the disease. The mean OPG levels in the saliva samples ranged from 4.33 to 204.33pg/mL with a mean of 139.03 pg/mL (± 51.19 SD). The mean levels were significantly high in cases without periodontitis or in cases with milder grade of periodontitis ($F =19.031, p<0.001$). Consequently, a strong positive correlation was established between RANKL/OPG ratio and the severity of periodontal disease ($(r_s = 0.759, p< 0.001$).

Conclusion

The levels of RANKL and OPG in saliva and their relative ratio have strong association with the severity of periodontal disease.

Recommendation

Salivary levels of RANKL and OPG and their relative ratio should be considered as a potential adjunctive diagnostic tool for evaluating periodontal disease. However, there is need for more salivary proteomic studies and randomized controlled trials in Kenyan setting to fully exploit the potential application in periodontal diagnosis.

CHAPTER 1: INTRODUCTION

Periodontal diseases are some of the commonest oral health problems. They affect every race worldwide and their prevalence has been found to vary from region to region [1]. The Kenya National Oral Health Survey Report 2015 [2] recognizes that, although preventable, the prevalence of these diseases is still relatively high in Kenya. In a literature review done by Ng'ang'a, the prevalence of gingivitis among the adult population is reported at 90% while 1-10% suffers from chronic periodontitis [3].

An important aspect of tissue breakdown in periodontal disease is alveolar bone loss. The disease alters the microenvironment of alveolar bone thus compromising its structural integrity. The process is modulated by a number of molecular events including an interplay between two important biomarkers - receptor activator of nuclear factor ligand (RANKL) and osteoprotegerin (OPG) [4].

RANKL is a ligand expressed by osteoblasts, activated T and B cells as well as fibroblasts. It is responsible for bone destruction by activating osteoclsats. OPG on the other hand is a decoy receptor for RANKL. By binding it, OPG prevents activation of osteoclasts thus inhibiting bone destruction [5-7]. A relative RANKL/OPG ratio is thus established. The ratio is significantly increased in periodontitis compared to health or gingivitis. It may therefore be a good pointer to the state of periodontal health [8-9].

Saliva has gained significant recognition as a sample for the detection of biological changes in the oral cavity. Buduneli and colleagues reported detectable levels of RANKL and OPG in saliva [10]. Collection of saliva is easy and safe. It is also non-invasive as opposed to the cumbersome and invasive conventional periodontal diagnosis. This formed the basis of the study with focus on the two biomarkers in saliva (RANKL and OPG) whose levels and ratio vary with the state of periodontal disease. The study therefore aimed at determining the association of salivary levels of RANKL and OPG with periodontal clinical status. The association may in future provide a platform for easy and non-invasive periodontal diagnosis.

Benefits from this study included partial fulfillment for the award of masters of dental surgery in periodontology at the University of Nairobi, adding new information to the existing body of knowledge and assessment of the periodontal health of the participants. The study has formed a basis for future randomized control trials aimed at providing easy, safe, cost-effective and non-invasive diagnostic approaches to periodontal disease.

CHAPTER 2: LITERATURE REVIEW

2.1 Periodontium and Periodontal diseases

Periodontium refers to specialized tissues that surround and support teeth, maintaining them in the maxillary and mandibular bones. It is composed of the gingival connective tissue, the periodontal ligament, the cementum, the alveolar bone and the associated neurovasculature. Although unique in structure and location, all of these components function as a single unit. Their biologic adaptation and renewal processes maintain a harmonious relationship. The junction between the tooth and the gingival tissues is known as dentogingival junction. It is formed by the junctional epithelium which is attached to the tooth through the dental lamina. This junction provides a potentially weak barrier via which bacteria and bacterial products may enter the underlying connective tissue and vasculature [(11)].

Periodontal diseases are a group of diseases which affect one or more of the periodontal tissues. They have been defined and classified mainly based on clinical manifestations, including location, degree of tissue loss and rate of destruction. Currently, classification is based on recommendations by the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions organized by the American Academy of Periodontology [(12)]. In terms of severity, periodontal diseases are classified as mild, moderate or severe based on modification of the clinical case definitions by the Centers for Disease Control (CDC) for use in population based surveillance of periodontitis 2007 [(13)].

Periodontal diseases lead to enhanced breakdown of the periodontal ligament and alveolar bone resulting into pocket formation and attachment loss. The destructive changes are brought about by the interplay between the microbial biofilm at the gingival margin and the host response. Progression of the disease depends on risk factors and risk indicators including oral hygiene, genetic factors, smoking, race, diabetes, economic status and level of education [(14)]. The disease is also modified by endocrine changes seen in pregnancy, puberty and contraceptives.

2.2 Burden of Periodontal diseases

Periodontal diseases affect every race worldwide. The prevalence vary from region to region [1].The World Health Organization performed extensive surveys of the periodontal status of potentially under-served populations around the globe, especially in developing countries [15]. Using the Community Periodontal Index for Treatment Needs to assess prevalence, a majority of subjects examined had gingivitis and 10–15% of adults had periodontal pockets ≥6 mm deep.

Regional studies show that periodontal diseases are widespread. A study done in a Nigerian population reported an early development of pathological periodontal pockets with a prevalence of 15-58% in individuals aged above 15 years [16]. In Uganda, there was a higher prevalence of calculus, plaque (causative agent of periodontal diseases) and gum bleeding in adults compared to children [17].

The Kenya National Oral Health Survey Report 2015 [2] recognizes that the prevalence of these diseases is relatively high. In an overview of epidemiological and related studies undertaken on common dental diseases and conditions in Kenya from 1980–2000, the prevalence of gingivitis among the adult population was reported to be 90% while periodontitis was 1-10% [3]. Another study done on a group of trainees aged between 18 and 26 years showed that gingivitis was widespread recording a prevalence of 66% in males and 44% in females. Periodontitis with recession of more than 3mm was recorded in 12% of the individuals while pocket depths of more than 3mm were recorded in 2% of individuals [18].

2.3 Pathogenesis of Periodontal diseases

Microorganisms existing in dental plaque are the primary etiological factors for the initiation of periodontal diseases. The tissue type, location, and exposure to external shear forces are some of the conditions affecting colonization of periodontal tissues by microorganisms. Gingival sulcus especially the col region, offer an ideal environment for bacterial settling. The products of these bacteria, mainly lipopolysaccharides, initiate an inflammatory response responsible for periodontal tissue destruction [19].

As the lesion develops, there is increased vascular leakage with polymorphonuclear leukocytes (PMNs) dominating the infiltrate. The PMNs release pro inflammatory molecules including prostaglandins, proteases as well as reactive oxygen and nitrogen species. The molecules are released to contain the intruding pathogen. In doing so, they also cause collateral damage to gingival connective tissue for failure to discriminate between host and bacteria [18]. Greatest tissue breakdown is caused by a sub class of proteases known as matrix metalloproteinases (MMPs) together with cytokine driven osteoclast activity [20].

A successful inflammatory response eliminates the infectious agent and initiates tissue repair. However, if the infection prevails, macrophages and dendritic cells present antigens that activate the adaptive immune system. T-cells and B-cells start to accumulate and ultimately dominate the lesion. The lesion then takes a chronic course. The result is more clinical attachment loss with apical migration of junctional epithelium, pocket formation and bone loss [21].

2.4 Alveolar bone loss in periodontal disease

Bone is a specialized connective tissue with different types of bone cells. It is dynamic in nature with continuous remodeling to meet functional needs. While osteoblasts are involved in creation and mineralization of bone, osteoclasts are responsible for resorption of the bone matrix. The alveolar bone undergoes a similar process so as to maintain the dynamism. Remodelling of alveolar bone occurs in different environments. Physiologically, it occurs during tooth eruption as well as when subjected to occlusal forces. It also occurs as a result of clinical interventions as the case with orthodontic tooth movement. Pathological conditions that cause bone resorption include (but are not limited to) periodontal disease, periapical pathology or tumours [4].

Resorption of the alveolar bone is a key characteristic of periodontal diseases. It is triggered through immune responses resulting from inflammatory reactions directed against bacteria and their products. Osteoclasts are the cells responsible for bone resorption. They differentiate from macrophage/monocyte lineage cells, and are

activated by various cytokines including RANKL [22]. The osteoclasts line pits in the bone surface called Howship's lacunae (resorption bay). Resorption proceeds from these resorption bays causing thinning of the surrounding bone and reduction in bone height. The destruction can either be horizontal or vertical. Horizontal destruction presents with generalized reduction in bone height. However, the bone margins remain somewhat perpendicular to the tooth surface. In cases of vertical/angular bone loss, the defect occurs in an oblique direction forming a triangular area of missing bone. The base of the defect is usually apical to the adjacent sound bone [23].

2.4 RANKL/OPG Pathway

Several biomolecules are produced during inflammation. The biomolecules, otherwise known as biomarkers, lead to breakdown of connective tissue and invasion of alveolar bone. The levels of the biomarkers are readily detectable in GCF as well as saliva of individuals with periodontal disease [24]. As a result, their analysis offer an important platform for diagnosing periodontal diseases and fabricating appropriate treatment plan [25].

RANKL and OPG are two biomarkers with significant influence on alveolar bone resorption in periodontal diseases. RANKL is a ligand that belongs to Tumor Necrosis Factor (TNF) family. It is expressed by osteoblasts, activated T and B cells as well as fibroblasts [26]. Production is stimulated by cytokines found in the GCF/saliva as a result of inflammation in individuals with periodontal disease. The ligand binds directly to its cognate RANK receptor on the surface of preosteoclasts and osteoclasts. This results in differentiation of osteoclast progenitors and the activation of mature osteoclasts which mediates bone resorption [21].

There are two isoforms of RANKL produced by alternate splicing: a type II membrane protein (mRANKL), and a secreted molecule (sRANKL), lacking the cytoplasmic and transmembrane domain. Both forms are bioactive. However, production of mRANKL is homeostatic while that of sRANKL is pathological in nature [27].

Osteoprotegerin is a receptor like protein made up of 401 amino acids synthesized as a monomer of ~55 kDa [28]. Having structural homology to type-2 TNF receptor, OPG is produced by PDL cells, gingival fibroblasts and epithelial cells. Similar to RANKL, its production is dependent on cytokines produced during inflammation [29].

OPG has structural homology to RANK and is therefore a decoy receptor for RANKL. The ligand preferentially binds to it at the expense of its natural receptor RANK. Once the interaction between the ligand and the receptor is interrupted, differentiation of osteoclasts is prevented with reduction in bone resorption as shown in Figure 1 [30].

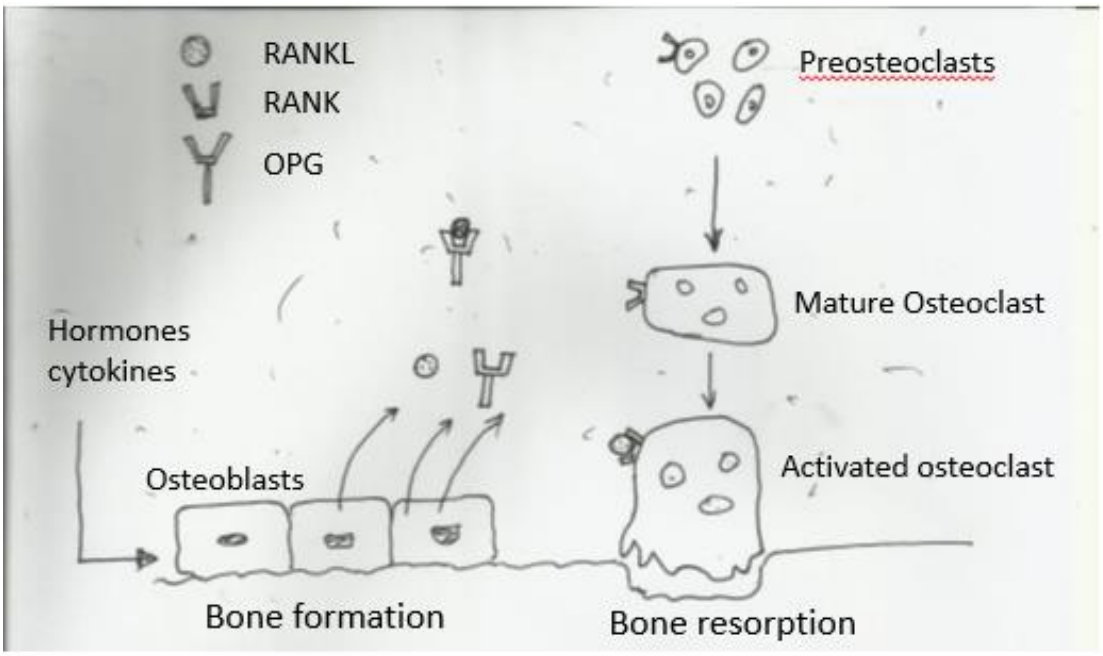


Figure 1: Illustration of RANKL/OPG Pathway

2.5 RANKL/OPG in saliva

Saliva contains numerous proteins and genetic materials that are easily accessed in a relatively noninvasive manner [31]. Most of the blood molecules including antibodies ,hormones and enzymes are available in saliva through passive diffusion, active transport or extracellular ultrafiltration [32]. As a result, saliva has gained significant recognition as a sample for the detection of biological changes in the oral cavity and the entire body in a manner similar to serum.

The collection of saliva and its handling is easy leading to its use application in the detection of caries, periodontal diseases and salivary gland disorders among others [33-35]. It is also important in the evaluation of systemic conditions including hepatitis and human immunodeficiency virus (HIV) [36-37].

Due to the numerous advances made in salivary diagnostics, authors who have reported detectable levels of RANKL and OPG in saliva with established relationship to the clinical manifestations of periodontal disease [10]. Other studies on the two molecules have been performed in GCF samples with similar trend of results. [38]. Collection of GCF is however difficult, technique sensitive and time consuming.

Generally, the existing literature indicates that there are higher RANKL levels in individuals with periodontitis compared to healthy individuals or those with gingivitis. The reverse is true regarding OPG levels. A relative RANKL/OPG ratio is thus established. Some studies have reported a 2.2-fold increase in the ratio during chronic periodontitis when compared with healthy subjects [39]. Sergio et al (2012) reported a median salivary RANKL concentration of 4.00 pg/ml (2.40–6.60) in the healthy control group. The median salivary RANKL concentration in those with chronic periodontitis was 6.00pg/ml (2.70–11.10). Salivary OPG concentration in the healthy control group was 131.60 (82.20–202.40) compared to 95.20 (49.80–145.20) in those with chronic periodontitis [40].

Apart from saliva, RANKL and OPG have been reported in GCF as well as serum. Mogi et al in 2004 reported higher GCF concentrations of RANKL in severe grades of periodontal disease (180 pg/mL).The levels were low in health (30 pg/ml). Conversely, OPG levels were high in health (400 pg/mL) and low in chronic periodontitis [41]. The relative RANKL/OPG ratio was higher in disease state compared to health. Concerning the serum levels, one study reported a range 96.0 – 135 pg/ml of serum OPG and a range 11.4 to 18.1 pg/ml in serum RANKL levels across the different groups of the disease [42].

2.6 Methods of saliva collection

Saliva is either gland-specific or whole in nature. Gland-specific saliva is obtained directly from the specific glands and is critical in the evaluation of gland-related pathologies. Whole saliva is a mixture glandular secretions (mainly from submandibular and parotid glands) and non-glandular elements including serum transudate, GCF, immune cells, food debris among others. Analysis of whole saliva is useful in detection of systemic pathologies [43].

The method of saliva collection and flow rate affect the composition of the sample to be analyzed. Collection of stimulated saliva is usually preceded by inducing mastication (chewing gum) or by stimulating the taste buds by applying something spicy on the tongue. While it increases the amount, stimulation dilutes some of the elements to undetectable levels and alters the pH of saliva. Due to the alteration in quality, stimulated whole saliva is less reliable for accurate diagnosis [44].

Unstimulated saliva is the most applied form in studies because it is harvested without any form of stimulation. The content therefore reflects the true levels of the constituents. Miller et al. 2010 discussed up to 21 different biomarkers in unstimulated whole saliva evaluating their role in diagnosing periodontal diseases and designing appropriate treatment plan [45]. Most of the harvesting of unstimulated saliva is done via draining method, spit method or suction [46]. For purposes of this study, whole unstimulated saliva that includes GCF from the gingival sulci was analyzed.

2.7 Techniques used in identification and quantification of RANKL and OPG

RANKL and OPG have been identified and quantified via different techniques including histochemical staining methods, In situ hybridization, gene expression analysis and Enzyme -linked Immunosorbent Assay (ELISA).

Histochemical staining methods reveal the distribution of the biomarkers and differentially expressed proteins of interest. Crotti et al in 2003 reported high degree of staining for RANKL and relatively lower degree of histochemical stains for OPG in tissue

affected by periodontitis. Histochemical stains for RANKL was predominantly associated with CD3 + lymphocytes of which only a few were associated with healthy sites [47].

RANKL and OPG have also been localized and quantified via In situ hybridization technique. The technique is applied in tissue sections where there is identification of particular mRNA series within respective cells, providing insights into physiological processes and disease pathogenesis. Using the technique, Liu and co-workers in 2003 reported a concentration of mRNA transcripts specific to RANKL located in inflammatory cells. The technique is however cumbersome with many steps required for optimal results [48].

These findings have been supported further by gene profiling analysis. Through the use of PCR to assess RANKL and OPG in the periodontal tissues, Bostanci et al. (2007) reported RANKL expression rate of 0–40% in health with higher figures being reported in CP and AP (54–100% and 75–100% respectively) [49]. Opposite was the case for OPG gene expression.

Other techniques used in identifying and quantifying RANKL and OPG include Western blot, Immunoflourometric method (IFMA), Enzyme -linked Immunosorbent Assay (ELISA) as well as Gelatin and Casein zymography among others [50]. The use of ELISA in detection of RANKL and OPG was demonstrated by Mogi and co-workers in 2004 using GCF samples [41]. It uses antibodies and color change to identify an antigen in a sample. ELISA technique was used in this study because of reliability, convenience and high detection specificity. The kits are commercially available and allows for assay of a large sample size.

2.8 ELISA

ELISA is a biochemical test that relies on the use of antibodies and change in color to correctly identify antigen. It involves series of steps that culminate in a colored end product whose intensity conforms to the concentration of the substance being analyzed [51].

ELISAs can be performed either as the basic procedure or with introduction of some changes in the protocol. The key step in the process is to immobilize the substance being analyzed (antigen). Immobilization of the antigen is achieved either by integration into the plate (Direct ELISA) or through a capture antibody bound to the plate (Indirect ELISA/sandwich technique). In the former method, there is only one antibody for direct detection of the antigen. The latter has two sets of antibodies. While the first set is used to bind the antigen to the plate, the second set of antibodies is labeled hence used for detection of the antigen. This study used Sandwich ELISA Technique because it is sensitive and robust. The analyte to be measured is bound between two primary antibodies, the capture antibody and the detection antibody, forming 'a sandwich' [(52)].

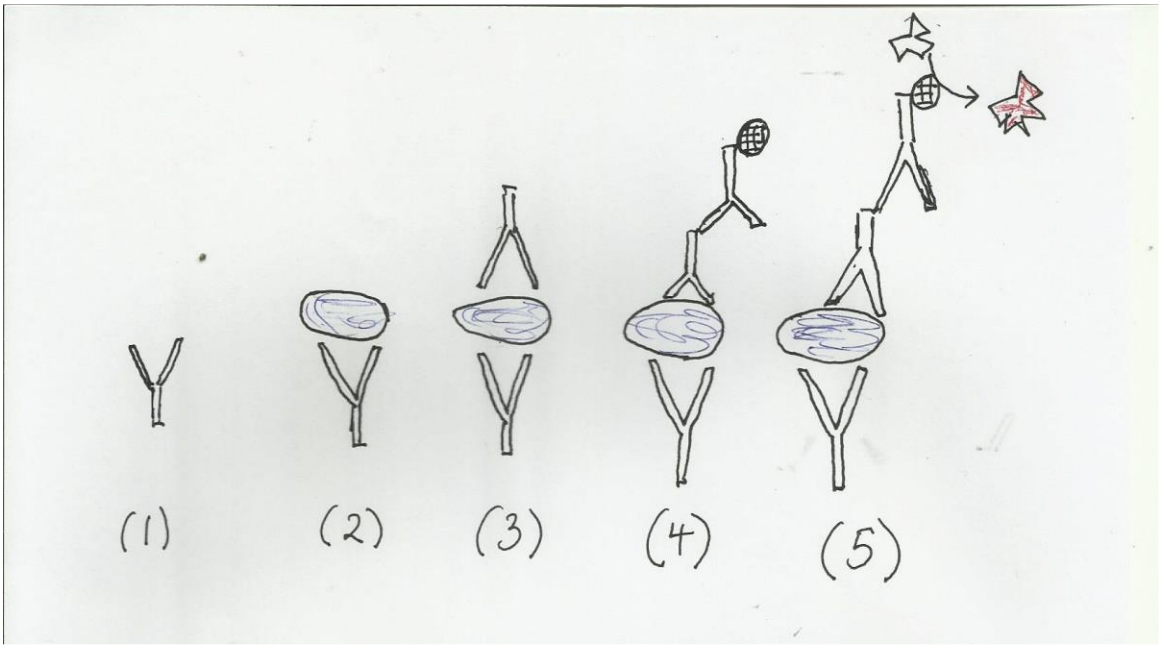


Figure 2: Diagrammatic representation of Sandwich ELISA Technique

The Sandwich ELISA diagram explained:

1. Thin layer of capture antibody applied onto the plates (Capture antibody was made of mouse anti-human RANKL or mouse anti-human OPG for this study).
2. Addition of sample or standard. Any antigen present in the sample or the standard will be bound to the capture antibody.
3. Addition of detection antibody and attachment the already bound antigen.
4. Addition of secondary antibody that is linked to an enzyme with subsequent binding to the antibody involved in detection.
5. Introduction of a substrate with subsequent enzymatic conversion to a form that is detectable through spectrophotometry.

In RANKL/OPG ELISA, the capture antibody is made of mouse anti-human RANKL or mouse anti-human OPG. The standards are made of recombinant human RANKL or recombinant OPG while detection antibody are biotinylated goat anti-human RANKL/OPG. The enzyme stage involves the use of streptavidin conjugated to horseradish-peroxidase. Streptavidin forms non covalent cross linkage to the biotinylated detection antibody due to the ability of biotin to bind streptavidin with high affinity and specificity forming a complex that is resistant to extremes of heat, pH and proteolysis [(53)].

With the addition of organic substrate, enzymatic reaction ensues with the formation of chromogenic end product located in areas with bound antigen. Tetramethylbenzidine in this case donates hydrogen as a result of which hydrogen peroxide is reduced to water by horseradish peroxidase enzyme. A diimine product is formed and turns the solution pale blue with intensity corresponding to the amount of bound antigen [(54)].

CHAPTER 3: PROBLEM STATEMENT, JUSTIFICATION, OBJECTIVES, HYPOTHESIS AND VARIABLES

3.1 Problem

Early detection of a disease plays a crucial role in successful therapy. In most cases, the earlier the disease is diagnosed, the more likely it is to be successfully cured or well controlled. Managing a disease in the early stage reduces its severity and impact on the patient's life.

Periodontal diseases are highly prevalent. In Kenya for example, the prevalence of gingivitis among the adult population is reported at 90% while 1-10% suffers from chronic periodontitis [(3)]. However, their diagnosis is cumbersome, invasive and in most cases done when the diseases are advanced. Finding disease biomarkers in a medium that is readily available like saliva is vital in overcoming these challenges.

RANKL and OPG are two biomarkers whose molecular interplay control bone resorption. Since alveolar bone loss is one of the most important hallmarks of periodontal breakdown, the levels of the two biomarkers and their ratio in GCF and saliva vary depending on the state of periodontal disease. This can be used as a diagnostic marker for the disease.

3.2 Justification

Saliva has gained significant recognition as a sample for the detection of biological changes in the oral cavity. Collection of saliva is easy and safe. It is also non-invasive as opposed to the cumbersome and invasive conventional periodontal diagnosis. This formed the basis of the study which focused on two biomarkers in saliva (RANKL and OPG) whose levels and ratio vary with the state of periodontal disease. The study therefore aimed at determining the association of salivary levels RANKL and OPG with periodontal clinical status. The association may in future provide a platform for easy and non-invasive periodontal diagnosis.

3.3 Main Objective

To determine the association of salivary levels of the bone remodelling regulators RANKL and OPG with periodontal clinical status.

3.4 Specific Objectives

1. To determine the periodontal clinical status of the participants.
2. To determine the salivary levels RANKL.
3. To determine the salivary levels of osteoprotegerin.
4. To determine the relationship between RANK/OPG ratio with periodontal clinical status

3.5 Null Hypothesis

There is no association between RANKL/OPG ratio with periodontal clinical status.

3.6 Study variables (Table 1)

Variables	**Measurement**
Socio-demographic variables	
Age	• Number of years
Gender	• Male or female
Residence	• Residence at the time of study
Education	• Highest level of education attained
Smoking Status	• Current smoker, non-smoker or previous smoker
Independent/Exposure Variables	
Periodontal status • Oral hygiene status • Severity of gingivitis • Severity of periodontitis	• Plaque score • Gingival Index • Clinical attachment Loss (CAL)
Dependent/Outcome Variables	
RANKL OPG	• picograms per milliliter (pg/mL) • picograms per milliliter (pg/mL)

CHAPTER 4: MATERIALS AND METHODS

This chapter describes in details the population from which data was collected, the methodologies used, the laboratory procedures and how the data was processed.

4.1 Study design

This was a hospital based analytical cross sectional study.

4.2 Study area

The study was carried out at the oral diagnosis clinic of University of Nairobi Dental Hospital. The hospital is located in Nairobi, the capital city of Kenya, approximately 2 kilometres from the city centre. It acts as a referral centre for patients from all parts of Kenya. It records a daily outpatient flow of about 25 patients. All the patients visiting the facility are attended to at the oral diagnosis where history is taken, examination done and diagnosis made. They are then referred to the respective clinics within the hospital for further management depending on the diagnosis.

4.3 Study population

The study population consisted of all adult patients who visited the University of Nairobi dental hospital during the period of study.

4.4 Inclusion criteria

Participants were individuals aged 18 years and above who consented to voluntary participation in the study. The individuals had at least 16 teeth present in the mouth including a premolar and a molar in each arch. This allowed adequate representation with results that can be extrapolated to the general population.

4.5 Exclusion criteria

Criteria for excluding an individual from the study involved the following:

- Failure to give consent

- Pregnancy
- Ongoing orthodontic therapy
- Systemic condition that could affect the host's periodontal status and bone metabolism e.g. osteoporosis, rheumatoid arthritis, diabetes
- Antibiotic therapy within 3 months prior to the study
- Use of antiseptic rinse within 3 months prior to the study
- History of periodontal therapy within 3 months prior to the study
- History of organ transplant or cancer therapy
- Need for antibiotics for infective endocarditis prophylaxis during dental procedures
- Corticosteroid therapy within 3 months prior to the study

4.6 Sample size determination

The prevalence of periodontal disease among adult Kenyans has been reported at 80% [55]Sample size was calculated based on Kish and Leslie formula for cross sectional studies as shown below[48].

$$N = \frac{Z^2 P(1-P)}{C^2}$$

N = desired sample size
Z = confidence level at 95% (standard value of 1.96)
P = estimated prevalence of periodontal disease among Kenyans (80%)
C = margin of error at 5% (standard value of 0.05)

Therefore;

$$N = \frac{1.96^2 \times 0.8 \times 0.2}{0.05^2}$$

$= 245.86 = \underline{246}$

The calculated sample size for a population more than 10, 000 was 246. However the average number of patients visiting the dental hospital at the time of study was roughly 25 patients per day giving a total of 400 in one month.

Using the correction formulae for a population of less than 10,000 [49]

$$n = \frac{n_0}{1 + \frac{(n_0 - 1)}{N}}$$

Where n = desired sample size (for population <10,000)

n_o = desired sample size (for population >10,000)

N = estimate of population size (400)

=153

5 more participants were included to allow for a dummy lab process to test the equipment and reagents.

Total sample size 158

4.7 Sample design and procedure

Participants were selected through systematic random sampling. The method was adapted due good spread across the population and its simplicity. The systematic random sampling method is useful when units in sampling frame are not numbered serially and when the sampling frame consists of a very long list. A screening form (appendix II) was given to every 3rd patient who visited the facility. The screening form was useful in identifying participants who fit the inclusion criteria. During the period of the study, an average of 25 patients attended the facility in a day.

4.8 Data collection tools, clinical examination and laboratory procedure

4.8.1 Data collection tools

A screening form (Appendix I) was used to identify participants who fit into the inclusion criteria. It was designed in English. Translation was done to those who do not understand English.

Concerning socio-demographic data, each participantfilled interviewer administered and serialized questionnaires giving information on age, gender, education and residence. (Appendix II).

Clinical findings were entered into clinical examination form (Appendix III).These included plaque score, gingival index probing pocket depths and recession at six points (mesiobuccal, buccal, distobuccal, lingual, mesiolingual, distolingual).

Unstimulated whole expectorated saliva of about 5 mL was collected from each subject using a plastic centrifuge tube. In the lab, concentrations of RANKL and OPG in the supernatants (obtained by centrifugation) were determined using a human ELISA Kit. The values were captured using ELISA worksheet (Appendix VI).

4.8.2 Periodontal parameters

The periodontal parameters were defined as follows:

Probing pocket depths (PPD)

This was the measure from the margin of the gingiva to the bottom of the pocket determined using Hu-Friedy periodontal probe.

Gingival recession

This was the distance between the cemento-enamel junction (CEJ) and the gingival margin. It was recorded using the Hu-Friedy periodontal probe to the nearest whole millimeter.

Clinical attachment loss (CAL)

The sum of probing pocket depth and the gingival recession gave the clinical attachment loss.

Gingival index /bleeding on probing (BOP)

BOP was deemed present if it occurred within 30 seconds after running the probe back and forth along the gingival margin. It will be recorded based on Gingival Index of Loe and Silness 1963 (Appendix IV a).

Plaque score

Plaque scores were taken using Silness-Loe index (1964) on the index teeth (FDI nomenclature, Appendix IV b).

Severity of periodontitis

Severity of periodontitis was quantified as per the CDC/AAP classification (Appendix V) [13].

4.8.3 Data collection – Preliminary phase

The preliminary phase of data collection began in April 2015 after obtaining approval from the Kenyatta National Hospital and University of Nairobi Ethics and Research Standards Board (Appendix XII). It began with obtaining permission from the relevant authorities within the University of Nairobi Dental Hospital – The Dean, School of Dental Sciences; The Chairman, Department of Periodontology/Community Dentistry and The Chairman, Department of Oral/Maxillofacial Surgery. Regular visits to the oral diagnosis clinic were conducted for familiarity. Discussions were held with the nursing officer in charge of the clinic to address logistic issues.

A pilot study was conducted to test the questionnaire and tolerance to the clinical examination. The principal investigator was then calibrated by the lead supervisor to evaluate inter examiner variability. Cohen's kappa score was set at 80%.

Permission to conduct the laboratory procedures at KAVI was granted by the director. An extensive tour of the lab was then conducted under the direction of the laboratory manager. During the tour, the availability and condition of the necessary equipment

were assessed. The ELISA protocol to be used was discussed in details as well as how the reagents and the samples would be stored.

Arrangements were then made with Bio-Techne Corporation, R&D Systems UK for importation of all the reagents necessary for the study.

4.8.4 Data collection - Clinical stage

The clinical stage of data collection involved filling the questionnaires, carrying out periodontal examination and collecting saliva. It lasted for 3 months from September 2015 to November 2015.

The stage began with explaining the purpose of the study to the participants. A screening form (Appendix I) was used to identify suitable study participants. Those who did not fit into the inclusion criteria were excluded. Those who fit into the inclusion criteria gave informed written consent (Appendix VIII) before duly filling in the questionnaire under the guidance of the principal investigator. Saliva was then collected followed by clinical examination.

Saliva collection

Unstimulated whole expectorated saliva of about 5 mL was collected from each subject between 7 and 8 AM before breakfast according to a method described by Navazesh.[(46))].In the method, there was neither a mouth rinse nor any dental hygiene procedure before collection. The participants were seated on the dental chair and allowed to relax. They were then requested to gently lean forward and allow saliva to passively pool in the anterior floor of the mouth without talking or swallowing. The amount of unstimulated whole saliva pooled after 5 minutes was then expectorated into a sterile plastic centrifuge tube (Eurotubo® Deltalab, Spain).The 50ml centrifuge tube was used to allow ease of saliva collection without spillage. The collected saliva was immediately placed in a cool box with ice pack for transportation to the lab within one hour.

Clinical evaluation

After saliva collection, periodontal clinical examination was done under illumination from dental chair light using disposable gloves, masks, gauze, a Hu-Freidy sterile periodontal probe and oral dental mirrors. Bleeding on probing was assessed by running the periodontal probe along the gingival margin and waiting for 30 seconds before visual inspection for areas of bleeding. Gingival recession was then recorded followed by pocket depths measurements to the nearest millimeter of all teeth at six points (mesiobuccal, buccal, distobuccal, lingual, mesiolingual, distolingual).The sum of probing pocket depth and the gingival recession gave the clinical attachment loss. The participant was then given a disclosing tablet (Produits Dentaire Vevey, Switzerland) to chew and roll against every tooth surface with the tongue before spitting. The plaque score was then graded based on visual inspection of the stained teeth surfaces.

Infection Control

Appropriate precautions were taken to protect the participants, the principle investigator and other users of the clinic from the risk of cross-infection. The dental chair was disinfected before ushering any participant. The principle investigator ensured thorough hand wash, use of clean lab coat, gloves and facemasks as shown in Figure 7 & 8. Each participant had a disposable bib covering and disposable plastic tumblers for mouth rinsing.

Only sterile dental mirrors, probes and tweezers packed in a sterile dental instrument tray were used for clinical evaluation. All the wastes were disposed according to the hospital's guidelines on waste disposal. Pre packed sterile centrifugation tubes used to collect saliva were large enough to avoid spillage. The tubes with samples were carefully closed tightly, packed into a clean cool box and transported to the lab. Handling of saliva was done in consultation with the supervisor (consultant) and a senior laboratory technologist to ensure compliance with biosafety protocols.

The used instruments were carefully returned to the tray, transported to the central sterilization unit for cleaning and decontamination. The instruments were then packaged, sterilized and stored for the next clinical session.

4.8.5 Data collection - laboratory stage

The laboratory stage involved a number of procedures that began with centrifugation and storage of saliva followed by series of ELISA assays to determine the respective concentrations of RANKL and OPG in each saliva sample. All of the procedures were performed at The Kenya AIDS Vaccine Initiative (KAVI), Institute of Clinical Research, College of Health Sciences, University of Nairobi.

Centrifugation and storage of saliva samples

In the laboratory, each saliva sample received was assigned a serial number and recorded. The samples were immediately clarified by centrifugation for 5 minutes at 1000 g (Heraeus Multifuge® 4KR Centrifuge).The supernatant was collected and aliquoted in 500 µL using micropipettes into clean microcap tubes (Micro tube 2ml,PP – Sarstedt, Germany). Two aliquots were made from each saliva sample and kept in ultra-low temperature freezer at -70°C until processing (U725 Innova® freezer, New Brunswick Scientific, last serviced by Biologic Solutions Limited in June, 2015).

The Assay Procedure

Salivary levels of RANKL and OPG were measured through an ELISA based protocol using DuoSet® ELISA Development Systemfrom Bio-Techne Corporation, R&D Systems UK (Human TRANCE/RANK L/TNFSF11, Catalog # DY626 and Human Osteoprotegerin/TNFRSF11B, Catalog # DY805). Each sample underwent double ELISA assay (one for RANKL and another for OPG). Appendices detailing materials provided, solutions required, plate preparation and assay procedure are included (Appendix IX, X, XI).

Plate Preparation

ELISA worksheet (Figure 3) was used to map out the 96 –well microplates (8 rows, 12 columns) clearly indicating the respective positions for standards and samples done in duplicates as shown below. A total of 8 plates were used for the whole assay process (4 for each analyte).

Plate No._____ Analyte _____ Date _____

Technologist _____ Principal Investigator: _____

	STANDARDS		SAMPLES									
	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK										
B												
C												
D												
E												
F												
G												
H												

Figure 3: ELISA worksheet

The reagents were then reconstituted as indicated in the manufacturer's instructions. All reagents were brought to room temperature before use. The components were allowed to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions were prepared and used immediately, unless otherwise noted.

Capture antibody made of mouse anti-human RANKL or mouse anti-human OPG was reconstituted by adding1.0 mL of phosphate buffer saline into the capture antibody vial with gentle agitation to make180 µg/mL stock solution for each of the analytes. Further dilution was done to a total volume of 40,000µL (40mL) at a working concentration of 1.0 µg/mL in PBS, without carrier protein. Using micropipettes (MRC micropipettes, MRC Lab Ltd), 100 µL of the diluted capture antibody was pipetted into each of the 96 wells. The coated microplates were covered with plate seals and put into incubation at room temperature overnight to allow the capture antibody to bind as much as possible onto the surfaces of the wells.

After overnight incubation a programmed and automated sequence of aspiration and washing was done using wash buffer in autowasher machine (Thermo Scientific Wellwash® 4 Mk 2, last serviced by Faram East Africa Ltd in August 2015) to remove any unbound capture antibody. The machine is programmed to wash by filling each well with 400 µLwash buffer followed by liquid aspiration from the wells, repeating the process two times for a total of three washes. Complete removal of liquid at each step was essential for good performance. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels.

300 µL of reconstituted reagent diluent was then added to each well and incubated at room temperature for 1 hour. The reagent diluent was made of 1% Bovine Serum Albumin (BSA) in phosphate buffered saline (pH 7.2 to 7.4, 0.2µm filtered).Aspiration and washing was then repeated as described above.

At this stage, the plates were ready for standards and sample addition.

Addition of standards and samples

The standards were made of recombinant human RANKL or recombinant OPG. They were reconstituted by adding 0.5 mL of Reagent Diluent (made of 1% Bovine Serum Albumin in phosphate buffered saline at a pH 7.2 to 7.4, 0.2µm filtered) into each standard vial to make 160 ng/mL stock solution**.** The stock solutions were allowed to sit for 15 minutes with gentle agitation prior to making dilutions. This was followed by 2-fold serial dilutions in reagent diluents with a high standard of 5000 pg/mL for RANKL or 4000 pg/mL for OPG. This stage was necessary for development of a seven point standard curve that was used to derive formulae for calculation of the analytes.

100 µL of reconstituted standards was pipetted into each well in the 1st 2 columns with blank wells in the 1st two wells of first row and high standards in the 1st 2 wells of the last row as indicated in the worksheet.100µL of undiluted saliva samples (allowed to thaw at room temperature after freezing at -70°C) was pipetted into each of the remaining wells in duplicate. The plates were then coveredwith an adhesive strip and

incubated for 2 hours at room temperature to allow binding of the standards and the analytes to the capture antibody coated wells.

Addition of Detection Antibody

Following a 2 hour period of incubation, then wells were aspirated and washed using the microplate autowaher machine in a sequence and manner described in the plate preparation section above.100 µL of detection antibody, diluted in reagent diluent, was added to each well.

Detection antibody for RANKL was made of 9 µg/mL biotinylated goat anti-human RANKL reconstituted with 1.0 mL of reagent diluent to a working concentration of 50ng/mL.The concentration of biotinylated goat anti-human OPG detection antibody in the vial provided was 36µg/mL. This was reconstituted by adding 1.0 mL reagent diluent to a working concentration of 200ng/mL.

The wells were sealed and plates put into incubation at room temperature for another 2 hours. The incubation period allowed the detection antibody to bind to the antigen (RANKL and OPG in the standards and samples).The antigen at this stage is stuck between two antibodies forming a sandwich.

The plates then went through the aspiration and washing cycles to remove the unbound detection antibodies.

Addition of streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP Enzyme)

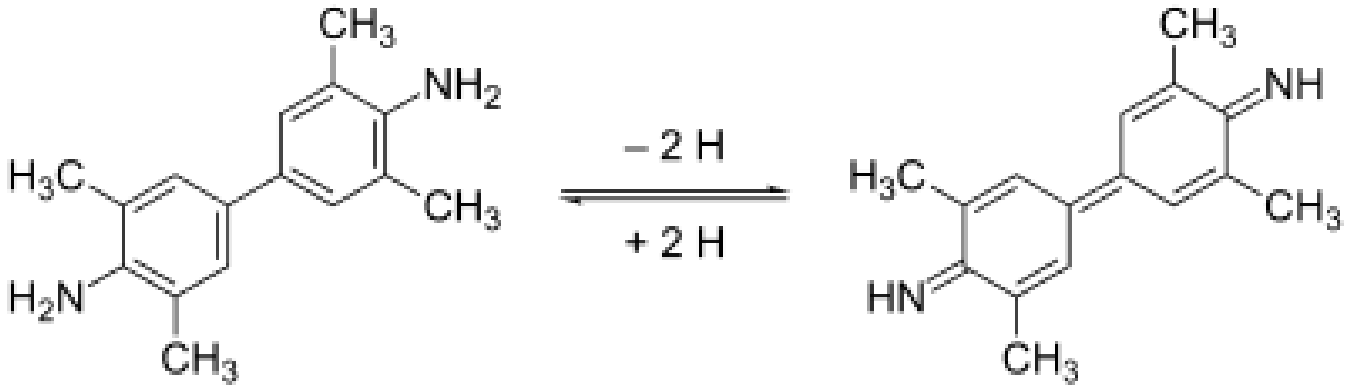
At this stage the wells were ready for addition of an enzyme -streptavidin conjugated to horseradish-peroxidase. The 1.0 mL streptavidin-HRP provided for each analyte underwent 200-fold dilution to a working concentration that was then used immediately.100 µL was pipetted into each well.
Incubation period for the plates after streptavidin-HRP enzyme addition was 20 minutes at room temperature away from direct light, period during which streptavidin formed non covalent cross linkage to the biotinylated detection antibody (biotin binds to streptavidin

with high affinity and specificity forming a complex that is resistant to extremes of heat, pH and proteolysis).

Addition of Substrate and Stop Solutions

Excess and unbound streptavidin- HRP were removed by aspiration and washing. A substrate solution for the streptavidin- HRP enzyme was then prepared by mixing stabilized hydrogen peroxide (supplied as Color Reagent A) and stabilized Tetramethylbenzidine (supplied as Color Reagent B) in a ratio of 1:1 then used within 15 minutes.100 µL of the prepared solution was then added to each well and incubated for 20 minutes at room temperature avoiding direct light.

With the addition of organic substrate, enzymatic reaction ensued with the formation of chromogenic end product located in areas with bound antigen. Tetramethylbenzidine donated hydrogen as a result of which hydrogen peroxide is reduced to water by horseradish peroxidase enzyme. A diimine product is formed and turns the solution pale blue with intensity corresponding to the amount of bound antigen (Figure 4).



Colorless **Pale Blue**

Figure 4: Oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) to 3,3',5,5'-tetramethylbenzidine diimine – Adapted from Wikipedia the free encyclopedia

At the end of incubation period, the wells did not go through the routine aspiration/washing cycles. Instead 50 µL of stop solution (2N sulfuric acid solution) was added to each well with gentle tapping to ensure thorough mixing. The addition of

sulfuric acid stopped the action of horseradish peroxidase enzyme on the substrate. The color changed from blue to yellow, intensity of which was then measured via spectrophotometry as shown in Figure 5.

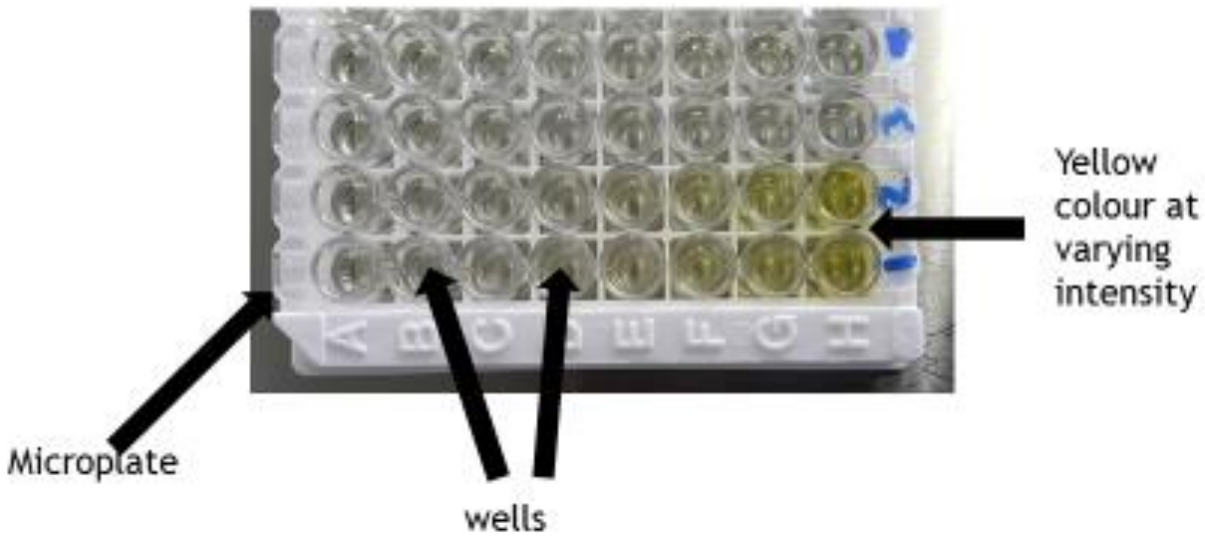


Figure 5: Photograph showing different intensities of yellow color in a plate

Determination of optical density

The optical density was determined immediately after the addition of stop solution using a microplate reader with inbuilt printer (Thermo Scientific Multiskan® EX, serviced by Faram East Africa Limited in August 2015) set at 450nm wavelength with correction wavelength at 620nm.The resulting optical density was the difference between optical density at 620nm and optical density at 450nm – a step necessary to correct optical imperfections in the plate.

The machine was turned on and left to warm for one minute. It was then programmed to determine optical density of a blank plate before determining the optical density of plates with reagent. A print out (Appendix VII) was obtained from the machine with optical density of each well corresponding to the plate map on ELISA worksheet.

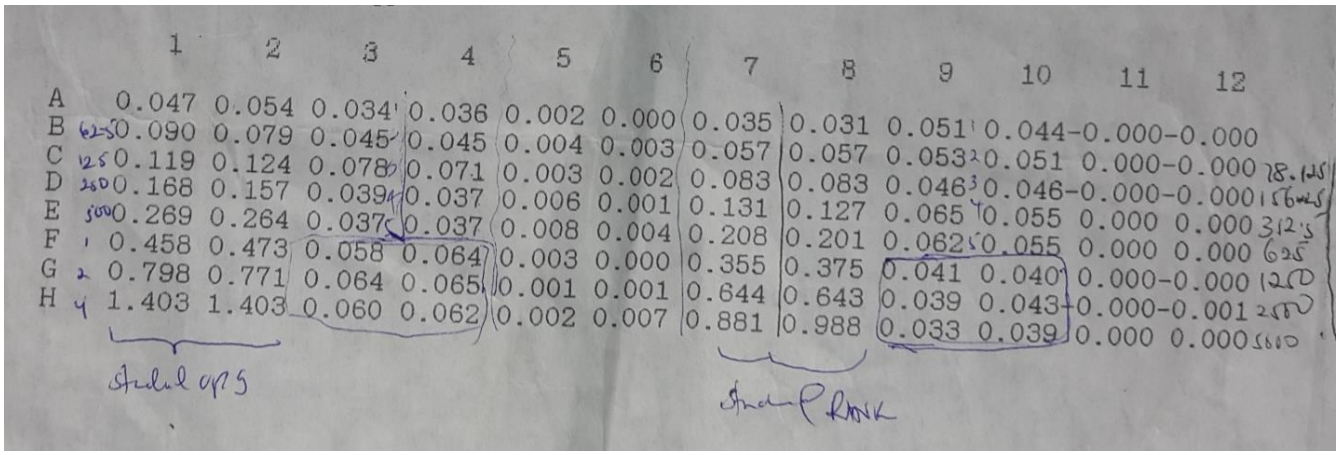


Figure 6: A photo showing a sample print out from the microplate reader

Calculation of RANKL and OPG Concentrations

The duplicate optical density readings for standards and samples were averaged and manually keyed into Microsoft Excel 2013. A standard curve was created using 4-parameter logistic (4PL) software by plotting the mean optical density for each standard on the y-axis against respective concentration on the x-axis. A best fit line was then determined by nonlinear regression analysis which is ideal for ELISA analysis. Formulae for the line of best fit was generated and used to calculate concentration of RANKL and OPG in each saliva sample in picograms per milliliter (pg/mL).

Minimizing laboratory errors

All procedures were done following manufacturer's instructions regarding reconstitution, working concentrations, storage conditions, incubation periods and assay procedures. The procedures were performed in a booth (Figure 7) using clean gloves to avoid contamination.

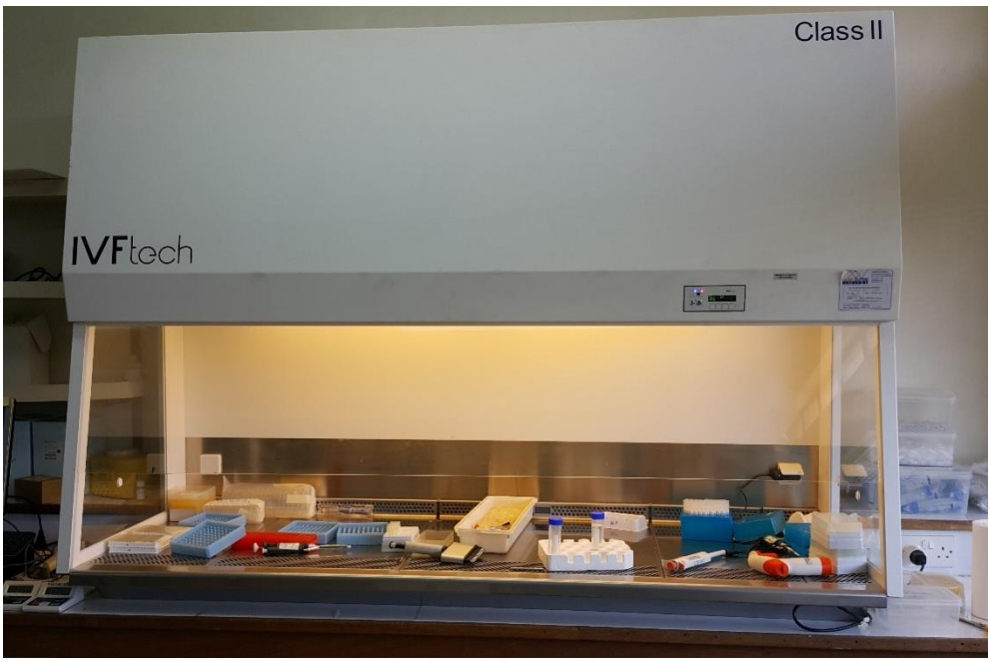


Figure 7: A photograph showing the laboratory booth where procedures were done

The aspiration and washing process for each plate using the autowasher machine was thorough and consistent. At each step, a fresh reagent reservoir and pipette tips were used to avoid cross contamination. New adhesive strips were also used at each step. Pipetting was done with great care and repeated at intervals to ensure accuracy. Reagents were reconstituted and used immediately to eliminate repeated thawing – freezing cycles. The assays were performed in duplicates.

4.9 Reliability and Validity

A number of measures were put in place to ensure that assessment tools produced stable, consistent and credible results. A pilot phase was carried out to ascertain the validity and reliability of questionnaires, clinical examination forms and instruments. Saliva collection protocol was also assessed in the pilot phase.

All the clinical measurements were carried out by the principal investigator. Intra examiner reliability was determined through double evaluation of every10th patient by the principal investigator. For inter- examiner reliability, the principal investigator was

calibrated by the supervisors who are consultants and experienced clinicians in the field of periodontology. Cohen's kappa score was used to calculate both inter- examiner and intra-examiner reliability. A score of 80% was accepted.

Transportation, processing and storage of saliva samples were done in consultation with a senior laboratory technologist to ensure safety and viability. All the equipment and machines used in the study were calibrated and passed quality assurance and quality control checks

Dummy samples were used for a test run before the actual assay to confirm that the analytical procedures employed were suitable for their intended use. The samples were assayed against standard reagents and in duplicates for reliability and trueness. Repeat tests were carried out at given intervals to assess reproducibility and validity.

All the standards and reagents were sourced from the same supplier for precision and reproducibility. The principal investigator was trained on ELISA and assisted by only one laboratory technician who was blinded to the clinical findings of the participants (the clinical data were not submitted to the lab).

Data processing included cleaning and validation with elimination of entries that were obviously erroneous. Extreme outliers were excluded from tests of association through systematic statistical tests.

4.10 Data entry, analysis and presentation

The collected data was entered, cleaned and validated. Coding and analysis was done by Statistical Packages for Social Sciences (SPSS) 20.0 for windows (SPSS inc. Chicago, Illinois, USA) and Microsoft Excel 2013. A 4-parameter logistic (4PL) nonlinear regression model was used to calculate concentration of RANKL and OPG in each saliva sample.

Descriptive statistics used in the analysis of categorical data like gender, frequency of brushing, smoking, and CAL included frequencies and percentages. Continuous data like age, salivary levels of RANKL, OPG were described using mean, range and standard deviation

Comparison of means and proportions were done using chi square and independent t tests. Analysis of variance (ANOVA) and Spearman's rank correlation were also used where appropriate. Independence of the association of salivary levels of RANKL, OPG and RANKL/OPG ratio with the disease status was done through hierarchical multiple linear regression analysis, whilst adjusting for confounders such as age strata and smoking habit.

Cohen's kappa score was be used to calculate both inter- examiner and intra-examiner reliability. A score of 80% was accepted. Confidence level was set at 95% (α level 0.05). Presentation of findings was done using tables, graphs and box plots.

4.11 Ethical considerations

The permission to carry out the study was sought from the Kenyatta National Hospital/University of Nairobi Ethics and Research Standards Board and approval obtained (Appendix XII). Permission was also obtained from the relevant authorities at the School of Dental Sciences and at KAVI. The main reason for the study was explained to each participant who then gave their consent by signing the consent form (Appendix VIII). All the subjects who met the inclusion criteria had an equal chance of being included in the study. Participation in the study was however voluntary and no coercion or forceful inclusion of subjects into the study was done. The participants had the choice of terminating their participation at any time without victimization.

There were no financial benefits either to the investigator or to the participants from the study. However, any participant who required treatment was advised accordingly and referred to respective clinic for management. The information collected from each participant was treated with utmost confidentiality and used only for the purposes of this study.

There were no risks posed to participants during clinical examination and saliva collection. Appropriate precautions were taken to protect the participants, the principle investigator and other users of the clinic from the risk of cross-infection.

Proper saliva collection, transportation, processing and storage were observed to ensure compliance with international biosafety protocols and infection control standards.

CHAPTER 5: RESULTS

This is a chapter about the data obtained from questionnaires, clinical evaluation as well as the laboratory procedures. Key result areas include results from the preliminary phase, socio demographic characteristics, clinical characteristics and salivary concentrations of both RANKL and OPG. The key variables are presented individually followed by bivariate and multivariate analysis.

5.1 Results from the preliminary phase

A pilot study was conducted to pre-test the questionnaires, clinical examination and saliva collection. The questionnaire was found to be clear and well understood. All individuals involved answered the questions adequately. The clinical examination was bearable. Each individual was able to expectorate whole saliva sample into the centrifuge bottle with ease.

Intra examiner reliability was determined during the pilot phase through double evaluation of every 2nd patient by the principal investigator. As for inter examiner reliability, the principal investigator was calibrated by the supervisors. The Cohen's kappa score obtained were as follows: gingival index 1, plaque score 0.95, recession 0.95, probing pocket depth 0.90. All the values were well above the 80% mark set as the minimum allowed Cohen's kappa value and showed that the results were reliable.

A dummy ELISA assay was carried out in duplicate in the lab to test the instruments and the protocol to be used. The instruments were valid and in good condition as evidenced by positive and consistent results. The protocol was well understood.

5.2 Socio-demographic characteristics

A total of 158 participants were included in the study. Of the 158, 92 (58.2%) were females while 66 (41.8%) were males. The age of the participants ranged between 18-75 years with a mean of 37 years ($\pm$12.74 SD). The males were slightly older with a mean of 38.18 ($\pm$14.20SD) compared to females with a mean of 35.64, ($\pm$11.56SD).The

difference was however not statistically significant (t (122) = 1.199, p = 0.233). Table 2 below summarizes the socio demographic characteristics of the participants.

Table 2: Socio-demographic characteristics of participants

Variable		Gender		X^2	*p-value*
		Male *n (%)*	**Female** *n(%)*		
Age	18 – 30 Years	25(37.9)	33(35.9)	2.846	0.241
	31 – 45 Years	21(31.8)	40(43.4)		
	Above 46 Years	20(30.3)	19(20.7)		
Education level	Primary + Secondary	25(37.9)	47(51.1)	2.703	0.100
	Tertiary	41(62.1)	45(48.9)		
Marital Status	Married	39(59.1)	48(52.2)	2.298	0.317
	Single	25(37.9)	36(39.1)		
	Other	2(3.0)	8(8.7)		
Occupation	Self employed	26(39.4)	36(39.1)	0.033	0.984
	Employed	25(37.9)	34(37.0)		
	Unemployed	15(22.7)	22(23.9)		

5.3 Oral hygiene practices

Tooth brushing was reported by all the participants. Majority 90(57%) brushed their teeth twice daily with 60(38%) brushing once daily. The rest brushed their teeth more than two times in a day. The association between the frequency of brushing and level of education was statistically significant (table 3). Participants with higher level of education brushed more frequent as compared to those with lower level of education.

Table 3: Oral hygiene practices of participants

Variable		Frequency of Brushing		X^2	*p-value*
		Once Daily *n(%)*	**Twice Daily** *n(%)*		
Age	18 – 30 Years	26(43.4)	32(32.7)	4.323	0.115
	31 – 45 Years	17(28.3)	44(44.9)		
	Above 46 Years	17(28.3)	22(22.4)		
Gender	Male	30(50.0)	36(36.7)	2.692	0.101
	Female	30(50.0)	62(63.3)		
Education	Primary + Secondary	37(61.7)	35(35.7)	10.106*	0.001
	Tertiary	23(38.3)	63(64.3)		
Marital Status	Married	38(63.4)	49(50.0)	3.270	0.195
	Single	20(33.3)	41(41.8)		
	Other	2(3.3)	8(8.2)		

**where p<0.05 – less than 1 in 20 chances of being wrong (less than 5%)*

5.4 Oral hygiene status

Oral hygiene status of participants was assessed using plaque score. The plaque score ranged between 0.58 – 4.33 with a mean of 2.27$\pm$.77 SD showing that every participant had some degree of plaque deposits on teeth surfaces. The scores were put into three categories and tested against various variables for association (table 4).There was statistical significance in the association between the plaque score and frequency of tooth brushing ($X^2 = 10.146, p=0.006$).The association between the plaque score and level of education was also significant ($X^2 = 6.183, p=0.045$).

Table 4: Plaque score among the participants

Variable		Plaque Score			X^2	*p-value*
		≤1.5	≤2.5	>2.5		
		n(%)	*n(%)*	*n(%)*		
Age	18 – 30 Years	12(60.0)	26(33.8)	20(32.8)	4.323	0.115
	31 – 45 Years	6(30.0)	35(45.4)	20(32.8)		
	Above 46 Years	2(10.0)	16(20.8)	21(34.4)		
Smoking Status	Non Smokers	19(95.0)	65(84.4)	52(85.2)	1.541	0.463
	Smoker	1(5.0)	12(15.6)	9(14.8)		
Brushing	Once Daily	6(30.0)	27(35.1)	27(44.3)	10.146*	0.006
	Twice Daily	14(70.0)	50(64.9)	34(55.7)		
Education	Primary + Secondary	5(25.0)	33(42.9)	34(55.7)	6.183*	0.045
	Tertiary	15(75.0)	44(57.1)	27(44.3)		

**where p<0.05- less than 1 in 20 chances of being wrong (less than 5%)*

5.5 Gingival inflammation (gingivitis)

The degree of gingival inflammation was assessed using the gingival index. The gingival index ranged between 0.42 – 2.75 with a mean of 1.56±.44 SD showing that every participant had some degree of gingivitis. Majority, 92(58.2%), had moderate gingival inflammation while 64(40.5%) had mild gingival inflammation. The rest had severe level of gingivitis. Association between gingival index and age was statistically

significant *(X^2=14.268, p= 0.006)* with lower degree of gingival index seen in lower age groups. Lower level of education was associated with significantly high level of gingival inflammation *(X^2=12.568, p= 0.014).*Though not statistically significant, individuals who brushed more frequently had lower gingival inflammation than those who brushed less frequently. The same trend was observed in education where most individuals with higher level of education presented lower degree of gingival inflammation. Summary is in table 5 below.

Table 5: Gingival index among the participants

Variable		Gingival Index			X^2	p-value
		1 n(%)	2 n(%)	3 n(%)		
Age	18 – 30 Years	25(39.1)	33(35.9)	0	14.268*	0.006
	31 – 45 Years	31(48.4)	30(32.6)	0		
	Above 46 Years	8(12.5)	29(31.5)	2(100.0)		
Smoking Status	Non Smokers	59(92.2)	76(82.6)	1(50.0)	5.089	0.079
	Smoker	5(7.8)	16(17.4)	1(50.0)		
Brushing	Once Daily	21(32.8)	37(40.2)	2(100.0)	4.187	0.123
	Twice Daily	43(67.2)	55(59.8)	0		
Education	Primary + Secondary	25(39.1)	45(48.9)	2(100.0)	3.896	0.143
	Tertiary	39(60.9)	47(51.1)	0		
Dental Visit	<1 Year ago	24(37.5)	27(29.3)	0	12.568*	0.014
	>1 Year ago	33(51.6)	49(53.3)	0		
	Never	7(10.9)	16(17.4)	2(100.0)		

**where p<0.05- less than 1 in 20 chances of being wrong (less than 5%)*

5.6 Periodontitis

The presence or absence of periodontitis and the severity thereof was assessed using consensus CDC/AAP definitions. Seventy seven participants (48.7%) did not have periodontitis, 39(24.7%) had mild periodontitis, 24(15.2%) had moderate periodontitis while 18(11.4%) had severe periodontitis. The association between periodontitis and age was statistically significant *(X^2=53.845, p= 0.001)* – severity of periodontitis was more in individuals in older age groups. Association with level of education was also statistically significant *(X^2=11.416, p= 0.010).* How different grades of periodontis relate to other variables is summarized in table 6 below.

Table 6: Periodontitis with other study variables

Variable		Periodontitis				X^2	p-value
		1 n(%)	2 n(%)	3 n(%)	4 n(%)		
Age	18 – 30 Years	43(55.8)	11(28.2)	2(8.3)	2(11.1)	53.845*	0.001
	31 – 45 Years	29(37.7)	20(51.3)	7(29.2)	5(27.8)		
	Above 46 Years	5(6.5)	8(20.5)	15(62.5)	11(61.1)		
Gender	Male	26(33.8)	17(43.6)	13(54.2)	10(55.6)	5.004	0.172
	Female	51(66.2)	22(56.4)	11(45.8)	8(44.4)		
Smoking Status	Non Smokers	70(90.9)	33(84.6)	19(79.2)	14(77.8)	3.560	0.313
	Smoker	7(9.1)	6(15.4)	5(20.8)	4(22.2)		
Brushing	Once Daily	25(32.5)	14(35.9)	11(45.8)	10(55.6)	4.054	0.256
	Twice Daily	52(67.5)	25(64.1)	13(54.2)	8(44.4)		
Education	Primary + Secondary	27(35.1)	17(43.6)	15(62.5)	13(72.2)	11.416*	0.010
	Tertiary	50(64.9)	22(56.4)	9(37.5)	5(27.8)		

**where p<0.05- less than 1 in 20 chances of being wrong (less than 5%)*

5.7 Salivary levels of RANKL AND OPG

Salivary concentrations of RANKL and OPG were obtained from a standard curve constructed using a 4-parameter logistic (4-PL) calibration curve-fit as shown below.

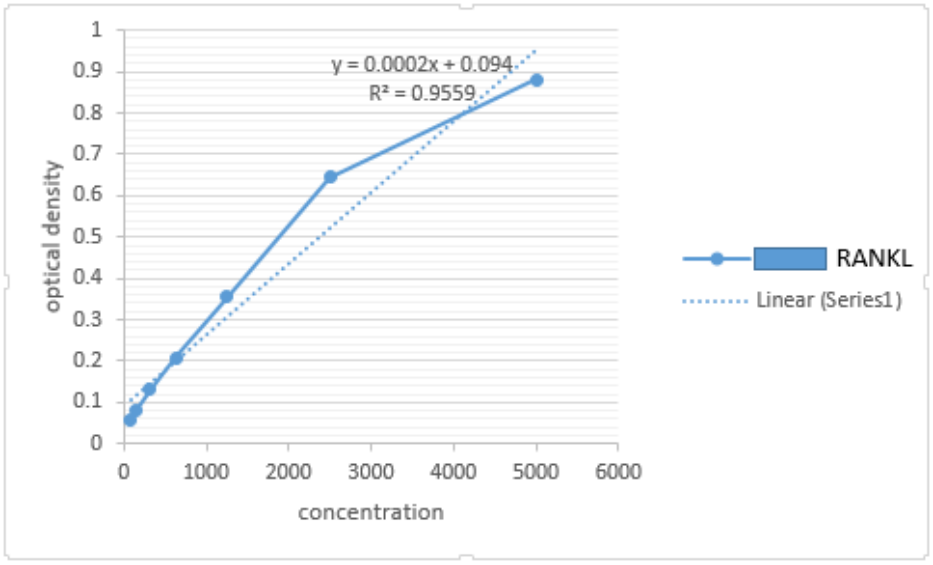


Figure 8: RANKL standard curve

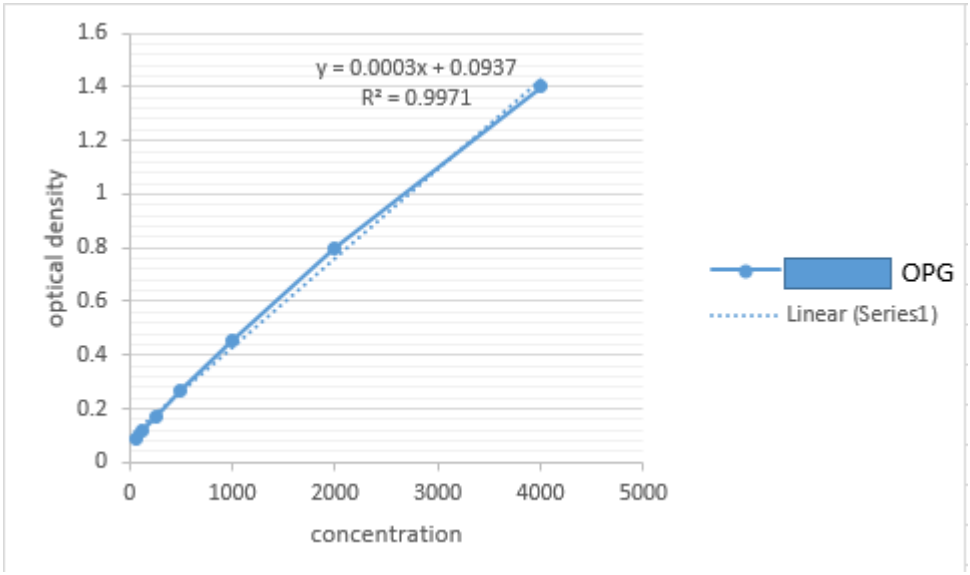


Figure 9: OPG standard curve

The formula obtained from each curve was used to derive the respective concentrations of the molecules.

Out of the 158 samples tested, 5 had RANKL and OPG levels that were either too low or too high. Through the use of box plots for data distribution display (Fig. 11, 12), the 5 samples were deemed outliers hence excluded from statistical analysis. Including them in the analysis would have skewed the data unfavorably. Their exclusion did not interfere with the general characteristics of parameters under study since the number recruited exceeded the calculated sample size. The negative values were undetectable hence adjusted to zero.

5.10.1 Salivary RANKL

Of the 153 saliva samples analyzed, the levels of RANKL ranged from undetectable levels to 60 pg/mL with a mean of 14.65 (± 18.72SD). As shown in Table 7, there was a statistically highly significant level of association between salivary RANKL levels and the severity of periodontal disease (*F = 64.82, p < 0.001*).

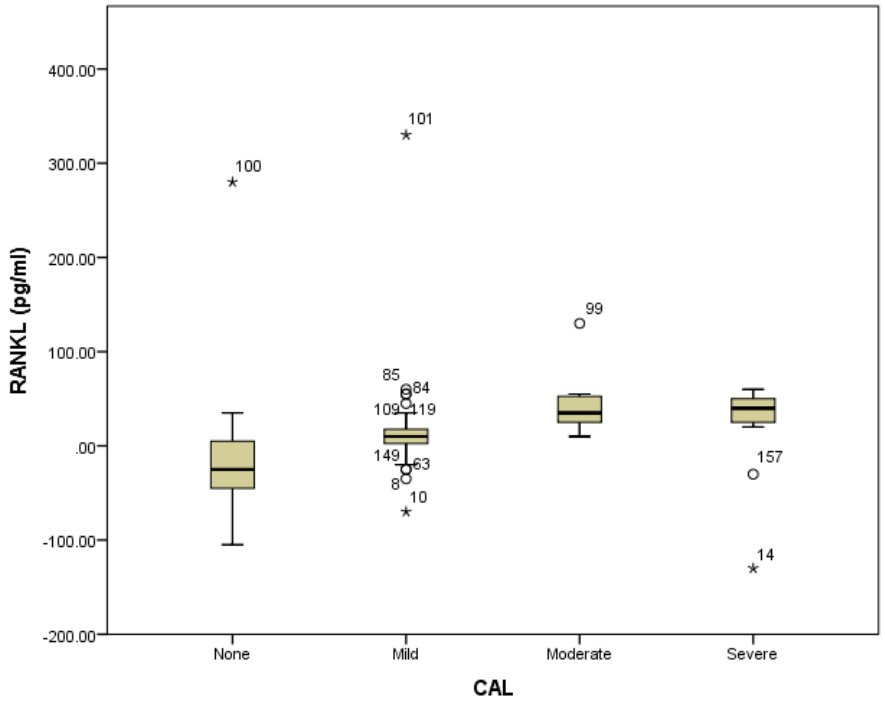


Figure 10: Box plot showing outlier RANKL values (outliers plotted as individual points)

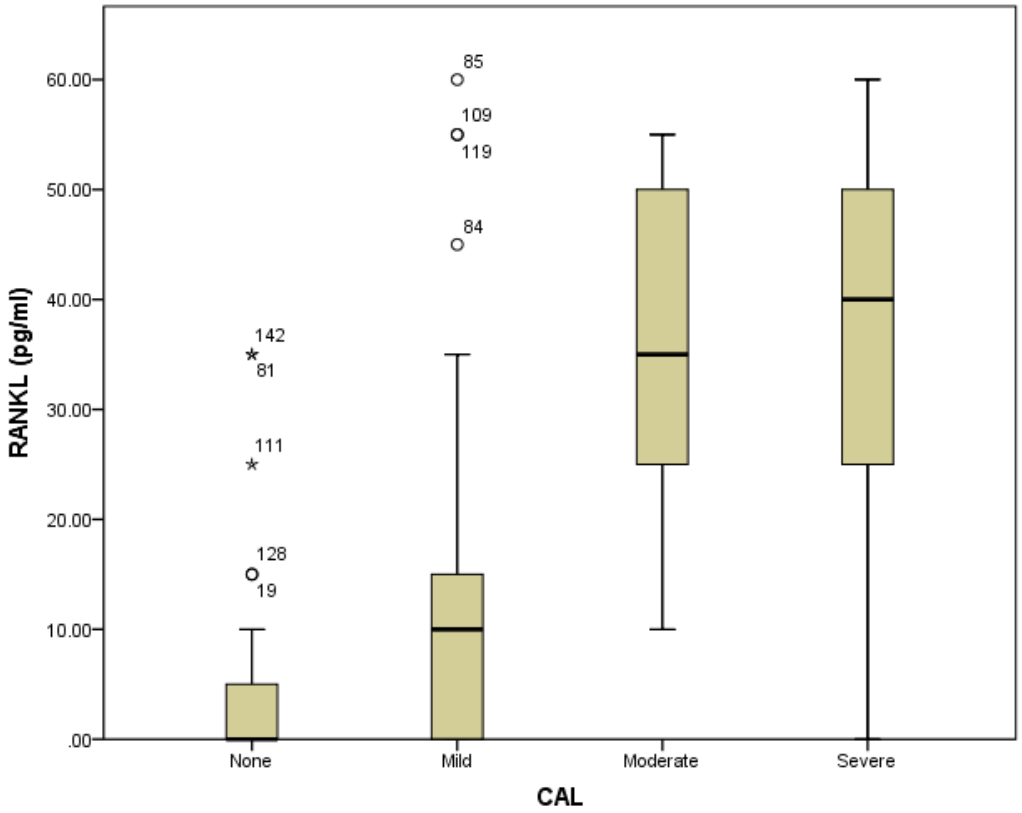


Figure 11: Box plot showing adjusted spread of RANKL values

In individuals without periodontitis or with milder degree of the disease, the RANKL levels were either undetectable or significantly low. An increase in severity of the disease correlated with significant increase in RANKL levels. Correlations between RANKL levels and other variables are summarized in Table 7 below.

Table 7: Salivary RANKL levels by study variables

Characteristics	n (%)	RANKL (pg/ml) (M±SD)	95% CI	Df	Test	*p*-value
Age						
18 – 30 Years	58 (36.7)	9.20±15.25	5.11 – 13.28	2, 152	F = 10.377**	<0.001
31 – 45 Years	61 (38.6)	12.79±17.62	8.27 – 17.30			
> 46 Years	39 (24.7)	25.66±20.83	18.81 – 32.51			
Gender						
Male	66 (41.8)	20.23±20.10	3.72 – 15.73	123.674	t = 3.205*	0.002
Female	92 (58.2)	10.51±16.55				
Brushing						
<= Once Daily	60 (38.0)	17.33±18.19	-1.83 – 10.41	153	t = 1.384	0.168
>= Twice Daily	98 (62.0)	13.04±18.94				
Smoking						
Non Smokers	136 (86.1)	11.73±16.40	-30.79 – -10.30	24.877	t = -4.132**	<0.001
Smokers	22 (13.9)	32.27±22.35				
Plaque Score						
1	20 (12.7)	6.25±11.34	0.94 – 11.56	2, 152	F = 4.979*	0.008
2	77 (48.7)	12.76±17.21	8.83 – 16.70			
3	61 (38.6)	19.92±21.16	14.40 – 25.43			
Gingival Index						
1	64 (40.5)	8.44±14.58	4.80 – 12.08	2, 152	F = 7.591*	0.001
2	92 (58.2)	18.60±20.13	14.36 – 22.84			
3	2 (1.3)	37.50±3.54	5.73 – 69.27			
Periodontitis						
None	77 (48.7)	3.22±7.10	1.60 – 4.85	3, 151	F = 64.818**	<0.001
Mild	39 (24.7)	13.42±16.36	8.04 – 18.80			
Moderate	24 (15.2)	36.96±14.12	30.85 – 43.06			
Severe	18 (11.4)	36.94±17.80	27.99 – 45.89			

Analysis of variance (ANOVA) and Independent-samples t tests were used.
**p<0.05 - less than 1 in 20 chances of being wrong (less than 5%)*
***p<0.001 – less than 1 in a thousand chance of being wrong*

5.10.2 Salivary OPG

The OPG levels in the saliva samples ranged from 4.33 to 204.33pg/mL with a mean of 139.03 (± 51.19 SD). The mean levels were significantly high in cases without periodontitis or in cases with milder grade of periodontitis while a decrease was observed with increase in the severity of periodontitis (Figure 13). The group with no periodontitis had a mean of 172.74 (± 23.97SD), those with mild periodontitis had a mean of 145.53 (± 35.28SD), while those with moderate periodontitis had a mean 89.84(±23.04SD).Individuals with severe periodontitis had significantly low mean of 45.90 (± 21.96SD). However, few individuals with mild or no periodontitis had relatively low levels of OPG. Analysis of variance (ANOVA) test revealed a strong level of association between salivary OPG levels and the different grades of periodontitis (*F =19.031, p<0.001)*. Correlations between OPG levels and other variables are summarized in Table 8.

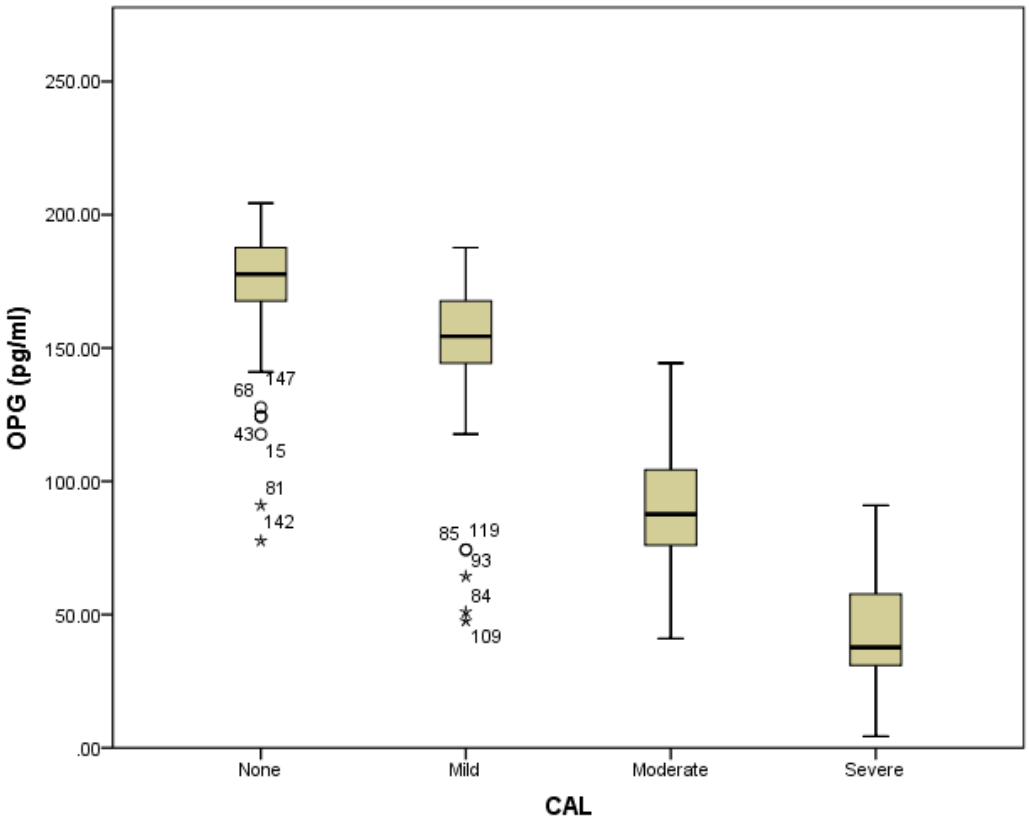


Figure 12: Box plot showing the distribution of salivary OPG levels in different grades of periodontal disease

Table 8: Salivary OPG levels by study variables

Characteristics	n (%)	OPG (pg/ml) (M±SD)	95% CI	Df	Test	*p*-value
Age						
18 – 30 Years	58 (36.7)	159.30±37.72	149.11 – 169.50	2, 149	F = 18.404**	<0.001
31 – 45 Years	61 (38.6)	144.06±48.28	131.58 – 1563.53			
> 46 Years	39 (24.7)	100.73±53.49	82.90 – 118.56			
Gender						
Male	66 (41.8)	123.37±55.67	-43.33 – -9.57	112.806	t = -3.104*	0.002
Female	92 (58.2)	149.81±45.09				
Smoking						
Non Smokers	136 (86.1)	147.69±48.28	41.12 – 84.33	150	t = 5.737**	<0.001
Smokers	22 (13.9)	84.97±32.76				
Plaque Score						
1	20 (12.7)	159.07±40.89	139.36 – 178.78	2, 149	F = 6.600*	0.002
2	77 (48.7)	148.03±49.20	136.63 – 159.43			
3	61 (38.6)	121.28±51.98	107.74 – 134.83			
Gingival Index						
1	64 (40.5)	158.26±39.83	144.14 – 168.37	2, 149	F = 10.691**	<0.001
2	92 (58.2)	127.44±53.48	116.11 – 138.77			
3	2 (1.3)	52.67±21.21	-137.93 – 243.26			
Periodontitis						
None	77 (48.7)	172.74±23.97	167.14 – 178.33	3, 148	F = 131.314**	<0.001
Mild	39 (24.7)	145.53±35.28	134.09 – 156.97			
Moderate	24 (15.2)	89.84±23.04	79.88 – 99.80			
Severe	18 (11.4)	45.90±21.96	34.61 – 57.19			

Analysis of variance (ANOVA) and Independent-samples t test were used.

**p<0.05 - less than 1 in 20 chances of being wrong (less than 5%)*
***p<0.001 – less than 1 in a thousand chance of being wrong*

5.10.3 RANKL/OPG Ratio

Although the ELISA analysis revealed sample-to-sample variations in the salivary levels of RANKL and OPG, it is noteworthy that significantly higher levels of RANKL were detected in severe grades of periodontitis in comparison to those with milder grades of the disease or those without disease at all. The reverse observation was made regarding the mean OPG values where significantly higher levels were detected in those without periodontitis or those with milder grades of the disease. A curve estimation linear regression model elicited a statistically significant association between RANKL and OPG, $F(1, 147) = 204.809$, $R^2 = 0.582$, $n = 158$, $p < 0.001$.

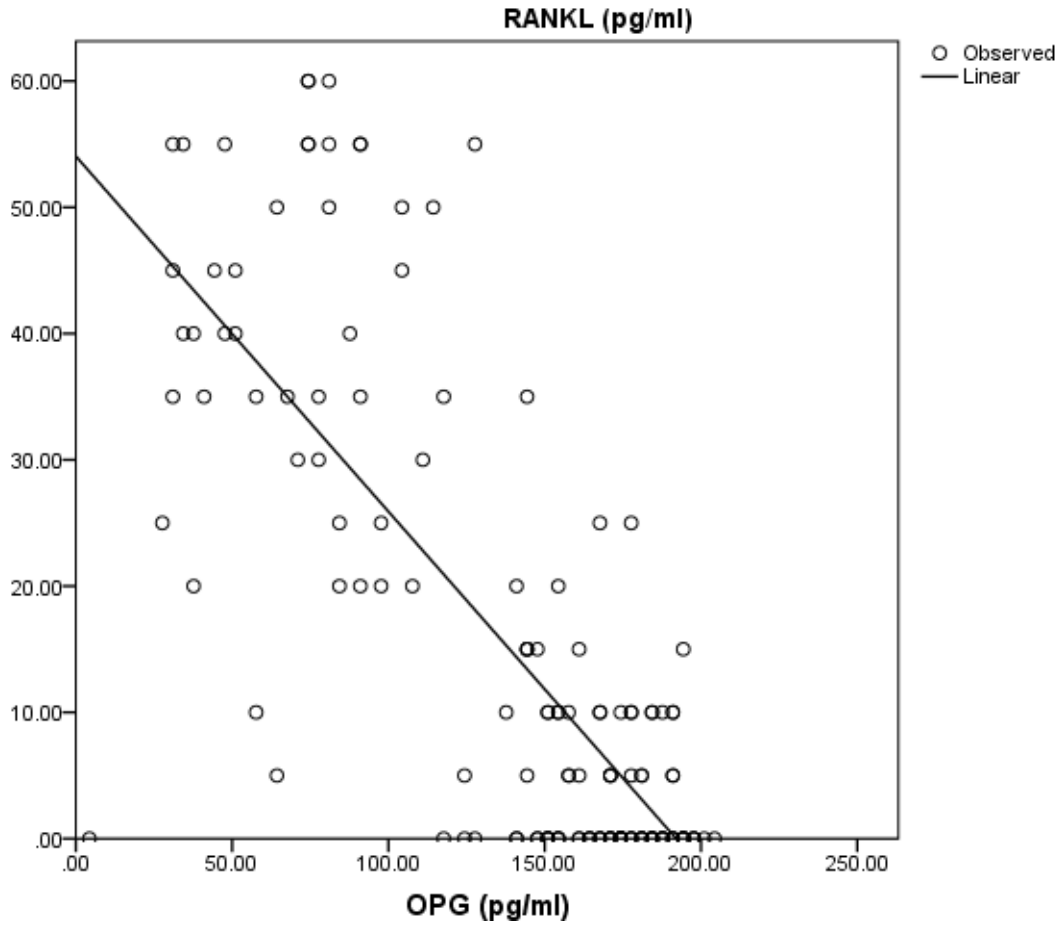


Figure 13: Plot model of RANKL and OPG.

As consequence, a relative ratio (RANKL/OPG ratio) whose association with the severity of periodontitis was statistically significant got established. A Spearman's rank order correlation revealed a strong, positive correlation between the ratio and disease severity ($r_s = 0.759, p < 0.001$) as shown in table 9 below. The null hypothesis was thus rejected.

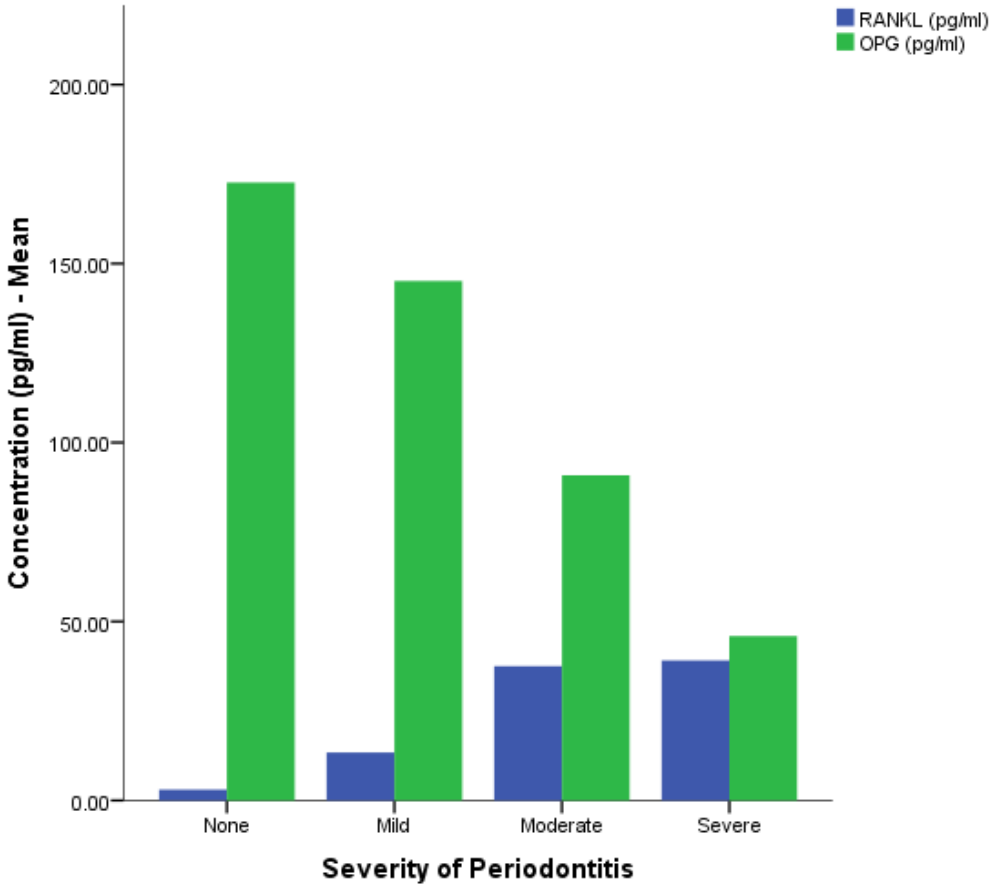


Figure 14: Respective concentrations of RANKL and OPG in each category of disease severity

Table 9: Correlation between RANKL/OPG ratio with demographic and clinical parameters

		Ratio	
Characteristics	**n (%)**	r_s	***p*-value**
Age			
18 – 30 Years	58 (36.7)	0.310**	<0.001
31 – 45 Years	61 (38.6)		
> 46 Years	39 (24.7)		
Gender			
Male	66 (41.8)	-0.247*	0.002
Female	92 (58.2)		
Smoking			
Non Smokers	136 (86.1)	0.308**	<0.001
Smokers	22 (13.9)		
Plaque Score			
1	20 (12.7)	0.212*	0.009
2	77 (48.7)		
3	61 (38.6)		
Gingival Index			
1	64 (40.5)	0.274*	0.001
2	92 (58.2)		
3	2 (1.3)		
Periodontitis			
None	77 (48.7)	0.759**	<0.001
Mild	39 (24.7)		
Moderate	24 (15.2)		
Severe	18 (11.4)		

**p<0.05 - less than 1 in 20 chances of being wrong (less than 5%)*

***p<0.001 – less than 1 in a thousand chance of being wrong*

5.10.4 Multiple regression analysis

Hierarchical Multiple Regression elicited a statistically significant association between severity of periodontitis and RANKL/OPG Ratio ($\beta = 0.759$, $t(157) = 12.330$, $p < 0.001$) controlling for age ($\beta = -0.099$, $t(157) = -1.634$, $p = 0.104$), smoking ($\beta = 0.215$, $t(157) = 3.973$, $p < 0.001$) and gender ($\beta = -0.019$, $t(157) = -0.345$, $p = 0.730$).

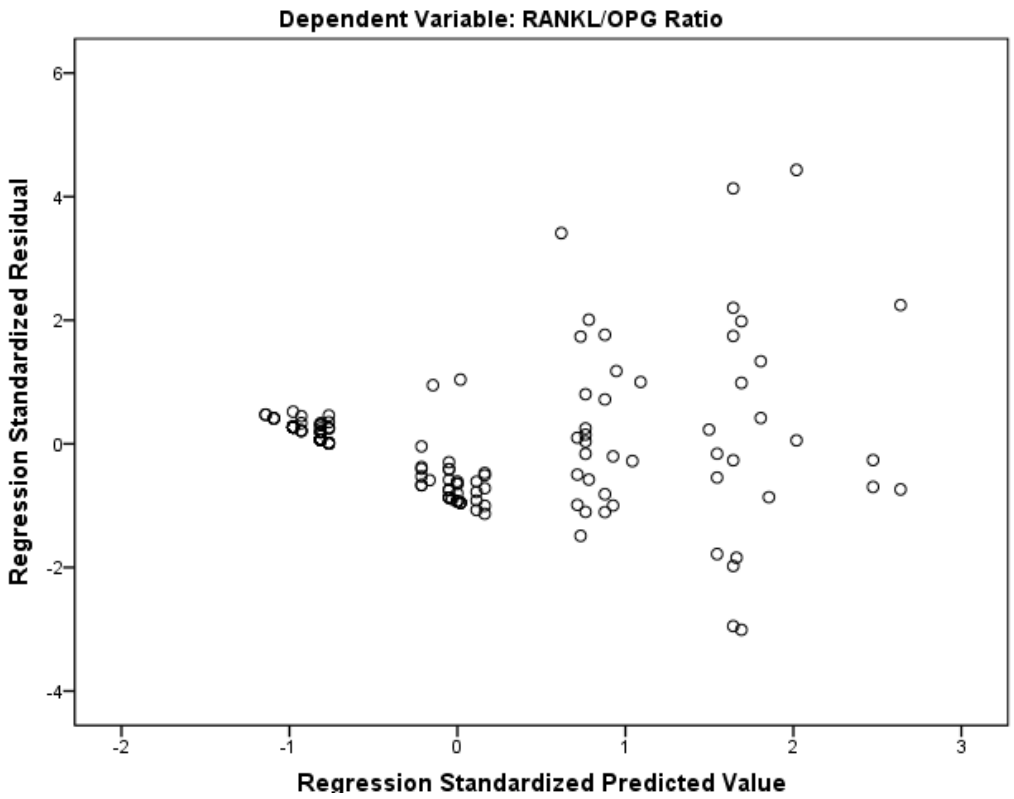


Figure 15: Hierarchical multiple regression analysis for variables predicting changes in RANKL/OPG ratio

5.10.5 Specificity and sensitivity of RANKL/OPG ratio as a potential diagnostic test

Receiver operating characteristic (ROC) test was used to determine the performance of RANKL/OPG ratio as a potential diagnostic test. A statistically significant area of 0.932 was reported with a 0.1613 cut-off ratio at 95% sensitivity and 6.2% specificity levels. The test correctly identified 95% of the patients with periodontitis as true positives and 6.2% of the patients without periodontitis as true negatives at a ratio level of 0.1613**.** (Table 10, Figure 17)

Table 10: ROC test for RANKL/OPG Ratio in periodontitis

			RANKL/OPG Ratio						
	+ n	- n	Area	SE	95% CI	Sensitivity	Specificity	Ratio	p value
Periodontitis	40	118	0.932**	0.028	0.878 – 0.987	0.950	0.062	0.1613	< 0.001

*Where ** p<0.001*

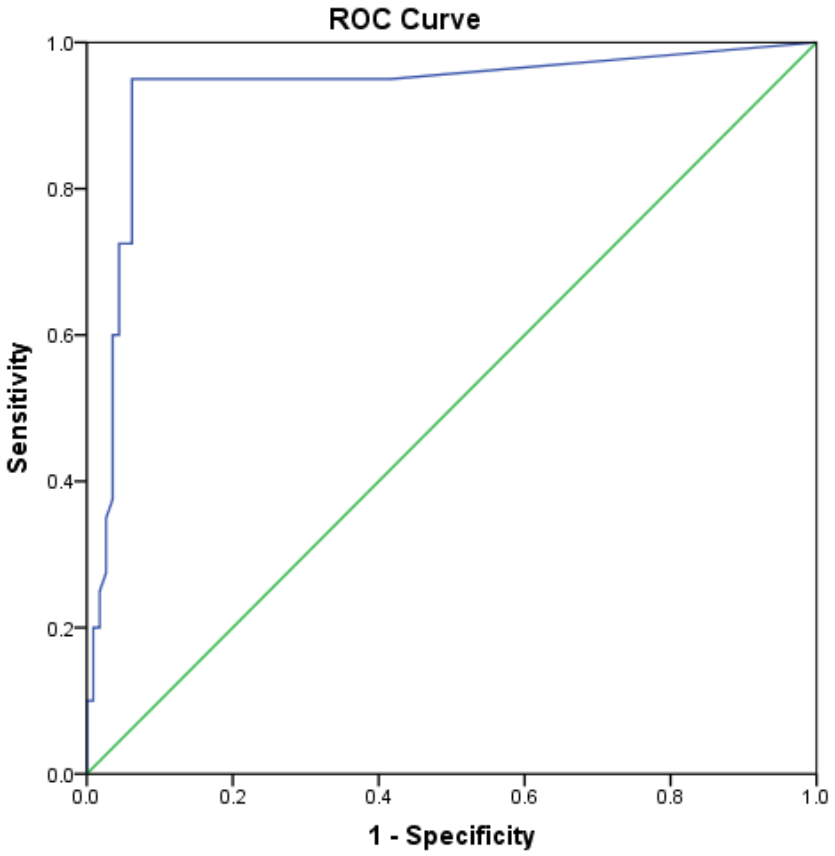


Figure 16: ROC Curve for RANKL/OPG Ratio in periodontitis

CHAPTER 6: DISCUSSION

This is a chapter dedicated to discussing the major findings of the study as highlighted in the result section in line with the study objectives and existing knowledge. The chapter interprets the findings and provides explanation that accounts for the findings linking them to the existing literature. The chapter also highlights some of the limitations of the study, provides concluding remarks and give recommendations.

6.1 Socio-demographic characteristics

The age of the participants ranged between 18-75 years with a mean of 36.71 (±12.74 SD).This implied that only adults who could give informed consent participated in the study. It also implied variability and diversity in ages of individuals seeking dental treatment at the hospital and general population by extension. There were more females (58.2%) than males (41.8%) in this study indicating better health seeking behavior among females and in agreement with the findings of a study done by Maubi in 2013 [56].

Concerning the level of education, majority of the participants had tertiary education (college, undergraduate or postgraduate education). This could be due to the fact that the study was carried out in urban setting whereby the urban population is probably more educated. 23.42% of the participants were unemployed. This could be a true reflection of the unemployment rate experienced in the entire country [57].

6.2 Oral Hygiene practices

All participants in this study brushed their teeth. Tooth brushing is a form of mechanical plaque control and is the most relied upon oral hygiene practice worldwide [58]. Concerning the frequency of brushing, majority of the participants in this study 90(57%) brushed their teeth twice daily with 60(38%) brushing once daily and rest more than two times in a day. This is in agreement with a study done in a similar urban setting in Germany where majority brushed their teeth twice daily. The findings are, however, in contrast to a local study done on a rural Kenyan population which found out that

majority brushed their teeth once daily [59]. The disparity is attributed to difference in study population setting, one being urban while the other being rural.

Although majority brushed their teeth twice daily, the relatively high percentage of those brushing once daily (38%) underscores the need for more oral health education in the population. The statistically significant association between the frequency of brushing and level of education is due to increased awareness that comes with education.

6.3 Oral hygiene status

Oral hygiene status of participants was assessed using plaque score. The mean plaque score was 2.27 (±.77 SD) showing that every participant had some degree of plaque deposits on teeth surfaces. The statistically significant association between the plaque score and frequency of tooth brushing is attributed to the fact that tooth brushing is the most relied upon form of mechanical control [56]. Participants with higher level of education are more enlightened on oral hygiene practices. This could explain the lower plaque score levels observed in individuals with higher level of education.

6.4 Gingivitis/Periodontitis

The mean gingival index was 1.56 (±.44 SD) showing that every participant had some degree of gingivitis. The statistically significant association between gingival index and plaque scores confirmed the role of dental plaque in the pathogenesis of gingival inflammation [58]. Lower degree of gingival inflammation was seen in lower age groups. This increased as the age advanced a fact attributable to longer exposure time to etiological factor (dental plaque). Individuals with higher level of education presented lower degree of gingival inflammation due to increased awareness in oral hygiene practices.

Periodontitis was assessed using consensus CDC/AAP definitions [13].The significant positive association between periodontitis and age is as a result of longer duration of exposure to risk factors. Increased periodontal breakdown in older age groups could as

well be attributed to the possible influence of undiagnosed systemic diseases that increase with age as reported by Grossi and colleagues [60].

6.5 Salivary levels of RANKL, OPG and their respective ratio

The salivary levels of RANKL ranged from undetectable levels to 60 pg/mL with a mean of 14.65 (±18.72SD) while that of OPG ranged from 4.33 to 204.33pg/mL with a mean of 139.03 (± 51.19 SD). As a consequence, a relative ratio (RANKL/OPG ratio) whose association with other parameters was statistically significant got established. The existence of the relative ratio is explained by the fact that RANKL and OPG are interrelated. They exist in inverse proportions and their molecular interplay control alveolar bone resorption in periodontal disease [26]. Production of RANKL is stimulated by inflammatory cytokines found in body fluids such as saliva. Its presence mediates alveolar bone destruction by stimulating osteoclasts. On the other hand, OPG is the natural inhibitor of alveolar bone resorption. Being a decoy receptor; RANKL binds to it (instead of binding to RANK) preventing osteoclast differentiation [30]

Compared to levels reported by Buduneli and group in 2008 (20 – 200pg/ mL) [10], the RANKL levels in this study were lower. This could be attributed to difference in population sampled as well as differences in the sensitivity of instruments used. The OPG levels however compared well.

In this study, a statistically significant, positive correlation was found between increasing age and RANKL/OPG ratio. The correlation is in agreement with earlier studies which reported significant age-dependent patterns in the expression of RANKL and OPG [61].The pattern is representative of the ageing-cumulative characteristics of periodontal damage due to prolonged exposure to risk factors.

Another significant correlation was between RANKL/OPG ratio and smoking status – the ratio was higher in smokers than non smokers. The study did not, however, elicit a difference between current smokers and former smokers. Confirming the above findings, Cesar-Neto et al in 2007 reported that the expression of OPG gene in the gingival tissues was 1.4-fold lower, with RANKL/OPG ratio that was 1.6-fold higher in smokers compared to non-smokers [62].They however used polymerase chain reaction

method (PCR) and gingival tissue. Buduneli and group (using the same technique as the one in this study) reported similar findings in saliva through ELISA [10]. The altered RANKL/OPG ratio is attributed to major histopathological changes that occur in the oral cavities of smokers. The changes include altered immune response, altered vascular system and lowered oxygen tension [63].

The study, within its limits, did not find strong correlation between RANKL/OPG ratio and gender.

6.6 Association of RANKL/OPG ratio and periodontal clinical status

The periodontal clinical status in this study was described using plaque score, gingival index and CDC periodontal disease classification. While plaque score gives state of the oral hygiene, gingival index corresponds to degree of inflammation (gingivitis).CDC periodontal disease classification describes the severity of the disease based on probing pocket depth and clinical attachment loss.

Positive correlations were found between RANKL/OPG ratio and plaque score as well as gingival index. Similar findings were reported Belibasakis and Bostanci in 2012 [22].The correlations were however weak. The weak correlation may be attributed to limited changes in alveolar bone during gingivitis (gingival inflammation without tissue loss).

The study found a strong positive correlation between RANKL/OPG ratio and severity of periodontitis ($r_S = 75.9\%$, $p < 0.001$). Hierarchical multiple regression was used to control for the confounders (age, smoking status). Severe grade of periodontitis corresponded to higher RANKL/OPG ratio, null hypothesis thus rejected. The present findings concur with most of the previous studies [22], [40], ([64]. Collectively, the studies indicate that the relative RANKL/OPG ratios increase as the severity of periodontitis advances. The inflammatory pathway in the periodontium explains this occurance. As inflammation advances due to persistent microbial challenge, macrophages and dendritic cells present antigens that activate the adaptive immune system. T-cells and B-cells then accumulate and ultimately dominate the lesion. The lesion then takes a chronic course [21]. Activated T and B cells are considered the major cellular sources of RANKL

[65].Increased production of RANKL causes an increase in the relative RANKL/OPG ratio resulting into more periodontal destruction. OPG on the other hand is a decoy receptor for RANKL and its presence is protective against periodontal breakdown. RANKL binds to it (instead of binding to RANK) preventing osteoclast differentiation [30].

Some studies have however failed to report significant correlations between RANKL/OPG ratio and severity of periodontitis. Their findings were therefore inconsistent with findings of the present study. Lu et al in 2006 failed to draw significant correlations between the ratio and clinical measurements of periodontal disease in terms of probing pocket depth, clinical attachment loss, extent and severity of tissue breakdown [66]. Mogi and co-workers in 2004 suggested further studies to conclusively establish significant correlations between RANKL, OPG, their relative ratios in GCF with clinical measurements [41]. The failure to report significant correlations could be attributed to the differences in protocols, methods of sampling and processing technique. Differences in study populations, sample size as well as sensitivity of the assays could as well justify their findings.

6.7 Specificity and sensitivity of salivary RANKL/OPG ratio as a potential diagnostic test

Saliva is easy to collect, handle and test. This has led to significant recognition as a diagnostic fluid for detecting changes in the oral cavity and the rest of the body. By establishing a strong positive correlation between salivary RANKL/OPG ratio and different grades of periodontal disease, this study has reaffirmed the diagnostic utility of saliva [24].

In determining the performance of salivary RANKL/OPG ratio as a potential diagnostic tool, Receiver operating characteristic (ROC) test reported a statistically significant area under curve value of 0.932.The test correctly identified 95% of the patients with periodontitis as true positives and 6.2% of the patients without periodontitis as true negatives (95% sensitivity and 6.2% specificity) at a RANKL/OPG ratio level of 0.1613. The findings herein are in agreement with another study done to determine host-response markers correlated with periodontal disease [67].Though designed to analyze more biomarkers than the current study, the authors reported area under curve value of

0.9 similarly reported by the current study. Within the limits of this study, salivary RANKL/OPG ratio is therefore robust and sensitive enough to be considered a diagnostic test. Ebersole and colleagues however reported a lower area under curve value (0.7).This could be attributed to the fact that their study focused on a different biomarker (matrix metalloproteinases 8) [68].

6.8 Limitations of the study

The study was carried out in a hospital set up. Extrapolation of the findings to the rest of the population may thus be a challenge. Moreover, the setting did not allow appropriate randomization due to the fact that the investigator did not have control over those who visited the facility for treatment. As such a potential selection bias may have been introduced. Being a cross sectional study, the snapshot timing may not have been fully representative as the study only captured the population at a single point in time. The study design also lacked the ability to make causal inference between the variables. Lastly, the study was conducted parallel to other post graduate academic activities with heavy cost implications. As such, there were both time and financial limitations.

6.9 Conclusion

The study, within its limits, has provided further evidence that the interplay between the levels of RANKL and OPG in saliva had a relationship with periodontal clinical status. While the RANKL levels increased with increase in disease severity, OPG levels decreased as the severity increased. This resulted in a relative RANKL/OPG ratio which had a strong, statistically significant positive correlation with the degree of periodontal tissue breakdown. The null hypothesis was thus rejected. These findings provide a platform for easy and non-invasive adjunct to periodontal diagnosis as well as host response modulation therapies. The mutual confounding effects of age and smoking should however be ruled out.

6.10 Recommendation

Salivary levels of RANKL and OPG and their relative ratio should be considered as a potential adjunctive diagnostic tool for evaluating periodontal disease. The biomarkers

should as well be considered as part of host response modulation therapies for periodontal disease. However, there is need for more salivary proteomic studies and randomized controlled trials in Kenyan setting to fully exploit the potential of these biomarkers.

6.11 Conflict of interest

The study was carried out as a partial fulfillment for the award of Masters of Dental Surgery in Periodontology at the University of Nairobi as well as for scientific purposes. The cost of the study was solely met by the principal investigator. There was no related conflict of interest.

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APPENDICES

Appendix I: Screening form

Date: _____ Serial No._____

	Yes	No
Diabetes		
Heart disease		
Osteoporosis		
Rheumatoid arthritis		
Antibiotics in the last 3 months		
Tooth cleaning in the last 3 months		
Use of antiseptic mouth wash in the last 3 months		
Pregnant		
Any steroid drug in the past 3 months		

Appendix II A: Questionnaire /biodata form

Title: Association of salivary RANKL and OPG levels with periodontal clinical status.

Date: _____ Serial No._____

Age (Years): _____

Gender: Male ☐ Female ☐

Highest level of education: Primary ☐ Secondary ☐ Tertiary ☐

Tooth brushing habits: Once daily ☐ Twice daily ☐ Thrice daily ☐

What do you use to brush your teeth?

☐ Commercial toothbrush ☐ Chewing stick

☐ Fingers ☐ Others (specify)

Last dental visit: ☐ 3-6 months ago ☐ 6 months -1 year ago ☐ >1 year ago

☐ > 5 years ago ☐ >10 years ago ☐ Never been to a dentist

Smoking Habit: ☐ Smoker ☐ Nonsmoker ☐ Previous smoker

Appendix II B: Hojaji

Tarehe: _____ Nambari ya usajili:._____

Umri: _____

Jinsia: Mwanaume ☐ Mwanamke ☐

Kiwango cha elimu: shule ya msingi ☐ shule ya upili ☐ Chuo kikuu ☐

Je,unasafisha meno mara ngapi: mara moja kwa siku ☐ Mara mbili kwa siku ☐
Mara tatu kwasiku ☐

Je,unasafisha meno ukitumia nini?

☐ mswaki ☐ kijiti

☐ vidole ☐ chengine,eleza…..

Mara ya mwisho kuona daktari wa meno

: miezi 3-6 ☐ mwaka 1 ☐ zaidi ya mwaka 1 ☐

Je,unavurutasigara: ☐ Ndio ☐ sijawahi ☐ nilitumia mbeleni,nimewacha

Appendix III: Clinical examination form

Date: _____ Serial No._____

Age: _____

GINGIVAL INDEX: Loe-Silness Index – 1963

Tooth	16		21		24		36		41		44	
Surface	F	L	F	L	F	L	F	L	F	L	F	L
Score												

PLAQUE SCORE: Silness- Loe Index – 1964

Tooth	16		21		24		36		41		44	
Surface	F	L	F	L	F	L	F	L	F	L	F	L
Score												

SIX POINT CHAT - MAXILLA

Tooth	**17**			**16**			**15**			**14**			**13**			**12**			**11**			**21**			**22**			**23**			**24**			**25**			**26**			**27**		
Palatal																																										
Recession																																										
Pocket depth																																									
CAL																																									
Facial																																									
Recession																																									
Pocket depth																																									
CAL																																									

SIX POINT CHART - MANDIBLE

Tooth	**47**			**46**			**45**			**44**			**43**			**42**			**41**			**31**			**32**			**33**			**34**			**35**			**36**			**37**		
Palatal																																										
Recession																																										
Pocket depth																																									
CAL																																									
Facial																																									
Recession																																									
Pocket depth																																									
CAL																																									

Appendix IV a:

Gingival Index, Loe and Silness 1963

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

Appendix IV B:

Plaque score, Silness and Loe1964

Scores	Criteria
0	No plaque
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.
2	Moderate accumulation of soft deposit s within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin

Appendix V: Consensus CDC/AAP Periodontal disease classification

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	

Gingivitis severity

0 No gingivitis

0.1– 1 Mild gingivitis

1.1-2 Moderate gingivitis

2.1-3 Severe gingivitis

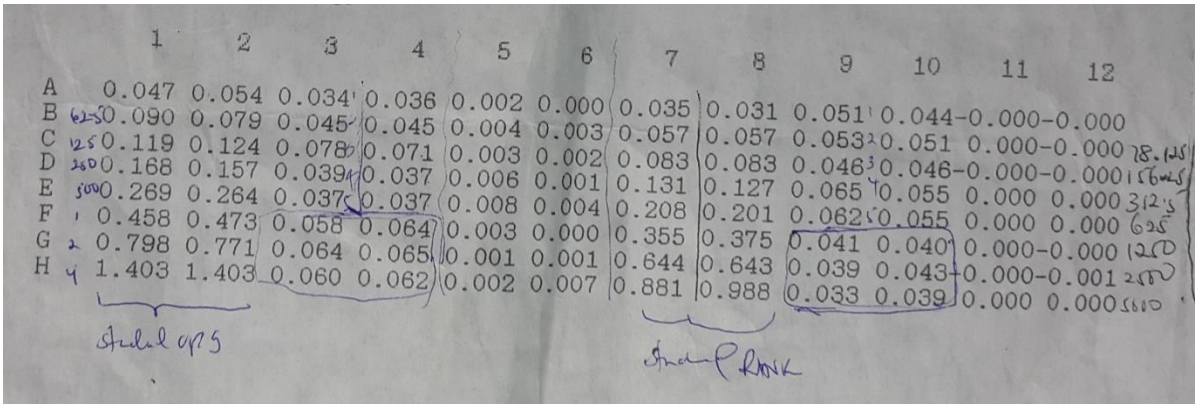
Appendix VI: ELISA Worksheet

Plate No._____ Analyte _____Date _____

Technologist _____ Principal Investigator: _____

	STANDARDS		SAMPLES									
	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK										
B												
C												
D												
E												
F												
G												
H												

Appendix VII: A sample print out from the microplate reader



	1	2	3	4	5	6	7	8	9	10	11	12
A	0.047	0.054	0.034	0.036	0.002	0.000	0.035	0.031	0.051	0.044	-0.000	-0.000
B	0.090	0.079	0.045	0.045	0.004	0.003	0.057	0.057	0.053	0.051	0.000	-0.000
C	0.119	0.124	0.078	0.071	0.003	0.002	0.083	0.083	0.046	0.046	-0.000	-0.000
D	0.168	0.157	0.039	0.037	0.006	0.001	0.131	0.127	0.065	0.055	0.000	0.000
E	0.269	0.264	0.037	0.037	0.008	0.004	0.208	0.201	0.062	0.055	0.000	0.000
F	0.458	0.473	0.058	0.064	0.003	0.000	0.355	0.375	0.041	0.040	0.000	-0.000
G	0.798	0.771	0.064	0.065	0.001	0.001	0.644	0.643	0.039	0.043	-0.000	-0.001
H	1.403	1.403	0.060	0.062	0.002	0.007	0.881	0.988	0.033	0.039	0.000	0.000

Appendix VIII A: Consent information document

Title of Study

Association of salivary receptor activator of nuclear factor ligand and osteoprotegerin levels with periodontal clinical status.

Description of the study

You are invited to participate in a research study conducted by Dr. Ochanji Allan Aldoh, a postgraduate student at the University of Nairobi. The study aimsat determining the levels of two molecules (RANKL and OPG) found in saliva and relating them to periodontal health**.**Your participation will involve providing a saliva sample by spitting into a sterile plastic tube. This will be followed by a non-invasive periodontal clinical examination using a dental probe and mirror.

Risks and discomforts

There are no anticipated risks associated with this research as it is non-invasive in nature. Minimal discomfort in the gums and slight bleeding maybe encountered. However care will be taken to minimize any possible discomfort.

Perceived benefits

Perceived benefits from this study will include partial fulfillment for the award of masters of dental surgery in periodontology at the University of Nairobi, adding new information to the existing body of knowledge and assessment of the periodontal health of the participants. The study will also form a basis for future randomized control trials aimed at providing easy, safe, cost-effective and non-invasive diagnostic approaches to periodontal disease.

Confidentiality

The information collected will be treated with utmost confidentiality and no name will be included in the questionnaire. Your identity will not be revealed in any publication resulting from this study.

Voluntary participation

Your participation in this research study is entirely voluntary. You may choose not to participate and you may withdraw your consent to participate at any time. You will not be penalized in any way should you decide not to participate or to withdraw from this study. No monetary compensation or otherwise is expected.

Appendix VIII B: Consent form

I _____

Having understood the nature of study as explained to me by Dr. Ochanji Allan Aldoh of The University of Nairobi; give my consent to participate in this study**.**

Name _____ **signed** _____ **Date** _____

Patient

I confirm that I have explained the nature of the study to the patient.

Name _____**Signed** _____ **Date**

Principal Investigator:

For more clarifications and enquiries on the consent please contact any of the following.

The Principal Investigator,
Dr. Ochanji Allan Aldoh
Phone: 0725441927.
Email: ochanjiaa@yahoo.com
Department of Periodontology/Community and Preventive Dentistry
University of Nairobi.

Or,
The Supervisors,
Dr. Nelson K Matu. BDS (Nrb), MSc.Dent (Periodontology) (UWC)
Senior Lecturer, Department of Periodontology/Community and Preventive Dentistry,
School of Dental Sciences, University of Nairobi.
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Dr. Tonnie K Mulli.BDS (Nrb),MClin.Dent-Periodontology (Lon), PhD (Lon), GCAP (Lon), AHEA (UK)
Lecturer, Department of Periodontology/Community and Preventive Dentistry,
School of Dental Sciences, University of Nairobi.
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Or,

The KNH/UoN Ethics and Research Standards Committee Secretariat
Email: uonknh_erc@uonbi.ac.ke
Telephone Number +254-20 2726300 Ext 44355

Kiambatisho VIII C:

Idhini kutoka kwa wahusika katika utafiti

Kichwa cha Utafiti

Uhusiano wa viwango vya salivary receptor activator of nuclear factor ligand na osteoprotegerin kwenye mate na hali ya ufizi.

Jinsi utakavyohusika

Unaalikwa kushiriki kwenye utafiti uliotajwa hapo juu utakaofanywa na daktari Ochanji Allan Aldoh. Utafiti huu unanuia kuchunguza uhusiano wa viwango vya RANKL na OPG kwenye mate na hali ya ufizi. Utahusika kwenye utafiti huu kwa kupeana kiwango kidogo cha mate na baadaye daktari ataangalia na kutathmini hali ya ufizi.

Mathara

Hakuna mathara yeyote inayotarajiwa kwa afya yako kutokana na utafiti huu isipokuwa maumivu kidogo kwenye ufizi. Daktari atahakikisha ya kwamba haya maumivu ni kidogo iwezekanavyo.

Manufaa

Utafiti huu utawezesha daktari kuhitimu shahada ya ‘Masters’ katika chuo kikuu cha Nairobi.Pia utaongeza ufahamu kwenye nyanja za utabibu wa magonjwa ya mdomo. Matokeo haya pia yatatumika katika utafiti zingine zitakazofanyika siku zijazo. Mahitaji yeyote ya dharura ya kimatibabu yatashughulikiwa na wataalamu katika hospitali hii.

Hifadhi ya Nakala ya Habari Utakayotoa

Habari zote zitakazokusanywa kutoka kwako zitahifadhiwa kwa siri na kutumiwa tu katika utafiti huu. Majina yako hayataandikwa mahali popote wakati wowote. Nakala zote za habari kukuhusu zitafungiwa katika makabati maalum wakati wote wautafitihuu. Habari hizi zitawekwa kwenye komputa na mchunguzi peke yake ndiye atakayetumia kitambulisho cha siri ili kufikia habari hizi.

Kushiriki kwahiari

Utashiriki kwa utafiti huu kwa hiari yako bila kushurutishwa au kulazimishwa na ye yote. Pia unaweza kujiondoa kutoka kwa utafiti huu wakati wo wote bila vitisho au madhara yoyote. Hakuna malipo ya kifedha au aina nyingine ambayo washirika watapewa kwa kushiriki katika utafiti huu.

Kiambatisho VIII D:

Fomu ya idhini

Mimi _____

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

Jina_____sahihi_____ Tarehe_____

Mshiriki

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

Jina_____sahihi_____ Tarehe____

Mchunguzu Mkuu

Kwa habari zaidi,tafadhali wasiliana na:

Mchunguzii Mkuu

Daktari Allan Aldoh Ochanji

Nambariyasimu: 0725441927.

Baruapepe: ochanjiaa@yahoo.com

Chuo kikuu cha Nairobi.

Au,

Wasimamizi

Dr. Nelson K Matu.BDS (Nrb), MSc.Dent (Periodontology)
Mhadhiri, hali ya ufizi
Chuo kikuu cha Naiorobi.
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Au,

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Barua Pepe: uonknh_erc@uonbi.ac.ke
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Appendix IX: DY626 RnD Systems – DuoSet® ELISA Development manual.

human TRANCE/RANK L/ TNFSF11

Catalog Number: DY626

MATERIALS PROVIDED

Bring all reagents to room temperature before use.

Capture Antibody (Part 841987, 1 vial) - 180 µg/mL of mouse anti-human TRANCE 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 1.0 µg/mL in PBS,[4] without carrier protein.

Detection Antibody (Part 841988, 1 vial) - 9 µg/mL of biotinylated goat anti-human TRANCE 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 50 ng/mL in Reagent Diluent.[4]

Standard (Part 841989, 3 vials) - Each vial contains 160 ng/mL of recombinant human TRANCE when reconstituted with 0.5 mL of Reagent Diluent (see Solutions Required section). Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Aliquot and store reconstituted standard 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 5000 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).[4]

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered.

Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems Catalog # WA126).

Reagent Diluent[1] - 1% BSA[5] 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 (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems Catalog # DY994).

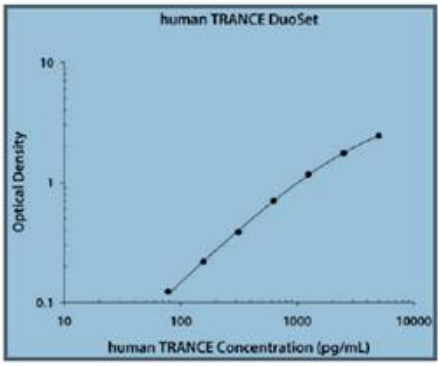

Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate[6] 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, 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. Avoid placing the plate in direct light.
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. Avoid placing the plate in direct light.
8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well 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.

Appendix X: DY805 RnD Systems - **DuoSet® ELISA Development manual.**

DuoSet® ELISA
DEVELOPMENT SYSTEM

Human Osteoprotegerin/TNFRSF11B

Catalog Number: DY805 (15 plates)

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

DESCRIPTION	PART #	# VIALS	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human OPG Capture Antibody	840369	1 vial	Refer to the lot-specific Certificate of Analysis (C of A) for storage conditions.
Human OPG Detection Antibody	840370	1 vial	
Human OPG Standard	840371	1 vials	
Streptavidin-HRP	890803	1 vial	

REAGENT PREPARATION

Bring all reagents to room temperature before use. Allow all components to sit for a minimum of 15 minutes with gentle agitation after initial reconstitution. Working dilutions should be prepared and used immediately, unless otherwise noted.

Streptavidin-HRP: 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Dilute to the working concentration specified on the vial label using Reagent Diluent.

Mouse Anti-Human OPG Capture Antibody: Refer to the lot-specific C of A for amount supplied. Reconstitute with 1.0 mL of PBS. Dilute in PBS without carrier protein to the working concentration indicated on the C of A.

Biotinylated Goat Anti-Human OPG Detection Antibody: Refer to the lot-specific C of A for amount supplied. Reconstitute with 1.0 mL of Reagent Diluent. Dilute in Reagent Diluent with 2% heat inactivated normal goat serum (NGS) to the working concentration indicated on the C of A. Prepare 1-2 hours prior to use.

Recombinant Human OPG Standard: Refer to the lot-specific C of A for amount supplied. Reconstitute each vial with 0.5 mL of Reagent Diluent. A seven point standard curve using 2-fold serial dilutions in Reagent Diluent is recommended. Prepare 1000 µL of high standard per plate assayed at the concentration indicated on the C of A.

OTHER MATERIALS & SOLUTIONS REQUIRED

DuoSet Ancillary Reagent Kit 2 (5 plates):
(R&D Systems, Catalog # DY008) containing 96 well microplates, plate sealers, substrate solution, stop solution, plate coating buffer (PBS), wash buffer, and Reagent Diluent Concentrate 2.

The components listed above may be purchased separately:

96 well microplates: (R&D Systems, Catalog # DY990).

Plate Sealers: (R&D Systems, Catalog # DY992).

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Catalog # DY006).

Wash Buffer: 0.05% Tween® 20 in PBS, pH 7.2-7.4
(R&D Systems, Catalog # WA126).

Reagent Diluent: 1% BSA 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 (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution: 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

Normal Goat Serum: (R&D Systems, Catalog # DY005).

Appendix XI: Human RANKL/OPG DuoSet® Certificate of Analysis

Human Osteoprotegerin/TNFRSF11B DuoSet, 15 Plate

Catalog Number: DY805
Expiration: 17 May 2019
Lot: 329315

Certificate of Analysis

SPECIFICATIONS

REAGENT	PART NUMBER	# OF VIALS	AMOUNT PER VIAL	WORKING CONCENTRATION	LOT #
Capture	840369	1	360 µg	2.00 µg/mL	CUZ0715021
Detection	840370	1	36.0 µg	200 ng/mL	CJA0915021
Standard	840371	3	35.0 ng	62.5-4000 pg/mL	1365190
Streptavidin-HRP	890803	1	N/A	200-fold dilution	326955

PREPARATION & STORAGE

Store unopened kit at 2-8 °C. Do not use past kit expiration date.

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REAGENT	PREPARATION	STORAGE OF OPENED/RECONSTITUTED MATERIAL
Capture	Reconstitute with 1 mL of PBS	Store at 2-8 °C for up to 8 weeks or aliquot and store at -20 °C to -70 °C in a manual defrost freezer for up to 24 weeks.*
Detection	Reconstitute with 1.0 mL of Reagent Diluent	
Standard	Reconstitute with 0.5 mL of Reagent Diluent	Aliquot and store reconstituted standard at -70 °C for up to 8 weeks.*
Streptavidin-HRP	Dilute with Reagent Diluent	Store undiluted at 2-8 °C for up to 24 weeks. DO NOT FREEZE.*

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

It is hereby certified that the above product has been tested for proper performance and function under our established Quality Control Testing criteria. It is authorized by our Quality Assurance program to be released for sale.

Diane R. Wotta, Ph.D.
Sr. Director, Quality and Regulatory Affairs
This C of A was updated on 5/15/2015, Rev. 0

bio-techne | Insert# 750627 | MS# 17450 | FOR RESEARCH USE ONLY. NOT FOR USE IN HUMANS.

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Bio-Techne is a trading name for R&D Systems

Appendix XII: Ethical approval

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Ref: KNH-ERC/A/148

2nd April, 2015

Dr. Ochanji Allan Aldoh
Dept. of Periodontology/Community and Preventive Dentistry
School of Dental Sciences
University of Nairobi

Dear Dr. Ochanji

Research Proposal: Association of Salivary Rankl and OPG Level with Periodontal Clinical Status (P710/12/2014)

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 2nd April 2015 to 1st April 2016.

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.erc.uonbi.ac.ke**

KENYATTA NATIONAL HOSPITAL APPROVED 02 APR 2015 KNH/UoN-ERC P.O. Box 20723

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 Chair, KNH/UoN-ERC
The Dean, School of Dental Sciences, UoN
The Chairman, Dept. of Periodontology/Community and Preventive Dentistry, UoN
Supervisor: Dr. Nelson K. Matu, Dr. Tonnia K. Muli