THE INFLUENCE OF VITAMIN D STATUS ON PERIODONTAL SURGERY OUTCOMES: A PROSPECTIVE ANALYSIS

By

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DEDICATION

This thesis is dedicated to my family – Mom, Jason, Aunty, TJ, Kennedi and Dad – your unconditional love has meant more to me than anything in the world. Thank you a million times over. I love you all.

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Abstract

Introduction: Periodontal disease is characterized by alveolar bone loss resulting from the host immune response to bacterial insult. With its important role in bone maintenance and immunity, there is biologic rationale to suggest that a deficiency in vitamin D may negatively affect periodontal treatment outcomes. The purpose of this double-masked, prospective observational study was to evaluate the influence of vitamin D levels on surgical outcomes. Methods: Sixty-five patients (aged 30-85) with moderate to severe chronic periodontitis received flap surgery. Enrolled patients had ≥1 tooth with probing depth (PD) ≥6mm and bleeding on probing (BOP); this served as the surgical (SX) site. Serum 25-hydroxyvitamin D (25[OH]D) samples and gingival crevicular fluid (GCF) samples were collected immediately prior to surgery (baseline [BL]) and analyzed for bone metabolism biomarkers. PD, clinical attachment level (CAL) and BOP were recorded at BL, 3, 6 and 12 months after surgery. Radiographic bone level was evaluated at BL and at 12 months. Patients were then divided into vitamin D deficient (DEF, <20ng/mL) and sufficient (SUF, ≥20ng/mL) groups, and differences between groups were analyzed. Results: Fifty-one patients (DEF, n=13; SUF, n=38) reported for the 12-month follow up and no BL differences were noted between groups. SUF patients had significantly greater PD reduction compared to DEF patients at both 6 months (3.1 +/- 1.3 mm vs. 1.8 +/- 1.1mm, p=0.001) and 12 months (2.8 +/- 1.3mm vs. 1.8 +/- 1.1mm, p=0.01). Linear regression analysis revealed significant a correlation between 25[OH]D levels and PD reduction at 6 months (p=0.001) and 12 months (p=0.005), CAL gain at 6 months (p=0.02) and radiographic bone gain at 12 months (p=0.04). At 6 months, PD

reduction was significantly correlated with BL GCF levels of IL-10 ($p=0.02$) and IL-6sR ($p=0.04$). At 12 months, CAL gain was significantly correlated with BL levels of IL-6sR in the GCF ($p=0.03$) and in the serum ($p=0.005$), and radiographic bone gain was significantly correlated with BL GCF levels of IL-10 ($p=0.01$). Additionally, significant positive correlations between serum 25[OH]D levels and BL GCF levels of MER ($p<0.00001$) and SDF-1 ($p=5x10^{-11}$) were identified. Conclusions: These results suggest that a deficient vitamin D level at the time of periodontal surgery negatively affects clinical outcomes for up to 1 year.

Chapter 1

I. Introduction

Periodontal disease is characterized by the loss of bone and supporting tissues as a result of the host immune response to chronic bacterial challenge. Non-surgical treatment for periodontal disease involves mechanical removal of pathogenic bacteria, whereas surgical therapy involves reshaping the soft tissue and bone to provide an environment that facilitates easy removal of pathogenic bacteria. In order to achieve optimal periodontal wound healing, proper immune function and control of systemic factors are essential. The importance of this relationship can be seen through the proven adverse effects on periodontal healing from uncontrolled diabetes, cigarette smoking, and other systemic influences (i.e. alcohol, osteoporosis, stress/coping) (1).

Vitamin D has long been associated with bone homeostasis through its regulatory effects on calcium; together, they have a documented role in establishing bone strength, preventing demineralization and encouraging remineralization of deficient areas (2,3). Additionally, recent evidence suggests that vitamin D plays a significant role in immune function (4). Vitamin D impacts innate antimicrobial activity through cathelicidins and beta defensins, decreases extracellular matrix enzyme activity, and modulates T-lymphocyte production and function (5–7). Additionally, Vitamin D has been shown to impact expression of inflammatory biomarkers such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-

6) in monocytes, and receptor activator of nuclear factor kappa-B ligand (RANKL) in osteoclasts (8–10). These biomarker levels can be detected in a number of body tissues, including gingival crevicular fluid—a transudate found between the tooth and gingival tissue—as well as serum.

With its role in bone maintenance and immunity, it is biologically plausible to expect a link between vitamin D levels and periodontal healing. Vitamin D deficiency is generally accepted as a serum 25-hydroxyvitamin D concentration of less than 20 ng/ml, whereas a level of 20ng/ml would be considered as sufficient (3). In cross-sectional studies, vitamin D deficient patients demonstrated more clinical attachment loss and gingival inflammation compared to vitamin D sufficient patients (11,12). Additionally, supplementation with vitamin D has been associated with decreased tooth loss in elderly patients and improved periodontal health parameters in a periodontal maintenance population (13,14).

Despite its relationship with periodontal disease, there is little information regarding the effect of Vitamin D on periodontal surgery outcomes (12,15). A 2010 randomized controlled trial compared the effect of the anabolic peptide teriperatide versus placebo in periodontal regeneration; both treatment groups were evaluated for pre-surgical serum vitamin D concentrations and received vitamin D and calcium supplementation throughout the study (16). One year later, a post-hoc analysis of this same study population was conducted and revealed that placebo patients who were vitamin D deficient (DEF) at the time of surgery had less clinical

attachment level gain (CAL) and less probing depth (PD) reduction when compared to vitamin D sufficient patients (SUF) (17). The study population of placebo patients was small, however, and vitamin D status was not the primary focus of the study. To ameliorate this issue, we conducted a study examining the impact of baseline serum 25-hydroxyvitamin D concentrations on periodontal clinical parameters over a one-year period. The purpose of this double-masked, prospective observational study was to determine the effect of vitamin D deficiency or sufficiency on periodontal surgical outcomes. Additionally, we sought to analyze trends in biomarkers of bone metabolism and inflammation from the oral cavity and serum with respect to serum vitamin D level.

II. Materials and Methods

a. Study Population and Overview

Sixty-five patients scheduled for periodontal flap surgery were enrolled in a double-masked, single-center, prospective, observational study at the University of Michigan Graduate Periodontics clinic. Patient enrollment occurred from September 2011 through December 2012, with the last patient follow-up examination occurring December 2013. The primary objective of this study was to compare clinical outcomes (PD reduction, CAL gain, and bleeding on probing [BOP]) of periodontal surgery between DEF and SUF patients. Secondary objectives

included evaluating radiographic changes, local bone turnover markers, and determining the influence of systemic conditions and demographic variables on the above outcomes. Additionally, due to the heterogeneity of the study population, we aimed to confirm the results of our primary and secondary objectives through sub-group analyses of surgical type, tooth type and tooth location.

Patients scheduled for periodontal flap surgery were given verbal and written information describing the nature and duration of the study. Those interested in the study were then asked to read and sign an informed consent form. The study was approved by and complied with the University of Michigan Institutional Review Board.

A verbal review of the patients' medical history was completed by one of the investigators in order to confirm the patients' medical qualification for the study. Inclusion criteria included being aged 30 to 85 years, having localized or generalized chronic periodontal disease and at least one tooth with probing depth ≥6mm and bleeding on probing. Exclusion criteria included patients younger than 30 years, pregnant or lactating patients, heavy smokers (>1 pack per day), uncontrolled diabetic patients, or patients in whom surgery was contraindicated.

After obtaining informed consent, study investigators verified that full mouth manual probing measurements had been taken within 90 days of the baseline (surgical) appointment. The following parameters were determined for each tooth

in the mouth: probing depth (PD): measured from the free gingival margin to the bottom of the sulcus; clinical attachment level (CAL): measured from the cemento-enamel junction to the bottom of the sulcus; recession (REC): measured as the distance from the free gingival margin to the cemento-enamel junction; bleeding on probing (BOP): measured as yes or no. Six sites were measured around each tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual). Periapical and bitewing radiographs were taken of each surgical site (2 films/site) if the patient did not have them taken within 6 months of the surgical appointment. A full mouth radiographic series was taken if the patient had not had one within the past 24 months.

b. Study Design

i. Serum and Gingival Crevicular Fluid Collection

Immediately prior to periodontal surgery, blood samples were obtained from each patient by venipuncture and centrifuged to separate serum from blood cells. The serum was then separated into two test tubes and stored in an -80°C freezer until completion of patient enrollment.

After venous blood collection, gingival crevicular fluid (GCF) was collected from one healthy and one diseased site from each patient. A diseased site was defined as having a PD ≥ 6mm, CAL ≥ 6mm and BOP; this site was later designated as the site

for study analysis ("study site"). A healthy site was considered as having a PD ≤ 4mm with no BOP. Prior to collection, the site was isolated and dried, and supragingival plaque was removed using a sterile instrument. A methylcellulose strip (Periopaper® Gingival Fluid Collection Strips, Pro flow, Inc., Amityville, NY, USA) was inserted one millimeter into the sulcus/pocket for 10 seconds. GCF volume was immediately determined using a calibrated Periotron 6000 (Harco Electronics, Tustin, CA, USA). GCF strips were then placed in vials, labeled and stored at -80°C until processing.

ii. Surgical Procedure

Each patient had periodontal surgery performed according to his or her needs. The surgical procedure consisted of either resective therapy (open flap debridement, apically positioned flap, distal wedge procedure) or regenerative therapy (bone replacement graft ± barrier membrane). Detail regarding each patient's surgical procedure was collected and recorded by the study investigator; these included: type of surgery, type of osseous procedure, bone graft utilization, and barrier membrane utilization.

iii. Follow-Up Visits

Patients returned for examination at 3, 6, and 12 months following baseline. Clinical measurements (PD, REC, CAL, and BOP) were recorded at each visit. Periodontal

maintenance therapy was conducted at 3-month intervals throughout the study period. Radiographs of the surgical area (one periapical film, one bitewing film) were taken at 12 months.

iv. 25-Hydroxyvitamin D and Biomarker Assessment

After the last patient enrollment was complete, one stored serum sample from each patient was sent for laboratory analysis of 25-hydroxyvitamin D concentration. Resultant serum vitamin D concentration levels for each respective patient were kept hidden from clinical examiners until the last 12-month follow-up visit was completed. After the last 12-month follow up visit, serum 25-hydroxyvitamin D concentrations were revealed for each patient and patients were categorized into one of two groups, vitamin D sufficient (SUF = ≥20 ng/ml) or vitamin D deficient (DEF = <20 ng/ml).

GCF samples were also processed and analyzed following completion of patient enrollment. Proteins within the harvested crevicular fluid were extracted from the GCF strips using an extraction method adapted from Giannobile et al. (18), involving a series of washes and centrifugations. The elution buffer used for GCF protein extraction contained the following: 24.5 mL Phosphate Buffered Saline, pH 7.4; 125µL phenylmethylsulfonylfluoride (PMSF; Sigma Chemical, St. Louis, MO), 200mM in MeOH; 250 µL Aprotinin (Sigma Chemical, St. Louis, MO), 1mg/ml in water; 83.5µL of 30% Human Serum Albumin (Sigma Chemical, St. Louis, MO). This was

made fresh and kept on wet ice throughout the entire extraction process to inhibit protease activity. Each strip was washed with 20μL and then centrifuged at 2000 rpm for 5 minutes at 4°C. This was done a total of five times to yield 100μL total volume.

The extracted GCF volume and remaining serum sample were quantitatively analyzed by a Quantibody Human Cytokine Array (RayBiotech, Inc., Norcross, GA, USA) for the concentration of the following: C-reactive protein low (CRP-low), Insulin-like Growth Factor 1 (IGF-1), Interleukin 10 (IL-10), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-6 soluble receptor (IL-6 sR), Monocyte Chemoattractant Protein 1 (MCP-1), MER Tyrosine Kinase (MER), Osteoprotegerin (OPG), Stromal cell-derived factor 1 (SDF-1), Sclerostin (SOST), Transforming Growth Factor Beta 1 (TGF-β1), TNF-related activation induced cytokine (TRANCE), Vascular Cell Adhesion Molecule 1 (VCAM-1), Vascular Endothelial Growth Factor (VEGF), and C-reactive protein high (CRP-high).

c. Radiographic Analysis

Emago™ (Oral Diagnostic Systems, Amsterdam, Netherlands) software was utilized in the radiographic analysis. Selection of radiograph type (i.e. bitewing or periapical) for analysis was determined by two examiners (EJ and JB), with inter-examiner agreement for which film produced the best visualization of the surgical study site defect. Prior to analysis, radiographs were standardized with respect to

density, via gamma correction using the enamel surface as a reference, and angulation, via reconstruction methods. Linear measurements of the defect were made from the CEJ to the deepest portion of the defect (CEJ-ACH).

d. Statistical Analysis

Of the original data set, a number of participants failed to report for the 3, 6 and 12 month time points. Because of this, their data was dropped from the analysis at each respective time point. The resulting sample size for the 3, 6, and 12 month time points were n=26, n=47, and n=51, respectively.

The outcome variable of this analysis was considered as the change from baseline to subsequent time points at the study site for each clinical outcome (PD, CAL, ACH-CEJ). Measurements were averaged across the study sites of all subjects to compute means of PD, CAL, and ACH-CEJ within each vitamin D group (SUF and DEF). Two sample t-tests were applied to assess the difference in PD, CAL, and ACH-CEJ between groups and differences with a p value less than 0.05 were considered significant. The impact of serum Vitamin D concentration on changes in each outcome variable was evaluated by linear regression analysis. For both the t-test and linear regression analysis, PD and CAL were assessed at 3-, 6-, and 12-month time points compared to baseline; ACH-CEJ was assessed at only the 12-month time point compared to baseline. BOP was categorized dichotomously as improvement or no improvement at each time point compared to baseline. Improvement was

defined as baseline = "1" and time point$_n$ = "0". Chi-square analysis and Fisher's exact test were used to determine the differences between groups for the proportion of "improved" surgical sites at each time point. Logistic regression analysis was used to evaluate the influence of serum vitamin D concentration (as a continuous variable) on the chance of observing "improved" surgical sites at each time point.

Log transformation of the serum and GCF values was performed to correct the non-normality. If the biomarker value exceeded the lab-determined limits, the value was reduced to the upper bound; if the value was below the lower limit, it was considered inaccurate and was replaced with a value equal to half the lower limit. The rationale for this approach reflects the assumption of a uniform distribution of all unknown values below the lower limit. With this in mind, the expected mean of these values is equal to half of the lower limit. For each biomarker, the mean of the log-transformed values between groups (SUF and DEF) were compared using two-sample t-tests. Additionally, linear regression analysis was performed to evaluate the impact of vitamin D as a continuous variable on the log-transformed value of each biomarker from each patient within the study population.

In a study of this type it is typical to see significant reductions in PD and CAL at 3 months (average reduction of 1.3 mm from baseline). A power calculation was done and, assuming a 30% prevalence of vitamin D deficiency, 70 patients would be

required to detect a 1mm difference in CAL. Sixty patients are the minimum necessary to detect a 1mm difference in PD reduction.

i. Sub-Group Analysis

Study sites were sub-categorized with respect to the following features: (1) resective surgery site or regenerative surgery site, single-rooted tooth or multiple-rooted tooth, and maxillary tooth or mandibular tooth. For each of the 6 resultant sub-groups, two sample t-tests, linear regression analysis, chi-square analysis and Fisher's exact test were repeated as described above.

III. Results

a. Patient Enrollment and Demographics

Fifty-one patients returned for the 12-month follow-up. Baseline demographic, clinical, and smoking characteristics of this group are presented in Table 1. Comparisons of patient groups (sufficient vs. deficient) yielded no significant differences with respect to baseline characteristics, with the exception of mean 25-hydroxyvitamin D serum concentrations ($p<0.05$). Fifteen patients were lost to follow-up during the study observation period. One patient passed away from

natural causes during the study period; to compensate for this loss, an additional patient was enrolled and the initial patient's data was discarded. Out of the remaining 14 lost patients, three patients required extraction of the tooth containing the study site, two patients moved out of state, and 9 patients were lost due to non-compliance. With the exception of the patient who passed away, 3- and 6-month time point data of lost patients were included in the analysis, as these time points were considered independently of one another.

b. Clinical Outcomes Related to Vitamin D Status

There was an overall improvement in clinical parameters after periodontal surgery. SUF patients, on average, demonstrated greater PD reduction than DEF patients at all time points (Figure 1). At the six-month time point, SUF patients had significantly more PD reduction compared to DEF patients, with SUF patients demonstrating a probing depth reduction of 3.1 +/- 1.3 mm compared to only 1.8 +/- 1.1mm observed in the DEF group ($p=0.001$). This effect was sustained throughout the 12-month time point, with SUF patients exhibiting a 2.8 +/- 1.3mm reduction in PD, compared to only 1.8 +/- 1.1mm in the DEF group ($p = 0.01$). There were no other significant differences noted between groups with regard to clinical outcomes for neither PD reduction and CAL gain at other times points, nor BOP (data not shown).

To explore the dose-dependent impact of vitamin D levels with regard to clinical outcomes, we evaluated the relationship between each patient's serum vitamin D level and clinical parameter improvements using linear regression analysis (Figures 2 and 3). Serum vitamin D level was positively correlated with PD reduction at the 6-month ($p = 0.001$) and 12-month ($p = 0.005$) time points. Additionally, we found significant correlations between serum vitamin D level and 6-month CAL gain ($p = 0.02$), as well as 12-month radiographic linear bone gain ($p = 0.04$). There were no other significant correlations for other time points or other clinical outcomes.

c. GCF and Serum Biomarkers Related to Vitamin D Status

We wanted to determine if biomarkers levels within the local oral environment or within systemic circulation were influenced by serum vitamin D concentration. Linear regression analysis revealed that baseline serum vitamin D levels were highly significantly correlated to GCF levels of MER ($p<0.001$) and SDF-1 ($p<0.001$). Analysis of the other biomarkers from the GCF and biomarkers from the serum failed to show significant correlations. See Tables 2 and 3.

d. Sub-group Analysis

Because of the heterogeneity within the study population with regard to type of surgery, surgical tooth type, tooth location, we wanted to test if the outcomes from the pooled population remained evident within sub-groups.

i. Surgical Type: Resective or Regenerative

Within the resective surgical group, PD reduction was significantly greater in the SUF group (2.8 ± 1.1 mm) compared to the DEF group (1.4 ± 0.8 mm) at the 6-month time point ($p = 0.002$). Additionally, we found that PD reduction significantly correlated with serum vitamin D concentration at 6 months ($p = 0.01$, $r = 0.19$) and 12 months ($p = 0.048$, $r = 0.07$). Considering the group receiving regenerative surgery, SUF patients had significantly more CAL gain at 6 months compared to DEF patients (3.1 ± 1.9 mm vs. 0.5 ± 0.7 mm, $p = 0.03$). Linear bone gain (ACH-CEJ) was also significantly positively correlated with serum vitamin D levels at 12 months ($p = 0.01$, $r = 0.80$).

ii. Tooth Location: Maxillary or Mandibular

For study sites located in the maxilla, SUF patients had significantly greater PD reduction at 12 months when compared to DEF patients (2.5 ± 1.4 mm vs. 1.4 ± 0.9 mm, $p = 0.02$). PD reduction from maxillary study sites was significantly positively correlated to serum vitamin D level at 12 months ($p=0.03$, $r = 0.13$).

For study sites in the mandible, significantly greater ($p = 0.002$) PD reduction was seen at 6 months for SUF patients (3.6 ± 1.6 mm) when compared to DEF patients

(1.8 ± 0.5 mm). Additionally, it was observed that vitamin D levels significantly positively correlated with both PD reduction at 6 months (p=0.02, r=0.26) and linear bone gain at 12 months (p=0.007, r=0.35).

iii. Tooth Type: Multiple-rooted or Single-rooted

Multi-rooted teeth, the predominant tooth type in the study, demonstrated very robust results within the study objectives. Within this sub-group, SUF patients showed significantly greater PD reduction at the study site when compared to DEF patients at both 6 months (3.1 ± 1.4 mm vs. 1.4 ± 0.8 mm, p <0.001) and 12 months (2.8 ± 1.4 mm vs. 1.6 ± 1.1 mm, p = 0.01). We also found that serum vitamin D concentration significantly correlated with PD reduction at 6 months (p=0.001, r=0.26) and 12 months (p=0.004, r=0.16), and linear bone gain at 12 months (p=0.04, r=0.10). There was also a trend towards significance for CAL gain at 6 months (p=0.05, r=0.09). Within the single-rooted sub-group, although the SUF group had greater improvement in clinical parameters, these results did not reach significance.

IV. Discussion

The results of this double-masked, prospective, observational study demonstrate that vitamin D deficiency at the time of periodontal surgery resulted in less PD

reduction for up to 1 year. Vitamin D levels were positively correlated to gains in CAL, PD reduction and radiographic linear bone gain in patients with chronic periodontitis. These findings remained significant after adjusting for varying characteristics in the study population such as surgical type, tooth type and tooth location. Twenty-eight percent of patients in this study population presented with a vitamin D serum concentration less than 20ng/ml, with more than half of this group being non-white (56%). The prevalence and distribution of vitamin D deficiency in our study was comparable to that observed in the general population (19,20).

To our knowledge, this is the first prospective, observational study examining the influence of serum concentration of vitamin D on clinical outcomes after periodontal therapy. Dietrich (11,12) utilized data from NHANES III to relate serum concentrations of 25-hydroxyvitamin D to attachment loss and gingival inflammation, determining that patients in the highest quintile of serum vitamin D concentration demonstrated less mean attachment loss (0.26 - 0.39mm) and 20% less likelihood of exhibiting BOP than those in the lowest quintile. Owing to their large sample sizes, these studies reflect appropriate study powering to adjust for potential confounding factors; however, the data is cross-sectional in nature and only reflects one measured time point. More recent studies have used a prospective, observational approach to examine periodontal parameters in response to vitamin D supplementation (14,15). At one year, the authors found that among patients supplementing with calcium and vitamin D, mean probing depths were 12% better, mean attachment levels were 13% better, and mean CEJ-AC distances were 12%

better (14); however, no evaluation of serum 25-hydroxyvitamin D levels were completed. In this study, patients taking vitamin D (≥400 IU/day) and calcium (≥1000mg/day) were considered as supplement "takers"—a category used as a surrogate representation of vitamin D status. However it has been shown that up to 1000-4000 IU/day of Vitamin D may actually be required to reach the physiologic range of serum vitamin D concentration (21). Without evaluation of serum 25-hydroxyvitamin D concentration, actual vitamin D status can only be implied. A post-hoc analysis of a previous randomized clinical trial that did evaluate serum 25(OH)D concentration revealed that placebo patients with vitamin D deficiency at the time of periodontal surgery showed less PD reduction and less CAL gain when compared to vitamin D sufficient placebo patients at 12 months (17).

In our study, patients with at least 20ng/ml of serum 25-hydroxyvitamin D demonstrated roughly 3 mm of PD reduction after periodontal surgery, for all time points measured. This is in comparison to the 1.8 mm of PD reduction in the group with less than 20ng/ml. CAL gain was noted over time in both groups, with gains ranging from 0.5-1.3mm over the three time points in the deficient group, and 1.1-1.9mm over the three time points in the sufficient group. Although the difference between groups did not reach statistical significance, SUF sites presented with more CAL gain at all time points. These findings of clinical improvement after periodontal surgery are consistent with previous reports (22–24), which cite gains in CAL ranging from 0.6-1.5mm and reduction in PD ranging from 1.4-2.3mm one year after surgery.

The analysis of vitamin D sufficient and deficient patients within the sub-groups provided interesting results. The observations seen at multi-rooted study sites with respect to vitamin D sufficiency (i.e. greater PD reduction, positive relationship of PD reduction, CAL gain, and linear bone gain with serum 25(OH)D concentration) did not reach significance when evaluated at single-rooted study sites. These findings may indicate that sufficient vitamin D levels may be beneficial for healing at sites with greater surface area, or where potential confounders (i.e. furcations, root concavities) may impact healing. Additionally, when looking within maxillary versus mandibular surgical sites, linear bone gain at mandibular surgical sites were positively correlated to serum 25(OH)D concentration, whereas maxillary sites did not demonstrate this relationship. Although animal studies point to a positive influence of vitamin D in mandibular bone (25), the outcomes of our study are likely related to the fact that most regenerative sites were located in the mandible—a fact that reflects the lower degree of predictability of grafting in maxillary molar furcation sites (26,27). These findings are preliminary and because each sub-category within each sub-group was not appropriately powered to draw significance, these findings must be interpreted with caution, but nonetheless give potential insight for future study directions.

Periodontal healing after surgical therapy is a result of connective tissue healing and long junctional epithelium formation. During healing, fibroblast cells from the periodontal ligament migrate to the root surface to form attachment to the root

(28,29). Additionally, epithelial cells migrate in an apical direction from surrounding oral epithelium and form the junctional epithelium layers, which maintain an intimate contact to the root surface through hemi-desmomosal attachments (30,31). This healing occurs with the help of cells such as gingival fibroblasts and PDL stem cells. Studies suggest that vitamin D may play a role on the activity of these cells in the oral cavity. The biologically active form of vitamin D, $1,25(OH)_2D_3$, has been shown to enhance anti-bacterial defense of epithelial cells in the oral cavity (32). 25-hydroxyvitamin D is converted to $1,25(OH)_2D_3$ by the enzyme 1α-hydroxylase. Evidence suggests that human gingival fibroblasts (hGFs) and human periodontal ligament stem cells (hPDLSCs) can convert 25(OH)D to $1,25(OH)_2D_3$ through their 1α-hydroxylase activity, thereby enacting a joint autocrine/paracrine function of vitamin D in the oral cavity (33). Additionally, it has been shown that $1,25(OH)_2D_3$ inhibited the expression of IL-8, a pro-inflammatory mediator, in hPDLSCs after exposure to the classic periodontal pathogen *Porphyromonas gingivalis* (34). Matrix metalloproteinase 9 (MMP-9) is a collagen-degrading enzyme that has been detected in high levels in the presence of gingival inflammation and chronic periodontitis (35,36). Studies have highlighted that vitamin D deficiency is associated with increased circulating levels of MMP-9 (37) and exposure to $1,25(OH)_2D_3$ decreases the formation of MMP-9 by pathogen-affected immune cells (38). Together these mechanisms may provide increased resistance to the destructive immune response that would be mounted by invading microorganisms during periodontal healing. In vitamin D deficient patients, these protective anti-bacterial and anti-enzymatic effects would be decreased, thereby

allowing a greater immune response, more tissue destructive activities and less periodontal healing.

Linear bone gain differences between the SUF and DEF group in our study was not appreciable, in part because the majority of surgeries performed involved simply open flap debridement or even minimal bone recontouring; these procedures are known not to produce any significant bone gain (39,40). However, the sub-group analysis revealed that within the group receiving regenerative therapy, increasing 25(OH)D concentration was positively correlated with increasing linear bone gain ($p = 0.01$, adj $r^2 = 0.80$). Vitamin D impacts skeletal maintenance by influencing RANKL expression by osteoblasts, thereby modulating serum calcium concentration and regulating calcium homeostasis. Additionally, $1,25(OH)_2D_3$ has been shown to regulate osteoblast differentiation through modulation of gene expression (41,42) and enhance collagen cross-linking in osteoblastic cell cultures (43). Thus, it appears that during periodontal bone regeneration, adequate levels of vitamin D will influence bone formation and bone quality.

Our study found that the levels of two biomarkers, stromal cell-derived factor 1 (SDF-1) and Mer tyrosine kinase (MER), as measured in the GCF of diseased periodontal sites, were positively correlated with serum 25-hydroxyvitamin D levels. SDF-1, also referred to as CXCL12, is a chemokine and potent chemoattractant of hematopoietic cells, including lymphocytes and neutrophils. It is expressed in areas of inflammatory bone destruction (44) and has recently been

implicated as a potential indicator of periodontal disease progression (45). MER is a part of the receptor protein tyrosine kinase family TAM (TYRO3, AXL, MER), which plays an important role in innate immunity and autoimmunity through the clearance of apoptotic cells, inhibition of the inflammatory response of dendritic cells and macrophages, maturation of natural killer cells, and inhibition of toll-like receptors (TLRs) and their cytokine receptor cascades (46,47). Mer on macrophages has been implicated in the downregulation of pro-inflammatory cytokines such as TNF-α (48) and also increased phagocytosis of apoptotic cells (49). Vitamin D ($1,25(OH)_2D_3$) has been shown to inhibit the proliferation and maturation of dendritic cells (50). Given both of their roles in blocking inflammatory cascades, it is plausible that in an instance of chronic pathogen invasion, vitamin D may work in concert with Mer to help suppress the inflammatory response. Although the relationship of SDF-1 to vitamin D is unclear, SDF-1 has been linked to regulation of dendritic cell migration (51); thus, there conceivably may be an intimate relationship of SDF-1, Mer, and vitamin D as it relates to regulation of immune response in areas of chronic inflammation.

The results of this prospective, observational clinical study highlight the significance of Vitamin D as it relates to periodontal soft tissue and bone healing, as well as interaction with the host's inflammatory response. The implications of these findings are significant in that Vitamin D is a readily available and easily accessible therapeutic modality. The effects of supplementation as it relates to oral health have been studied, with cross-sectional and observational data revealing

improvement in oral health parameters and even decreased tooth loss among older populations (13,52). The current recommended dietary allowances for vitamin D in adults ranges from 600-800 IU per day. Dixon et al. (53) showed that among patients in a periodontal maintenance populations, only 7% were meeting published guidelines for recommended vitamin D intake. While initiating supplementation will be a first step towards raising serum concentrations of vitamin D, this must be done well in advance of periodontal surgery, as vitamin D concentration at baseline seems to be more important than subsequent time points (16,17). With supplementation, it may take anywhere from 6 weeks to 3 months for serum concentrations to reach sufficient levels for bone health (17,21).

Our study had several limitations, including small sample size, heterogeneity of operators and examiners, non-standardized radiographs, and heterogeneity of defects and surgery type. As described earlier, a number of patients failed to report for clinical examination at each of the three time points. The study was powered at sixty-five patients to detect a 1mm difference between baseline and each time point, however, there were 26, 47, and 51 patients available for the 3-, 6-, and 12-month time points, respectively. Previous studies have shown the greatest improvement in clinical parameters after surgery at 3 months. Our study detected no significance in any parameter at this time point, which may be in part due to the small sample size available for data analysis.

There was a moderate degree of heterogeneity among operators and examiners. To limit inter-examiner variability, every effort was made have the same examiner perform clinical measurements at each time point for any given patient. When this was not possible, all measurements were performed by one calibrated examiner (EJ).

Radiographic analysis was performed to evaluate changes in radiographic bone level. Our radiographs were not standardized, however, we performed gamma correction and image reconstruction to correct for variation in density and angulation. Previous studies indicate that estimation of bone changes over time can be accomplished on non-standardized radiographs, with relatively low error, provided the same reference points are used in analysis (54,55).

Our inclusion criteria required a PD and CAL of 6mm or greater with the presence of BOP. The surgical therapy performed on these sites included pocket elimination surgery, open flap debridement with or without osteoplasty and ostectomy, and periodontal regeneration using bone grafts and/or membranes. Surgical therapy was chosen by the operator based on individual patient needs and details regarding the surgery were recorded. Despite this heterogeneity, significance with respect to PD reduction, CAL, and radiographic linear bone gain was retained when analysis was performed within sub-groups. This fact lends itself to the generalizability of these results within the greater population, as reaching significance in the presence of heterogeneity and smaller sample size amplifies the importance of these findings.

V. Conclusion

The results of this single-center, double blind, prospective, observational study indicate that vitamin D sufficiency at the time of periodontal surgery improves clinical outcomes for the treatment of chronic periodontal disease. Specifically, having a serum concentration of 25-hydroxyvitamin D of 20ng/ml or greater at the time of surgery provides greater probing depth reduction for up to 1 year. Additionally, there appears to be a significant positive relationship with serum vitamin D level as it relates to probing depth reduction, clinical attachment level gain, radiographic linear bone gain and certain GCF biomarkers related to regulation of the immune response. As vitamin D serum concentration is relatively easily modifiable, the implications of these findings are far-reaching. Future directions should include the use of larger-scale, randomized controlled trials examining the effects of supplementation on serum levels over a longer study period, as it relates to periodontal therapeutic outcomes.

Chapter 2

I. Introduction

The initiating factor in chronic periodontal disease is widely accepted as bacterial plaque in a susceptible host. Researchers have corroborated bacterial groups and clusters that are associated with both periodontal health and disease (56). Additionally, because we recognize that it is actually the hosts' immune response to a bacterial challenge that causes periodontal bone and soft tissue destruction, researchers have begun examining biological markers (*"biomarkers"*) that may be reflective of certain immunologic processes within the host. Varying degrees and types of gingivitis and periodontal disease have been associated with biomarkers, which include matrix-metalloproteinases, growth factors, and other indicators of soft and hard tissue destruction and repair (57). RANK, RANKL and IL-8 have been correlated with periodontal disease severity (58,59), while MMP-8 has been shown to predict periodontal treatment outcomes in smokers and differentiate between sites of severe bone loss versus those with slight bone loss (60,61).

Traditionally, biomarkers of periodontal disease have been measured in both the saliva, as well as the gingival crevicular fluid (GCF). Because the information is garnered from the site of action, these fluids are highly likely to reflect biological activity within the oral cavity and directly around the tooth. However, as the oral cavity is closely linked with the entire body, systemic biomarkers from the serum and blood may also be affected by periodontal disease activity. Recently, researchers have associated biomarkers such as C-reactive protein, MCP-1, TNF-α,

and IL-6 in the serum with periodontal disease and non-surgical therapeutic outcomes (62–64).

There has been little to no investigation into oral and serum biomarkers as they relate to periodontal outcomes. A 2012 study demonstrated that certain salivary biomarkers were able to predict severity of gingival inflammation response in an experimental gingivitis study (65). A recent study longitudinally compared biomarker levels between groups receiving periodontal surgery with or without initial scaling and root planing (66), but found no differences between groups. To date, the relationship of baseline levels of biomarkers to periodontal surgical outcomes has not been examined. We previously set out to evaluate the influence of vitamin D status on periodontal surgical outcomes and biomarkers from the GCF and serum. In this secondary analysis, the role of biomarkers as they relate to periodontal surgical outcomes was explored. The aim of the current investigation was to determine if baseline levels of GCF and serum biomarkers have an association with clinical parameter change after periodontal surgery.

II. Review of Biological Markers

C-Reactive Protein

C-reactive protein is considered a marker of systemic inflammation. It is an acute-phase protein that has been associated atherosclerosis and increased risk of cardiovascular events (67). Its relationship to periodontal disease has also been elucidated, with studies suggesting elevated levels of CRP in chronic periodontitis, with or without the presence of cardiovascular disease (68,69).

IGF-I

Insulin-like growth factor 1 (IGF-I) is a part of a family of growth factors that play an important role in cell proliferation, differentiation and transformation during embryonic stages and later developmental stages. Specifically, IGF-I has been shown in vitro to enhance PDL and fibroblast migration and differentiation, as well as impacting osteoblast migration and proliferation (70).

IL-10

Interleukin-10 is a pleiotropic anti-inflammatory cytokine. Some of its effects include limiting the release of pro-inflammatory cytokines such as IL-2, TNF-α, and IFN-γ, as well as down-regulation of Th1 cytokines (71). There is evidence that IL-10 may play an important role in modulating the inflammatory response in periodontal disease, with polymorphisms being shown to result in more severe chronic periodontitis (72).

IL-1β

The pro-inflammatory cytokine, IL-1β, is secreted by monocytes and has multiple effects on both soft and hard tissue. It is considered to be a bone resorptive cytokine, with increased levels being demonstrated in the presence of periodontal disease (73,74). In an early study, levels of IL-1β were shown to decrease after non-surgical periodontal therapy, suggesting that this cytokine may be useful as an indicator of periodontal inflammation (75,76)

IL-6 and IL-6sR

Although IL-6 is considered to be an anti-inflammatory myokine, with respect to periodontal disease and other tissues within the body, it is a pro-inflammatory cytokine. It is secreted by gingival fibroblasts, T lymphocytes and macrophages and induces the production of RANKL, thereby stimulating osteoclastic activity (4). The activity of IL-6 is mediated through binding to a receptor composed of a cognate IL-6 receptor (IL-6R) and a signal-transducing element (gp130). IL-6R is located on only select cell types (i.e. monocytes, neutrophils, T lymphocytes), whereas gp130 is located on almost all tissues within the body. Researchers have identified a soluble form of the IL-6 receptor (IL-6sR), which forms a complex with IL-6. This complex (IL-6/IL-6sR) is then able to interact with cells that express the gp130, thereby enabling activation of cells that normally would not respond to IL-6 alone (77).

MCP-1

Monocyte chemotactic protein 1 is synthesized in areas of inflammation by mononuclear cells. It is a potent chemoattractant for monocytes and cellular events associated with chemotaxis, such as calcium influx and integrin expression, and has been seen in increased levels in periodontal disease (78,79).

MER

Mer tyrosine kinase is a part of the receptor protein tyrosine kinase family TAM (TYRO3, AXL, MER), which plays an important role in innate immunity and autoimmunity. Although its effects on periodontal disease have not been studied,

MER influences the clearance of apoptotic cells, inhibition of the inflammatory response of dendritic cells and macrophages, maturation of natural killer cells, and inhibition of toll-like receptors (TLRs) and their cytokine receptor cascades (46,47).

OPG

OPG, or osteoprotegerin, is a receptor decoy for RANK Ligand. RANK, or receptor activator of nuclear kappa B ligand, is located on osteoclast progenitor cells and when bound to RANKL, induces osteoclast maturation. OPG is produced by osteoblasts in response to high osteoclastic activity and binds to RANKL, thereby preventing differentiation and maturation of the osteoclast precursors. Patients exhibiting periodontal disease have demonstrated low levels of OPG in oral fluid in comparison to periodontally healthy controls (80,81).

SDF-1

SDF-1, also referred to as CXCL12, is a chemokine and potent chemoattractant of hematopoietic cells, including lymphocytes and neutrophils. It is expressed in areas of inflammatory bone destruction (44) and has recently been implicated as a potential indicator of periodontal disease progression (45).

SOST

Sclerostin is a protein expressed by osteocytes, which shows anti-anabolic effects on bone formation. Sclerostin binds to receptors and inhibits the Wnt signaling pathway, leading to decreased bone formation (82). Relatively higher levels of

Sclerostin have been detected in gingival tissues of periodontitis patients versus healthy controls (83), and inhibition of Sclerostin has been associated with greater potential for periodontal regeneration (84).

TGF-β

TGF-β is a cytokine protein that has many biological activities. Some of its roles that are featured in periodontal disease activity include stimulating the formation of collagen, fibronectin, osteonectin and other components of the extracellular matrix (85–88). In addition, it inhibits the action of matrix metalloproteinases (i.e. collagenases) and is said to have significant effects on bone formation (88).

TRANCE

TRANCE, or TNF-related activation-induced cytokine, as another term for RANK Ligand. As described above, RANKL binds to RANK on osteoclast precursors, stimulating differentiation, maturation and proliferation of osteoclasts, thereby modulating bone regeneration and remodeling.

VCAM-1

Vascular cell adhesion molecule 1 mediates the adhesion of immune cells (i.e. lymphocytes, monocytes, basophils) to vascular endothelium and can be upregulated in response to TNF-α and IL-1 (89).

VEGF

Vascular endothelial growth factor (VEGF) plays a role in the formation of new blood vessels. It is often expressed by oxygen-deficient cells as a means to increase blood circulation and oxygenation. Its overexpression can lead to disease, with high levels of VEGF being demonstrated at periodontal disease sites when compared with healthy sites (90). In addition to its vascular effects, VEGF may play a role in osteoblast differentiation (91).

III. Materials and Methods

The University of Michigan Institutional Review Board approved the study, which was conducted from September 2011 through December 2013. All participants signed written informed consents prior to participation in the study. The details of the full investigation are provided in a previous section of this manuscript. In brief, 65 patients received periodontal flap surgery and were evaluated for one year. The primary outcome variable was the change in clinical parameters (i.e. probing depth [PD] reduction, clinical attachment level [CAL] gain, gingival recession [REC], and linear bone gain), comparing vitamin D sufficient (SUF) patients to vitamin D deficient (DEF) patients. A secondary analysis was then completed to evaluate the impact of baseline serum and gingival crevicular fluid biomarker levels on clinical outcomes (PD reduction, CAL gain, and REC) after periodontal surgery.

a. Serum and Gingival Crevicular Fluid Collection

Immediately prior to periodontal surgery, venous blood samples were obtained from each patient, centrifuged, separated into two test tubes and stored in an -80°C freezer until completion of patient enrollment. GCF was then collected from one healthy (≤4mm PD with no BOP) and one diseased site (≥6mm PD + BOP; designated as "study site") from each patient. Prior to collection, the site was isolated and dried, and supragingival plaque was removed using a sterile instrument. A methylcellulose strip (Periopaper® Gingival Fluid Collection Strips, Pro flow, Inc., Amityville, NY, USA) was inserted one millimeter into the sulcus/pocket for 10 seconds. GCF volume was immediately determined using a calibrated Periotron 6000 (Harco Electronics, Tustin, CA, USA). GCF strips were then placed in vials, labeled and stored at -80°C until processing.

b. Surgical Procedure and Follow-Up Visits

Surgical therapy was performed and consisted of either resective or regenerative therapy. Clinical parameters (PD, REC, CAL, and bleeding on probing [BOP]) were recorded at 3, 6 and 12 months visits following surgery. Radiographs of the surgical area (one periapical film, one bitewing film) were taken at 12 months.

c. Radiographic Analysis

Radiographs were standardized with respect to density, via gamma correction using the enamel surface as a reference, and angulation, via reconstruction methods. Linear measurements of the defect were made from the CEJ to the deepest portion of the defect (CEJ-ACH).

d. Assessment of Serum and GCF Biomarkers

After the last patient enrollment was complete, GCF samples were processed in the following manner. Proteins within the harvested crevicular fluid were extracted from the GCF strips using an extraction method adapted from Giannobile et al. (18), involving a series of washes and centrifugations. The elution buffer used for GCF protein extraction contained the following: 24.5 mL Phosphate Buffered Saline, pH 7.4; 125μL phenylmethylsulfonylfluoride (PMSF; Sigma Chemical, St. Louis, MO), 200mM in MeOH; 250 μL Aprotinin (Sigma Chemical, St. Louis, MO), 1mg/ml in water; 83.5μL of 30% Human Serum Albumin (Sigma Chemical, St. Louis, MO). This was made fresh and kept on wet ice throughout the entire extraction process to inhibit protease activity. Each strip was washed with 20μL and then centrifuged at 2000 rpm for 5 minutes at 4°C. This was done a total of five times to yield 100μL total volume.

The extracted GCF volume and one serum sample from each patient were

quantitatively analyzed by a Quantibody Human Cytokine Array (RayBiotech, Inc., Norcross, GA, USA) for the concentration of the following: C-reactive protein low (CRP-low), Insulin-like Growth Factor 1 (IGF-1), Interleukin 10 (IL-10), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-6 soluble receptor (IL-6 sR), Monocyte Chemoattractant Protein 1 (MCP-1), MER Tyrosine Kinase (MER), Osteoprotegerin (OPG), Stromal cell-derived factor 1 (SDF-1), Sclerostin (SOST), Transforming Growth Factor Beta 1 (TGF-β1), TNF-related activation induced cytokine (TRANCE), Vascular Cell Adhesion Molecule 1 (VCAM-1), Vascular Endothelial Growth Factor (VEGF), and C-reactive protein high (CRP-high).

e. Statistical Analysis

Of the original data set, a number of participants failed to report for the 3, 6 and 12 month time points. Because of this, their data was dropped from the analysis at each respective time point. The resulting sample size for the 3, 6, and 12 month time points were n=26, n=47, and n=51, respectively.

Log transformation of the serum and GCF levels was performed to correct the non-normality. If biomarker level exceeded the lab-determined limits, the level was reduced to the upper bound; if the level was below the lower limit, it was considered inaccurate and was replaced with a value equal to half the lower limit. The rationale for this approach reflects the assumption of a uniform distribution of all unknown values below the lower limit. With this in mind, the expected mean of

these values is equal to half of the lower limit. Linear regression analysis was used to evaluate the relationship between the log-transformed biomarker levels at baseline and the mean change in outcome variable (PD, CAL, REC, ACH-CEJ) at each time point for the study population. The outcome variables PD, CAL, and REC were assessed at 3-, 6-, and 12-month time points compared to baseline; ACH-CEJ was assessed at only the 12-month time point compared to baseline. BOP was categorized dichotomously as improvement or no improvement at each time point compared to baseline. Improvement was defined as baseline = "1" and time point$_n$ = "0". Logistic regression analysis was used to evaluate the relationship of the log-transformed biomarker levels at baseline with regard to the proportion of "improved" surgical sites at the 3-, 6-, and 12-month time points.

IV. Results

In general, clinical parameters improved after periodontal surgery at each time point. Improvement of clinical parameters over time was correlated with baseline levels of biomarkers from the serum and from GCF diseased sites ("study sites") (Table 3). Specifically, baseline concentration of IL-10 from GCF was correlated with PD reduction at 6 months ($p = 0.02$, $r^2 = 0.0908$) and linear bone gain at 12 months ($p = 0.007$, $r^2 = 0.1630$). The levels of the soluble receptor for IL-6 (IL-6sR) in the GCF of study sites were correlated with 6-month PD reduction ($p = 0.04$, $r^2 = 0.0748$) and 12-month CAL gain ($p = 0.03$, $r^2 = 0.0744$), while the same from the serum was correlated with 12-month PD reduction ($p = 0.005$, $r^2 = 0.1306$). The

remaining biomarkers failed to show significant associations with clinical parameter changes at any time point (data not shown).

V. Discussion

The results of this secondary analysis demonstrate that baseline serum and GCF biomarkers are associated with improvement in periodontal clinical parameters following periodontal surgery. Previous reports examining the relationship of biomarker levels as they relate to periodontal therapeutic outcomes are limited, with many studies evaluating levels of a specific biomarker throughout the healing course. For example, a 2014 study by Aljateeli and colleagues compared periodontal surgical therapy, with (control group) and without (test group) prior scaling and root planing (66). In addition to evaluating clinical parameter changes over time, these researchers sought to investigate changes in biomarker levels over time within groups and between groups. They evaluated a number of GCF biomarkers (IL-1β, IL-6, MMP-8, MMP-9 and VEGF) at 1, 2, 4, and 12 weeks after periodontal surgery and found no differences in biomarker levels between groups for any time point (66). The focus of that investigation, however, was to compare biomarker levels over time, as opposed to looking at baseline levels and their impact on treatment outcomes. In a 2012 study, however, Lee and colleagues did just that. This group evaluated salivary biomarkers during an experimental gingivitis study and found that baseline levels of salivary IL-6 and IL-8 demonstrated the best ability to discriminate between participants exhibiting a high inflammatory response or

low inflammatory response during the experimental gingivitis period, as measured by gingival index (GI) (65). Additionally, this group found that high levels of MMP-1 and IL-6 at baseline showed the strongest ability to predict a high inflammatory response during the experimental gingivitis period. The focus of the aforementioned study is similar to the present study in that baseline biomarker levels were compared to clinical outcomes; however, Lee's study instigated disease (experimental gingivitis), whereas our study eliminated disease (periodontal therapy), a fact that precludes outcome comparison because of the difference in immune response of these two processes. The relationship of baseline biomarker levels in response to periodontal therapy was recently reported, however, by Leppilahti and colleagues. They found that in smokers, high levels of baseline GCF levels of MMP-8 were related to weaker treatment responses (60). Although impressive, this trend was not observed in non-smokers, thereby limiting the applicability of their findings.

Our study found that two biomarkers (IL-6sR and IL-10) showed significant associations with PD reduction, CAL gain and radiographic linear bone gain. IL-10 is an anti-inflammatory cytokine, whereas IL-6-related entities are typically involved in promoting inflammatory cascades. IL-10 levels at baseline were significantly positively correlated to clinical parameter (PD, linear bone level) improvement at 6 and 12 months. This cytokine has been associated with lower disease severity in chronic periodontitis (92) and has demonstrated an inhibitory effect on matrix metalloproteinases through the upregulation of tissue inhibitors of matrix

metalloproteinases (TIMPs) (93). This inhibition of extracellular matrix degradation enzymes could enable undisturbed collagen formation during periodontal healing, leading to increased PD reduction. Additionally, a positive association of IL-10 levels to radiographic bone gain may be explained through IL-10's stimulating effects on the production of OPG (71,94). Greater OPG production would, in effect, decrease the amount of RANK/RANKL complex, thereby limiting osteoclastic activity. Both GCF and serum levels of IL-6sR were also correlated to the level of clinical improvement at 6 and 12 months. The IL-6sR is essentially an IL-6 agonist, enabling a biologic response from cells that normally would not respond to IL-6 through a process called trans-signaling (95). It is interesting to note that increasing levels of IL-6sR were correlated to periodontal healing at the same time points at which IL-10 levels were correlated. Although IL-6 is traditionally a pro-inflammatory cytokine, in certain tissue cells, it has been shown to be anti-inflammatory through stimulation of IL-10 (96). It is plausible that increased levels of IL-6sR and its agonistic effect on IL-6 may trigger an increased production of anti-inflammatory cytokines such as IL-10, thereby suppressing inflammation and promoting healing.

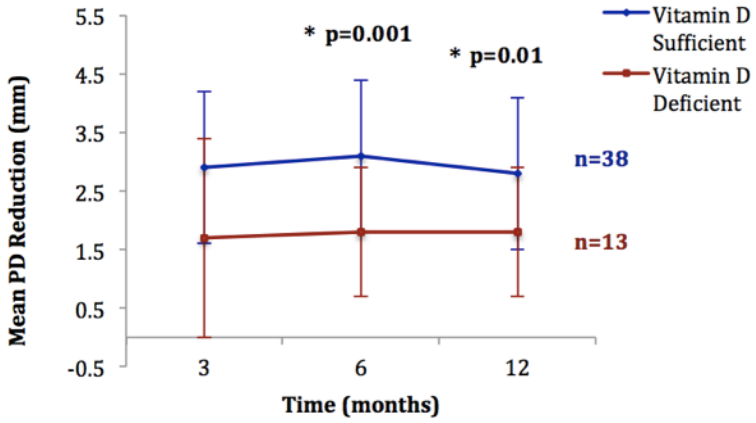
VI. Conclusion

The results of this secondary analysis suggest a weak, but significant positive relationship between serum and gingival crevicular fluid biomarkers and clinical

outcomes of periodontal surgery. These relationships may be helpful in predicting patient response patterns after surgical therapy. However, our study sample was powered to detect differences in clinical parameters with respect to vitamin D levels and not biomarkers, therefore some associations may have been missed due to inadequate powering. In the future, large-scale, appropriately-powered clinical investigations would be necessary to further examine this relationship.

Figure 1. Comparison of clinical parameter improvement over time between Vitamin D sufficient and deficient groups.

(a)



(b)

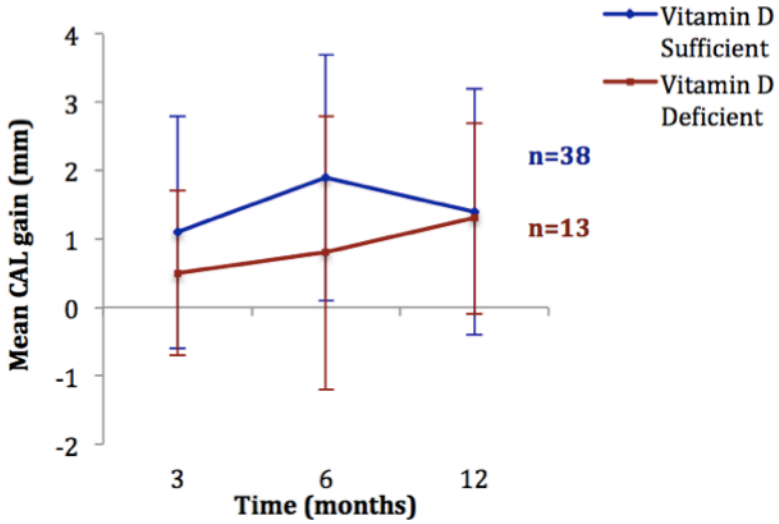
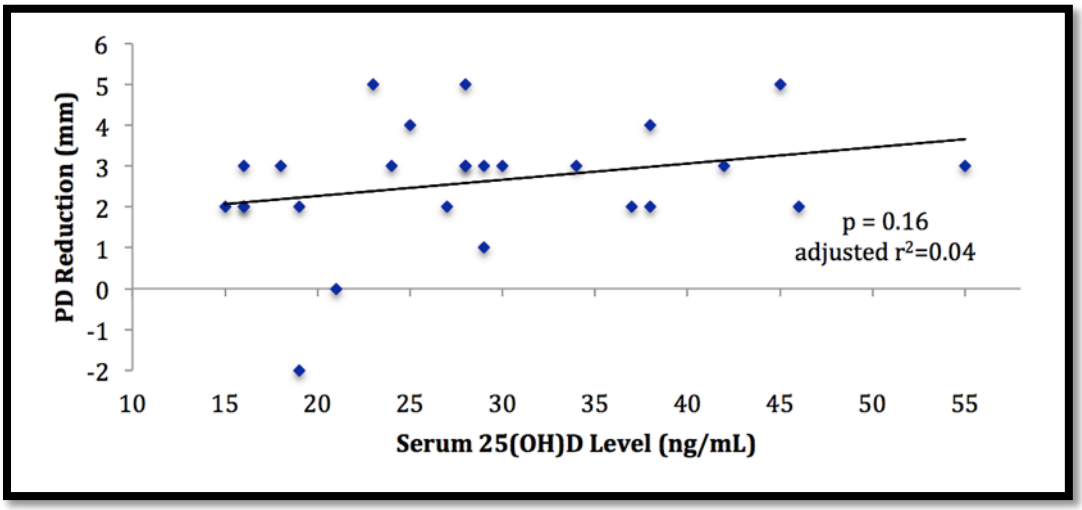


Figure 2. Scatter plots demonstrating change in clinical parameter (i.e. PD and CAL) versus serum 25(OH)D level for each time point.

3 months

(a)



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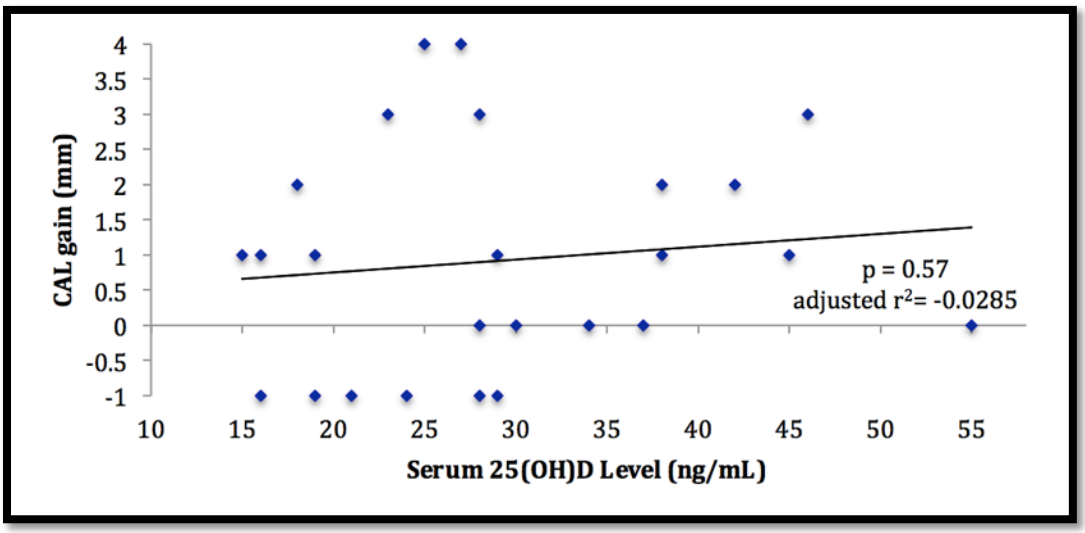
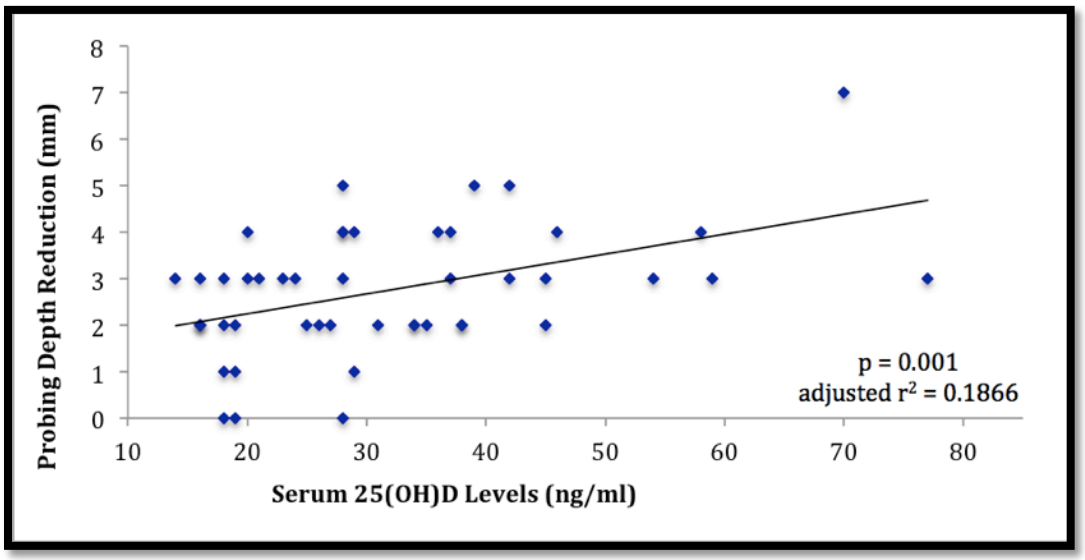
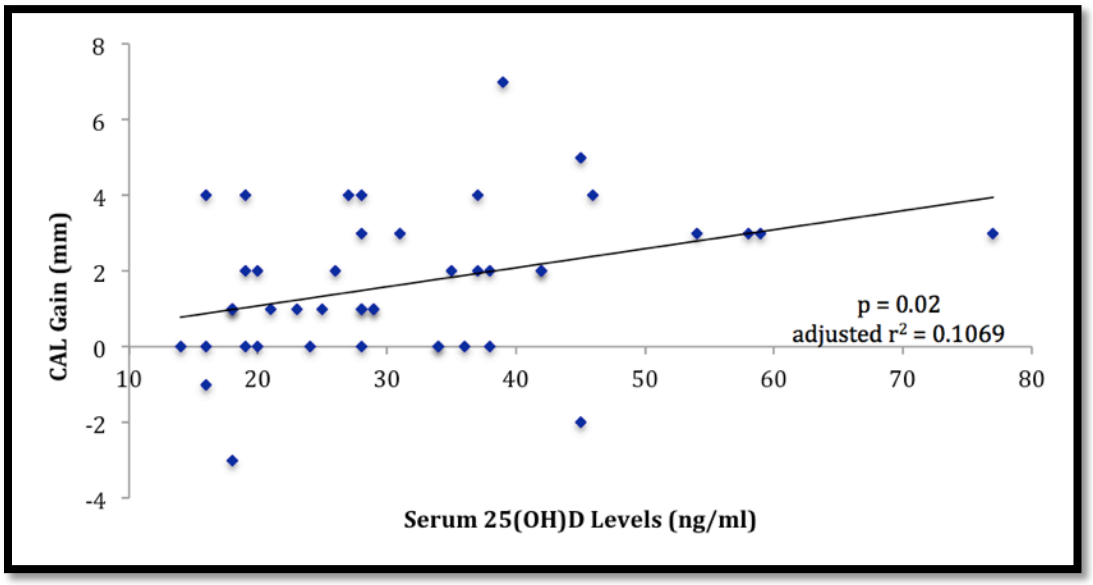


6 months

(c)

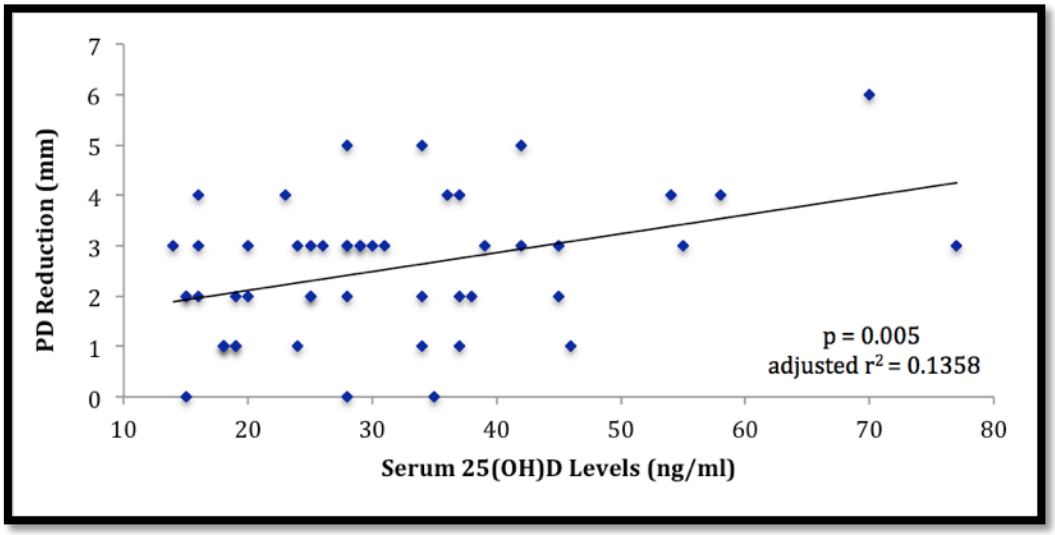

Probing Depth Reduction (mm)
8
7
6
5
4
3
2
1
0
10
20
30
40
50
60
70
80
Serum 25(OH)D Levels (ng/ml)
p = 0.001
adjusted r² = 0.1866

(d)


CAL Gain (mm)
8
6
4
2
0
-2
-4
10
20
30
40
50
60
70
80
Serum 25(OH)D Levels (ng/ml)
p = 0.02
adjusted r² = 0.1069

12 months

(e)


PD Reduction (mm)
Serum 25(OH)D Levels (ng/ml)
p = 0.005
adjusted r² = 0.1358

(f)

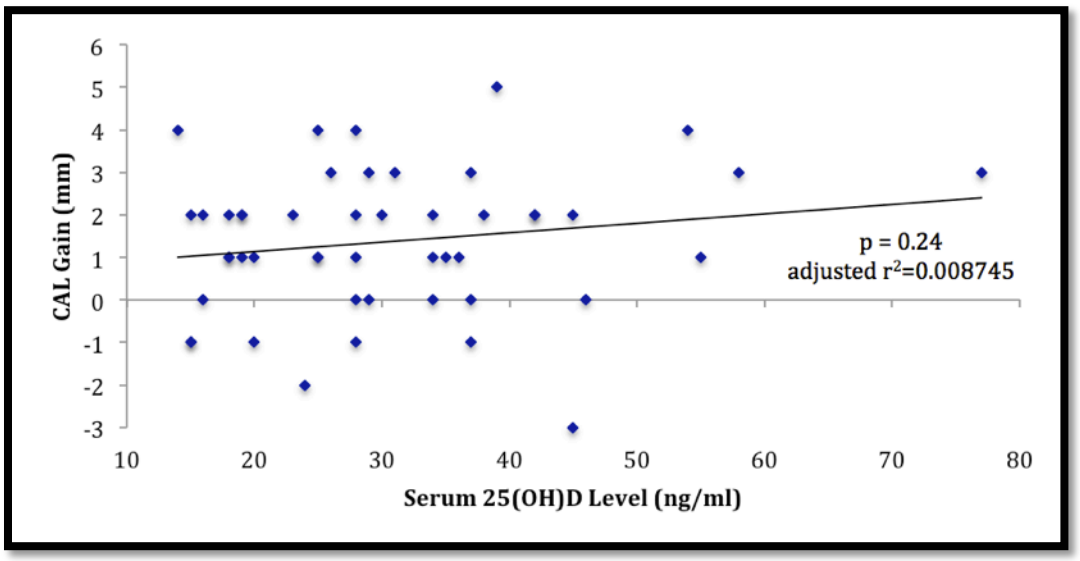
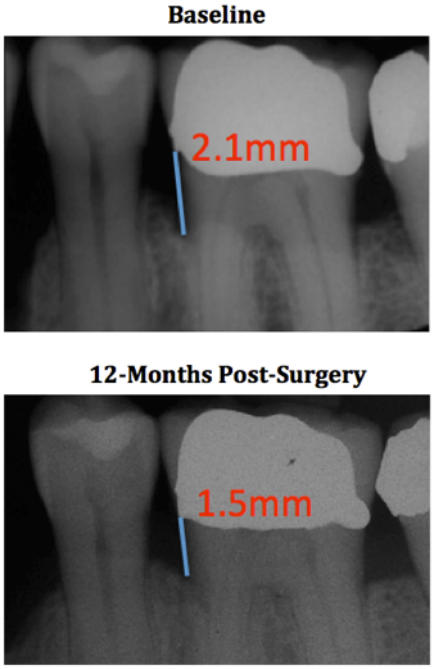

CAL Gain (mm)
Serum 25(OH)D Level (ng/ml)
p = 0.24
adjusted r²=0.008745

Figure 3. (a) Representative radiograph showing linear bone gain from baseline to 12 months post-surgical therapy. (b) scatter plot demonstrating linear bone gain (ACH-CEJ change) at 12 months versus serum 25(OH)D level at baseline.

(a)



(b)

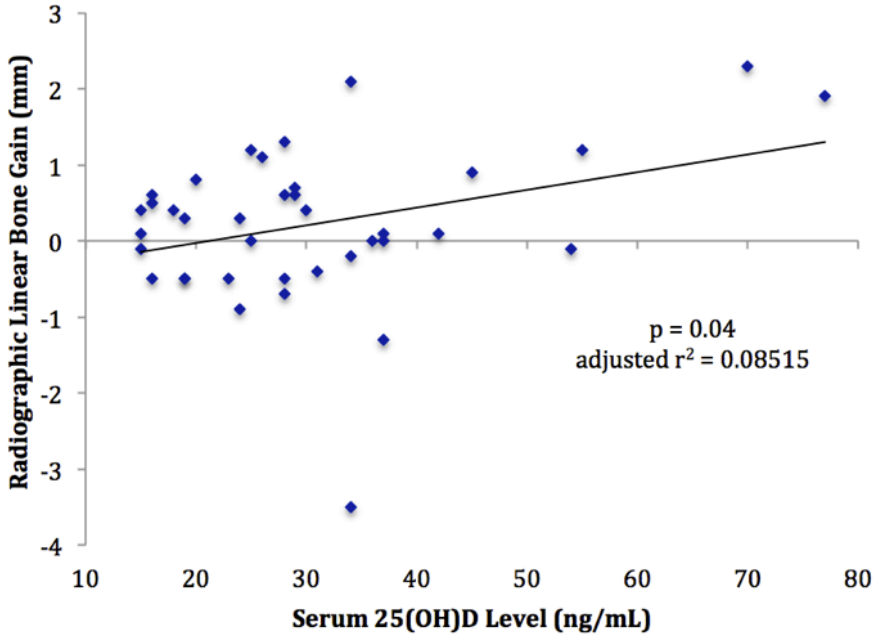


Table 1. Baseline Demographics and Mean Examination Time of Vitamin D Sufficient and Deficient Groups.

Characteristic	Vitamin D Deficient (N=18, [27.7%])	Vitamin D Sufficient (N=47, [72.3%])	
Age (mean, yrs)	54.1 ± 10.6	59.8 ± 9.9	NSD
Gender			
Male	10 (55.6%)	29 (61.7%)	NSD
Female	8 (44.4%)	18 (38.3%)	NSD
Race			
Caucasian	8 (44.4%)	41 (87.2%)	NSD
African-American	7 (38.9%)	3 (6.4%)	NSD
Asian	2 (11.1%)	--	NSD
Hispanic	--	2 (4.3%)	NSD
Other	1 (5.6%)	1 (2.1%)	NSD
25-Hydroxyvitamin D (mean, ng/mL)	16.7 ± 1.8	35.1 ± 12.9	$p<0.05$
Smoking Status			
Current smoker	4 (22.2%)	12 (25.5%)	NSD
Former/Never smoker	14 (77.8%)	35 (74.5%)	NSD
Clinical Measurements			
Probing Depth	6.5 ± 1.2	6.8 ± 1.0	NSD
Recession	0.8 ± 2.1	0.3 ± 1.8	NSD
Clinical Attachment Level	7.4 ± 2.2	7.1 ± 2.0	NSD
Radiographic Defect Depth	2.8 ± 1.0	2.6 ± 1.3	NSD
Mean Examination Time			
3-Month	3.3 ± 0.7	3.5 ± 0.6	NSD
6-Month	6.7 ± 1.2	6.7 ± 1.1	NSD
12-Month	12.5 ± 1.0	12.8 ± 1.3	NSD

Table 2. GCF Biomarkers Related to Serum 25(OH)D Level.
^values below detectable limit.

Relationship of GCF Biomarker with Serum 25(OH)D Concentration		
Biomarker	p-value	adjusted r^2
CRP (low)	0.78	-0.0147
IGF-1	0.09	0.0293
IL-10	0.62	-0.0120
IL-1β	0.82	-0.0151
IL-6	0.69	-0.0133
IL-6sR	0.39	-0.0038
MCP-1	0.97	-0.0159
MER	<0.001**	0.4918
OPG	0.23	0.0069
SDF-1	<0.001**	0.4884
SOST	^	^
TGF-β	0.61	-0.0117
TRANCE	0.89	-0.0156
VCAM-1	0.13	0.0214
VEGF	0.80	-0.0148
CRP (high)	0.73	-0.0140

Table 3. Biomarkers Related to Clinical Outcomes

Relationship of Biomarkers with Clinical Outcomes			
Clinical Parameter	**Biomarker**	**p-value**	**Adjusted r^2**
GCF Biomarkers			
6-month PD Reduction	**IL-10**	0.02	0.0908
	IL-6sR	0.04	0.0748
12-month CAL Gain	**IL-6sR**	0.03	0.0744
12-month CEJ-ACH	**IL-10**	0.007	0.163
Serum Biomarkers			
12-month PD Reduction	**IL-6sR**	0.005	0.1306

APPENDIX 1. Informed Consent Form

UNIVERSITY OF MICHIGAN
CONSENT TO BE PART OF A RESEARCH STUDY

INFORMATION ABOUT THIS FORM

You may be eligible to take part in a research study. This form gives you important information about the study. It describes the purpose of the study, and the risks and possible benefits of participating in the study.

Please take time to review this information carefully. After you have finished, you should talk to the researchers about the study and ask them any questions you have. You may also wish to talk to others (for example, your friends, family, or other doctors) about your participation in this study. If you decide to take part in the study, you will be asked to sign this form. Before you sign this form, be sure you understand what the study is about, including the risks and possible benefits to you.

1. GENERAL INFORMATION ABOUT THIS STUDY AND THE RESEARCHERS

1.1 Study title:

The influence of vitamin D status on periodontal surgery outcomes: A prospective analysis.

1.2 Company or agency sponsoring the study:

Advance Grant Crosby Award.

1.3 Names, degrees, and affiliations of the researchers conducting the study:

Principal Investigator: Jill Bashutski, DDS, MS; Clinical Assistant Professor of Periodontics
Co-Investigator: Laurie K. McCauley, DDS, PhD; Professor, School of Dentistry
Co-Investigator: Ebone' Jordan, DDS; Graduate Periodontics Resident, School of Dentistry

2. PURPOSE OF THIS STUDY

2.1 Study purpose:

Vitamin D is important for calcium regulation and proper immune function, which is important for good bone healing. The purpose of this study is to compare clinical outcomes of periodontal surgery between vitamin D deficient and sufficient patients. This research study is being done to learn what effect of

different vitamin D levels will have on clinical outcomes for patients receiving periodontal surgery.

3. INFORMATION ABOUT STUDY PARTICIPANTS (SUBJECTS)

Taking part in this study is completely **voluntary**. You do not have to participate if you don't want to. You may also leave the study at any time. If you leave the study before it is finished, there will be no penalty to you, and you will not lose any benefits to which you are otherwise entitled.

3.1 Who can take part in this study?

Healthy males and females, ages 30 to 85, with a diagnosis of a localized or generalized periodontal disease and a need for periodontal surgery can take part in this study. If you take part in this study, you must be able and willing to follow study procedures and instructions. In addition, you must have read, understood and signed the informed consent form. Female patients who are pregnant or breast-feeding will not be able to participate in this study.

Patients who are heavy smokers (>1 pack per day), uncontrolled diabetes, or who are unable to attend regular follow up examinations are not eligible to participate in this study.

It is very important that you provide complete and accurate information about your health condition/history in order to ensure that you are safe and appropriate candidates for participation.

3.2 How many people (subjects) are expected to take part in this study?

65 people are expected to take part in this study.

4. INFORMATION ABOUT STUDY PARTICIPATION

4.1 What will happen to me in this study?

Non-experimental procedures: You are a candidate for periodontal surgery. This surgery would be performed on you whether you participate in the study or not.

You will receive a full dental examination, including having x-rays taken as necessary.

All patients will receive periodontal surgery as was treatment planned by your health care provider.

You will return for a **post-operative visit** 1-2 weeks after surgery. **Follow-up visits** to assess if adequate healing has occurred will take place 3, 6, and 12

months after surgery.

These procedures are the same regardless of whether you were in a study or not.

Experimental procedures: The following procedures will be performed only as part of the study, and not as part of the standard of care for periodontal surgery. Intra-oral photographs may be taken at all appointments. These photographs will only capture images of the inside of your mouth and will not be identifiable to non-dental personnel.

At the **surgery appointment** the Investigators will thoroughly explain the study and discuss study details with you. If you agree to participate in the study, you will have a blood draw. Up to 5cc of blood will be drawn (about 1 teaspoon).

4.2 How much of my time will be needed to take part in this study?

Your participation will require about 30 minutes on the day of your surgical appointment. In addition, we will be using the clinical information obtained from your follow up appointments over the next 12 months, although these procedures would be done whether or not you were in the study.

4.3 When will my participation in the study be over?

Your participation in the study will terminate the day of your periodontal surgery.

5. INFORMATION ABOUT RISKS AND BENEFITS

5.1 What risks will I face by taking part in the study? What will the researchers do to protect me against these risks?

Having a routine blood draw is a well-established procedure that you could have done whether you were in this study or not. The researchers will do their best to perform the blood draw in a manner that limits the risk of side effects. Any side effects will be managed by the Investigator and other clinical care providers.

Some side effects associated with blood draws include pain, bruising, redness, and/or swelling at the blood draw site. Rarely, an infection can occur after having blood taken.

As with any research study, there may be additional risks that are unknown or unexpected.

5.2 What happens if I get hurt, become sick, or have other problems as a result of this research?

The researchers have taken steps to minimize the risks of this study. Even so, you may still have problems or side effects, even when the researchers are careful to avoid them. Please tell the researchers listed in Section 10 about any

injuries, side effects, or other problems that you have during this study. You should also tell your regular doctors**.**

5.3 If I take part in this study, can I also participate in other studies?

Being in more than one research study at the same time, or even at different times, may increase the risks to you. It may also affect the results of the studies. You should not take part in more than one study without approval from the researchers involved in each study.

5.4 How could I benefit if I take part in this study? How could others benefit?

You may not receive any personal benefits from being in this study. However, you will receive $40 compensation for your time.

Possible benefits of the research for society (or for future patients with periodontal disease) include understanding the impact of vitamin D deficiency on healing in the oral cavity. The results of this study may contribute to our understanding of how healing occurs in the oral cavity and may change in the way millions of patients are treated, resulting in a significant improvement in their quality of life.

5.5 Will the researchers tell me if they learn of new information that could change my willingness to stay in this study?

Yes, the researchers will tell you if they learn of important new information that may change your willingness to stay in this study. If new information is provided to you after you have joined the study, it is possible that you may be asked to sign a new consent form that includes the new information.

6. OTHER OPTIONS

6.1 If I decide not to take part in this study, what other options do I have?

Your alternative option is to continue with your dental care plan without the blood draw associated with this research project.

7. ENDING THE STUDY

7.1 If I want to stop participating in the study, what should I do?

You are free to leave the study at any time. If you leave the study before it is finished, there will be no penalty to you. You will not lose any benefits to which you may otherwise be entitled. If you choose to tell the researchers why you are leaving the study, your reasons for leaving may be kept as part of the study

record. If you decide to leave the study before it is finished, please tell one of the persons listed in Section 10 “Contact Information” (below).

7.2 Could there be any harm to me if I decide to leave the study before it is finished?

No, although we may contact you by telephone to ask if you are having any adverse events as a result of your participation in the study.

7.3 Could the researchers take me out of the study even if I want to continue to participate?

Yes. There are many reasons why the researchers may need to end your participation in the study. Some examples are:

- ✓ The researcher believes that it is not in your best interest to stay in the study.
- ✓ You become ineligible to participate.
- ✓ Your condition changes and you need treatment that is not allowed while you are taking part in the study.
- ✓ You do not follow instructions from the researchers.
- ✓ The study is suspended or canceled.

8. FINANCIAL INFORMATION

8.1 Who will pay for the costs of the study? Will I or my health plan be billed for any costs of the study?

There will be very little cost to participating in this study. All procedures related to the study will be performed at no cost to you. Ask the researcher if you have any questions about bills, fees, or other costs related to this study.

The study will pay for research-related items or services that are provided only because you are in the study. If you are not sure what these are, see Section 4.1 above or ask the researchers for a list. If you get a bill you think is wrong, call the researchers’ number listed in section 10.1.

You or your health plan will pay for all the things you would have paid for even if you were not in the study, like:

- Health care given during the study as part of your regular care
- Items or services needed to give you study drugs or devices
- Monitoring for side effects or other problems
- Treatment of complications
- Deductibles or co-pays for these items or services.

If you do not have a health plan, or if you think your health plan may not cover these costs during the study, please talk to the researchers listed in Section 10 below or call your health plan's **medical reviewer.**

By signing this form, you do not give up your right to seek payment if you are harmed as a result of being in this study.

The study will pay for research-related items or services that are provided only because you are in the study. If you are not sure what these are, see Section 4.1 above or ask the researchers for a list. If you get a bill you think is wrong, call the researchers' number listed in section 10.1.

You or your health plan will pay for all the things you would have paid for even if you were not in the study, like:

- Health care given during the study as part of your regular care
- Items or services needed to give you study drugs or devices
- Monitoring for side effects or other problems
- Deductibles or co-pays for these items or services.

If you do not have a health plan, or if you think your health plan may not cover these costs during the study, please talk to the researchers listed in Section 10 below or call your health plan's medical reviewer**.**

By signing this form, you do not give up your right to seek payment if you are harmed as a result of being in this study.

8.2 Will I be paid or given anything for taking part in this study?

Yes. You will receive $40 cash immediately after the blood draw.

8.3 Who could profit or financially benefit from the study results?

The company whose product is being studied: vitamin D (multiple manufacturers).

9. CONFIDENTIALITY OF SUBJECT RECORDS AND AUTHORIZATION TO RELEASE YOUR PROTECTED HEALTH INFORMATION

The information below describes how your privacy and the confidentiality of your research records will be protected in this study.

9.1 How will the researchers protect my privacy?

Research records will be kept in a separate research file that does not include names, registration numbers, or other information that is likely to allow someone other than the researchers to link the information to you. Your research

information will be stored in a locked cabinet and will not be made a part of your regular medical record. However, if the researcher orders any tests, the order and results may become part of your regular medical record.

9.2 What information about me could be seen by the researchers or by other people? Why? Who might see it?

Signing this form gives the researchers your permission to obtain, use, and share information about you for this study, and is required in order for you to take part in the study. Information about you may be obtained from any hospital, doctor, and other health care provider involved in your care, including:

- Hospital/doctor's office records, including test results (X-rays, blood tests, urine tests, etc.)
- All records relating to your condition, the treatment you have received, and your response to the treatment
- Billing information

There are many reasons why information about you may be used or seen by the researchers or others during or after this study. Examples include:

- The researchers may need the information to make sure you can take part in the study.
- The researchers may need the information to check your test results or look for side effects.
- University, Food and Drug Administration (FDA), and/or other government officials may need the information to make sure that the study is done in a safe and proper manner.
- Study sponsors or funders, or safety monitors or committees, may need the information to:
    - Make sure the study is done safely and properly
    - Learn more about side effects
    - Analyze the results of the study
- Insurance companies or other organizations may need the information in order to pay your medical bills or other costs of your participation in the study.
- The researchers may need to use the information to create a databank of information about your condition or its treatment.
- Information about your study participation may be included in your regular UMHS medical record.
- If you receive any payments for taking part in this study, the University of Michigan accounting department may need your name, address, social security number, payment amount, and related information for tax reporting purposes.
- Federal or State law may require the study team to give information to government agencies. For example, to prevent harm to you or others, or for public health reasons.

The results of this study could be published in an article, but would not include any information that would let others know who you are.

9.3 What happens to information about me after the study is over or if I cancel my permission?

As a rule, the researchers will not continue to use or disclose information about you, but will keep it secure until it is destroyed. Sometimes, it may be necessary for information about you to continue to be used or disclosed, even after you have canceled your permission or the study is over. Examples of reasons for this include:

- To avoid losing study results that have already included your information
- To provide limited information for research, education, or other activities (This information would not include your name, social security number, or anything else that could let others know who you are.)
- To help University and government officials make sure that the study was conducted properly

As long as your information is kept within the University of Michigan Health System, it is protected by the Health System's privacy policies. For more information about these policies, ask for a copy of the University of Michigan Notice of Privacy Practices. This information is also available on the web at http://www.med.umich.edu/hipaa/npp.htm. Note that once your information has been shared with others as described under Question 9.2, it may no longer be protected by the privacy regulations of the federal Health Insurance Portability and Accountability Act of 1996 (HIPAA).

9.4 When does my permission expire?

Your permission expires at the end of the study, unless you cancel it sooner. You may cancel your permission at any time by writing to the researchers listed in Section 10 "Contact Information" (below).

10. CONTACT INFORMATION

10.1 Who can I contact about this study?

Please contact the researchers listed below to:

- Obtain more information about the study
- Ask a question about the study procedures or treatments
- Talk about study-related costs to you or your health plan
- Report an illness, injury, or other problem (you may also need to tell your regular doctors)
- Leave the study before it is finished

- Express a concern about the study

Principal Investigator: Jill Bashutski, DDS, MS
Mailing Address: University of Michigan School of Dentistry, 3349 Dent, Box 1078
Ann Arbor, Michigan 48109-1078
Telephone: 734-763-3759

You may also express a concern about a study by contacting the Institutional Review Board listed below, or by calling the University of Michigan Compliance Help Line at 1-888-296-2481.

University of Michigan Medical School Institutional Review Board (IRBMED)
2800 Plymouth Road
Building 200, Room 2086
Ann Arbor, MI 48109-2800
Telephone: 734-763-4768 (For International Studies: US Country Code: 001)
Fax: 734-763-1234
e-mail: irbmed@umich.edu

If you are concerned about a possible violation of your privacy, contact the University of Michigan Health System Privacy Officer at 1-888-296-2481.

When you call or write about a concern, please provide as much information as possible, including the name of the researcher, the IRBMED number (at the top of this form), and details about the problem. This will help University officials to look into your concern. When reporting a concern, you do not have to give your name unless you want to.

11. RECORD OF INFORMATION PROVIDED

11.1 What documents will be given to me?

Your signature in the next section means that you have received copies of all of the following documents:

- This "Consent to be Part of a Research Study" document. (*Note: In addition to the copy you receive, copies of this document will be stored in a separate confidential research file and may be entered into your regular University of Michigan medical record.)*
- Other (specify):_____

12. SIGNATURES

Research Subject:

I understand the information printed on this form. I have discussed this study, its risks and potential benefits, and my other choices with _____. My questions so far have been answered. I understand that if I have more questions or concerns about the study or my participation as a research subject, I may contact one of the people listed in Section 10 (above). I understand that I will receive a copy of this form at the time I sign it and later upon request. I understand that if my ability to consent for myself changes, either I or my legal representative may be asked to re-consent prior to my continued participation in this study.

Signature of Subject: _____ *Date:* _____

Name (Print legal name): _____

*Patient ID:*_____ *Date of Birth:* _____

Principal Investigator (or Designee):

I have given this research subject (or his/her legally authorized representative, if applicable) information about this study that I believe is accurate and complete. The subject has indicated that he or she understands the nature of the study and the risks and benefits of participating.

Name: _____ *Title:* _____

Signature: _____ *Date of Signature:* _____

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