SALIVARY INFLAMMATORY BIOMARKERS IN A STENT-INDUCED BIOFILM OVERGROWTH MODEL

Michael Stella

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Approved by:

Dr. Steven Offenbacher

Dr. Silvana Barros

Dr. Thiago Morelli

ABSTRACT

MICHAEL STELLA: Salivary Inflammatory Biomarkers in a Stent-Induced Biofilm Overgrowth Model
(Under the direction of Dr. Steven Offenbacher)

Introduction: Salivary diagnostics is an emerging field that has promise for improving understanding, diagnosis, and treatment of periodontitis. Recent studies showed that inflammatory biomarker levels present in saliva have the potential to discriminate between disease states and predict disease progression.

Objective: To determine whether baseline salivary inflammatory biomarkers could discriminate between different clinical levels of disease and/or predict clinical progression over a 3-week stent-induced biofilm overgrowth (SIBO) period.

Methods: 165 subjects ranging from healthy to periodontitis were recruited, 33 or 34 subjects per group: Healthy (BGI-H), Gingivitis (BGI-G), Mild (BGI-P1), Moderate (BGI-P2), and Severe (BGI-P3) Periodontitis. Subjects wore stents to prevent plaque removal during brushing over one maxillary and one mandibular posterior dental sextant for 21 days. Clinical periodontal parameters and unstimulated saliva samples were collected at screening, baseline, and each week during biofilm overgrowth induction. Saliva samples were assessed for levels of selected inflammatory biomarkers.

Results: Higher salivary levels of IL-1β, MMP-3, MMP-8, MMP-9, and NGAL were found in diseased groups compared to healthy at baseline. Higher IL-1ra was found in healthy

subjects at baseline. In addition, MMP-1, TIMP-1, TIMP-2, and TIMP-4 levels increased during induction across all subject groups.

Conclusion: Salivary inflammatory biomarkers differ at baseline by periodontal status and show changes in the immunoregulators of host response during an experimental disease progression model.

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LIST OF ABBREVIATIONS

A.a. = Aggregatibacter actinomycetemcomitans

BOP = bleeding on probing

CAL = clinical attachment loss

CEJ = cementoenamel junction

CP = chronic periodontitis

CRP = C-reactive protein

GCF = gingival crevicular fluid

GM = gingival margin

IFNγ = interferon-gamma

IL = interleukin

MIP = macrophage inhibitory protein

MMP = matrix metalloproteinase

NGAL = neutrophil gelatinase-associated lipocalin

OPG = osteoprotegerin

PD = probing depth

PGE_2 = Prostaglandin E_2

-ra = receptor antagonist

SIBO = stent-induced biofilm overgrowth

SRP = scaling and root planing

TIMP = tissue inhibitor of matrix metalloproteinase

TNFα = tumor necrosis factor alpha

CHAPTER 1. INTRODUCTION

Background

Periodontal diseases are a heterogeneous group of inflammatory conditions that affect tooth-supporting tissues[1]. These diseases are broadly grouped into two major categories: gingivitis and periodontitis. Gingivitis is reversible and limited to superficial, gingival tissues, whereas periodontitis is non-reversible and features tissue destruction extending to deeper tissues such as periodontal ligament and alveolar bone. A recent study estimated the prevalence of periodontitis in the US greater than 47% for those aged over 30 and 70% for subjects over 65 years old[2]. Clinical measurements reflect history of disease and have poor reliability for predicting disease activity and progression[3]. Furthermore, differentiating periodontitis from gingivitis for the purpose of treatment recommendations has proven difficult. Ideally, inexpensive, non-invasive tests should allow detection and characterization of disease state, aiding in treatment of an under-diagnosed, under-treated disease.

Periodontitis is known to be a bacterial biofilm and host-response mediated disease. It is characterized by periods of activity and remission, which complicates diagnosis and adequate prognosis. As dental plaque collects and matures, its initial gram-positive, aerobic coccal colonizers become more numerous. After several days, filamentous and rod forms appear with a mild leukocytic immune response. At 4-10 days, neutrophils enter the tissues resulting in the intensification of gingivitis with edema and spontaneous bleeding, increased probing pocket depths, and a switch to a plaque mainly colonized by vibrio and spirochetal species.

As the immune response intensifies, macrophages and neutrophils degranulate releasing toxic compounds, causing apoptosis and eventually necrosis. The pocket lining ulcerates, and the bacteria invade the inner soft tissues, eventually reaching the vascular bed and disseminating throughout the body. Monocytes activated by the bacterial lipopolysaccharide (LPS) secrete inflammatory mediators such as prostaglandin E_2 (PGE_2), thromboxane B_2, interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNFα). Macrophages and T-helper cells secrete receptor activator of nuclear factor –kappa B ligand (RANKL), leading to an increased ratio of RANKL to osteoprotegerin (OPG) and increased binding of RANKL to RANK on macrophage precursor cells. This promotes differentiation and activation of the precursor cells into osteoclasts, which begin to resorb the adjacent alveolar bone[4]. Simultaneously, matrix metalloproteinases (MMPs) are activated and break down proteins in the connective tissue surrounding the lesion. In part, the ratio of these MMPs to tissue inhibitors of matrix metalloproteinases (TIMPs) determines the relative speed and magnitude of tissue destruction[4-6]. The importance of the MMP/TIMP ratio is underscored by studies demonstrating tetracycline-like compounds that block MMPs reduce bone and attachment loss[7].

Data derived from animal models indicate that gingivitis is a precursor to periodontitis[8]. However, human population studies clearly demonstrate that not all individuals with gingivitis linearly progress to periodontitis [9, 10]. The host response of an individual is thought to be the major determinant of the onset and progression of periodontitis. In a landmark study of Sri Lankan tea workers with virtually no oral hygiene, Löe *et al*. evaluated periodontal disease progression and found that 8% of subjects experienced rapid tooth loss, 82% moderate loss, and 10% no appreciable periodontitis[9]. Hirschfield and Wasserman

categorized their patients by response to therapy, as determined by number of teeth lost over more than 15 years of maintenance (average 22, median 20 years), finding that 83% of patients were "well-maintained," losing three or fewer teeth, but that 12% of patients' status trended "downhill," losing 4-9 teeth, and that 4% were "extreme downhill" cases, losing 10-23 teeth despite ongoing treatment[11]. Thus, there is a gradient of host response, with some individuals exhibiting hyperresponsive, average, or hyporesponsive phenotypes.

Furthermore, data from cohort studies indicate that a subset of individuals appear to exhibit more pronounced local inflammatory changes to the etiologic plaque biofilm as compared to others who fail to respond[12-14]. These differences in host susceptibility to periodontal disease may be traced to inter-individual differences in environmental exposures or genetic risk factors[15]. Accordingly, evidence from studies with human twins indicates that approximately 50 percent of the inter-individual variability in the expression of periodontal disease may be explained by genetic factors[16]. Observational studies suggest that risk for advanced periodontitis may be elevated among humans with certain genetic biomarkers or polymorphisms (e.g., Interleukin-1B gene)[17, 18]. However, the risk conferred by these markers is not consistent among different populations[18] and may be modified by environment and behaviors such as smoking[19]. Genome-wide association studies are identifying new candidate loci for investigation and show great promise[20].

Papapanou *et al.* have conducted a pilot study of gene expression signatures in gingival tissues obtained from two subsets of periodontitis patients, chronic versus aggressive[21]. The results from this study indicate that there is good overall intra-individual agreement in gene expression. However, no significant difference in gene expression signatures was noted between the two periodontitis cohorts. Nevertheless, the group concluded that gene

expression profiling techniques might be useful in the identification of different subclasses of periodontal diseases and for distinguishing between health and disease. More recently, the same group[22] characterized differential mRNA expression corresponding to pathogen colonization and experimental gingivitis demonstrating upregulation of immune cell response pathways. Cumulatively, these studies provide evidence for the potential utility of using biochemical mediators for periodontal diagnosis and activity characterization.

In an *in-vitro* study, Molvig *et al*. showed that peripheral monocyte secretion of IL-1, TNFα, and PGE_2 when stimulated by LPS was variable between subjects but generally stable, and that there was an association between low-responder status and human leukocyte antigen (HLA) type, establishing a possible genotype/phenotype linkage[23]. In 1992, Pociot *et al*. found a single nucleotide polymorphism (SNP) in the IL-1B gene that led to four-fold greater IL-1β production[24]. When combined with another allelic variant of IL-1A, this SNP conferred 18.9-fold greater risk for non-smoking subjects to have severe disease. Identification of hyper- and hypo-responsive individuals could allow establishment of treatment regimens based on risk profiles and improve patient outcomes. Genotype studies are expensive and slow, and are but an indirect predictor of the outcome in question. Therefore, measurement of the inflammatory mediators themselves is likely to prove more robust for predicting current immediate risk of progression. These mediators, in addition to bacteria and bacterial products such as LPS, can be found in gingival crevicular fluid (GCF). GCF has been widely analyzed to study disease status and disease progression, since it is relatively easy and painless to collect. In addition, GCF is site-specific and requires multiple samples for a reliable subject-level analysis. Conversely, saliva provides a combined, whole-mouth sample for analyses and includes mediators found in the GCF, which pass into the

saliva. Recent advances in assay methodologies have overcome the assay problems associated with measuring low levels of inflammatory mediators in a complex, viscous glycosaminoglycan-rich bodily fluid.

Salivary diagnostics

The potential application of new lab-on-chip methods for the saliva-based diagnosis of periodontal disease represents an exciting new opportunity that has the potential to move the assay from the laboratory to chairside. Combining fast turnaround with non-invasive sampling will enable clinicians to stratify patients by risk and allocate treatment accordingly. Among potential markers for periodontitis diagnosis and disease progression, interleukins, MMPs, and their modifiers have shown promising results.

IL-1β, a marker of destructive inflammation, is produced by macrophages in response to bacterial insult. Along with TNFα, it promotes diapedesis and acute phase response, and acts to upregulate inflammatory pathways[25]. Its expression is well known to be upregulated in chronic periodontitis, and application of exogenous IL-1β in animal models enhances bone loss[26]. Studies have found increased salivary levels of IL-1β and MMP-8 in chronic periodontitis patients[27, 28], and IL-1β increased in aggressive and chronic periodontitis compared to healthy patients, and correlated with clinical measures[29]. Recently, Rocha *et al*.[30] found that in IL-1β levels in dental implant patients differed between patients with healthy and inflamed sites, irrespective of whether they were otherwise edentulous. Perhaps the most compelling findings for progression come from a five-year longitudinal case-control study by Scannapieco *et al*., who found higher salivary levels of IL-1β in subjects who lost alveolar bone[31]. Kaushik found IL-1β elevated in periodontitis patients and decreases after initial therapy, though not to the levels of healthy patients [32].

IL-1 receptor antagonist (IL-1ra) inhibits the destructive action of IL-1β through competitive binding to the IL-1 receptor[33]. In an in-vitro study, the monocytes of subjects with chronic periodontitis produced significantly less IL-1ra on pathogen stimulation compared to those of patients with other types of periodontitis[34]. It can be found in GCF[35], and Ishihara *et al*. found that the ratio of IL-1 to IL-ra was correlated with alveolar bone loss score in periodontitis subjects, though total amount of IL-1ra showed only a non-significant decrease in severe bone loss sites.[36] This ratio has since been confirmed to be correlated with clinical signs of disease[37]. Roberts *et al*. also found increases in both IL-1β and IL-1ra in connective tissue biopsies of periodontitis patients[38], but other studies have found decreased GCF IL-1ra levels in diseased sites compared to healthy sites[39, 40]. Holmlund *et al*. showed significant decreases in IL-1β and IL-1ra in GCF following periodontal therapy[41]. In saliva, Chan *et al* found IL-ra levels to be lower in diabetic periodontitis subjects.[42]

Interleukin 8 (IL-8) is a cytokine released by monocytes and other cells, including fibroblasts[43] and endothelial cells, upon stimulation by lipopolysaccharide (LPS), IL-1α, IL-1β, and TNFα. It functions to recruit neutrophils to sites of inflammation and activate them to degranulate once there[44]. It is therefore decidedly pro-inflammatory and pro-destructive[45]. It has recently been shown to be increased in GCF during experimental gingivitis in smokers[46] and in diseased patients.[47, 48]

Monocyte chemotactic protein-1 (MCP-1, also referred to as chemokine ligand 2 CCL-2) is a small chemoattractant cytokine produced by phagocytes and endothelial cells that attracts immune cells, chiefly monocytes, to sites of inflammation. Non-inflamed tissues express little to no MCP-1, but inflamed tissues show MCP-1-positive cells in numbers that correlate to the severity of the inflammation.[26] MCP-1 has been shown to be increased in GCF in both

aggressive periodontitis[49, 50] and chronic periodontitis[48, 50, 51]. In a mouse model, experimental periodontitis was inhibited by blocking the receptor for MCP-1, CCR-2.[52] It has also been shown to decrease to near-healthy levels in the GCF of treated patients.[51, 53] Recently, MCP salivary levels were shown to be higher in disease and to decrease with therapy.[51]

MMPs are divalent cation-dependent proteases that digest extracellular matrix proteins and have been broadly implicated in tissue remodeling in health and disease[54]. Their relative abundance compared to their inhibitors (TIMPs) appears to determine the severity and progression of tissue destruction.[4] MMP production by neutrophils, macrophages, epithelial cells, and fibroblasts is stimulated by IL-1, IL-8, TNFα, and prostaglandins such as PGE_2.[45] PCR analysis of gingival biopsies has shown higher MMP levels in periodontitis, with higher TIMP levels in subjects exhibiting chronic disease rather than aggressive disease pattern.[6] Saliva studies are limited, with two studies showing higher salivary TIMP-1 levels in periodontitis subjects.[27, 55] Other TIMPs do not appear to have been studied in saliva.

MMP-1 is a collagenase thought to be involved in much general tissue remodeling. MMP-3, or stromelysin-1, targets basement membrane components and may also activate other MMPs from their inactive proMMP form. Reddy et al. found higher GCF levels of MMP-3 in periodontitis patients compared to controls, and SRP reduced those levels significantly.[56] MMP-8 is also termed neutrophil collagenase due to its major source. Along with MMP-8, MMP-9, or neutrophil gelatinase, is thought to be secreted in a "reactive" rather than maintenance pattern, possibly provoked by IL-8.[5] Yakob et al. recently found the periodontal pathogen *Treponema denticola* to be associated with MMP-8 and -9 in GCF,[57] and Sorsa et al. have proposed MMP-8 as a point-of care marker for periodontitis and cardiovascular disease due to its putative predominance as a reactive rather than maintenance MMP[58].

Sexton *et al.* studied a group of patients assigned to oral hygiene instruction control or scaling and root planing (SRP) treatment group. Salivary IL-1β and MMP-8 decreased only in SRP patients, and MMP-8 differentially reduced in high responders. Compared to other markers, MMP-8 had greatest area under the curve, indicating best predictive value[59].

Neutrophil gelatinase-associated lipocalin (NGAL) is an iron-binding glycoprotein that functions as part of the innate immune defense to bacterial infection[60]. It is most commonly used as a marker of acute kidney injury, but it is elevated (along with MMP-9) in gingival tissue in periodontitis, apparently secreted by extravasated neutrophils.[61] It is thought to complex with MMP-9, preventing its degradation and, thereby, prolonging MMP-9's gelatinase activity. The lone reports in the periodontal literature by Sorsa's group in 1996[61, 62] have not since been revisited.

In a longitudinal study of periodontitis patients, the Giannobile group[63] investigated combining salivary and serum biomarkers with biofilm pathogen levels. In their study, subjects who displayed greater than 2mm of attachment loss over a six-month monitoring phase clustered into a "progression" group, and models could predict progression or stability of clinical signs of disease. They also found that after treatment, salivary levels of MMP-8, MMP-9, OPG, and IL-1β were significantly reduced. Those four markers, especially MMP-8, were previously described by the same group[64] as showing high predictability for disease severity.

Experimental gingivitis

Since the first report in humans in 1965[65], authors have used a biofilm overgrowth-induced experimental gingivitis model to investigate gingival response to undisturbed plaque growth. In that study, twelve healthy subjects abstained from oral hygiene measures until overt

gingivitis developed due to plaque accumulation, after which time the subjects resumed home care, leading to a reversal in clinical and microbiological parameters. In most subjects, the induction phase lasted between 15 and 21 days. In one quarter of the subjects, however, gingivitis was reached in 10 days or fewer. This provides early evidence for biologic phenotypes governing host response to plaque. This phenomenon of "high- and low-responders" may have some bearing on the transition from gingivitis to periodontitis. However, most studies included only patients without periodontitis.

Recent experimental gingivitis studies[66-68] have incorporated stents to encourage compliance, a method termed stent-induced biofilm overgrowth (SIBO). One such study[66] analyzed GCF samples collected prior to the induction of experimental gingivitis, at the 21-day peak of gingival inflammation, and again at resolution of gingivitis and attainment of gingival health one week later. The results from this study identified specific inflammatory mediators (including MMP-1, IL-1ra and CRP as primary with secondary mediators resistin, IFNγ, Complement D, MCP-1, MMP-13, TPO and IL-8) that were predictive of clinical response in gingivitis either at baseline or upon initial presentation and illustrated that the dynamics of inflammatory mediator change were different among those subjects that were "high responders" (i.e. developed more clinical disease) as compared to "low responders" (i.e. displayed little change in clinical phenotype, despite plaque overgrowth). Thus, these inflammatory biomarkers were demonstrated to change during the induction and resolution of gingival inflammation. Furthermore, the changes in inflammatory biomarkers differed with regard to the magnitude of the clinical changes seen in this gingivitis induction model. Thus, the goal of this study is to understand whether salivary diagnostics (assaying key inflammatory mediators in saliva) can be used to discriminate among health, gingivitis, and

three levels of periodontal disease and whether baseline mediator values can predict worsening of clinical parameters during SIBO. This study represents the first report of using the SIBO model among patients with gingivitis or periodontitis and enables us to explore the changes in salivary mediators during disease progression in a short-term, reversible clinical model.

CHAPTER 2. MATERIALS AND METHODS

Study design and enrollment

Five groups of subjects categorized as health, gingivitis, mild, moderate, and severe periodontitis were enrolled, stratifying into groups until 33 or 34 subjects were reached per group (total subjects, 168), using the biofilm-gingival interface (BGI) criteria previously described in 2007 by Offenbacher et al[69]. Subjects with no pockets greater than 3mm were classified as healthy (BGI-H) if bleeding scores were less than 10% or gingivitis (BGI-G) if their bleeding scores were 10% or greater. Subjects with 4mm or greater probing depths (PD) were also sorted by based on whether their bleeding scores were less than 10% (BGI-P1), 10-50% (BGI-P2), or greater than 50% (BGI-P3). Exclusion criteria included gross oral pathology, treatment with antibiotics or chronic treatment with medication known to affect periodontal status within 1 month prior to enrollment, beginning a new medication for a chronic condition less than three months prior to enrollment, clinically significant organ disease, severe unrestored caries, or pregnancy or nursing. Full inclusion/exclusion criteria and the criteria for subject safety/study exit are listed in appendix C.

All groups were subjected to a stent-induced biofilm overgrowth model (SIBO) that experimentally induced a short-term biofilm overgrowth. In brief, acrylic stents were made covering one maxillary and one mandibular posterior sextant of each subject's dentition. Subjects wore the stents while performing oral home care for three weeks to allow biofilm accumulation. Subjects were monitored each week for safety to provide escape therapy in the

event of more than 2mm PD increase. Subjects exhibiting progression were to be exited from the study and given scaling and root planing treatment as a rescue therapy. Subjects were also to be exited from the study for changes in medical status or medications, use of mouth rinses, or use of non-study dentifrices, toothbrushes, or floss during the induction or resolution phases. At the completion of 3 weeks, subjects were instructed to begin brushing again. At the end of the study, subjects were given a dental prophylaxis and referred for further care.

Examiners were masked, and patients were forbidden from using antiseptic rinses. At each visit, plaque index[70], gingival index[71], probing depths, clinical attachment levels, and bleeding on probing were measured, and unstimulated saliva samples were acquired on days -14, 0, 7, 14, 21, 35, and 49. At each of these time points, approximately 3 mL of unstimulated saliva were collected into a 15 mL plastic conical tube. Participants refrained from eating, drinking, chewing gum, breath mints, etc., or performing oral hygiene procedures for at least one hour prior to saliva collection. This request was made after consenting for the first sample collection. The collection vessel was kept at room temperature, centrifuged and aliquoted as three samples of .5 ml in each tube. Remaining saliva was aliquoted into a fourth tube. Saliva was stored at -80^0C.

These samples were assessed for levels of IL-1β, IL-1ra, IL-8, MCP-1, MMP-1, MMP-3, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 using commercially available assay kits on the BioPlex 200 by multiplex immunochemistry according to manufacturer's instructions. NGAL levels were measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., Minneapolis, MN). Each saliva sample was assayed in duplicate.

Statistical analysis

A pre-study power analysis indicated a minimum sample size of eight test/control pairs to yield power of 90% with alpha of 5% for IL-1β changes. Other outcome thresholds were unknown, so 33 subjects were enrolled per group with the goal of completing 30, for a total of 150 subjects. Clinical and biochemical changes were analyzed across groups using a mixed-models analysis. For mediators that showed overall significance between groups, generalized linear modeling allowed analysis of each mediator independent of changes of the others.

Changes in clinical signs were used to identify high and low responders dichotomizing on the median change in clinical sign at 21 days as compared to baseline.

CHAPTER 3. RESULTS:

Demographics and safety

Subjects did not differ significantly between groups for most variables, but there were some differences. Healthy and gingivitis groups had more African Americans, were younger, and had lower BMI than the periodontitis groups. No subjects were exited due to increasing of PD>2mm. Subjects with increasing PD during the SIBO were found to respond to therapy at exit with PD returning to baseline or improving from baseline. No subjects experienced irreversible loss of attachment.

Salivary mediators at baseline and during SIBO

Significant differences ($p < 0.05$) were found at baseline between groups for IL-1β, MMP-3, MMP-8, MMP-9, TIMP-3, IL-1ra, and NGAL (table 4). P3 subjects had significantly higher log mean values (see figures 2-5, 8) than other groups for IL-1β, MMP-3, MMP-8, MMP-9, and NGAL. Compared to healthy subjects, P3 subject levels were more than two-fold elevated for IL-1β, MMP-3, MMP-9, and TIMP-3, and more than five-fold elevated for MMP-8.

Mean TIMP-3 was significantly lower for P2 than for other groups (fig 6). Mean IL-1ra was higher in H than for G or P1 and higher in P2 than in G or P1 (fig 7).

Significant increases in saliva values from baseline to peak induction were found for MMP-1, TIMP-1, TIMP-2, and TIMP-4 (table 5) pooling all subjects. When subdivided by disease

category there were no significant changes in salivary biomarker level during SIBO induction. No mediator showed a significant decrease during the induction phase.

Clinical changes

Subjects showed significant increases in plaque, gingival index, and bleeding on probing across all groups (table 4). The gingivitis subjects also showed slight but significant increase in attachment loss (0.11 ± 0.05mm, p=0.03) during SIBO. P1 subjects showed small but significant increase in pocket depth (0.11 ± 0.05mm, p=0.047).

Grouped together, Health + Gingivitis subjects showed increases in pocket depth (0.11 ± 0.05mm, p=.04) and attachment loss (0.09 ± 0.03mm, p=.008).

Baseline predictors of clinical changes

A stepwise linear regression model using all salivary biomarkers demonstrated that IL-1ra (p=0.0044) and IL-6 (p=0.0093) were the two best predictors of change in probing depths during the induction phase, with an overall r^2 value of 0.37. The effect slope for IL-1ra was positive (0.675) and negative for IL-6 (-0.246). There were no significant salivary predictors of change in bleeding or change in gingival index.

CHAPTER 4. DISCUSSION:

This study has shown significant baseline differences between subject groups, but no differences in response to SIBO. There is some controversy over saliva should be stimulated prior to collection, as studies have demonstrated[72] differences in lactoferrin and other anti-bacterial compounds by collection strategy. In this study, since the goal was to study local accumulation of cytokines from GCF washout into saliva, a stimulated collection strategy would unnecessarily dilute samples, potentially increasing error. Therefore, unstimulated saliva was collected for analysis.

Differences between groups may have influenced the results obtained. The severe periodontitis group (P3) had fewer African Americans. The periodontitis subjects were older, as a group, than the healthy/gingivitis subjects were. Prevalence of periodontitis increases with age, so this is not surprising. They also had a higher mean BMI. Saito et al.[73] first described a Japanese population in which periodontitis was associated with obesity (BMI > 30), with an odds ratio of OR = 8.6. Obesity is a source of oxidative stress and leads to increased serum levels of IL-6 and CRP, as does periodontitis.[74]

The BGI case definitions, designed to differentiate between subject phenotypes, segregate subjects into disease categories by bleeding levels rather than by the more traditionally used percentage of deep pockets and attachment levels or bone loss. For this reason, some subjects categorized P1 would fit under the American Academy of Periodontology definition of

gingivitis rather than periodontitis.[1] The BGI groups purposefully ignore attachment loss to allow investigators to concentrate on inflammatory burden and host response to present disease rather than its history.

The most obvious differences at baseline were between the P3 group and all other subjects. The finding of significant differences in IL-1β, MMP-3, MMP-8, MMP-9, TIMP-3, IL-1ra, and NGAL between groups is in contrast to that of Teles *et al,* who found no significant differences in salivary granulocyte-macrophage colony-stimulating factor, IL-1β, IL-2 IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, or TNFα between healthy and periodontitis subjects, though there were some differences when analyzed based on pocket depths, bleeding, and attachment levels.[75] Their study might have shown differences between groups had they grouped by BGI classification rather than traditional definition. However, our results confirm those of several other studies showing higher levels of IL-1β[27-29, 31, 32, 59, 76-78], MMP-8[27, 28, 55, 59, 64, 78], and MMP-9[64, 79] in the saliva of periodontitis subjects. Westerlund et al. also found NGAL in periodontitis subject saliva, but no statistical analysis was presented.[61]

Increases in plaque, gingival index, and bleeding on probing across all groups are to be expected in an experimental gingivitis study. Several mediators, including MMP-1, TIMP-1, TIMP-2, and TIMP-4 increased significantly from baseline to peak of induction (table 5). To our knowledge, this is the first human study demonstrating changes in TIMPs during experimental gingivitis. These increases ranged from 1.12-1.48 –fold changes, smaller than the baseline differences for the mediators that were significantly different between groups. The increased levels of TIMPs may be due to a local response to dampen inflammation and prevent protein degradation. MMPs are generally produced in an inactive (proMMP) form and must be cleaved to become functional. It is possible an assay of the collagenolytic

activity of saliva would be more useful than one of collagenase concentration alone in showing tissue response to bacterial stimulation. One study found significant differences in GCF MMP-8 activity by diagnosis and by time point during treatment.[80] The same group[81] showed the activity levels did not correlate significantly with Western blot analysis of zymogen vs. active form. MMP-3, MMP-8, and MMP-9 did not significantly increase during induced gingivitis in this study. Possible explanations include the possibility that initial changes are mostly expressed by enzymes being cleaved into their active forms, a three-week study may be too short to induce measurable changes in the amounts of these enzymes, or the effect size may be too small when covering only two sextants and diluting in saliva.

Baseline IL-1ra and IL-6 levels were found to be significant predictors of change in probing depths. The r^2 value of 0.37 is rather low, implying that they do not alone predict the bulk of changes, as might be expected in a chronic, complex disease system. IL-1ra is the antagonist for IL-1β receptor, blocking its action. As IL-1β is a pro-inflammatory molecule, high levels of its receptor antagonist could indicate a compensatory mechanism of negative feedback or the release of molecules that are normally tissue-bound. IL-6 was not significant between groups at baseline. However, high levels at baseline predicted an increase in PD. IL-6 is known to be a pro-inflammatory molecule that shift the immune response from toward a cell-mediated reaction, is elevated in chronic periodontitis and decreases with periodontal therapy.[74]

Future directions:

The markers chosen for this study have good evidence for showing baseline differences and changes during induction. However, some other cytokines could have been used as well. TNFα is a macrophage-produced pyrogen that stimulates acute inflammation, apoptosis,

neutrophil chemotaxis, and bone resorption. It has been shown experimentally to accelerate bone loss in mice, and knockout mice have much lower levels of other inflammatory cytokines and less bone loss[26]. In addition, TNFα antagonists have been shown to reduce bone loss in experimental periodontitis[4]. Frodge *et al*. [82] found increased salivary TNFα levels in periodontitis patients compared to healthy controls. In their study, TNFα levels 2 standard deviations above median were predictive for increased probing depth, bleeding on probing, and attachment loss. Fine *et al.* found *A.a.* –positive subjects who developed aggressive periodontitis to have had 50-fold elevated macrophage inflammatory protein (MIP-1α) six to nine months prior to radiographic evidence of bone loss.[83] Analysis of these salivary samples for TNFα and MIP-1α levels might yield data helpful for diagnosis and gauging risk of progression.

Several studies[36, 37] have found the ratio of IL-1β to IL-1ra to be more meaningful in periodontitis than absolute values of either mediator, and efforts to change that ratio through supplying exogenous IL-1ra have promise, as shown by the use of human recombinant forms of IL-1ra (anakinra) to limit host response in other chronic inflammatory diseases such as rheumatoid arthritis,[84] gout,[85] osteoarthritis,[86] and cardiovascular disease.[87] Perhaps analysis of this ratio in our cohort would yield informative data.

Recent studies have begun to profile the salivary metabolome. In a longitudinal study, Nomura *et al*. [88] found aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase to be increased at baseline in patients who later experienced progression. Parwani *et al*. showed higher salivary levels of nitric oxide in gingivitis and periodontitis patients versus healthy controls. After treatment, these numbers were reduced as compared to pre-treatment levels[89]. Combining cytokines like those assayed in this study with salivary

compounds, such as enzymes and bacterial breakdown products, may increase the predictive power of salivary analysis. Elsewhere, our group will investigate the metabolomes of these samples.

Salivary diagnostics is emerging as a valuable possible method of determining disease presence, severity, and activity. In this study, there were significant differences at baseline between the severe periodontitis group and all other groups. There were also limited increases in markers during gingivitis induction, notably MMP-1 and TIMPs. More study is needed to determine whether techniques and markers can be combined to improve the diagnostic and prognostic abilities of salivary testing.

Table 1: Study Design

PHASES	LEAD-IN ORAL HYGIENE PHASE		NO ORAL HYGIENE IN STENT AREA PHASE			
Procedure	**Screen**	**Day-14**	**Baseline (Day 0)**	**Day 7**	**Day 14**	**Day 21**
Visit	***1***	***2***	***3***	***4***	***5***	***6***
Informed Consent	X					
Medical History/Updates	X	X	X	X	X	X
Vital Signs	X	X	X	X	X	X
Height and weight / Demographics	X					
Inclusion/Exclusion Criteria - pregnancy test if applicable	X					
Stent Impressions	X					
Deliver Stent			X			
Discontinue Stent						X
Continuance Criteria		X	X	X	X	X
Oral Cancer Screening, Plaque and Gingival Indices, PD, CAL & BOP	X	X	X	X	X	X
Adverse Events		X	X	X	X	X
Unstimulated Saliva Collection		X	X	X	X	X
GCF collection		X	X	X	X	X
Subgingival Plaque Collection		X	X			X

Table 2: Demographics by BGI Category

	H	G	P1	P2	P3	p-value
Female	23 (20.5%)	23 (20.5%)	23 (20.5%)	23 (20.5%)	20 (17.9%)	
Male	10 (17.9%)	11 (19.6%)	10 (17.9%)	11 (19.6%)	14 (25.0%)	0.87
African American	21 (20.8%)	26 (25.7%)	23 (22.8%)	16 (15.8%)	15 (14.9%)	
Caucasian	7 (13.7%)	6 (11.8%)	7 (13.7%)	17 (33.3%)	14 (27.5%)	
Other	5 (33.3%)	2 (13.3%)	3 (20.0%)	1 (6.7%)	4 (26.7%)	0.03
Diabetic	3 (42.9%)	0 (0.0%)	1 (14.3%)	1 (14.3%)	2 (28.6%)	
No	30 (18.6%)	34 (21.1%)	32 (19.9%)	33 (20.5%)	32 (19.9%)	0.41
Current Smoker	3 (14.3%)	5 (23.8%)	7 (33.3%)	3 (14.3%)	3 (14.3%)	
No	30 (20.6%)	29 (19.9%)	26 (17.8%)	31 (21.2%)	30 (20.6%)	0.47
Mean (StdErr) Age	30.3 (1.9)	29.6 (1.9)	36.5 (1.9)	37.0 (1.9)	34.4 (1.9)	0.01
Mean (StdErr) BMI	28.1 (1.3)	24.5 (1.2)	27.4 (1.3)	30.7 (1.2)	29.6 (1.2)	0.008

Table 2: Significant differences were found between groups for ethnic background (p=0.03), age (0.01), and BMI (0.008).

Table 3: Baseline log10 Mean (StdErr) Saliva Mediator by BGI Category.

	Health	G1	P1	P2	P3	Overall p-value
IL-1β	2.43 (0.07)	2.46 (0.07)	2.45 (0.07)	2.47 (0.07)	2.76 (0.07)	0.004
IL-8	2.75 (0.06)	2.80 (0.06)	2.77 (0.06)	2.78 (0.06)	2.98 (0.06)	0.07
MCP-1	2.28 (0.06)	2.44 (0.06)	2.45 (0.06)	2.49 (0.06)	2.48 (0.06)	0.07
MMP-1	1.50 (0.17)	1.35 (0.17)	1.59 (0.18)	1.18 (0.15)	1.13 (0.17)	0.24
MMP-3	1.94 (0.11)	1.94 (0.11)	1.88 (0.11)	1.94 (0.10)	2.34 (0.11)	0.02
MMP-8	4.70 (0.10)	4.85 (0.10)	4.76 (0.10)	4.94 (0.09)	5.42 (0.10)	<0.0001
MMP-9	4.92 (0.07)	4.98 (0.07)	4.93 (0.08)	5.05 (0.08)	5.39 (0.08)	<0.0001
TIMP-1	5.26 (0.05)	5.24 (0.05)	5.18 (0.05)	5.08 (0.05)	5.21 (0.05)	0.15
TIMP-2	4.53 (0.04)	4.78 (0.04)	4.50 (0.04)	4.45 (0.04)	4.62 (0.04)	0.06
TIMP-3	1.49 (0.24)	1.93 (0.24)	1.56 (0.24)	0.67 (0.24)	1.81 (0.24)	0.003
TIMP-4	1.11 (0.04)	1.11 (0.04)	1.08 (0.04)	1.05 (0.04)	1.14 (0.04)	0.67
IL-1ra	5.79 (0.07)	5.58 (0.07)	5.57 (0.07)	5.83 (0.07)	5.72 (0.07)	0.03
NGAL	2.51 (0.05)	2.48 (0.05)	2.60 (0.05)	2.54 (0.05)	2.75 (0.05)	0.005

Table 3: Significant differences were found between groups for IL-1β (p=.004), MMP-3 (p=.02), MMP-8 (p<.0001), MMP-9 (p<.0001), TIMP-3 (p=.003), IL-1ra (p=.03), and NGAL (p=.005).

Table 4: Mean (StdErr) Delta Clinical Sign (Day 21-Day 0) During Induction by BGI Group

BGI (Health)		p value
Plaque	0.85 (0.11)	<0.0001
Gingival Index	0.44 (0.06)	<0.0001
Bleeding On Probing	19.4 (4.03)	<0.0001
Probing Depth	0.05 (0.06)	0.46
Attachment Level	-0.08 (0.05)	0.12
BGI (Gingivitis)		
Plaque	0.79 (0.09)	<0.0001
Gingival Index	0.29 (0.05)	<0.0001
Bleeding On Probing	20.5 (3.49)	<0.0001
Probing Depth	0.18 (0.09)	0.052
Attachment Level	-0.11 (0.05)	0.03
BGI (P1)		
Plaque	0.70 (0.10)	<0.0001
Gingival Index	0.21 (0.05)	0.0001
Bleeding On Probing	21.0 (4.47)	<0.0001
Probing Depth	0.11 (0.05)	0.047
Attachment Level	0.01 (0.05)	0.91
BGI (P2)		
Plaque	0.82 (0.10)	<0.0001
Gingival Index	0.28 (0.06)	0.0001
Bleeding On Probing	15.7 (4.05)	0.0005
Probing Depth	0.03 (0.06)	0.67
Attachment Level	0.03 (0.06)	0.63
BGI (P3)		
Plaque	0.65 (0.08)	<0.0001
Gingival Index	0.15 (0.04)	0.002
Bleeding On Probing	8.30 (4.47)	0.07
Probing Depth	-0.06 (0.06)	0.30
Attachment Level	0.02 (0.08)	0.79
BGI (Health + Gingivitis)		
Plaque	0.82 (0.07)	<0.0001
Gingival Index	0.37 (0.04)	<0.0001
Bleeding On Probing	19.9 (2.67)	<0.0001
Probing Depth	0.11 (0.05)	0.04
Attachment Level	-0.09 (0.03)	0.008
BGI (P1-P3)		
Plaque	0.73 (0.05)	<0.0001
Gingival Index	0.22 (0.03)	<0.0001
Bleeding On Probing	15.8 (2.51)	<0.0001
Probing Depth	0.02 (0.03)	0.48
Attachment Level	0.02 (0.04)	0.66

Table 4: Clinical parameters for teeth in SIBO sextants, stratified by BGI group.

Table 5: Mixed Models: Log10 Mean (StdErr) Saliva Mediator by time point.

	Baseline	Peak	-fold change	p-value B/L-Peak
IL-1b	2.51 (0.03)	2.50 (0.03)	0.98	0.60
IL-8	2.81 (0.03)	2.82 (0.03)	1.02	0.73
MCP-1	2.43 (0.03)	2.44 (0.03)	1.02	0.39
MMP-1	1.34 (0.08)	1.51 (0.08)	1.48	0.002
MMP-3	2.01 (0.05)	1.97 (0.04)	0.91	0.39
MMP-8	4.94 (0.05)	4.89 (0.05)	0.89	0.20
MMP-9	5.05 (0.04)	5.07 (0.04)	1.05	0.55
TIMP-1	5.20 (0.02)	5.28 (0.02)	1.20	0.0002
TIMP-2	4.52 (0.02)	4.60 (0.02)	1.20	<0.0001
TIMP-3	1.50 (0.11)	1.56 (0.11)	1.15	0.12
TIMP-4	1.10 (0.02)	1.15 (0.02)	1.12	0.03
IL-1ra	5.70 (0.03)	5.72 (0.02)	1.05	0.42
NGAL	2.58 (0.02)	2.57 (0.02)	0.98	0.76

Table 5: Clinical parameters for teeth in SIBO sextants, stratified by BGI group.

Figure 1: Study Design

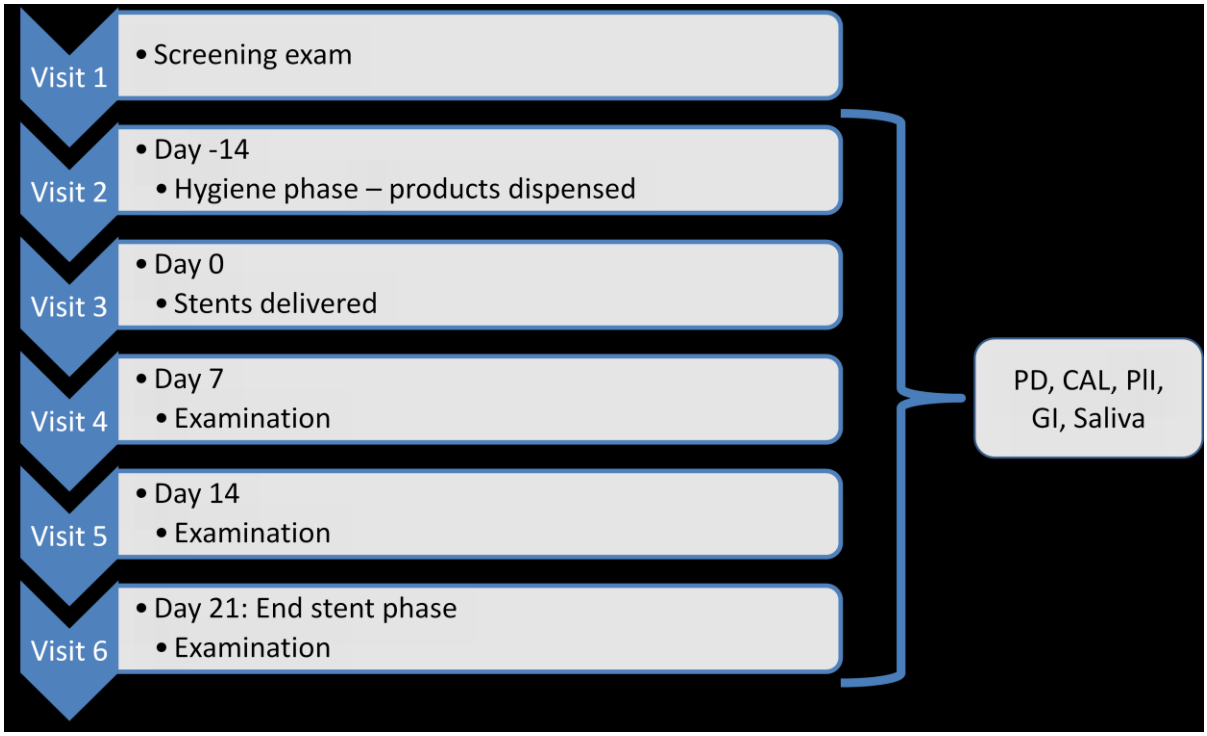

Figure 1. Study design. At screening visit, inclusion/exclusion/continuance criteria were reviewed and subjects were enrolled. At each visit, saliva was collected and examinations were conducted, with measurements of probing depths, clinical attachment loss, plaque index, and gingival index. At day 21, subjects resumed brushing.

Figure 2: IL-1β Baseline Values Box Plot

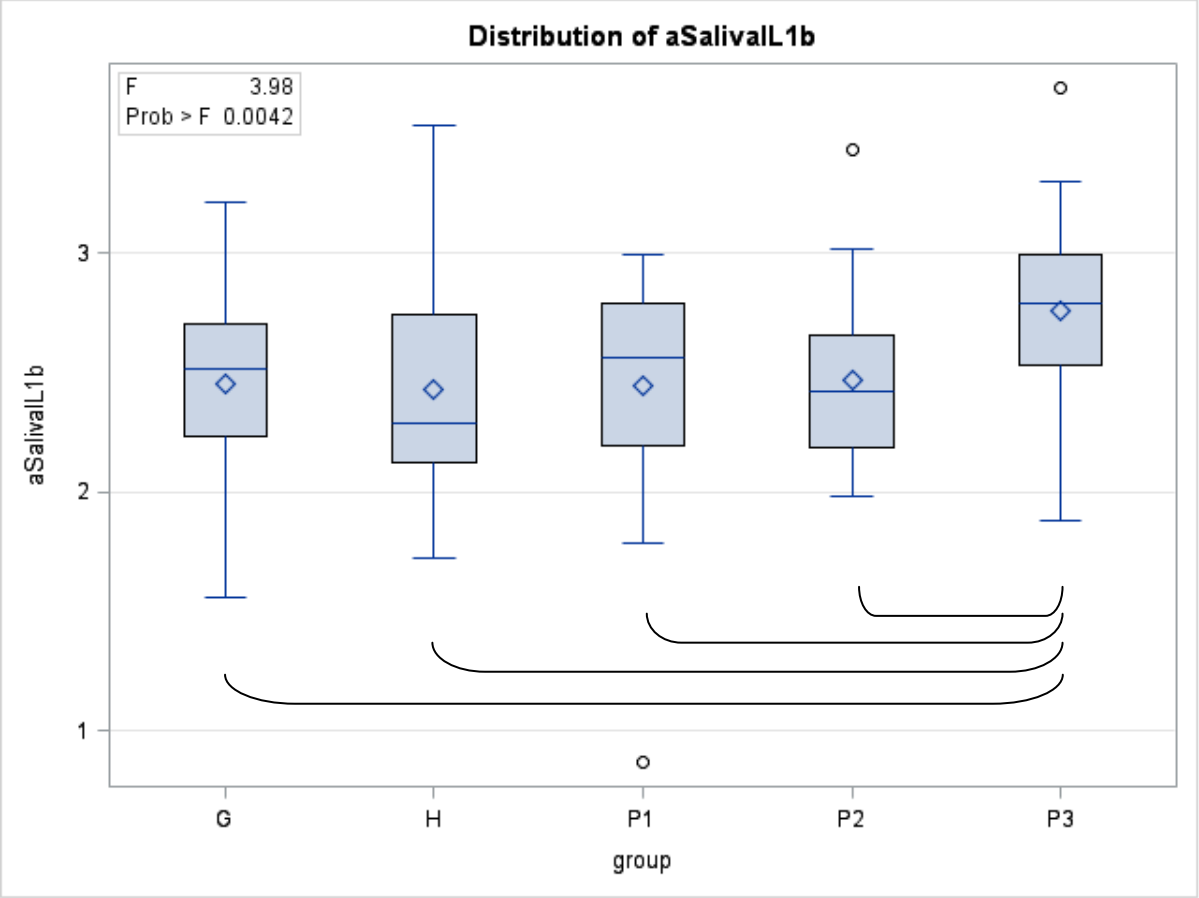

Figure 2. IL-1β baseline differences were significant overall between groups and individually for P3 vs. H (P=0.0008), G (P = 0.0024), P1 (P = 0.0021), and P2 (P = 0.0035).

Figure 3: MMP-3 Baseline Values Box Plot

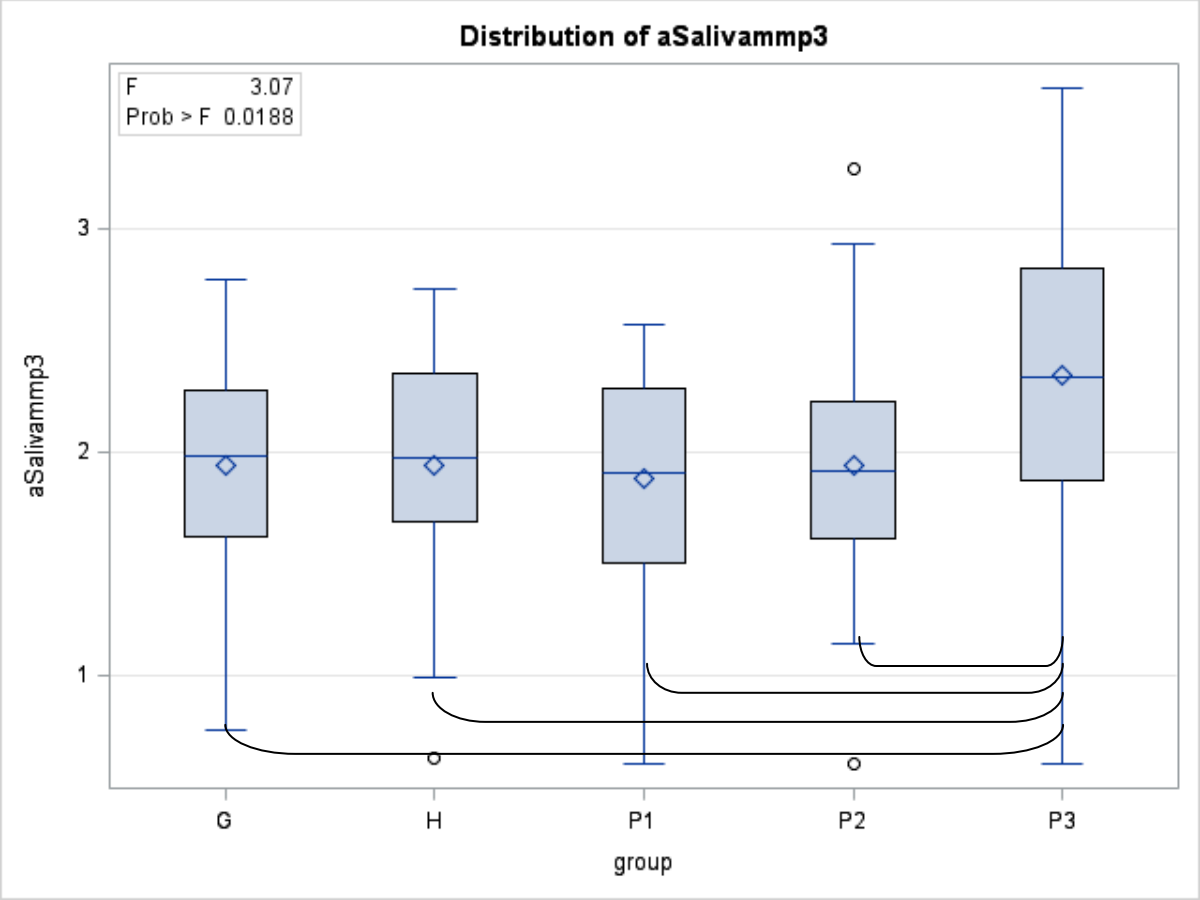

Figure 3. MMP-3 baseline differences were significant overall between groups and individually for P3 vs. H (P = 0.0093), G (P = 0.0096), P1 (P = 0.0039), and P2 (P = 0.0061).

Figure 4: MMP-8 Baseline Values Box Plot

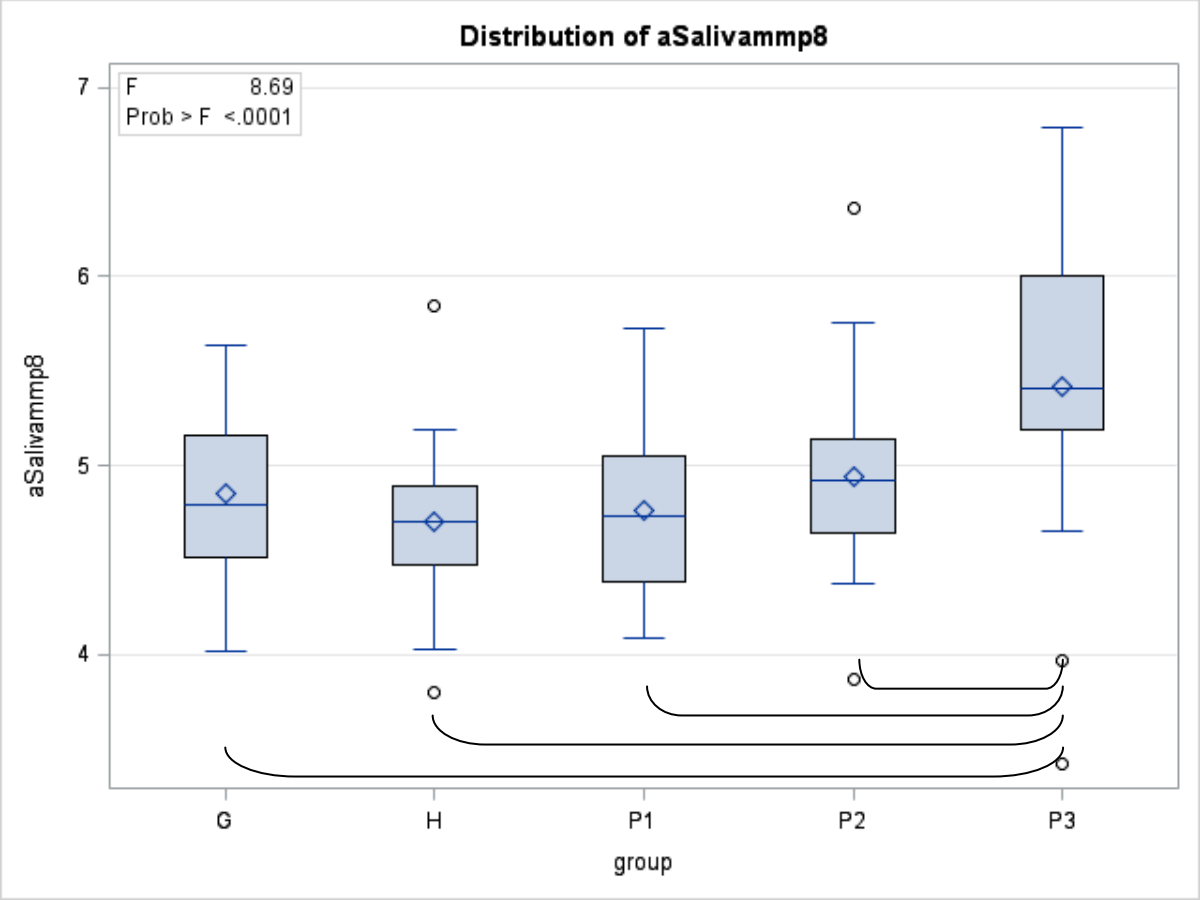

Figure 4. MMP-8 baseline differences were significant overall between groups and individually for P3 vs. H ($P < 0.0001$), G ($P < 0.0001$), P1 ($P = < 0.0001$), and P2 ($P = 0.0004$).

Figure 5: MMP-9 Baseline Values Box Plot

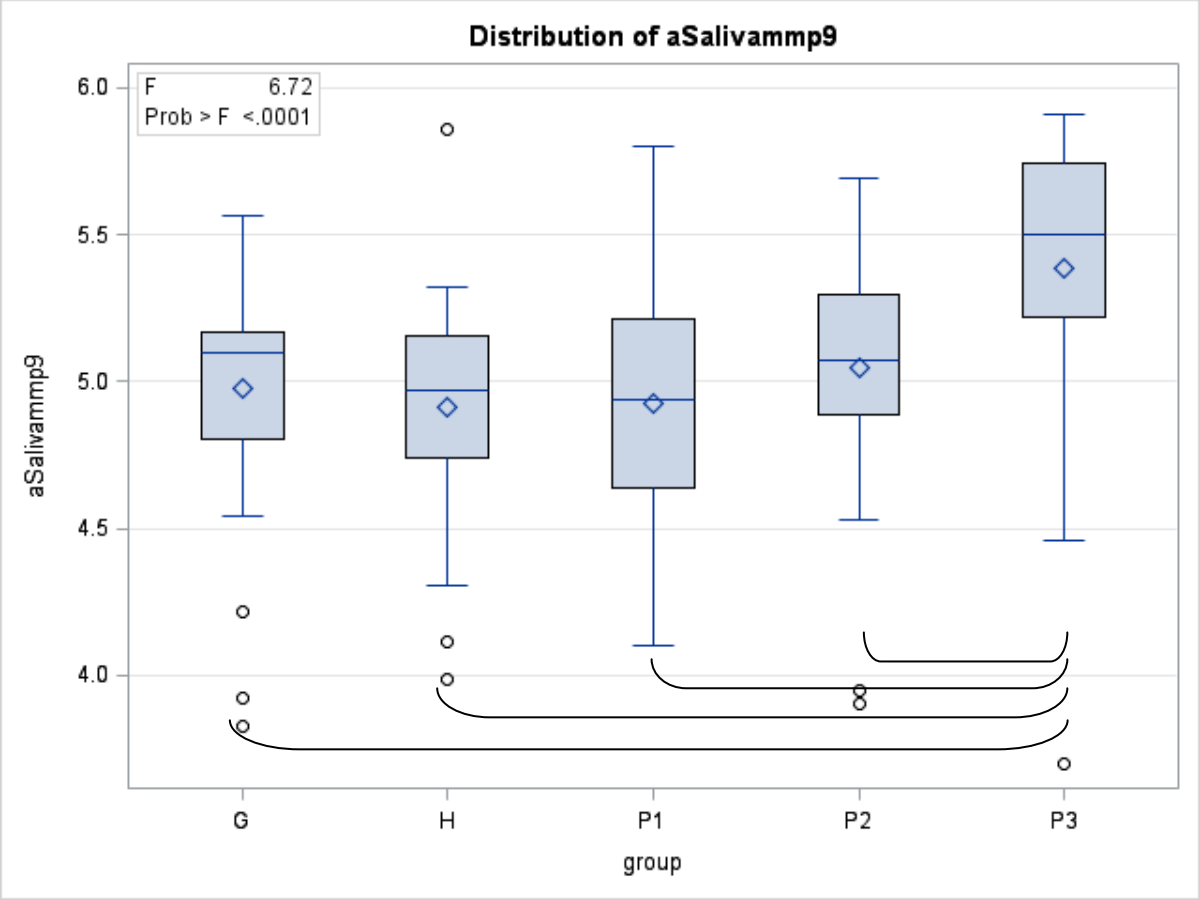

Figure 5. MMP-9 baseline differences were significant overall between groups and individually for P3 vs. H ($P < 0.0001$), G ($P = 0.0002$), P1 ($P < 0.0001$), and P2 ($P = 0.0019$).

Figure 6: TIMP-3 Baseline Values Box Plot

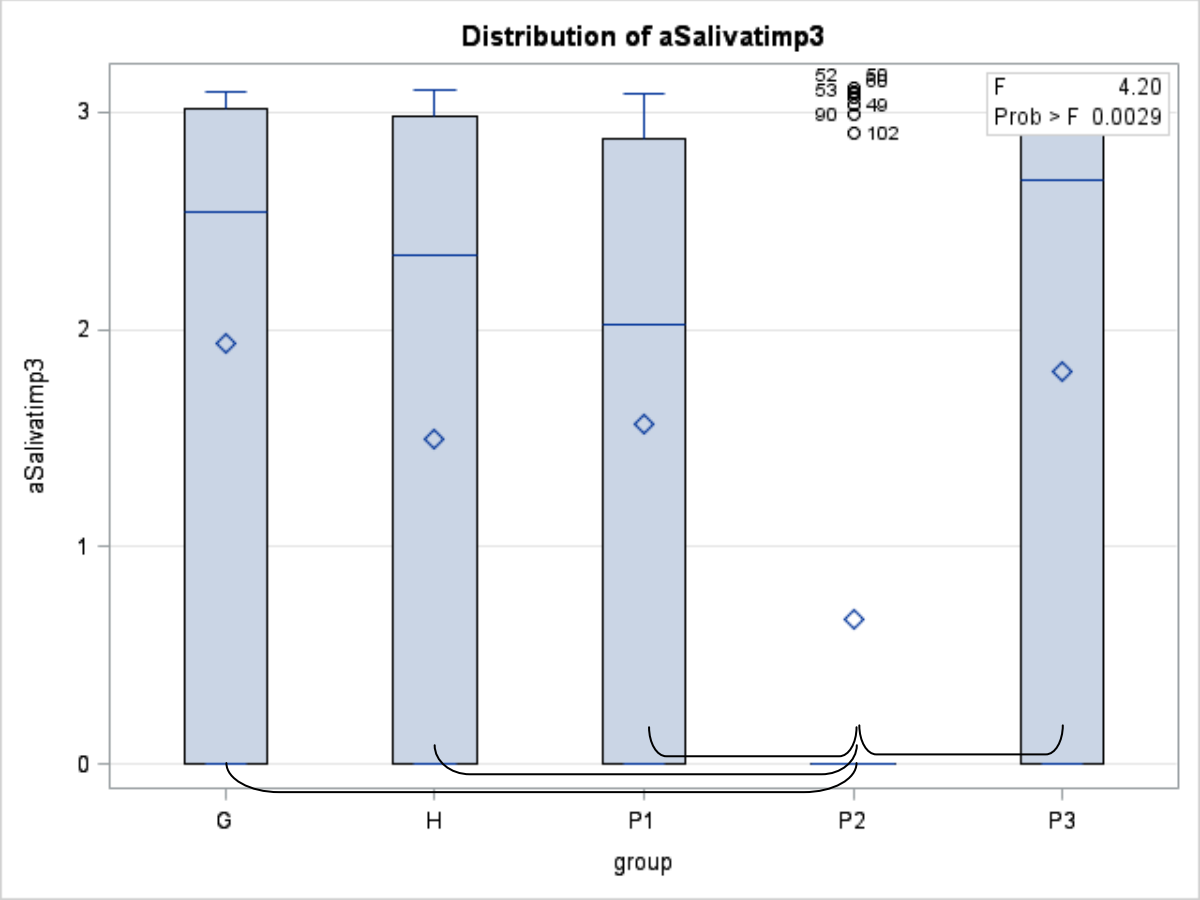

Figure 6. TIMP-3 baseline differences were significant overall between groups and individually for P2 vs. H (P = 0.0154), G (P = 0.0003), P1 (P = 0.0097), and P3 (P = 0.0011).

Figure 7: IL-1ra Baseline Values Box Plot

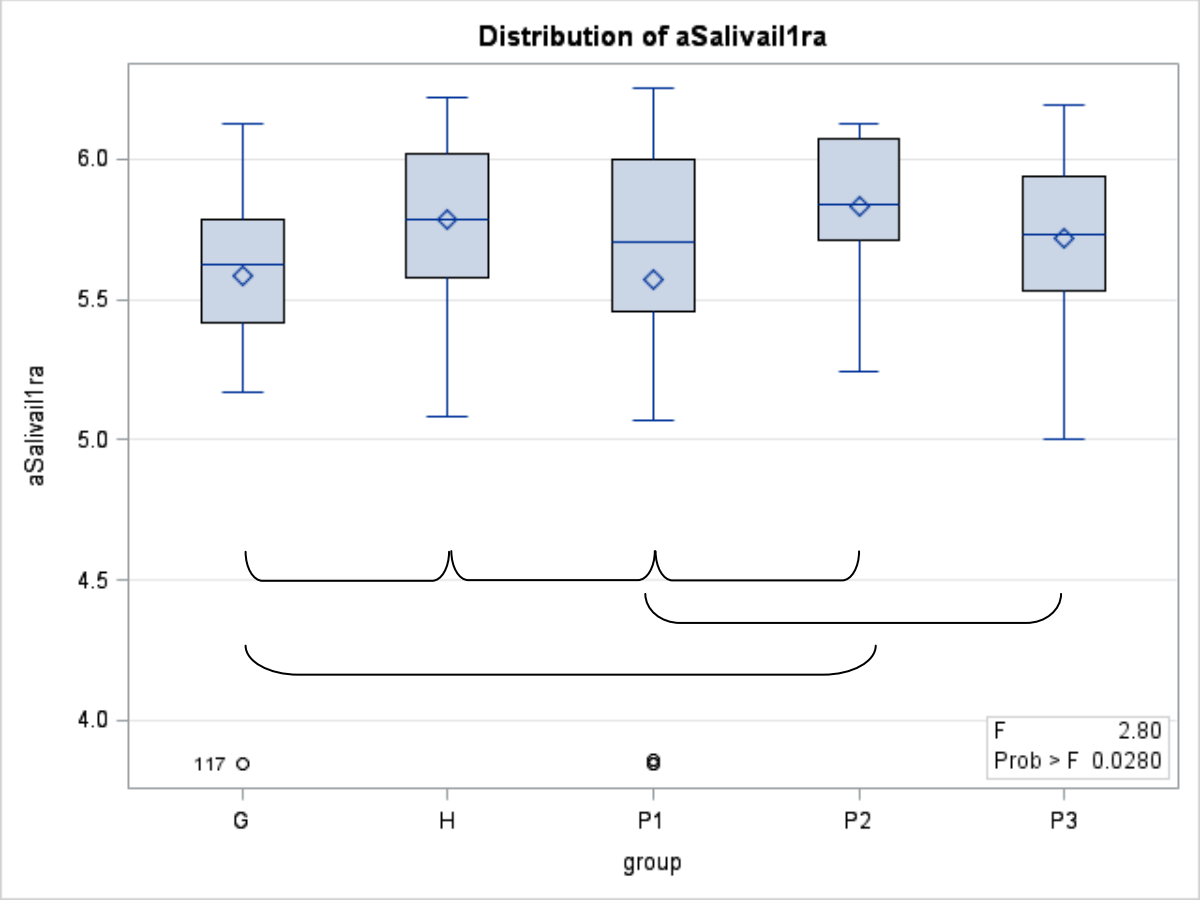

Figure 7. IL-1ra baseline differences were significant overall between groups and individually for H vs. G (P = 0.0418), H vs. P1 (P = 0.0303), G vs. P2 (P = 0.0141), and P1 vs. P2 (P = 0.0099).

Figure 8: NGAL Baseline Values Box Plot

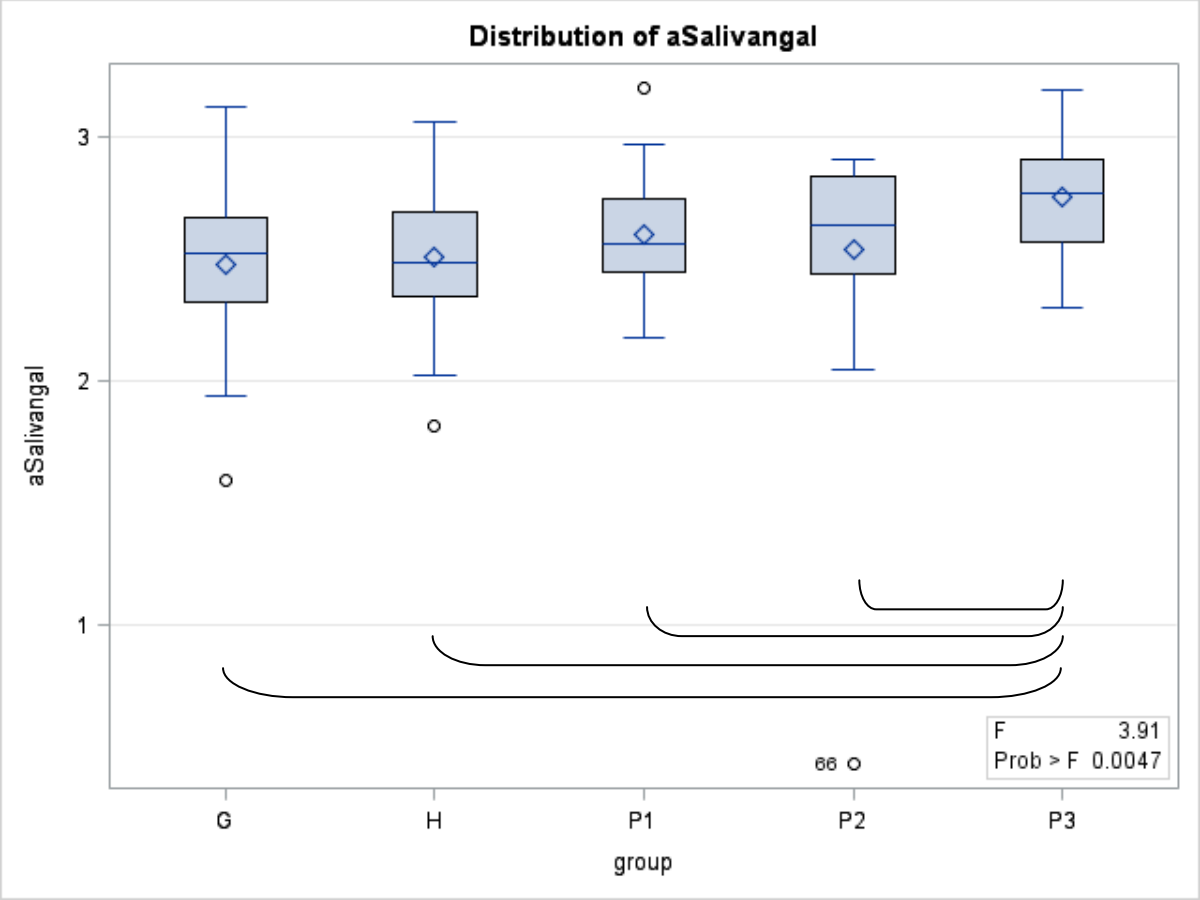

Figure 8. NGAL baseline differences were significant overall between groups and individually for P3 vs. H (P = 0.0017), G (P = 0.0005), P1 (P = 0.0475), and P2 (P = 0.0076).

Inclusion criteria:

- Participants must be adult males or females aged 18-75.
- Participants must be willing and able to follow study procedures and instructions.
- Participants must have read, understood, and signed informed consent.
- Participants must present with at least eight teeth in the functional dentition with a minimum of three adjacent teeth with interproximal papilla in each posterior sextant that will have the stent.
- Participants must be in good general health.
- Participants must present with one of the following five categories to be considered for enrollment
    - BGI-Health (all PD≤3mm, BOP<10%)
    - BGI-Gingivitis (all PD≤3mm, BOP≥10%)
    - BGI-P1 (1+ site with PD>3mm, BOP≤10%)
    - BGI-P2 (1+ site with PD>3mm, BOP>10% but BOP≤50%)
    - BGI-P3 (1+ site with PD>3mm, BOP>50%)

Exclusion criteria

- Individuals who have a chronic disease with oral manifestations or active infectious diseases such as hepatitis, HIV, or tuberculosis.
- Individuals who exhibit gross oral pathology.
- Treatment with antibiotics for any medical or dental condition within 1 month prior to the screening examination.
- Chronic treatment (i.e., two weeks or more) with any medication known to affect periodontal status (e.g., phenytoin, calcium antagonists, cyclosporine, anticoagulants, non-steroidal anti-inflammatory drugs, high dose aspirin such as >100mg per day) within one month of the screening examination.
- Ongoing medications initiated less than three months prior to enrollment (i.e., medications for chronic medical conditions must be initiated at least three months prior to enrollment).
- Participants with clinically significant organ disease including impaired renal function, and/or any bleeding disorder.
- Severe unrestored caries or any condition that is likely to require antibiotic treatment during the study, including the need for prophylactic antibiotic.
- Individuals who are pregnant, or expect to become pregnant within the next three months and individuals nursing.

Exit Criteria

- Changes in participant's medical status or medications
- Use of mouth rinses (e.g. chlorhexidine, Listerine®, or hydrogen peroxide products)
- Use of non-study dentifrices, toothbrush, dental floss (e.g. triclosan or stannous fluoride products) during the study
- Use of irrigating devices or other interdental aids
- Inability or noncompliance to wear stents over the selected no-hygiene sextants during daily brushing procedures
- Use of oral antibiotics or non-steroidal anti-inflammatory drugs
- Progression of periodontitis, defined as PD or CAL change >2mm or suppuration at any site

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