CLINICAL, MICROBIOLOGICAL, AND IMMUNOLOGICAL MARKERS OF GINGIVITIS AND PERIODONTITIS UNDER DISEASE INDUCTION AND PROGRESSION

Presented to the Faculty of
The University of Texas Health Science Center at San Antonio,
Department of Community Dentistry
In partial fulfillment of the requirements for the
Certificate in Dental Public Health

By

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August, 1999

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A. Abstract:

Clinical, Microbiological and Immunological Markers of the Induction and Progression of Gingivitis and Periodontitis

Objective: The impact of periodontal disease as a potential risk factor for systemic diseases presents challenges for health promotion and disease prevention strategies. This study examined the clinical, microbiological, and immunological correlates in a disease model to identify potential biomarkers that may be relevant in predicting either the onset or severity of both inflammatory and destructive periodontal disease. Methods: This project incorporated an historical cohort design that examined secondary data obtained on 47 female nonhuman primates over a 6 year period. The animals were in the control groups of five unique projects that utilized the ligature-induced model of periodontitis. Standardization of protocols for sample collection and analysis allowed for comparison over time. Statistical evaluation of biomarkers (bleeding, plaque, pocket depth, attachment, total microorganisms, black pigmented bacteria, Gram-positive bacteria, IgG and IgA) was accomplished using logistic regression analysis. Bleeding and pocket depth measures were selected as the dependent variables of human interest based upon the literature. Results: Significant differences were observed between both baseline or gingivitis measures and periodontitis for all variables. Bleeding-The final logistic model demonstrated a positive correlation with supragingival plaque, pocket depth, and IgG and a negative correlation with IgA. The fit, based upon the Hosmer-Lemeshow statistic, was good (0.7739) and demonstrates a high degree of sensitivity and specificity. Pocket Depth-This logistic model correlated with attachment loss, bleeding, total microorganisms, and IgG. The fit was good (0.5694). Although IgG did not maintain significance in the model, addition of this covariate maximizes the predictive value of the model. Conclusion: Utilization of this model to prospectively examine causal hypotheses can provide focus for human research into the mechanisms of progression from health to gingivitis to periodontitis and support for periodontal disease prevention and health promotion strategies.

B. Name of Project

Clinical, Microbiological and Immunological Markers of the Induction and Progression of Gingivitis and Periodontitis

C. Responsibility of the Resident

To successfully complete this project, the resident served as the manager for each project. In addition, the resident collected all of the samples, performed the assays, calculated adjusted values, analyzed the data for association, and completed statistical analysis of the data.

D. Statement of the Problem

With mounting evidence demonstrating the relationship between periodontal disease and multiple systemic diseases, including diabetes and cardiovascular disease, the public health implications could be profound. The impact of periodontal disease as a potential risk factor presents challenges for health promotion and disease prevention strategies. In an attempt to describe the molecular epidemiology of periodontal disease, models have been employed to explain complex mechanisms of progression from health to disease. To date, reliable mathematical or computer models have not been developed that explain the microbiologic and immunologic interactions reflected in the clinical disease presentation. Animal models, specifically the nonhuman primate, have been used with success to mimic the human disease process via the ligature-induced model. This model allows the ethical induction of disease to examine prospectively changes that occur, and can be used to develop hypotheses that can be further tested in humans.

The nonhuman primate (Nhp) model (Kornman, Holt, Robertson, 1981) characterizes the human periodontal disease experience, clinically, microbiologically, and immunologically (Ebersole, in press). Application of epidemiologic principles to disease model systems can be utilized to develop hypotheses that demonstrate the interrelationship between clinical disease and microbial and host response variables. Incorporation of a historical cohort design permits a prospective examination of this interrelationship. The etiology of periodontal disease progression

reflects a complex series of changes in the resident microbiota from predominantly aerobic to anaerobic, and in host response to the increasing pathogenic insult. This project elucidates this complex alteration in the nonhuman primate model. The purpose of this project is to utilize an *in vivo* model system to test potential associations between disease, microbial ecology and host response changes. This study examined clinical, immunological, and microbiological correlates of induced periodontal disease and its progression in the nonhuman primate model to identify potential biomarkers of its onset or severity.

E. Background

Although a reduction in the presence of pathogenic microorganisms is considered a prerequisite for the control of periodontal disease, the specific causal mechanism has yet to be established. On the other hand, the causal pathway of gingival inflammation has been elucidated. Oral plaque is the primary etiologic factor in gingival inflammatory disease (Silness, 1964) and effective removal of this inciting factor will result in the control of the disease. Woolf (1994) demonstrated that those subjects with poor oral hygiene had a greater number of pathogens compared to subjects with good oral hygiene. Since microorganisms in the dental plaque are the primary cause of experimentally induced gingivitis, variation in the degree of gingivitis may reflect differences in the oral microbiota (Lie, 1995), although this variation has yet to be elucidated. Colonization of the tooth surface is multifactorial, influenced by the inflammation of the gingiva, and plaque formation related to the gingival inflammatory exudate (Ramberg, 1995). Loe (1965) described that removal of supragingival plaque was effective in reducing the prevalence of gingival inflammation.

Schroeder (1980) indicated that inflammation of the gingival tissue is a prerequisite for the formation of periodontitis. Yet, Cappelli (1993) has demonstrated that such inflammation may not ultimately progress from gingivitis to periodontitis. This and similar observations have led to the theory that periodontitis and gingivitis may have separate causal pathways with limited similarities. While gingivitis predisposes one to a more destructive periodontal disease, all sites

exhibiting gingivitis do not develop a more severe disease. Infections caused by selected subgingival pathogens may indicate a predisposing factor on which to model disease severity. Increasing data suggest that periodontal diseases actually result from a mixed infection of periodontopathogenic bacteria (Haffajee, 1994). Supporting data stresses the requirement for these microorganisms to be present in the local microbial environment for periodontal disease progression to occur.

The mechanism of progression from gingival inflammation to frank periodontitis may be a result of the reaction of the host to bacterial insult present in the subgingival environment. The host response to periodontopathogenic microorganisms present in the subgingival environment has been demonstrated to contribute to a more severe disease (Cappelli, 1992). Alterations in the level of immunoglobulin detected in the gingival crevicular fluid have been associated with increasing periodontal disease severity. In this regard, IgG has been demonstrated as the predominant local immunoglobulin in periodontitis (Ogawa, 1989). Further analysis of the crevicular fluid and the subgingival environment may, in fact, establish potential markers for gingival inflammatory disease as well as more destructive periodontal disease by examination of the variability of the local and humoral immune parameters in relation to the microbial insult.

Recognition of the impact of gingivitis and periodontitis in humans has focused research on intervention methods to reduce the prevalence of this disease in the population. However, the lack of a universally accepted method of estimating periodontal treatment needs has hampered this effort (Oliver, 1989). Historically, secondary and tertiary preventive measures that incorporate behavior modification (i.e. smoking cessation), oral hygiene education and continued reliance on home care have been employed to control the prevalence of this disease in the population. Yet, primary preventive measures may be the optimal strategy to reduce the incidence of periodontitis within the population (Sheiham, 1991). Current research has examined the utility of immunization mechanisms as an intervention strategy, but with limited success (Ebersole, 1996) due to the complexity of the infectious process. Recently, potential genetic markers have

been investigated (Kornman, 1996) as an approach for identifying 'at-risk' persons. However, there is a paucity of data which identifies individuals within the population who may be 'at risk' for future development of periodontal disease. Clearly, variation in the host response, the complexity of the periodontal infection, and concommitant social and behavioral risk markers impede the ability to delineate a clear mechanism of the periodontal disease process.

F. Description of Project

This proposal utilized the nonhuman primate (Nhp) model of gingivitis and periodontitis (Ebersole, 1991; Holt, 1988; Kornman, 1981) to further understand the mechanisms of progression from health to inflammation and ultimately to destructive disease. This project examined potential biomarkers for the development of gingival inflammation and further progression to destructive periodontal disease in this nonhuman primate population. The ultimate goal was the transfer of information that can elucidate populations at risk for disease and the development of primary preventive strategies.

This project incorporates an historical cohort design to examine secondary data obtained from 1991 to 1997 on 47 female, nonhuman primates (*Macaca fascicularis*). The nonhuman primates in this study were control animals used in five unique projects. The study protocols, sample collection and analysis were performed under Good Laboratory Practice (GLP) guidelines as established by the Food and Drug Administration. Compliance to GLP insured standardization of methods of collection and analysis and permits comparison across studies.

Inclusion and exclusion criteria were established to insure the welfare of the colony. Nonhuman primates were excluded from any study for: i) high degree of certainty that participation in the study will result in morbidity/mortality; ii) chronic medical diagnosis that requires long term therapy and that may confound the outcome of the study; iii) severe oral disease requiring therapy that would interfere with the study outcome; iv) communicable disease of sufficient severity that continued maintenance in the colony would jeopardize the health and well-being of the remainder of the colony.

Since 1991, studies have been conducted in our facility using the nonhuman primate model originally defined by Kornman, Holt, and Robertson (1981). Although experimental treatment varied by study, the control or non-experimental groups were managed according to a standardized protocol and they were the subjects of this study. This protocol incorporated a three phase design: i) hygiene (healthy) phase, ii) gingivitis phase, and iii) periodontitis phase. The Nhp were stratified for each study based upon a pretreatment baseline survey of the combination of the serum antibody levels to *P. gingivalis* and the bleeding index. Both of these indices have been shown to correlate with each other (Moritz, 1998) for purposes of stratification.

i. Gingivitis Phase: After completion of hygiene, the Nhp were placed on a soft chow diet to facilitate the production of inflammation. The primate chow is allowed to soak in warm water for approximately 2 minutes, drained and fed. The animals were maintained on the chow for 30 days and samples were obtained 7, 14 and 28 days after the initiation of soft diet.

ii. Periodontitis Phase: At the conclusion of the Day 28 sample collection, ligatures were tied around the study teeth and periodontal disease was allowed to develop. Each study tooth was ligated with a 3-0 silk suture, tied in a surgical knot, and packed subgingivally. Ligatures remained for 90 days with measurements and samples collected at intervals of 30, 60 and 90 days.

G. Procedures and Methods

Variables: In both humans and nonhuman primates, the presence of bleeding upon gentle probing is the clinical determinant of gingivitis. The scoring of bleeding was based upon well-documented indices (Silness, 1963) that have gained acceptance as the clinical standard of the presence of gingivitis. Periodontitis, on the other hand, has less clearly defined clinical measures of detection. Attachment level changes and pocket depth have been utilized to define periodontal changes in both humans and nonhuman primates. However, the literature (Schou, 1993) suggests that variability in the attachment level measure, due to the the small increase in pocket depth, size of the teeth, and limitations of clinical measurement systems, makes this measure an unreliable

marker of disease progression in nonhuman primates. Pocket depth measures are considered a more reliable measure of periodontal disease in the nonhuman primate model (Table 1).

Since the causal pathway for the expression of gingivitis and periodontitis has yet to be elucidated, several exposure variables were examined to determine the association with disease progression and induction. The current body of knowledge indicates that periodontal diseases follow an infectious disease paradigm. Plaque has a complex microbial ecology, which is transformed from a predominantly aerobic to more anaerobic environment as the plaque accumulates and matures. This transformation is accompanied by an increase in the pathogenesis of the bacteria incorporated in the biofilm. Subsequent to the establishment of this pathogenic insult, the host responds, both locally and systemically, to this challenge. Increases in both local and systemic production of IgG and IgA support this observation (Ebersole, 1991). Microbiological products, in the form of endotoxin or lipopolysaccharide, are expressed which can lead to tissue destruction. Furthermore, the nonspecific immune response mounted by the host can lead to destruction of the gingival tissue and underlying bone. Ancillary clinical measurements that have been thought to indicate periodontal disease progression were examined to determine the potential association with accepted periodontal disease measures. Therefore, supragingival plaque, recession and attachment loss was compared to the outcome variables in an effort to detect an association. The outcome is determined clinically as inflammation of the surrounding tissue and/or an increase in probing depth, which are characteristics of gingivitis and periodontitis.

To follow this schema, several exposure variables were recorded to determine potential correlation with measures of gingivitis and periodontitis. Microbiological: Total bacteria, black pigmented bacteria, and Gram-positive microorganisms. These classifications have relevance in the initiation and progression of disease. Immunological: Local antibody measurements will be accomplished for analysis with the clinical outcome variables. Local antibody levels, IgG and

IgA were measured in crevicular fluid. These variables indicated the local immune response to the periodontopathogenic insult.

Sample Collection: Samples obtained at each time point included: i) clinical measurements of supragingival plaque formation, pocket depth, attachment level, recession, and bleeding upon probing. Scoring was performed on four sites of each of the study teeth: mesiobuccal, buccal, distobuccal and lingual. Study teeth included tooth numbers 18, 19, 20, 29, 30, and 31 (mandibular first and second molars and second bicuspids). Because of the homology to the human dentition, posterior teeth were preferred. Elongated canines and anterior diastemas in the nonhuman primate create sufficient deviation to the human structure that may impact the similarities to the human disease experience. Protocols for obtaining clinical measurements and collection and analysis of the microbiologic and immunologic samples appear in Appendix 1.

Animal Husbandry and Veterinary Care: All projects were conducted according to IACUC (Institutional Animal Care and Use Committee, UTHSCSA) approved protocols in the Laboratory Animal Resource (LAR) Facility under the supervision of the veterinarian. Animal handling and sedation methods were managed based on LAR Standard Operating Procedures. Husbandry duties were conducted by LAR personnel. Untoward reactions during the course of the studies were noted and became a part of the study records.

Data Collection and Management: After data collection, the data was coded and entered into a database. Data analysis was performed using the STATA® or Minitab® software packages. Parameters included periodontal assessments of visible supragingival plaque and bleeding as categorical variables. Pocket depth and attachment were recorded as continuous variables. Laboratory findings were entered into the database as continuous variables.

Data Analysis: Initial comparisons were made based upon exposure variables (initiation of soft diet, ligature placement). Increase in disease severity, from gingivitis to periodontitis, was determined.using mean values, as a measure of central tendency, for each

animal at each time point. Means and standard error was calculated for attachment loss, pocket depth, and levels of subgingival plaque and ELISA values. Means were considered appropriate for this analysis because disease was induced in the model, in fact, standardizing the outcome. In a naturally occurring disease model, the worst site value may be more appropriate measure of tendency. Student's t-test analysis of the means was conducted. Data was plotted using incidence of disease activity as time 0 and examining changes in both the microbial environment and host response parameters before and after the event. This method of data analysis identified changes in both microbiological and immunological parameters prior to and after clinical identification of disease. Correlation coefficients were calcualated within each time point for all variables. A logistic regression analysis was performed on all of the data to evaluate potential associations and to control for potential confounders in the data. Odds ratios were calculated for each risk factor.

H. Findings

a. Dependent Variables: Bleeding exhibited a bimodal frequency distribution (Figure 1A). The distributions for both bleeding and pocket depth demonstrated distinct frequency distributions between disease states (Figure 1B, 1C). Bleeding measures during gingivitis ranged from 0-1.8 with the mode at 0.6 while for ligature-induced periodontitis, bleeding ranged from 0.5-3 with the mode at 1.5. Pocket depth measurements (Figure 2A) exhibited a similar frequency distribution between gingivitis and periodontitis (Figure 2B, 2C). The range of values during gingivitis was 1.0-3.0 with the mode at 1.6. During periodontitis, the pocket depth ranged from 1.8-3.8 with the mode at 2.8. Based upon these frequency distribution, cut points were set at 1.0 for bleeding and 2.0 for pocket depth.

b. Distribution of Variables over Time: Dependent and independent variables were examined to determine change between each time point and from baseline. Group means with standard errors are depicted in Figures 3, 4, 5. Time points labeled with an asterisk (*)/(**) indicate variations that are significant at a 0.5 level or less. Clinical indices, bleeding, plaque, and pocket depth, demonstrated an increase in severity from gingivitis to periodontitis (Figure 3). Bleeding

and pocket depth (3A, 3C) exhibited a bivariate distribution from gingivitis to periodontitis, while changes in plaque occurred in a linear configuration. Attachment loss (3D) was not significant at any time point. Microbiological changes over time demonstrated a similar pattern to the clinical parameters (Figure 4) with a relatively stable ecology through gingivitis and marked increases observed through periodontitis. While the clinical indices seem to stabilize by 90 days of ligature-induced disease, the microbiota appeared to decrease within the same time period. Immunological parameters of gingivitis and periodontitis (Figure 5) revealed a different pattern throughout the disease process. IgA (5B) appeared to be enhanced through the hygiene (baseline) process where the highest level is evidenced. A reduction in IgA levels from baseline was observed throughout gingivitis with an increase during the challenge of ligature-induced periodontitis. IgG levels (5A) tended to remain stable thoughout gingivitis and increase during the ligature phase.

c. Correlations at Individual Time Points: Variables at each time point were examined to determine trends through disease progression that may indicate potential associations (Table 2, 3). Table 2 outlines correlations at all three stages of gingivitis, G1, G2 and G3. At all time points, attachment loss and pocket depth level measurements were highly correlated. In early gingivitis (G1 and G2), microbiological variables, specifically total microorganisms and black pigmented bacteria, correlated with IgA levels. Gram-positive organisms correlated with pocket depth in early gingivitis and with total bacteria in late gingivitis. In late gingivitis (G3), a slightly different pattern emerged with a positive correlation between supragingival plaque levels and IgA.

At all periodontitis time points (L1-L3), attachment loss was correlated with changes in recession levels and levels of total bacteria were correlated with levels of black pigmented species in the subgingival plaque (Table 3) while pocket depth measures were inversely correlated to recession levels, as may be expected. Increases in both bleeding and plaque were associated with an increase in pocket depth at L3. Throughout ligature-induced periodontitis, increases in IgG levels correlated with increases in IgA levels. In early periodontal disease, increases in bleeding were associated with IgA levels.

d. Univariate Analysis of Variables: Dependent variables (bleeding, pocket depth) were examined against independent variables for association using both the Wald test (p) and the comparison of the odds ratio (Table 4). Of the nine variables, black pigmented bacteria did not demonstrate a statistical association against bleeding. When compared to pocket depth, only IgA did not retain significance according to the criteria.

e. Multivariate Analysis of Variables: The remaining variables were eligible for inclusion within the models. Variables were retained in the model based upon the fit and contribution to the outcome of the model (Tables 5 and 6). For the outcome variable bleeding, the final logistic model included the following covariates: supragingival plaque, pocket depth, IgG and IgA levels. Based upon the Hosmer-Lemeshow statistic, the fit was good (0.7739) and significance of the model was confirmed by both the odds ratio and the Wald statistic less than 0.05. Further analysis of the model indicated a high degree of sensitivity (78.22%) and specificity (82.41%) with 80.38% of the animals correctly classified as to disease status.

Development of the model to describe the relationship of pocket depth and several covariates, including attachment loss, bleeding, total microorganisms, and IgG suggested a complex relationship of microbiologic and immunologic parameters in presentation of clinical disease. The fit of this model was good (Hosmer–Lemeshow=0.5694) and this model provided a high level of sensitivity (89.09%) and specificity (86.25%). All but one of the covariates was significant at the 0.000 level. Although IgG does not achieve a $p \leq 0.05$ level of statistical significance in this model, inclusion was based upon optimization of sensitivity and specificity values.

I. Discussion

a. Dependent Variables: The bimodality demonstrated in bleeding appeared to be specific to gingivitis and periodontitis. Although bimodality was not evident in the pocket depth frequency distribution, distinct patterns emerged differentiating gingivitis values from periodontitis values, suggesting a different patterns exists between the disease states, gingivitis and periodontitis, for

this variable. This observation of the continuous variables permitted their dichotomization for further analysis by logistic regression methodologies.

b. Distribution of Variables over Time: Bleeding (Figure 3A) demonstrated statistically significant differences in this measure at each stage of disease, from health (baseline) through gingivitis, and periodontitis, which may make this variable the ideal marker to describe disease progression. Supragingival plaque (Figure 3B) levels exhibited a linear progression from health to disease. This progression achieved a statistically significant difference from baseline values at G3. The plateau effect observed at L2-L3 may be a result of the limitation of the scoring system which has an upper limit of 3. Pocket depth measurements (Figure 3C) presented with a distinct dichotomous distribution between gingivitis and periodontitis in this measure. This observation supported the use of this covariate as a dependent variable in model development. Both bleeding and pocket depth measures exhibited a dramatic increase between gingivitis and periodontitis in which further supports their use as dependent variables in creation of a model. Both bleeding and pocket depth measurements exhibit a plateau effect through late periodontitis (L2-L3). Analysis of the changes in attachment loss (Figure 3D) through health and disease did not demonstrate a statistical difference. Furthermore, the increasing size of the standard error suggested that this variable would not be reliable for use in model generation and supports observations in the literature (Schou, 1993). The divergent standard errors may indicate that as inciting factors increase, not all nonhuman primates respond to these risk factors in the same fashion, resulting in the generation of high and low responder groups.

Total microorganisms (Figure 4) in the subgingival plaque remained stable throughout the gingivitis phase. Although the supragingival plaque mass increased linearly, no change in the number of subgingival pathogens was observed. A dramatic increase in total bacteria (Figure 4A) was observed with the placement of the ligature that was concomitant with the increase in black pigmented species and Gram-positive microorganisms. These observations, along with clinical manifestation of periodontitis, support the specific plaque hypothesis. In all three microbiological

parameters examined, a plateau effect was observed between L2 and L3. This observation suggests that periodontal disease is self-limiting, possibly through the ability of the host to respond to the biologic challenge, resulting in a 'burst' of disease.

An increase in immunoglobulin levels at L1 and L2 may have contributed to the limiting of destructive disease that was demonstrated clinically and microbiologically (Figure 5). The timing of the increase in IgA followed by increasing IgG suggests an evolution from an acute to chronic response to the infection. The elevation of IgA levels (Figure 5B) at baseline may have been in response to a repeated insult to the gingival tissue through hygiene. Repeated cleaning can have an "immunization" effect by irritation of the tissues and introduction of small amounts of bacteria. This repeated procedure could have lead to stimulation of antibody production in response to the irritation and disruption of the biofilm.

c. Correlations at Individual Time Points: Patterns emerged in both gingivitis and periodontitis that may lead to molecular characterization of each disease state (Table 2 and 3). Increases in total microorganisms, black pigmented species, and supragingival plaque mass were highly correlated with IgA levels suggesting that the host mounts an acute response to the increasing challenge. The presence of increasing Gram-positive microorganisms in early gingivitis and this correlation with pocket depth preceded the shift to a more pathologic state noted by the increase in black pigmented species in later gingivitis. Consistent correlation between increasing attachment loss with pocket depth measures may be indicative of a progressing gingival disease process.

Following ligature placement and the induction of periodontitis in this model, several associations became apparent. The correlation between increases in total bacteria and increases in black pigmented bacteria indicate a change in the microbial ecology in both number, but also in pathogenicity. Recession was positively correlated with attachment loss and negatively correlated with pocket depth measures. This observation suggests that tissue inflammation is correlated to the loss of attachment which would indicate an active clinical disease process. This observation is

supported by the correlation between pocket depth and both increased bleeding and supragingival plaque accumulation. In addition, correlation between IgA and IgG levels may indicate the induction of the host response to an increasing biologic insult.

d. Univariate Analysis of Variables: Univariate analysis of the nine covariates against the two dependent variables (Table 4) provided supportive evidence of the biologic relevance to include these variables in the model system. Although human evidence for inclusion of these variables is documented in the literature, statistical significance indicates the applicability of this model system for examination of gingival disease and supports the biologic homology to the human.

e. Multivariate Analysis of Variables: Two models were prepared to reflect the recognized markers of disease for both gingivitis and periodontitis (Tables 5 and 6). Both models expressed similarities that may suggest applicability of a single model design. Gingival bleeding appeared to have a direct correlation with increasing supragingival plaque, pocket depth, and IgG. This observation supports current evidence of causal hypotheses related to gingivitis. Supragingival plaque mass contributes to a heightened inflammatory response, and possibly an increase in IgG production, with an ultimate increase in pocket depth related to the clinical inflammatory response. The inverse relationship of IgA is an interesting finding. Since IgA is related to an early response, the inability of the host to respond to this insult may contribute to disease severity. Sensitivity (78.22%) and specificity (82.41%) demonstrated the ability of this model to accurately identify individuals with disease and without disease.

The model for pocket depth included attachment, bleeding, total microorganisms, and IgG as covariates. As noted, IgG did not achieve statistical significance based upon the Wald test or analysis of the odds ratio and confidence interval, however, inclusion of this variable improved both the sensitivity and specificity of the model. The understanding of its biologic significance and its role in inflammation, along with the improvement of the utility of the model, was the reasoning for including this covariate in the model.

In developing this model, it became apparent that the clinical covariates assumed a primary role in this association. Bleeding and attachment loss were highly correlated. Interestingly, subgingival plaque correlated with increasing disease. While supragingival plaque mass was associated with gingivitis, subgingival colonization is correlated with increasing pocket depth. The observed improvement in the predictive value of the model with the inclusion of IgG may reflect the secondary role of the inflammatory response. The hypothesis that loss of supporting periodontal structure may be attributable to the microbiota and their by-products rather than the cytotoxic properties of the immune response may be supported in this model.

J. Conclusion

Both the model for bleeding and pocket depth provide statistically significant and biologically sound explanations for gingival disease induction and progression. These observations suggest that the nonhuman primate model has direct applicability to study the molecular epidemiology of both gingival and periodontal diseases. These findings support the use of the model to mimic disease initiation and progression in humans and suggests potential causal correlations that can be tested using these and additional biomarkers. The ability to use an *in vivo* induced disease model system to develop epidemiologic hypotheses that can be further studied in humans provides an opportunity to investigate not only causal associations, but also primary preventive strategies. By induction of periodontitis, linkages between periodontal disease and systemic diseases can be examined in a prospective manner to establish molecular mechanisms that delineate this association. Molecular model development can provide focus to human research of periodontal disease and support for primary prevention and health promotion strategies.

K. Suggested Changes to the Project:

Although these animals are all adults, determined by onset of menses and presence of third molars, an exact date of birth would have been useful to further examine the impact of these covariates on disease progression. The use of females is based upon scientific and historical

application of the model, however this practice reduces the generalizability of the model. Since an association between bone density and estrogen levels has become evident, the use of females may have introduced confounding.

L. References

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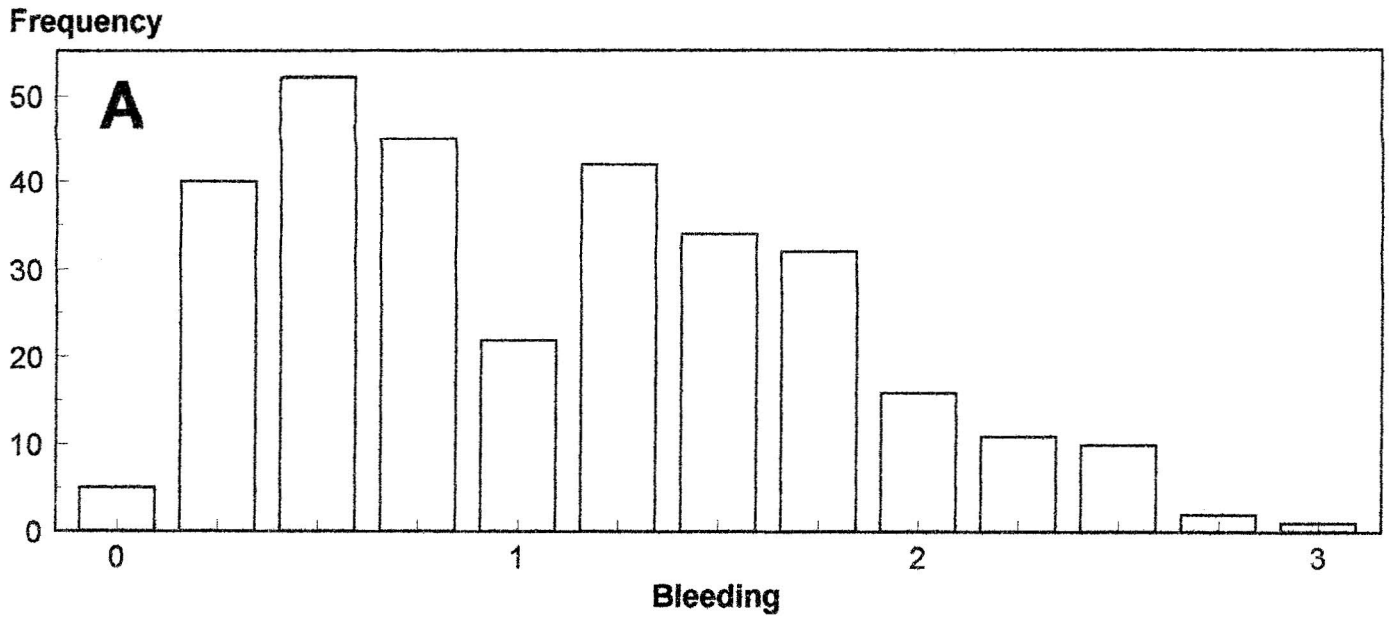
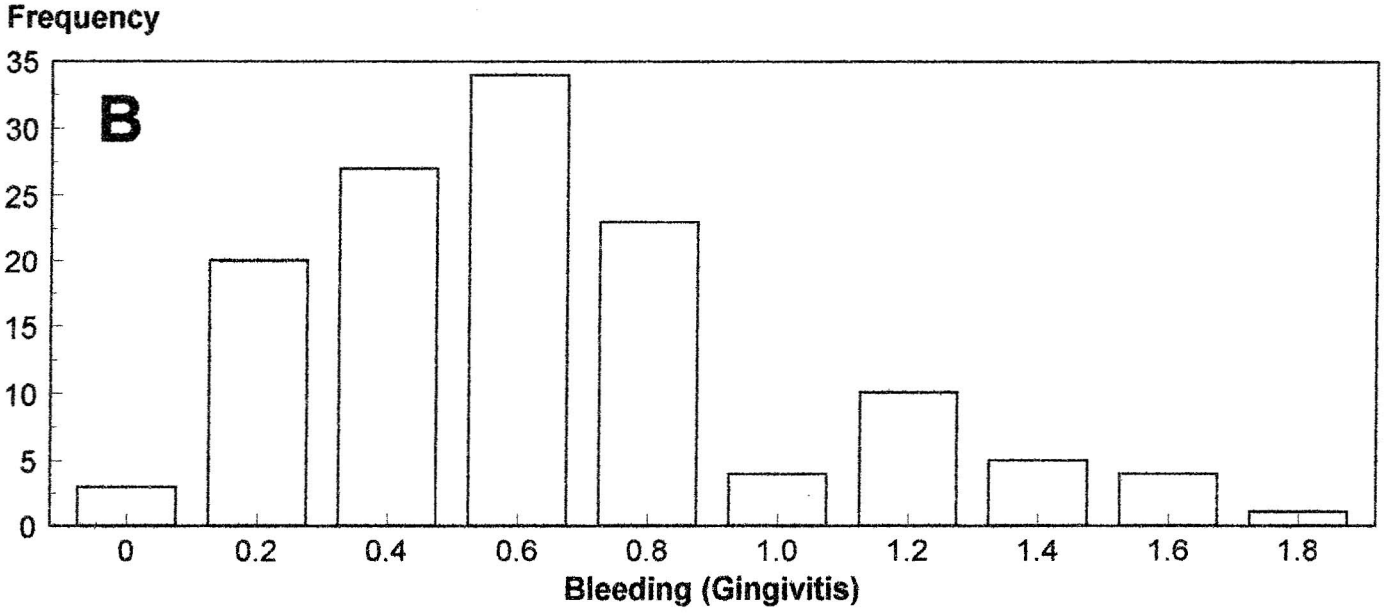
Table 1. Markers Utilized in Model Development. Variables analyzed in this study are listed by biological parameter. Dependent variables, labeled with an asterisk (*), are examined for association to the remaining covariates.

Clinical	**Microbiological**	**Immunological**
Bleeding on Probing (*)	Total Microorganisms	Total IgG
Pocket Depth (*)	Black Pigmented Bacteria	Total IgA
Supragingival Plaque	Gram-positive Bacteria	
Recession		
Attachment Loss		

Figure 1. Frequency Distribution for Bleeding. This distribution is based upon the mouth mean for each of the subjects. A: Illustration of the distribution of bleeding for this sample. A bimodal distribution is evident. B: Distribution of bleeding for gingivitis (G1, G2, G3) demonstrates a range of 0-1.8 with the mode of 0.6. C: The range of the mean values of 0.5-3.0 for bleeding during periodontitis (L1, L2, L3) is shown. The mode for this distribution is 1.5. Based upon these frequency distributions, a cut point of 1.0 was established.


Frequency
A
50
40
30
20
10
0
0
1
2
3
Bleeding


Frequency
B
35
30
25
20
15
10
5
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Bleeding (Gingivitis)

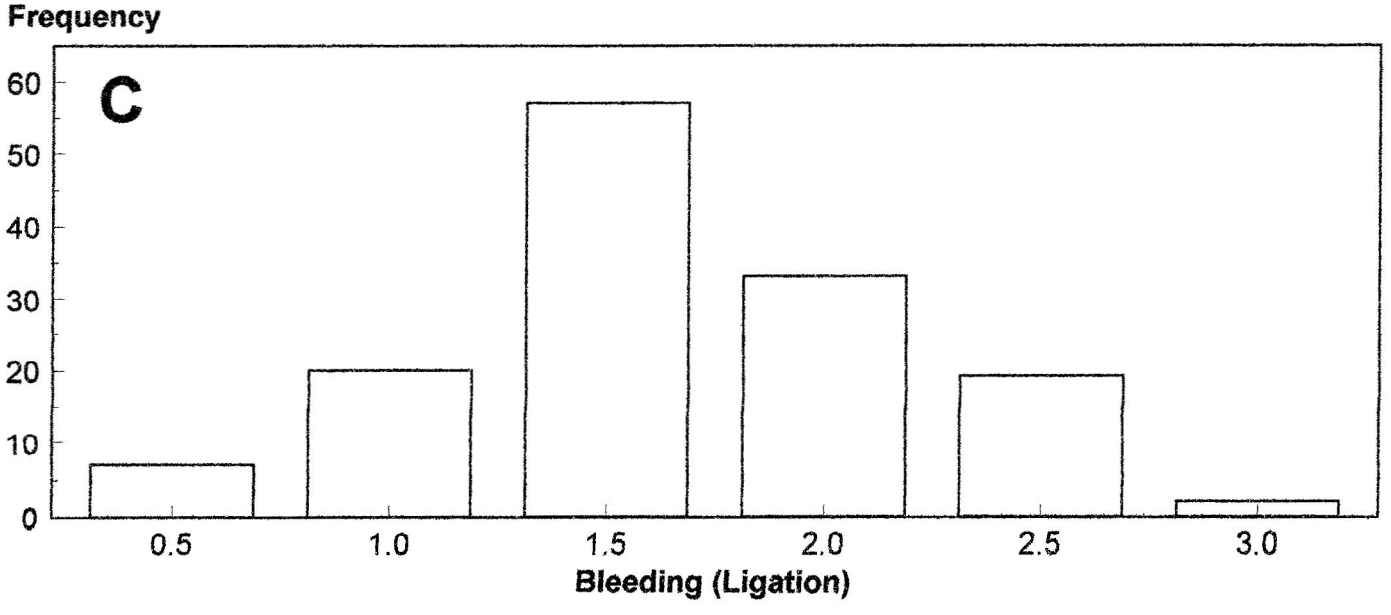
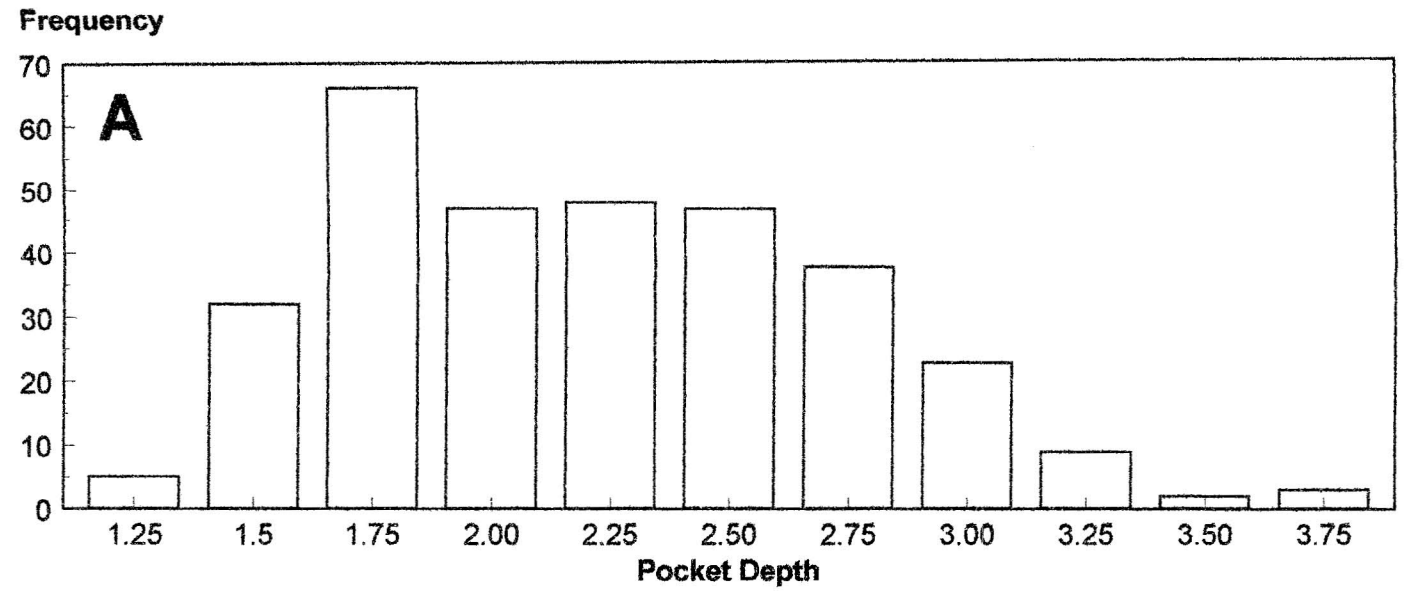
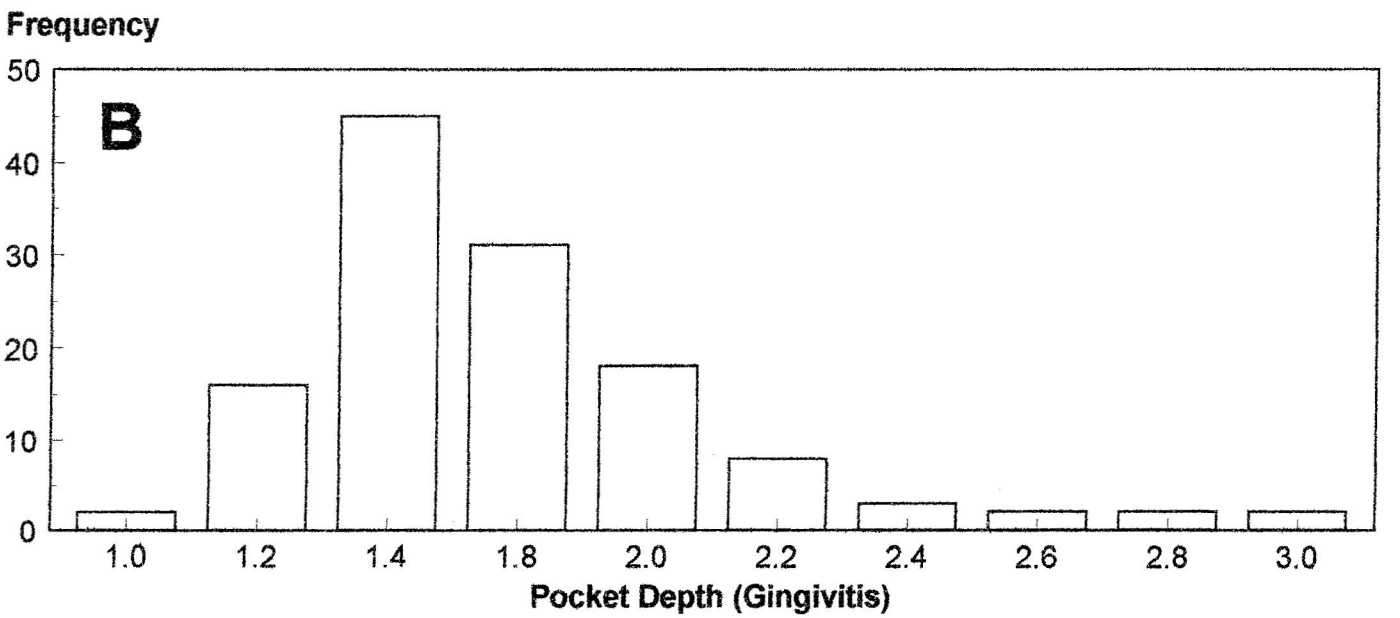

Frequency
C
60
50
40
30
20
10
0
0.5
1.0
1.5
2.0
2.5
3.0
Bleeding (Ligation)

Figure 2. Frequency Distribution for Pocket Depth. This distribution is based upon the mouth mean for each of the subjects. A: Illustration of the distribution of pocket depths for this sample. B: Distribution of pocket depths for gingivitis (G1, G2, G3) demonstrates a range of 1.0-3.0 with the mode of 1.6. C: The range of the mean values of 1.8-3.8 for pocketing during periodontitis (L1, L2, L3) is shown. Although the distribution is distinct for gingivitis and periodontitis, bimodality is not clearly demonstrated. The mode for this distribution is 2.8. Based upon these frequency distributions, a cut point of 2.0 was established.


Frequency
A
70
60
50
40
30
20
10
0
1.25
1.5
1.75
2.00
2.25
2.50
2.75
3.00
3.25
3.50
3.75
Pocket Depth


Frequency
B
50
40
30
20
10
0
1.0
1.2
1.4
1.8
2.0
2.2
2.4
2.6
2.8
3.0
Pocket Depth (Gingivitis)

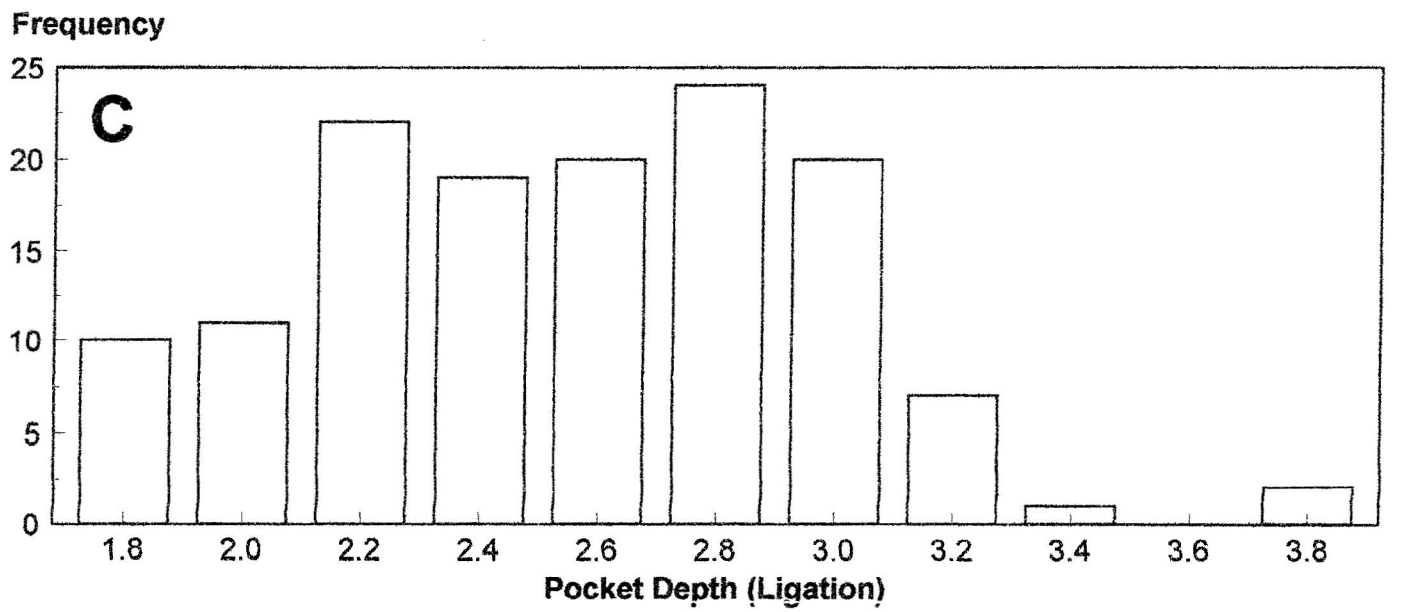
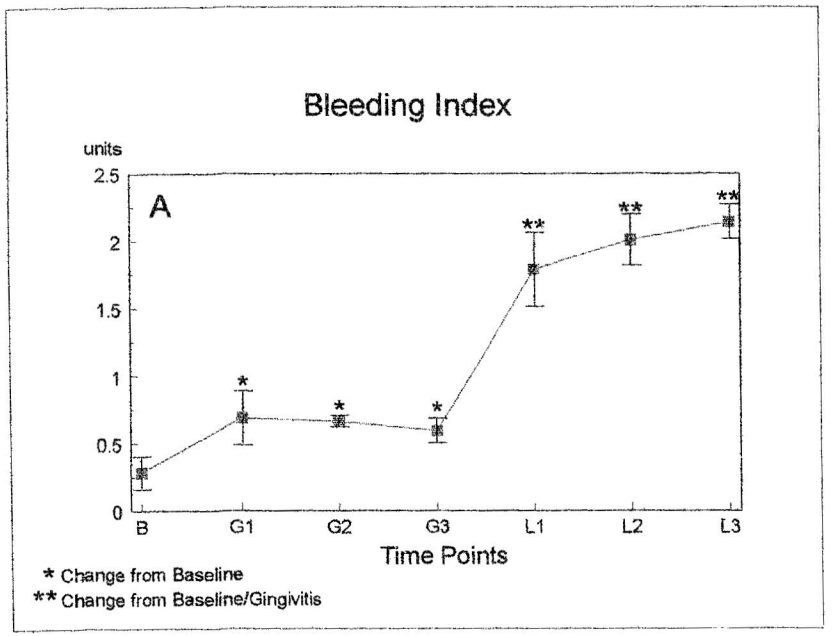
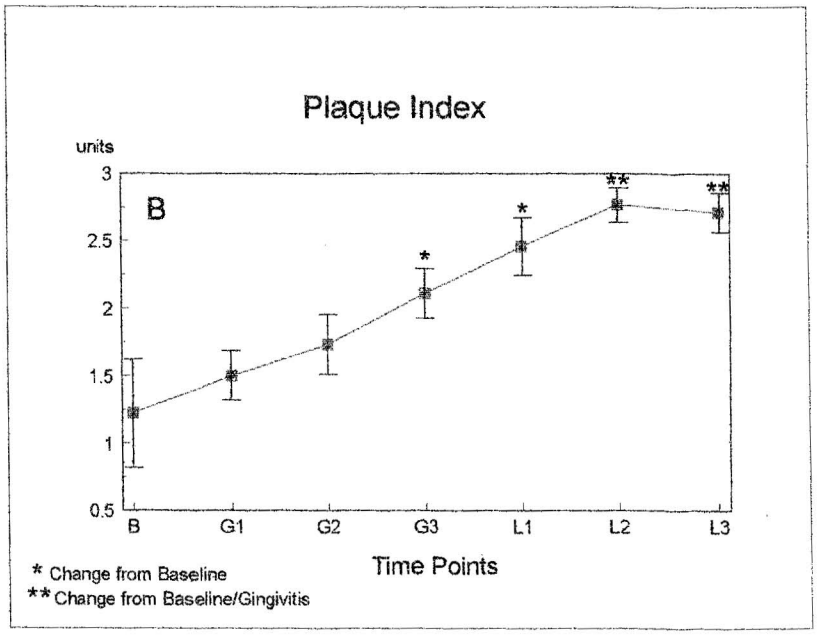
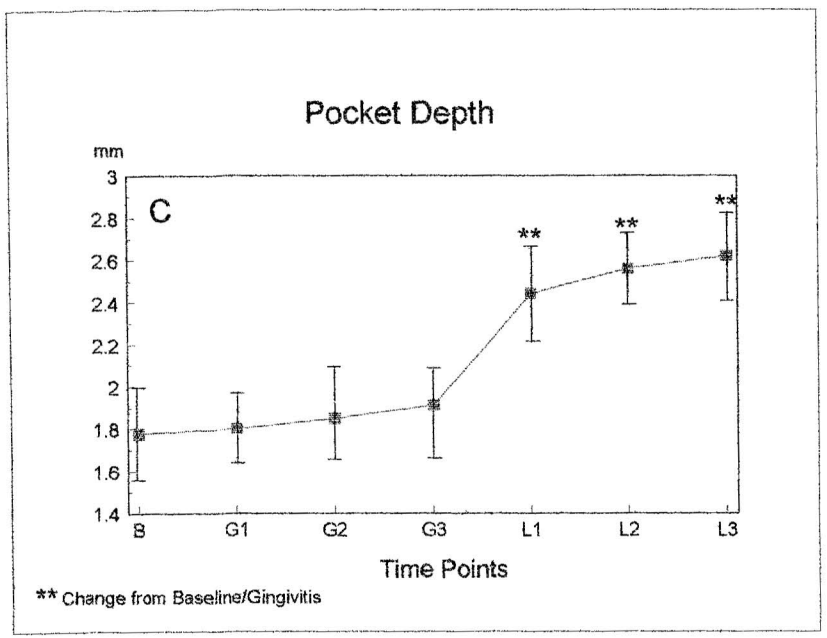

Frequency
C
25
20
15
10
5
0
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
Pocket Depth (Ligation)

Figure 3. Distribution of Clinical Variables over Time. Means of the values at each time point along with standard errors are presented for the bleeding index (A), plaque index (B), pocket depth (C), and attachment level (D). While pocket depth and attachment level are presented in millimeters, bleeding and plaque scores are recorded in units based upon the specific index value.


Bleeding Index
units
2.5
2
1.5
1
0.5
0
A
B
G1
G2
G3
L1
L2
L3
Time Points
* Change from Baseline
** Change from Baseline/Gingivitis


Plaque Index
units
3
2.5
2
1.5
1
0.5
B
B
G1
G2
G3
L1
L2
L3
Time Points
* Change from Baseline
** Change from Baseline/Gingivitis


Pocket Depth
mm
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
C
B
G1
G2
G3
L1
L2
L3
Time Points
** Change from Baseline/Gingivitis

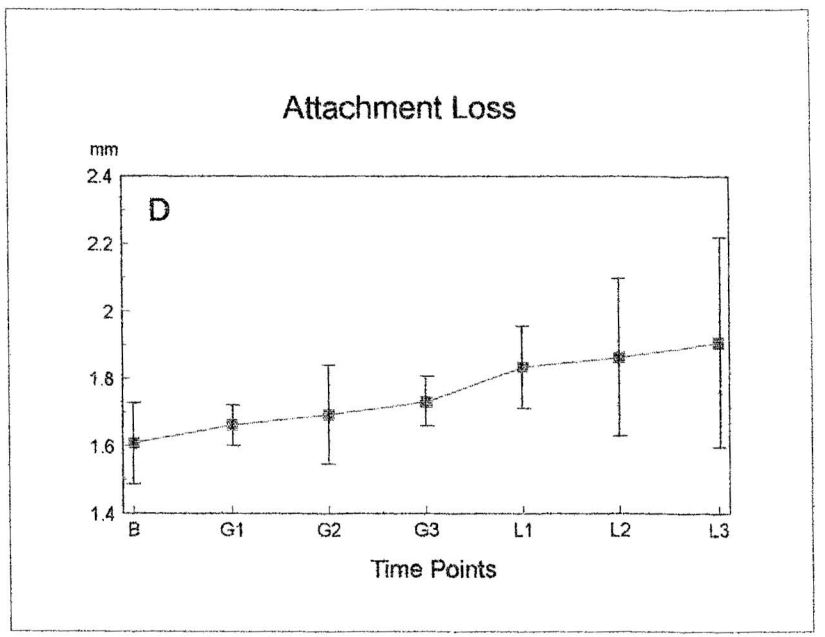
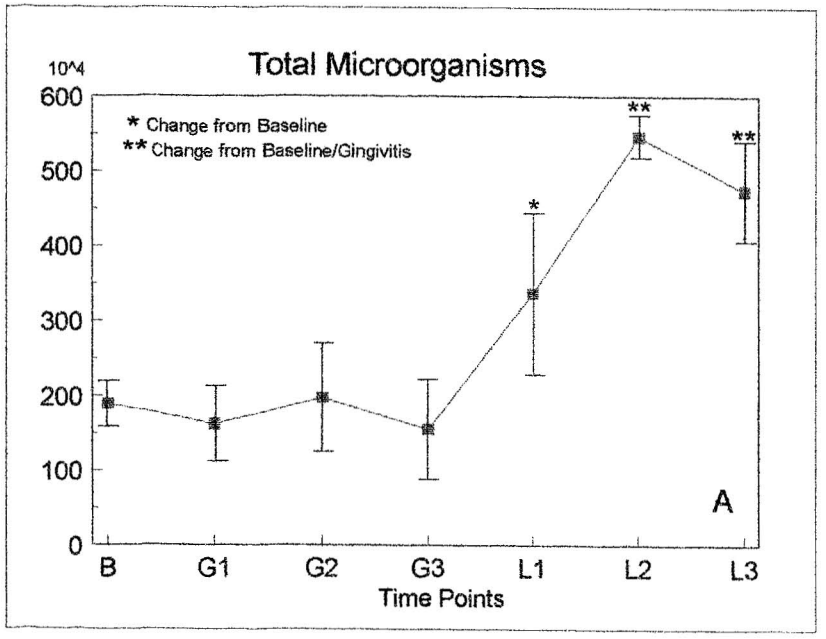
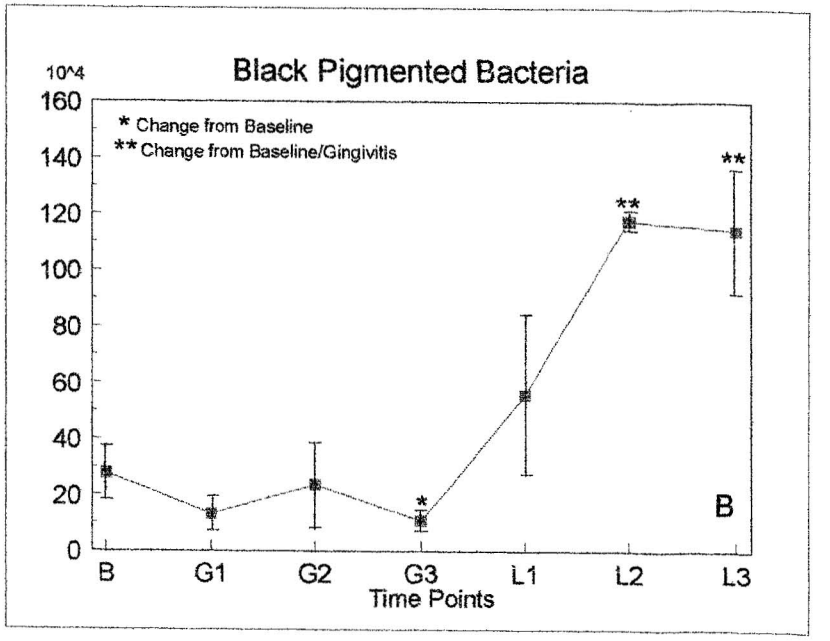

Attachment Loss
mm
2.4
2.2
2
1.8
1.6
1.4
D
B
G1
G2
G3
L1
L2
L3
Time Points

Figure 4. Distribution of Microbiological Variables over Time. Means of the values at each time point along with standard errors are presented for total microorganisms (A), black pigmented bacteria (B), and Gram-positive microorganisms (C). Values are presented as the number of colonies times 10^4.


Total Microorganisms
10^4
600
500
400
300
200
100
0
* Change from Baseline
** Change from Baseline/Gingivitis
*
**
**
A
B
G1
G2
G3
L1
L2
L3
Time Points


Black Pigmented Bacteria
10^4
160
140
120
100
80
60
40
20
0
* Change from Baseline
** Change from Baseline/Gingivitis
*
**
**
B
B
G1
G2
G3
L1
L2
L3
Time Points

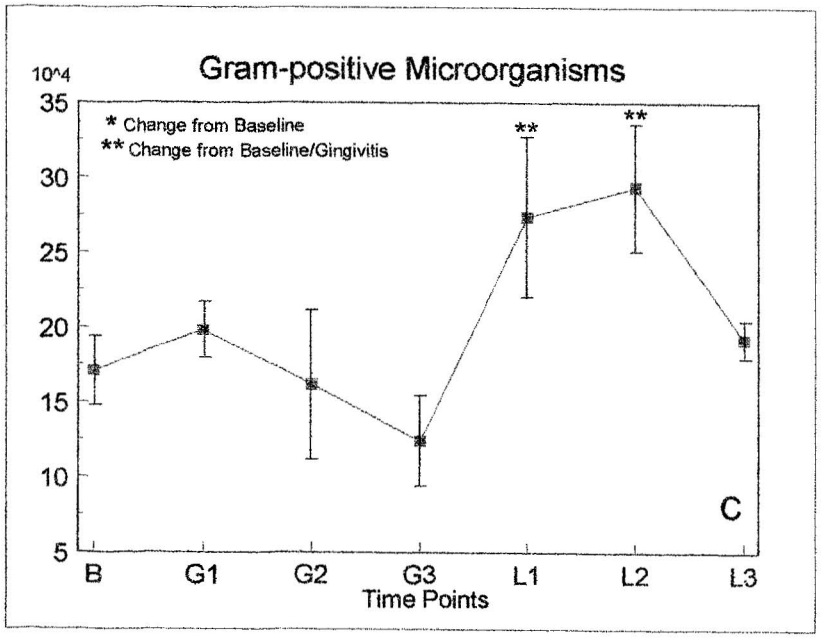
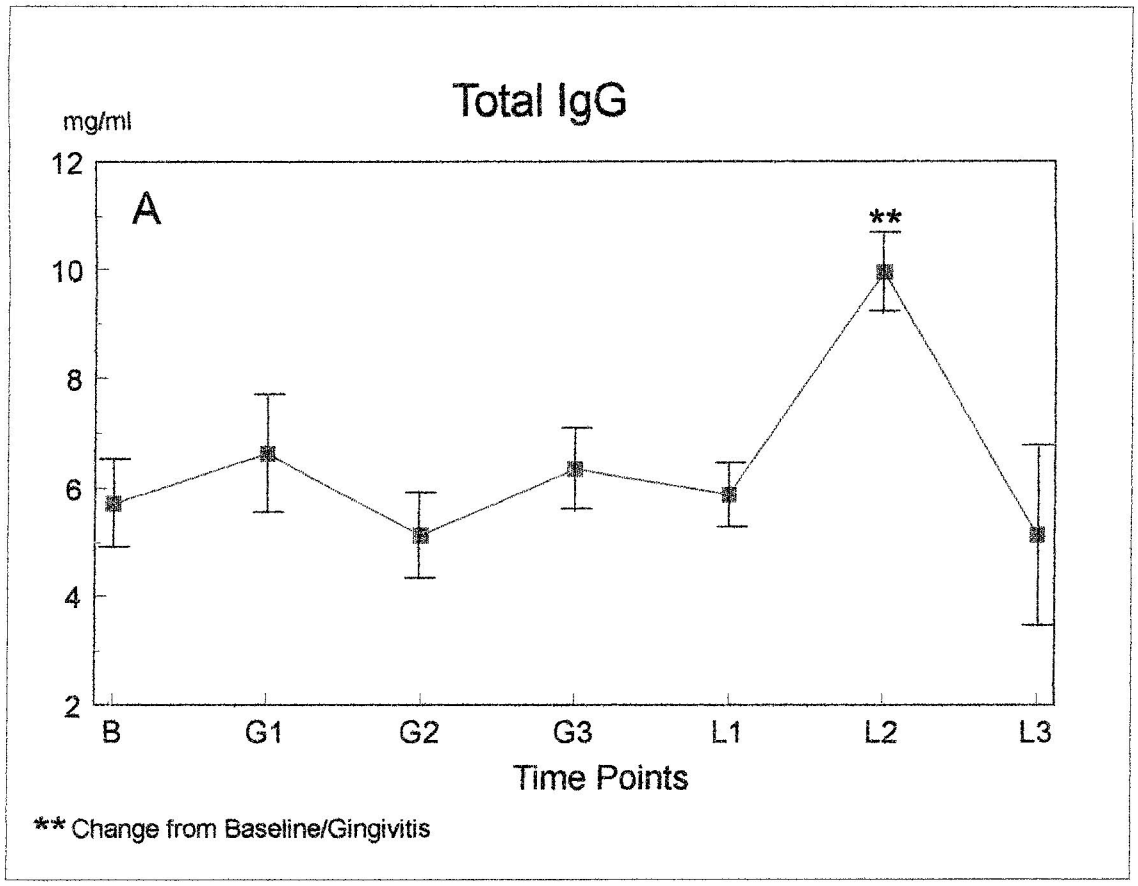

Gram-positive Microorganisms
10^4
35
30
25
20
15
10
5
* Change from Baseline
** Change from Baseline/Gingivitis
**
**
C
B
G1
G2
G3
L1
L2
L3
Time Points

Figure 5. Distribution of Immunological Variables over Time. Means of the values at each time point along with standard errors are presented for total IgG (A) and total IgA (B). Values are presented as ELISA units (EU). Change from baseline values are marked with an asterisk (*) while those values that demonstrated a change from baseline and gingivitis are annotated with a double asterisk (**).


Total IgG
mg/ml
A
12
10
8
6
4
2
**
B
G1
G2
G3
L1
L2
L3
Time Points
** Change from Baseline/Gingivitis

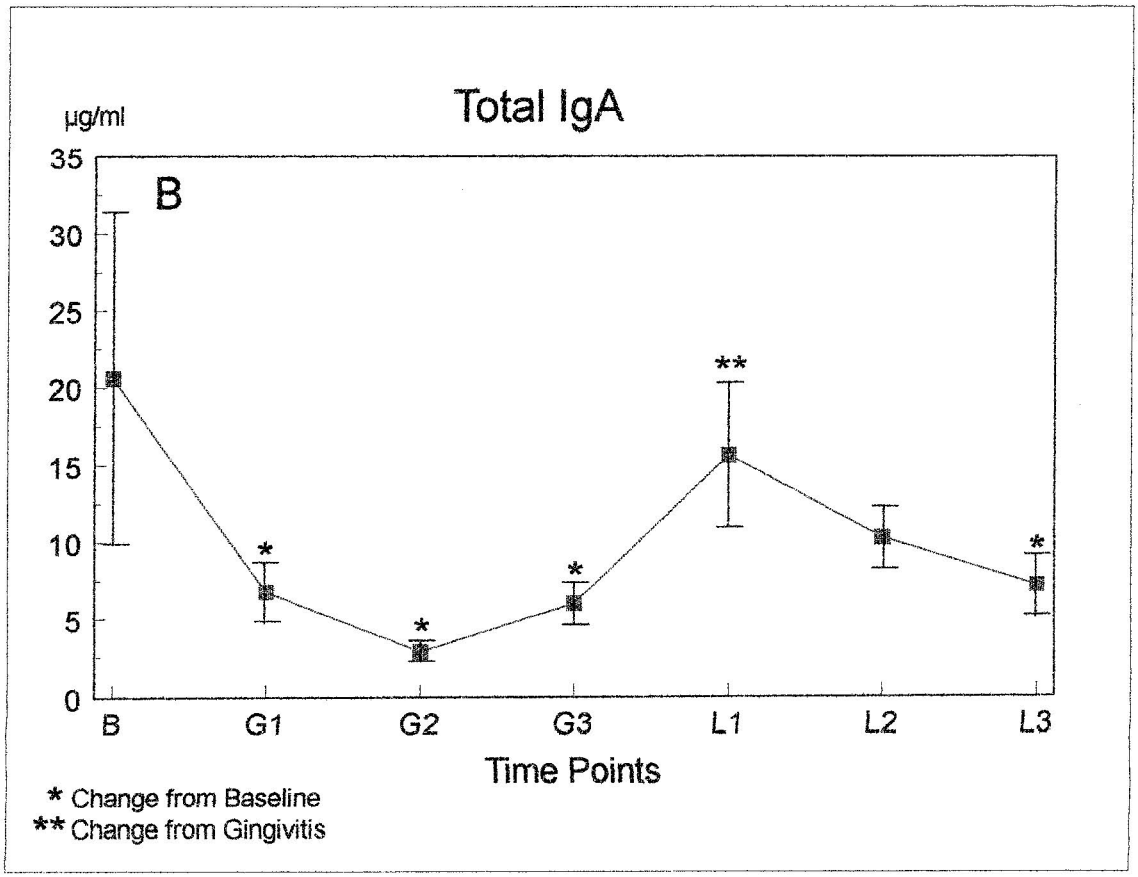

Total IgA
µg/ml
B
35
30
25
20
15
10
5
0
*
*
*
**
*
B
G1
G2
G3
L1
L2
L3
Time Points
* Change from Baseline
** Change from Gingivitis

Table 2. Correlation coefficients. Significant correlation coefficients are presented for each variable at each time point during gingivitis (G1, G2, and G3). Covariate abbreviations are described in the List of Abbreviations (page ii).

G1	PD	BPB	IgG
AL	0.790		
IgA		0.699	0.715

G2	PD	ETSA
AL	0.834	
G+	0.719	
IgA		0.634

G3	PD	Plaque	ETSA
AL	0.634		
IgA		0.611	
BPB			0.648

Table 3. Correlation coefficients. Significant correlation coefficients are presented for each variable at each time point during ligature-induced periodontitis (L1, L2, and L3). Covariate abbreviations are described in the List of Abbreviations (page ii).

L1	AL	Bleeding	ETSA	IgG
Recession	0.610			
BPB			0.701	
IgA		0.633		0.735

L2	PD	AL	ETSA	IgG
Bleeding	0.554			
Recession	-0.756	0.746		
BPB			0.971	
IgA				0.670

L3	PD	AL	ETSA	IgG
Bleeding	0.594			
Plaque	0.614			
Recession	-0.621	0.761		
BPB			0.708	
IgA				0.543

Table 4. *Univariate Model of Covariates for Bleeding and Pocket Depth.* Associations are presented as an odds ratio (OR), 95% confidence interval (95% CI), z statistic, and Wald statistic (p) for each covariate.

Bleeding

Variable	OR	95% CI	z	p
Plaque	9.19	5.34, 15.81	8.012	0.000
PD	36.32	16.63, 79.34	9.014	0.000
AL	6.05	2.99, 12.20	5.029	0.000
Recession	0.131	0.068, 0.25	-6.069	0.000
ETSA	1.0004	1.00007, 1.006	2.518	0.012
BPB	1.0007	0.999, 1.00	1.752	0.080
G+	1.01	1.004, 1.02	2.868	0.004
IgG	1.09	1.01, 1.18	2.394	0.017
IgA	0.092	0.016, 0.17	-2.058	0.04

Pocket Depth

Variable	OR	95% CI	z	p
Plaque	5.32	3.33, 8.53	6.974	0.000
Bleeding	14.70	8.06, 26.78	8.777	0.000
AL	24.65	9.92, 61.25	6.902	0.000
Recession	0.12	0.003, 0.043	-6.779	0.000
ETSA	1.0004	1.0001, 1.007	2.654	0.008
BPB	1.003	1.001, 1.004	3.616	0.000
G+	1.02	1.007, 1.02	3.362	0.001
IgG	1.11	1.02, 1.21	2.587	0.010
IgA	0.99	0.95, 1.05	-0.043	0.966

Table 5. Multivariate Model for Bleeding. Covariates associated with bleeding in this model include supragingival plaque, pocket depth (PD), IgG and IgA. Values for both the Pearson goodness-of-fit test and the Hosmer-Lemeshow test are presented. Both values indicate a good fit for this model. Evaluation of the predictive value of the model is presented and indicates an 80.38% correct classification of disease.

```
. logistic bleed plaque PD IgG IgA

Logit Estimates                                   Number of obs =    209
                                                  chi2(4)       =  92.95
                                                  Prob > chi2   = 0.0000
Log Likelihood = -98.274406                       Pseudo R2     = 0.3211

------------------------------------------------------------------------------
       bleed | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     plaque |   4.105467   1.456098     3.982   0.000     2.048635    8.227363
         PD |     11.104   5.696313     4.693   0.000      4.06271    30.34889
        IgG |   1.174686   .0727851     2.598   0.009     1.040352    1.326366
        IgA |   .6947992   .1076513    -2.350   0.019     .5128321    .9413332
------------------------------------------------------------------------------

. lfit

Logistic model for bleed, goodness-of-fit test

      number of observations =       209
 number of covariate patterns =       209
           Pearson chi2(204) =    362.96
                 Prob > chi2 =    0.0000

. lstat

Logistic model for bleed

             -------- True --------
Classified |         D              ~D |       Total
-----------+---------------------------+-----------
     +     |        79              19 |          98
     -     |        22              89 |         111
-----------+---------------------------+-----------
   Total   |       101             108 |         209

Classified + if predicted Pr(D) >= .5
True D defined as bleed ~= 0
-------------------------------------------------
Sensitivity                     Pr( +| D)   78.22%
Specificity                     Pr( -|~D)   82.41%
Positive predictive value       Pr( D| +)   80.61%
Negative predictive value       Pr(~D| -)   80.18%
-------------------------------------------------
False + rate for true ~D        Pr( +|~D)   17.59%
False - rate for true D         Pr( -| D)   21.78%
False + rate for classified +   Pr(~D| +)   19.39%
False - rate for classified -   Pr( D| -)   19.82%
-------------------------------------------------
Correctly classified                        80.38%
-------------------------------------------------

. lfit, group (10)

Logistic model for bleed, goodness-of-fit test
(Table collapsed on quantiles of estimated probabilities)

      number of observations =       209
            number of groups =        10
  Hosmer-Lemeshow chi2(8) =         4.85
                 Prob > chi2 =      0.7739
```

Table 6. Multivariate Model for Pocket Depth (PCD). Covariates associated with pocketing in this model include bleeding, attachment loss (AL), total microorganisms (ETSA), and total IgG. Values for both the Pearson goodness-of-fit test and the Hosmer-Lemeshow test are presented. Both values indicate a good fit for this model. Evaluation of the predictive value of the model is presented and indicates an 87.89% correct classification of disease.

```
. logistic PCD bleeding AL ETSA IgG

Logit Estimates                                   Number of obs =     190
                                                  chi2(4)       =  131.57
                                                  Prob > chi2   =  0.0000
Log Likelihood = -63.536062                       Pseudo R2     =  0.5087

------------------------------------------------------------------------------
         PCD | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
     bleeding |   7.437791   3.445707    4.331  0.000     2.999906    18.44082
           AL |   33.30748   28.89943    4.041  0.000     6.081237    182.4281
        ETSA |   1.004223   .0011182    3.785  0.000     1.002034    1.006418
          IgG |   1.135803   .0806969    1.792  0.073      .988158    1.305507
------------------------------------------------------------------------------

. lfit

Logistic model for PCD, goodness-of-fit test

      number of observations =       190
 number of covariate patterns =       190
           Pearson chi2(185) =    341.27
                 Prob > chi2 =    0.0000

. lstat

Logistic model for PCD

             -------- True --------
Classified |         D              ~D          |      Total
-----------+--------------------------+----------+----------
     +     |        98              11          |        109
     -     |        12              69          |         81
-----------+--------------------------+----------+----------
   Total   |       110              80          |        190

Classified + if predicted Pr(D) >= .5
True D defined as PCD ~= 0
-------------------------------------------------
Sensitivity                     Pr( +| D)   89.09%
Specificity                     Pr( -|~D)   86.25%
Positive predictive value       Pr( D| +)   89.91%
Negative predictive value       Pr(~D| -)   85.19%
-------------------------------------------------
False + rate for true ~D        Pr( +|~D)   13.75%
False - rate for true D         Pr( -| D)   10.91%
False + rate for classified +   Pr(~D| +)   10.09%
False - rate for classified -   Pr( D| -)   14.81%
-------------------------------------------------
Correctly classified                        87.89%
-------------------------------------------------

. lfit, group (10)

Logistic model for PCD, goodness-of-fit test
(Table collapsed on quantiles of estimated probabilities)

      number of observations =       190
            number of groups =        10
     Hosmer-Lemeshow chi2(8) =      6.70
                 Prob > chi2 =    0.5694
```

APPENDIX 1

PROTOCOLS FOR CLINICAL MEASUREMENTS, MICROBIOLOGIC AND IMMUNOLOGIC SAMPLE COLLECTION AND ANALYSIS

APPENDIX 1: PROTOCOLS FOR CLINICAL MEASUREMENTS AND COLLECTION AND ANALYSIS OF MICROBIOLOGIC AND IMMUNOLOGIC SAMPLES

I. CLINICAL MEASUREMENTS

Pocket depth and recession measures, presented as mm values, were obtained using a Michigan "O" probe. Recession measure was accomplished by palpating and determining the CEJ, placing the probe tip at that site and measuring to the free gingival margin. Attachment measures were calculated, in the database, as an additive function of the pocket depth and recession measures. Supragingival plaque determination was based on the Silness and Loe (1964) technique and evaluated at the sites identified in the body of the report. Plaque was categorized on a scale of 0-3. Bleeding on probing was given a categorical gingival bleeding score of 0-3, with 0: no bleeding after gentle probing, 1: pinpoint gingival bleeding with no flow, 2: isolated interdental bleeding that appears as a fine line, and 3: interdental triangle fills with blood, profuse bleeding or spontaneous bleeding.

II. SAMPLING PROTOCOLS

Microbiologic: Subgingival plaque samples were obtained from the mesiobuccal site of each study tooth. Two distinct methodologies were used to obtain the plaque samples. The first methodology, paper point sampling, was based upon the culture techniques employed in the early literature. With the advent of more sensitive techniques, a DNA hybridization methodology had been adopted. This change in isolation reflects changes in technology and reflects the 'state of the art' in detection of microbial changes in the subgingival environment. Initially, the paper point method of collection was used, the samples were plated and analyzed for total microorganisms, black pigmented bacteria, and Gram positive species. Both methodologies required initial removal of the supragingival pellicle and maintainance of a dry field. Results

were presented as colony forming units (CFU). Beginning in 1995, the experimental design incorporated the more current DNA hybridization technique of analysis, requiring sample collection using a curette.

Three fine paper points were inserted simultaneously into the mesiobuccal site of each study tooth in an apicolingual direction to the base of the pocket and permitted to remain there for a minimum of 10 seconds. After that time, the points were removed with sterile forceps and placed into glass vials containing 2 ml. reduced transport fluid (RTF) (Loesche, 1972) and 0.2 g of 1 mm glass beads.

Using a Gracey curette 1-2, the instrument was inserted into the base of the pocket by placing the angle of the curette at the gingival margin and directing the curette. With a single upward motion, the curette was extracted obtaining as much subgingival plaque as possible. Once the sample was collected, the curette was placed into an Eppendorf vial containing Tris EDTA buffer and the plaque was transferred by gentle shaking of the curette or physically removed using a sterile instrument. Upon transfer to the laboratory, 100 µl of 0.5M sodium hydroxide was added to each tube, vortexed, and stored at 4° C until analyzed using DNA hybridization technologies.

Immunologic: Gingival crevicular fluid (GCF) samples were obtained at each time point. After removal of gross debris, a strip of Periopaper® was inserted into the mesiobuccal site of each tooth and left until saturated or until the strip was no longer absorbing fluid. Each strip was removed and fluid volume determined by reading on a calibrated Periotron® prior to placement in a vial containing 250 µl of PBS buffer. Three strips were collected per tooth in the same manner described above and stored at -70° C until analyzed.

III. LABORATORY EVALUATION

Microbiological Determination: The purpose of this procedure was to determine, by colonization, the microbiota residing in the subgingival environment. Once plaque samples were

transferred to an anaerobic chamber, they were vortexed for 30 seconds and 0.1 ml of sample was added to 3.9 ml sterile RTF and mixed. Then, 0.1 ml of diluted sample was extracted and added to fresh 1.9 ml sterile RTF and vortex. After dilution of the sample, 50 µl of sample was plated onto an enriched trypticase soy agar (ETSA), CAP agar, CVE agar, TSBV agar, and *Mitis salivarius* agar using a spiral plater.

All samples were then incubated for 5 days under anaerobic conditions at 37° C for 5-7 days and read. The exact time of incubation was dependent on the media used. Subcultures were taken of all black pigmented bacteria and surface translocating bacteria for identification using standard microbiological protocols. Reagents and media were prepared according to standardized protocols. The sensitivity and specificity of this assay approached 95%. Validation of this assay was performed using repeated culture of a 'spiked' sample and multiple readings by different technical staff.

DNA Hybridization Methodology: Initially, samples were boiled for 5-10 minutes and then iced prior to adding 800 µl of ammonium acetate. Each sample was dispensed and distributed onto the slots of the miniblotter with Pasteur pipette. When the liquid had been absorbed, the membrane and filter paper were cross-linked using a UV stratolinker. After cycling, the membrane was placed into the assembly and 150 µl of each probe was added into the minislot. Making certain that the probe covered the entire lane, the membrane was suspended over a water bath and incubated overnight. The following day, the membrane was washed and incubated for 30 minutes in anti-DIG conjugate and CSPD. Once this process was complete, the film was developed in an automatic developer and scanned to determine presence of specific DNA.

Immunological Determination: Prior to assay, the fluid was extracted from the filter strips by centrifugation (Ebersole, 1984). Analysis of the serum for IgG and IgA levels was performed using enzyme linked immunosorbent assay (ELISA) techniques. The purpose of this assay was to determine levels of IgG and IgA antibody in crevicular fluid to antigenic challenge. Formalinized bacteria, prepared according to a standardized protocol, were diluted in phosphate buffered saline

(buffer) so that an optical density of 0.3 absorbance units was achieved when read using a spectrophotometer at 580 nm. Plates were coated using 0.2 ml of this solution per well of an Imumulon® plate. The plate was then covered and allowed to incubate for 4 hours at 37° C. After incubation, the plate was washed using approximately 30 ml of buffer per plate for 5 minutes. Plates were washed three times using this procedure.

GCF samples were diluted at 1:25 for IgA and 1:100 IgG. After vortexing, 0.1 ml of the diluted sample and serum standard were added to each plate using a multichannel pipette. The plate incubated for 2 hours on a rotator at 100 rpm, then, was washed using 30 ml of buffer per plate for 5 minutes per wash. Addition of 0.1 ml of dilute goat-anti-human IgG/IgA at a concentration of 1:500 was added and incubated for 2 hours at room temperature (25° C) on a rotator at 100 rpm. Rabbit-anti-goat IgG/IgA alkaline phosphatase (1:1000) was then added to each well, incubated overnight at 25° C on the rotator.

After washing the plate 3-6 times for 5 minutes each time with buffer, 0.2 ml of NPP substrate, diluted to 1 mg/ml with buffer, was added and incubated for one hour. The reaction product is yellow. Termination of the reaction was initiated by adding 1N sodium hydroxide. The plate was read at 405-410 nm on a ELISA plate reader.

Volumes were calculated using a standard curve derived from each plate. Each sample was analyzed in replicate and each replicate value was assessed for deviation. Differences greater than 15% in the replicates were reanalyzed. If the values fell within 10%, the replicates were averaged to produce a single value. Four negative controls appear on each plate and these values were averaged to produce a background level or minimum detectable dose. The optical density background was no greater the 0.2 amplification units. This data was then plotted using a linear regression program. Acceptable sensitivity was 90%, while the specificity of this assay was 85%.