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June 2004

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Interleukin-6 Levels in Generalized and Localized Aggressive Periodontitis Patients

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Abstract

INTERLEUKIN-6 LEVELS IN GENERALIZED AND LOCALIZED AGGRESSIVE PERIODONTITIS PATIENTS

By Bindu Reddy, D.D.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at the Virginia Commonwealth University

Virginia Commonwealth University, 2004

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Periodontitis is an inflammatory disease involving the supporting structures of the dentition. Many studies have shown that there is a relationship between periodontal disease, the presence of pro-inflammatory cytokines, and systemic disease such as cardiovascular disease and diabetes mellitus. The purpose of this study was to measure serum Interleukin-6 levels in generalized and localized aggressive periodontitis and non-periodontitis patients and look for relationships with measures of disease severity. We also examined variables known to have a relationship with IL-6. A total of 172 subjects, comprising three periodontal subgroups, non-periodontitis (NP=61), generalized

aggressive periodontitis (GAP=77), and localized aggressive periodontitis (LAP= 34), had serum samples evaluated for IL-6 levels using a highly sensitive ELISA test. The IL-6 levels were compared with clinical and demographic data including age, race, gender, number of teeth, probing depth, attachment loss, bleeding index, plaque index, gingival index, cotinine levels, smoking status, and CRP levels. Using multiple regression analysis, smoking status (p=0.0015) was the only variable found to have a significant relationship with IL-6 levels for all three groups.

Introduction

Periodontitis is an inflammatory disease involving the supporting structures of the dentition including the cementum, bone, and periodontal ligament. The initial presentation of the disease is localized to the gingival sulcus and coronal connective tissue. However, the disease process, if allowed to progress, can result in periodontal attachment loss and bone destruction.[1] Approximately 50% of the United States population has gingivitis around three or four teeth at any given time and 35% have periodontitis defined by the presence of one or more teeth with attachment loss and probing depth ≥ 3mm.[2]

Juvenile periodontitis, which we now classify under aggressive periodontitis, is a term which is defined by Baer[3] as a disease of the periodontium occurring in an otherwise healthy adolescent which is characterized by rapid loss of alveolar bone about more than one tooth of the permanent dentition. There are two basic forms in which it occurs. In the localized form, the only teeth affected are the first molars and incisors. In the generalized form, it may affect most of the dentition.[4] According to Baer, the amount of destruction is not related to the local irritants or amount of plaque present.[3] However, Burmeister *et al.*[5] refute this idea and have reported a positive correlation between plaque present and the degree of destruction seen in aggressive cases. Using

the 1991 National Institute of Dental Research national survey, Löe and Brown reported that the prevalence of localized juvenile periodontitis was 0.53% and generalized juvenile periodontitis was 0.13%. Males were slightly more likely to have the localized form of the disease, with blacks more likely than whites to have either form.[6]

Both chronic and aggressive forms of periodontitis are characterized by inflammation. Phagocytic cells are recruited to the site of infection through the release of cytokines and other inflammatory mediators. These cytokines include interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12), and tumor necrosis factor α (TNF-α). All of these mediators have important local and systemic effects.

The inflammation and destruction which comprise periodontal disease result from the host immune response to bacterial challenge. Bacteria and bacterial components such as lipopolysaccharide (LPS) result in the production of inflammatory mediators, such as cytokines, by activated macrophages and fibroblasts present in periodontitis lesions.[7,8] Several bacteria including *Actinobaccilus actinomycetemcomitans (Aa*) and *Camphylobacter rectus (Cr*) have been shown to amplify human host inflammatory reactions mediated by pro-inflammatory cytokines, including IL-6 and IL-8.[9] There have been several other pro-inflammatory cytokines implicated in the immunopathology of periodontitis, with the most convincing evidence for destruction of the periodontium for Interleukin-1 beta (IL-1β),[10,11,12,13] and TNF-α.[14,15,16] Elevated levels of pro-inflammatory cytokines have been found in inflamed periodontal tissues and systemically.[17,18,19] Due to the presence of bacterial plaque and cytokine activation, an inflammatory response occurs which may have global consequences throughout the body.

The cytokines IL-1, IL-6, and TNF-α are key mediators in inflammation. These cytokines cause systemic effects such as increased body temperature, neutrophil mobilization, and increased lymphocyte activation.[20] They are of particular importance due to their role in the acute-phase response.[20] This response involves the release of proteins by hepatocytes such as C-reactive protein (CRP) and mannan-binding lectin.[21] These two proteins are capable of initiating complement activation and opsonization of target bacteria.[20] Thus, the presence of molecules such as IL-1, IL-6, TNF-α, and CRP has become a marker of inflammation and the initiation of host defense mechanisms.

Cytokines play an important role in the pathophysiology of chronic inflammatory diseases, including periodontitis. IL-6 in particular is a major mediator of the host response to tissue injury, infection, and bone resorption. [20] IL-6 is a critical component of the acute-phase response because it triggers the release of CRP which is involved in immune response and pro-inflammatory host reactions. [20]

The role of IL-6 and periodontal bacteria, specifically, has been examined. One such study by Kesavalu *et al*.[22] looked at murine tissue expression of pro-inflammatory cytokines TNF-alpha, IL-6, and IL-1 beta in response to challenge with periodontal pathogens such as *Porphyromonas gingivalis (Pg*) and *Actinobacillus actimomycetemcomitans (Aa*). Results of the study showed that both *Pg* and *Aa* elicited elicited expression of all three cytokines with *Pg* being more potent than *Aa* in inducing cytokine expression. In another study by Nakamura *et al*.,[23] blood samples from seven healthy subjects were treated with *Aa* lipopolysaccharide (LPS) and tested for IL-6 and IL-1 beta expression. Blood cells pre-treated with *Aa* LPS showed increased production of IL-1 beta and IL-6 compared to non-LPS pre-treated cells. Also, further analysis

showed that the monocyte fraction of the blood was mostly responsible for the increased cytokine production seen. Collectively, this data shows that tissues do respond to bacteria and their byproducts by mounting an inflammatory reaction which includes activation of inflammatory cells and production of inflammatory cytokines.

Barthold *et al.*[18] examined IL-6 production by human gingival fibroblasts using immunohistochemistry. In the study, they were able to localize IL-6 production within the connective tissue and lower portions of gingival epithelium. They found that areas of inflammation showed a much more intense staining for IL-6 and concluded that there may be an association between this inflammatory cytokine and periodontal disease. In another study by Chen *et al.,*[24] levels of IL-6 and IL-1 beta were examined in both the gingival tissue and serum of patients with periodontal disease and of periodontally healthy patients. The levels of both cytokines were measured by ELISA. A positively significant relationship was found between levels of IL-6 and clinical assessments of periodontal destruction. IL-6 levels were found to increase in tissues of periodontitis patients.

Many studies have shown a correlation between periodontitis and systemic disease involving cytokines such as IL-6 as a shared mechanism of inflammation.[25,26,27,28,29,30,31,32,33] In one such study, gingival crevicular fluid (GCF) levels of IL-6 were determined in patients with type II Diabetes Mellitus (T2DM) with periodontitis, adult periodontitis, and healthy controls. The mean GCF IL-6 levels were highest in T2DM patients with periodontitis, followed by the adult periodontitis patients with the healthy controls having the lowest levels.[25] These findings suggest that GCF IL-6 levels were significantly higher in areas of inflammation and periodontal destruction.

Studies by Offenbacher *et al.*[26] have shown a relationship between periodontal disease and pre-term low birth weight (PLBW) infants. The inflammatory mediators that occur in periodontal disease also play a significant role in the initiation of labor and provide a plausible mechanism which may link the two together. Lopez *et al.*[27] investigated whether the maintenance of a mothers' periodontal health after 28 weeks gestation reduced the risk of PLBW infants. They found that the incidence of PLBW was 2.5% in periodontally healthy women, and 8.6% in women with periodontal disease ($p=0.0004$).

In the Physician's Health Study, Ridker *et al*.[28] found elevated levels of IL-6 in apparently healthy males predicted up to a 2.3-fold increase in myocardial infarction over 6 years. Among these men, the number of risk factors at baseline was correlated with the plasma concentration of IL-6. In another study, Ridker *et al*.[29] also reported that baseline IL-6 levels have been found to predict cardiovascular events in the Women's Health Study. Bermudez *et al.*[30] discuss several clinical cardiovascular risk factors which are related to circulating plasma levels of IL-6 and CRP in women. Specifically, age, BMI, smoking status, systolic blood pressure, alcohol use, presence of diabetes, and exercise frequency are all independent correlates of circulating plasma concentrations of IL-6.

Ciancio[31] reports many risk factors which are associated with both periodontal disease and cardiovascular disease including age, smoking, diabetes, obesity, stress and diet. According to Ciancio practitioners must consider these shared risk factors in diagnosing and treating periodontal disease. In addition to these associations, a pathway relating oral conditions with cardiovascular disease is discussed by Joshipura *et al*.[32] whereby periodontal disease can lead to increased tooth loss and inflammation followed

by dietary changes, stress, vascular injury, bacteremia and possible coronary vascular disease outcomes such as endocarditis, stroke and coronary heart disease. Iacopino and Cutler[33] show a linkage between infection and hyperlipidemia. In this model chronic periodontitis and/or acute infection cause bacteremia and endotoxemia which leads to increased serum pro-inflammatory cytokine production. The cytokines then cause enhanced lipogenesis and reduced lipid clearance with a resultant increase in free fatty acids, LDL, and triglycerides. This model is cyclic in nature and as lipid levels are further elevated, periodontitis is exacerbated leading to worse inflammation and lipid levels.

Although an exact causal mechanism has not yet been determined, the risk factors and associations between periodontal disease and systemic disease are strong. The key underlying shared characteristic is inflammation. Based on the biology of inflammation we know that pro-inflammatory cytokines are major mediators involved in inflammatory changes throughout the human body. IL-6 has been found in inflamed periodontal tissues at increased levels compared to healthy tissues. Several studies have demonstrated local increases in IL-6 levels in gingival crevicular fluid and gingival tissues in response to periodontitis.[18,24,25,34,35,36] However, at present, there is no published data which correlates serum levels of IL-6 with periodontal disease and oral inflammation. Thus, there is a need for studies examining the relationship between systemic IL-6 serum concentrations and periodontitis as well as studies on the effect of periodontal therapy on serum IL-6 concentrations. This information is of particular importance due to the potential therapeutic implications and close relationship between periodontal disease and systemic disease.

HYPOTHESIS: IL-6 levels are elevated in the serum of certain patient groups with periodontal disease. There is a link between inflammatory systemic conditions and the periodontal oral inflammation. Periodontal therapy could potentially help to alleviate and ameliorate certain inflammatory systemic diseases and their symptoms. The purpose of this study is to measure IL-6 serum levels in generalized and localized aggressive periodontitis patient populations and healthy controls and determine if there is a relationship between this variable and measures of periodontal health. We also will examine additional variables known to relate to IL-6 levels.

Materials and Methods

Patient Selection and Clinical Examination

All subjects were taken from the VCU Clinical Research Center for Periodontal Disease Database. The database included patients referred to the center and patients identified from the dental population at VCU School of Dentistry. All patients signed an informed consent form approved by VCU Committee on the Conduct of Human Research. A total of 172 subjects had a medical history obtained by questionnaire and interview, complete dental exam, and matching serum samples with clinical exam. Complete exams consisted of suppuration index, plaque index (APLI),[37] gingival index (AGI),[38] pocket depth (PKT Avg), bleeding index (ABI), attachment loss measurements (Aloss), and tooth mobility. Missing and deciduous teeth were also recorded. Smoking status, cotinine levels, CRP levels were also measured. Clinical exam was measured at 4 sites per tooth (mesio-buccal, buccal, distal-buccal, and lingual) with a Hu-Friedy color-coded probe (PCP 12). Diabetic subjects and subjects with reported arthritis were eliminated from the database. All examiners performing exams were calibrated to verify examiner reliability.[39]

Subject Groups

Aggressive Periodontitis. Encompasses distinct types of periodontitis that affect people who, in most cases, otherwise appear healthy. It tends to have a familial aggregation and there is a rapid rate of disease progression. Aggressive periodontitis occurs in generalized and localized forms.

Generalized Aggressive Periodontitis- A total of 77 subjects showing a generalized pattern of severe periodontal destruction with attachment loss of at least 5 mm on eight or more teeth, at least 3 of which are <u>not</u> first molars and incisors. Subjects ranged in age from 12 to 67 years.

Localized Aggressive Periodontitis- A total of 34 subjects. Clinical attachment loss pattern—must have at least 4 mm attachment loss (Aloss) on at least 2 permanent first molars and incisors (at least one molar must be affected) and no more than 2 teeth, which are not first molars or incisors that are affected by 5 mm AL or more. Subjects ranged in age from 8 to 46 years.

Non-Periodontitis (NP). A total of 61 subjects with no evidence of attachment loss at more than one site, except for recession on the buccal of anterior teeth, or pockets greater than 3 mm. That is, no detectable periodontitis; healthy periodontium. Gingival inflammation may be present in variable degrees of severity. Subjects ranged in age from 6 to 50 years of age.

IL-6 Assay

96-well microtiter plates were used which were coated with antibody for human IL-6. Standard serial dilutions of recombinant human IL-6 were prepared with a standard diluent 0.1% (w/v) sodium azide. IL-6 levels ranged from 0.63-20 pg/ml. 100µl of standard diluent was loaded into designated wells and 100 µl of sample was loaded into the appropriate wells on the 96 well microtiter plates. Plates were then covered and stored for 2 hours at room temperature (20-25°C) with continuous shaking. After this incubation period, each well was aspirated and washed thoroughly with wash buffer for a total of four washes. Complete liquid removal was assured after each wash step. After the wash cycles, 100 of biotinylated antibody reagent was pipetted into all wells. Plates were again covered and incubated for 30 minutes at room temperature (20-25°C) with continuous shaking. The wash and aspiration cycle was again repeated four times as described above. Next 100 µl of Amdex amplification reagent was added to all wells. Plates were covered with new adhesive strips and and incubated for 30 minutes at room temperature (20-25°C) with continuous shaking. The wash and aspiration cycle was again repeated four times as described above. Finally 100 µl of TMB substrate was added to each well and incubated for 30 minutes at room temperature (20-25°C) with continuous shaking. Plates were not covered during this step. Following the 30 minute incubation, 100 µl of stop solution was added to each well. Optical density was then determined for each well within 30 minutes using a spectrophotometer set to 450nm. The duplicate readings for each standard and sample were averaged and the zero standard optical density was subtracted from each value.

Statistical Analysis

One way ANOVA and bivariate analysis were used to calculate the significance of IL-6 levels against various continuous and dichotomous variables including race, sex, number of teeth, pocket depth (PKT Avg), attachment loss (Aloss), plaque index (APLI), gingival index (AGI), bleeding index (ABI), reported smoking status, cotinine levels, and CRP levels within each subject group. Multiple regression was used to calculate significance of IL-6 levels against these variables for all three subject groups combined. A significance level of 0.05 was used. Stepwise regression was then performed using variables which approached significance with $p < 0.2$.

Results

Patient demographics and parameter means are given in Tables 1 and 2. Table 1 shows mean values and standard errors of the continuous variables measured for each subject group. Table 2 shows percentages for dichotomous variables measured for each subject group.

We compared the IL-6 levels in each patient group to the variables found in Tables 1 and 2 (Table 3). In Table 3, a statistically significant correlation was found between IL-6 levels and smoking status within the LAP patient group ($p<0.0001$). No other significant differences were found.

We then combined the data for all 3 groups and examined the relationship between IL-6 and the variables in Tables 1 and 2 including attachment loss and periodontal classification (Table 4). Non-smokers had significantly lower IL-6 levels (odds ratio= -3.11, $p= 0.0024$) and smokers had significantly higher IL-6 levels (odds ratio= 3.26, $p= 0.0015$) (Table 4). A trend was noted between LAP periodontal status and IL-6 levels (odds ratio=1.88, $p=0.0627$) (Table 4). No significant results were noted.

Stepwise regression analysis was used for factors which approached significance using $p< 0.2$. The three variables tested were LAP status, smoking, and plaque index. Results showed that smokers again had significantly elevated IL-6 levels ($p< 0.0001$).

LAP patients showed increased IL-6 levels, but results were still not significant ($p=0.0587$). Plaque index was not significant ($p=0.1302$).

Table 1

Mean Values of Continuous Variables Measured for Patient Subgroups

	LAP	±Std error	GAP	±Std error	NP	±Std error
Age	22.7		31.3		31.0	
Number of Teeth	26.970588	±0.415	24.571429	±0.640	27.508197	±0.352
PKT Avg	2.6369407	±0.095	3.437879	±0.101	2.0684114	±0.256
Aloss	0.6489857	±0.075	2.5871881	±0.180	0.2468588	±0.036
APLI	0.8624218	±0.084	1.2406649	±0.057	0.5018255	±0.055
AGI	1.173042	±0.064	1.3570198	±0.048	0.6055005	±0.047
ABI	0.3527414	±0.038	0.4450448	±0.027	0.1833749	±0.025
Cotinine	64.909524	±26.597	202.22466	±34.115	35.3785571	±20.694
IL-6 mean	3.5356176	±2.015	1.258987	±0.252	2.2888852	±1.071
CRP mean	3.6125882	±0.638	3.7772987	±0.390	1.5823607	±0.270

Table 2

Dichotomous Variables Measured for Patient Subgroups

		LAP	GAP	NP
Race	Black	0.76471	0.62338	0.22951
	White	0.20588	0.37662	0.77049
Sex	Male	0.41176	0.33766	0.52459
	Female	0.58824	0.66234	0.47541
Smoker	Yes	0.32353	0.62338	0.52459
	No	0.67647	0.37662	0.47541

Table 3

IL-6 Levels of Patient Subgroups Based on Variables Measured

P-Values

	LAP	GAP	NP
Race	0.5072	0.3416	0.5217
Sex	0.8914	0.2048	0.9591
Number of Teeth	0.5158	0.3443	0.8787
PKT Avg	0.6975	0.2397	0.7560
Aloss	0.5770	0.6487	0.4245
APLI	0.1127	0.1284	0.9062
AGI	0.5825	0.9129	0.5224
ABI	0.8210	0.5060	0.5375
Cotinine	0.6725	0.4536	0.6687
Smoking Status	<0.0001*	0.4584	0.1190
CRP	0.1000	0.7714	0.3630

**Statistically significant P values obtained by bivariate analysis*

Table 4

Significance of Variables on IL-6 Levels-Comparisons Across All Patient Subgroups

	t ratio	Prob>⎮t⎮
Class (LAP)	1.88	0.0627
Class (GAP)	-0.18	0.8600
Class (NP)	-0.39	0.6958
Race (Black)	-0.32	0.7469
Sex (F)	-0.42	0.6759
Number of Teeth	-0.36	0.7214
PKT Avg	0.50	0.6172
Aloss	-0.35	0.7308
APLI	-1.58	0.1173
AGI	0.58	0.5614
ABI	-0.04	0.9700
COTININE	-0.21	0.8377
Smoker (Y)	3.26	0.0015*
Smoker (N)	-3.11	0.0024**
CRP mean	-0.01	0.9941

**statistically significant increase in IL-6 levels*
***statistically significant decrease in IL-6 levels*

Discussion

In this study we examined the relationship of IL-6 and measures of periodontal disease in LAP and GAP patients. We also examined variables known to relate to IL-6, including CRP. The only variable which was significantly related to IL-6 levels was smoking. It is not surprising that smoking was found to be significantly correlated to increased IL-6 levels. Several studies have also shown the mechanisms by which smoking may affect the periodontium including PMN chemotaxis problems, increased proportions of harmful periodontal microflora, increased bone andattachment loss, and poor wound healing.[40,41,42,43,44,45,46,47,48] Tomar *et al.*[49] researched the NHANES III database and found that current smokers were about 2-3 times as likely as non-smokers to have periodontitis. The odds ratios ranged from 2.79 for those who smoked < 10 cigarettes/day to 5.88 for those who smoke >30/day. They also calculated that smoking cigarettes may be responsible for over 50% of the cases of periodontitis among adults in the United States. Grossi *et al.*[46,47] assessed risk indicators for alveolar bone loss and attachment loss. They found that smokers had greater odds ratios than non-smokers for bone loss ranging from 3.25 for light smokers to 7.28 for heavy smokers and attachment loss ranging from 2.05 for light smokers to 4.75 for heavy smokers. These odds ratios are similar to the odds ratio we found between smoking and IL-6 of 3.26.

Giannopoulou *et al.*[36] looked at the effects of smoking and inflammation on gingival crevicular fluid cytokine levels in 40 patients with aggressive and chronic periodontitis and 20 patients with gingivitis compared to 20 healthy controls. They found that IL-6, IL-8 and IL-1 beta levels were significantly elevated in peridontally diseased subjects compared to healthy controls. They also reported that IL-4, IL-6, IL-8 and IL-1 beta were all positively correlated with probing depths. IL-4, IL-6 and IL-8 were found to be correlated to smoking. Therefore, we can see the relationship between smoking, inflammation, and periodontal destruction as evidence and support for our findings.

We noted that amongst the three subject groups, LAP status showed an increased odds ratio (t ratio=1.88, p=0.0627) of elevated IL-6 levels. The literature shows a relationship between inflammation and clinical parameters of periodontitis such as bleeding and increased probing depths which may explain this correlation.[50,51,52] Studies by Caton *et al*.[52,53] and Davenport *et al*.[54] have shown that bleeding is indicative of histologic inflammatory changes occurring within the tissues. Bleeding on probing along with suppuration or increased probing depths can be predictive of future attachment loss.[50,51] A 2002 VCU Master's thesis project by Fernandez[55] also examined the relationship between periodontal disease (local inflammation) and cardiovascular disease (systemic inflammation) using the NHANES III database. Results of the project showed that gingival bleeding was significantly related to a history of non-fatal heart attack with an odds ratio of 1.95. Discussion related bleeding to periodontal inflammation and the possible role of pro-inflammatory cytokines in leading to further systemic inflammation. A study by Willerhausen *et al.* [56] shows that there is a correlation between inflammatory periodontal disease and cardiovascular disease. They found that mean bleeding index

was significantly higher in coronary heart disease patients compared to healthy controls. Based on these previous studies we can see a possible relationship between clinical periodontitis and inflammation and would expect to see increased pro-inflammatory cytokines such as IL-6.

IL-6 is part of the acute phase response and causes CRP to be released by hepatocytes, which is then involved in initiating complement activation and opsonization of target bacteria.[20] We noted a correlation between LAP status and IL-6 cytokine levels. However, results did not show any increase or significance in IL-6 levels when measured against CRP in any of the subgroups. D'Aiuto *et al.*[57] conducted a pilot study on 94 subjects and assessed serum CRP and IL-6 levels at baseline and at 2 and 6 months following non-surgical periodontal therapy. They found significant reductions in CRP and IL-6 serum levels along with improvement in all clinical periodontal parameters with therapy. In another related study, Loos *et al.*[58] looked at CRP and IL-6 levels in periodontitis patients. They examined 53 localized periodontitis patients, 54 generalized periodontitis patients and 43 healthy controls and found significantly higher mean levels of CRP and IL-6 in periodontitis patients compared to controls. They also found that CRP and IL-6 correlated with each other, and both correlated with the number of neutrophils in the peripheral blood samples of the periodontitis patients. We would expect to see a correlation between IL-6 and CRP in the subjects. However, in our study, we did not note such a correlation.

It is possible that the reason we failed to find relationships for IL-6 reported by others was due to degradation or denaturation of the IL-6 in the samples used for our experiments. In our study, the serum samples were obtained over the last 20 years and

some have been repeatedly thawed and frozen during ongoing projects. We suspect that there may have been a problem with the stability of IL-6 in the sample serum. Kenis *et al.* [59] examined the stability of IL-6 in human serum under varying conditions. They looked at the concentration of serum IL-6 stored at 4ºC, 20ºC, 30ºC, and 40ºC for 1 to 21 days compared to triplicate samples of the same person frozen at -20ºC. They found that recovery of IL-6 did not decrease for up to 21 days at 4ºC, 20ºC or 30ºC and only decreased after 21 days at 40ºC. They found that the concentration of IL-6 did not change when incubated at 4ºC or 20ºC for up to 4 days before centrifugation. They also found that up to four repeated freeze-thaw cycles did not influence recovery of IL-6 in their study. These results do not corroborate those from Flower *et al*. [60] who found that IL-6 concentrations were affected by various treatments prior to experimental use. Serum samples were obtained from healthy subjects and examined for IL-6 levels under many conditions: (i) standing for 1, 2, 4, or 6 h prior to, or after separation, before freezing at -70 degrees C; (ii) taken into tubes with different anticoagulants or no anticoagulant, separated and frozen; and (iii) separated, and repeatedly freeze-thawed for up to six cycles prior to assay. Results showed that storage conditions can significantly alter the concentrations of the molecules and need to be standardized. Also, the type of anticoagulant used and the number of freezing cycles may alter the concentration of cytokines and should be considered when studies are being conducted. Another study by Thavasu *et al*. [61] looked at blood cytokine levels of many cytokines including IL-6 under different anticoagulant, processing, and storage conditions. The study showed that the stability and recovery of the cytokines was affected by the varying study parameters. Handling methods did influence cytokine levels. The recovery of IL-6 from serum

samples was found to be consistently lower than the amount recovered from plasma samples. Therefore, the properties and concentrations of the cytokine in the samples may have been altered by the treatment of the samples prior to use.

Interestingly, Sothern *et al.*[62,63] conducted two studies which examined the circadian characteristics of IL-6 in blood and urine samples of clinically healthy men. Because most human biologic activities and functions are governed by circadian rhythms, this group tested the activity of IL-6 during various daytime and nighttime hours. They found that average IL-6 values ranged from 1.66 to 5.38 pg/ml, with the lowest to highest values within 24 hours ranging from 1.20 to 7.58 pg/ml between subjects. On average, values were greater than the mean throughout the night, with a peak at 01:00 hours and less than the mean throughout the day, with the lowest value at 10:00 hours. The opposite trend was found for urine IL-6 levels with the highest values found during the day and the lowest values found at night. A significant time effect was found by ANOVA. They suggested that endogenous IL-6 levels may be significantly altered by their large day-night variations and the type of fluid collected. In our study, subjects were seen in the clinics during the daytime, during office hours only. Therefore, it is possible that IL-6 serum concentrations were affected by collection times.

In future studies, we will collect samples and use them within a given time period. Storage and processing protocols must be carefully laid out ahead of time. We will also avoid repeated freezing and thawing of samples for use. Samples should be aliquoted in small amounts so that fresh sample tubes could be used for each experiment and contamination would be avoided. We will thus possibly prevent some product loss due to improper handling conditions. It would also be interesting to investigate the relationship

between collection time and serum IL-6 concentrations in our patient population. Finally, in light of these past studies, it would seem wise to standardize collection times to certain parts of the day in order to prevent bias in the results.

We hypothesized that serum IL-6 concentrations, like gingival tissue concentrations,[18,24] would be increased and directly related to periodontal breakdown and disease in our patient population. The study by Loos *et al.*[58] previously discussed examined levels IL-6 and CRP in the peripheral blood of periodontitis patients. They found that CRP, IL-6 and neutrophil levels were elevated in periodontitis patients. The elevated pro-inflammatory markers may be related to the inflammation seen in cardiovascular disease and may lead to an increased risk for cardiac sequellae such as development of atherosclerotic lesions in this patient population. If there is indeed a relationship between systemic inflammation, cardiovascular disease, and periodontitis, then therapeutic measures aimed at reducing periodontal inflammation are warranted. In the future we would like to examine the relationship between periodontal therapy and serum IL-6 concentrations and hope to see that therapy will help in reducing the amount of this pro-inflammatory cytokine within the patient's system. Perhaps we can then explain a causal relationship amongst systemic inflammation, cardiovascular disease, and periodontal disease.

Some recent studies have investigated different avenues of therapy for combating periodontal inflammation and perhaps subsequent systemic inflammation. In a study by Tipton *et al.*,[64] the role of cyclooxygenase-2 (COX-2) inhibitors in periodontal therapy was examined. Gingival fibroblasts were cultured in medium with IL-1 beta, with or without COX-2 inhibitors. Results showed that COX-2 inhibitors caused dose-dependent

decreases in PGE-2 and IL-6 production by IL-1 beta stimulated gingival fibroblast and may be useful in controlling inflammation in these patients. Thus, a link is shown between systemic therapy and treatment of periodontitis. In a study last year, Takashiba *et al.*[65] discussed a possible role for anti-cytokine therapy, such as anti-IL-6 antibodies, in combating periodontal inflammation. If a direct relationship is found among serum levels of IL-6 and other inflammatory cytokines and periodontitis on a systemic level then systemic therapy such as vaccinations may be possible and warranted.

In this study, a significant correlation was found between serum IL-6 levels and smoking status. Also, a trend was noted between LAP periodontal status and IL-6 levels. Our future studies will aim to repeat this work with standardized handeling of serum samples as described above. We will also examine the relationship of periodontal therapy and IL-6 levels in patients with chronic and aggressive periodontitis.

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List of References

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