LOCAL AND SYSTEMIC INFLAMMATORY RESPONSE IN TYPE II DIABETIC PATIENTS WITH PERIODONTAL DISEASE

By

RUBEN FERNANDO MESIA

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2011

“To my wife, Dagmara, my son Alexander and my parents for their support during this journey”

ACKNOWLEDGMENTS

I thank my research mentor Dr. Luciana Shaddox as well as Dr. Shannon Wallet, for dedicating themselves and inspiring me with their passion for the search of deep knowledge. I also would like to thank to the entire faculty at the University of Florida, for their tireless commitment in providing an outstanding education in the field of periodontics and helping me to develop solid clinical skills.

TABLE OF CONTENTS

page

LIST OF TABLES

Table page

LIST OF FIGURES

LIST OF ABBREVIATIONS

AL	Attachment loss
BOP	Bleeding on probing
CAL	Clinical attachment levels
CRP	C-reactive protein
DBT	Diabetes or diabetic
FMPE	Full mouth periodontal examination
GCF	Gingival crevicular fluid
GM-CSF	Granulocyte macrophage colony-stimulating factor
HbA1c	Hemoglobin A1c
IDDM	Insulin-dependent diabetes mellitus
IL	Interleukins
LPS	Lipopolysaccharides
MIP1	Macrophage inflammatory protein 1
NDBT	Non-diabetes or Non-diabetic
PD	Pockets depths
PMN	Polymorphonuclear neutrophilic leukocytes
PMPE	Partial-mouth periodontal examination
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

LOCAL AND SYSTEMIC INFLAMMATORY RESPONSE IN TYPE II DIABETIC PATIENTS WITH PERIODONTAL DISEASE

By

Ruben Fernando Mesia

May 2011

Chair: Luciana Shaddox
Major: Dental Science

Numerous studies have shown a correlation between chronic inflammatory periodontal disease and diabetes, in which both of them influence the progression and response to treatment of the other. However, the mechanisms behind the association of these two diseases are not yet elucidated. The objective of this study was to quantify local and systemic inflammatory responses in Type II diabetic patients as compared to normoglycemic subjects with periodontal disease.

Gingival crevicular fluid (GCF) and blood samples were collected from 20 patients with chronic periodontal disease (10 Type II diabetes-DBT; 10 non-diabetic-NDBT). GCF samples were collected from a diseased site (pocket depth-PD≥5mm, attachment loss –AL≥2mm and bleeding on probing-BOP) and a healthy site (no AL, no BoP). Blood samples were stimulated with ultra-pure TLR-2 and TLR-4 agonists for 24h. Twenty-two cyto/chemokines were quantified in GCF and culture supernatants using Luminex (Milliplex®). Results were compared between groups using t-test.

DBT patients showed higher systemic unstimulated levels of IL-8, TNFα, IL-10, MIP1α and MIP1β; higher stimulated levels of IL-6, IL-8, IL-10, MIP1α and MIP1β; and

lower stimulated and unstimulated levels of GM-CSF when compared to NDBT ($p<0.05$). DBT patients also showed higher levels of MIP1β in GCF from healthy sites ($p<0.001$) than NDBT, while NDBT showed higher levels of IL-7 in GCF from diseased sites ($p<0.05$).

Among patients with chronic periodontitis, those with diabetes seem to have a higher systemic inflammatory response than those without diabetes, as measured by pro-inflammatory cytokine release. Lower levels of certain cytokines associated with wound healing in diabetes (DBT) patients upon stimulation with toll like receptors (TLRs) could also indicate an impaired immune response in this patient population. Longitudinal studies to assess the relationship between degree of inflammation, magnitude of diabetes control and extent/severity of periodontal disease are justified to better understand the role of inflammatory response and periodontal disease control.

CHAPTER 1
INTRODUCTION

Diabetes type II is a heterogeneous disease that arises from and interaction among environmental factors such as obesity, sedentary lifestyle, high calorie food intake, and genetic susceptibility that result in increased insulin resistance and the clinical manifestation of disease (Lopez, Valenzuela et al. 2009).

Periodontitis is a common disease; the clinical sings include a chronic, tissue-destructive inflammation, which degrades the attachment around teeth, resulting in tooth loss. The interpretation of epidemiological data of periodontal disease is difficult, due to inconsistencies in the methodology used. It is not possible, therefore, to accurately assess if the prevalence of periodontal diseases shows a worldwide decline (Papapanou 1996). The total true prevalence of periodontitis in the USA from the “gold standard” full mouth periodontal examination (FMPE) protocol was 22.4%, in which the most severe form of the disease is present in approximately 4.8% of the population, whereas the 17.5% exhibit moderate to mild signs of disease (Papapanou 1996; Eke, Thornton-Evans et al. 2010).

Several studies have reported association between periodontal disease and diabetes (Shlossman, Knowler et al. 1990; Emrich, Shlossman et al. 1991; Taylor, Burt et al. 1998); one possible mechanism for the reported association between periodontitis and diabetes could be the release of bacteria, bacterial products or pro-inflammatory cytokines from the chronic periodontal lesion into the blood stream. This might lead to a systemic inflammatory response in diabetic patients and may lead to a worsening of diabetic control.

New data support the concept that in diabetes-associated periodontitis, the altered host inflammatory response plays a critical role. Elevated circulating levels of interleukins, tumor necrosis factor alpha can worsen insulin resistant in individual with diabetes and thereby impair the glycemic control (Taylor, Burt et al. 1996). Thus, periodontal disease may have a significant impact on the metabolic state in diabetes (Mealey and Oates 2006). The extent of the local and systemic inflammation mediators' associate with diabetes remains unclear.

The aim of this study was to quantify local and systemic inflammatory responses in Type II diabetic patients as compared to normoglycemic subjects with periodontal disease. The levels of inflammatory mediators such as cytokines and chemokines were evaluated locally (GCF - gingival crevicular fluid) and systemically (serum levels) in both groups. We hypothesized that among patients with chronic periodontitis, those with diabetes have a higher systemic inflammatory response than those without diabetes, as measured by pro-inflammatory cytokine release upon stimulation with toll like receptors (TLR s) agonists.

CHAPTER 2
BACKGROUND

Diabetes

Diabetes is a syndrome in which chronic hyperglycemia leads to long-term damage to various organs including the heart, eyes, kidneys, nerves and vascular system (Mealey and Oates 2006). Diabetes mellitus affects over 21 million Americans, including >9% of the adult population (Harris, Hadden et al. 1987; Mokdad, Bowman et al. 2001). The current classification of diabetes is based upon the pathophysiology of each form of the disease. Type I diabetes is a cellular mediated auto-immune destruction of the insulin producing β-cells of the pancreas resulting in life-long dependence on exogenous insulin. Type II diabetes results from insulin resistance in which the use of endogenously produced insulin is altered at the target cells (Mealey and Oates 2006). Type II diabetes is the most prevalent type, affecting 85 to 95% of all diseased patients. While Type II diabetic patients have some altered insulin production, the patients retain the capacity to produce insulin. However, stresses from additional illnesses, such as infection can exacerbate the insulin resistance such that it results in ketoacidosis, a life-threatening condition (DeFronzo and Ferrannini 1991). Therefore appropriate treatment of these secondary illnesses is imperative to the diabetic patient's long-term health.

Elevated systemic proinflammatory mediators in subjects with diabetes have a tremendous impact. Adipose tissue has been found to release pro-inflammatory cytokines, this cytokines are involved in an Insulin regulation. Insulin resistance are strongly linked to the actions of the proinflammatory cytokines IL-6 and TNFα (Crook 2004). IL-6 stimulates TNFα production; therefore, increased production of IL-6 from

adipocytes in obese individuals causes elevated TNFα production, which may further exacerbate insulin resistance (Natali, Toschi et al. 2006).

Traditionally, diabetics have been considered more susceptible to severe periodontal disease due to metabolic and host response that results in impaired wound healing. People with diabetes often have a shift in monocyte/macrophage phenotype, which results in the overproduction of these same inflammatory cytokines in response to periodontal pathogens (Salvi, Yalda et al. 1997). Diabetic patients who also have periodontitis may present with an even greater systemic inflammatory condition with elevated serum levels of IL-6, TNFα, which can worsen insulin resistance and thereby aggravate the glycemic response. This could confirm the hypothesis that severe periodontitis in persons with non-insulin-dependent diabetic increases the risk of poor glycemic control (Taylor, Burt et al. 1996).

Periodontal Disease

Periodontitis is a multifactorial polymicrobial infection initiated by the presence of Gram-negative bacteria, which accumulates in the gingiva crevice region. Bacterial plaque accumulation on the tooth surface leads to inflammation of the marginal tissues, which may lead to destruction of periodontal ligament (clinical attachment loss) and the adjacent supporting bone, causing tooth loss. The prevalence of periodontal disease varies among the adult population depending on severity and extent. The most severe form of the disease is present in approximately 10-15% of the population, whereas 35% exhibit moderate to mild signs of disease (Papapanou 1996). Recently, the National Health and Nutrition Examination Survey (NHANES) reviewed the data for the assessment of national prevalence of periodontitis in the US. Historically, because of time, labor and cost constraints variations of a partial-mouth periodontal examination

(PMPE) have been the clinical protocol of choice for large-scale studies. However, because periodontitis is not evenly distributed in the mouth PMPE protocols do underestimate prevalence. The accuracy of previous NHANES periodontal examination protocols was questioned and compared with the prevalence determined by the "gold standard" full mouth periodontal examination (FMPE) and it was found that both PMPE protocols used in previous studies (NHANES III and 2001-04) substantially underestimate the true prevalence of periodontitis by 50% or more. The total true prevalence of periodontitis from the "gold standard" FMPE protocol was 22.4% (4.8% severe and 17.5% moderate) *vs.* 8.9% found in the NHANES III protocol (Eke, Thornton-Evans et al. 2010).

Periodontal destruction is considered as a result of the response of a susceptible host to bacterial challenge. Periodontal disease progression by tissue destruction is host-mediated by locally produced pro-inflammatory cytokines in response to the bacterial flora and its products. Lipopolysaccharides (LPSs) of Gram-negative microorganisms are recognized by host receptors such as toll-like receptors (TRLs). Toll-like receptors (TLRs) are germ-line- encoded pattern recognition receptors expressed on cells of the innate immune system that recognize structural components conserved among classes of microorganisms, also called pathogen-associated molecular patterns. Inflammation in the gingival sulcus is initiated and maintained by the host defense against bacteria migrating from dental plaque. Toll-like receptor (TLR) 2 and TLR4 are constitutively expressed in periodontal tissue and the TLRs can recognize a variety of bacterial components. Cell walls of Gram-positive and Gram-negative bacteria are the ligands for TLR2, and lipopolysaccharide (LPS), an outer membrane of

Gram-negative bacteria, is the ligand for TLR4 (Yoshioka, Yoshimura et al. 2008).

The interactions between the LPS and the toll-like receptors stimulate the production of cytokines. Cytokines are soluble proteins, secreted by cells involved in both the innate and adaptive host response. Interleukins are important members of the cytokine group and are primarily involved in communication between leukocytes and others cell such us epithelial cells, endothelial cells, and fibroblasts engaged in the inflammatory process. There is clear evidence that the cell wall components of gram-bacteria in the periodontitis such as *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Prevotella intermedia, Fusobacterium nucleatum* and *Tannerella forsythensis*, stimulate, via TLR2 and TLR4, the production of proinflammatory cytokines from the host, such as interleukin-1β and tumor necrosis factor, which induce alveolar bone resorption and the production of matrix metalloproteases (Kikkert, Laine et al. 2007).

It is possible that the production of local cytokine and/or low-level asymptomatic bacteremia affects the plasma concentrations of biomarkers (Loos, Craandijk et al. 2000). Elevate levels of proinflammatory cytokines, such as TNF-alpha, IL-6 and IL-8 can stimulate a numbers of events that occur during periodontal disease, including the induction of adhesion molecules, amplification of inflammatory responses, and stimulation of matrix metalloproteinases and bone resorption. Anti-inflammatory cytokine, such as IL-10, plays a major roll in the suppression of inflammatory responses by inhibiting the antigen-presenting capacity of macrophages and T helper 1 cell differentiation (Yamaguchi, Yoshimura et al. 2009).

Diabetes and Periodontal Disease

Research has shown that periodontal disease is closely associated with diabetes

mellitus (Rylander, Ramberg et al. 1987; Shlossman, Knowler et al. 1990; Emrich, Shlossman et al. 1991; Novaes, Pereira et al. 1991; Seppala, Seppala et al. 1993; Nishimura, Takahashi et al. 1998; Taylor, Burt et al. 1998). Indeed a meta-analysis using 3,500 diabetic adults concluded that the majority of studies demonstrate more severe periodontal disease in diabetic patients than in adults without diabetes, confirming a significant association between periodontal disease and diabetes (Papapanou 1996). Further studies have shown that compared to non-diabetic individuals, type II diabetics were 2.81 times more likely to have clinical attachment loss and 3.43 times more likely to have radiographic bone loss than normoglycemic controls (Emrich, Shlossman et al. 1991). Also, a 2-year longitudinal study demonstrated that diabetic subjects had a significantly increased risk for alveolar bone loss compared to non-diabetic individuals, with an odds ratio of 4.2. Among these patients, poorly controlled diabetics had an odds ratio of 11.4 compared to 2.2 of well-controlled diabetics (Taylor, Burt et al. 1998). The strength of evidence on the relationship between diabetes and periodontal disease have led some to suggest that periodontal disease should be listed among the “classic” complications of diabetes (Loe 1993). Yet, the relationship between metabolic control of diabetes and periodontal disease is still unclear.

Just as diabetes contributes to increased incidence and severity of periodontal disease, periodontal disease can have a significant impact on the metabolic state in diabetes (Mealey and Oates 2006). For instance, in a 2-year longitudinal trial, diabetic subjects with severe periodontitis at baseline had a six-fold increased risk of worsening of glycemic control compared to diabetic subjects without periodontitis (Taylor, Burt et

al. 1998). An additional study reported that 82% of diabetic patients with severe periodontitis experienced the onset of one or more diabetic complications such as major cardiovascular, cerebrovascular or peripheral vascular events compared to only 21% of diabetic subjects without periodontitis (Thorstensson, Kuylenstierna et al. 1996). These and other studies support the notion that the presence of periodontal disease in diabetic patients may increase insulin resistance and contribute to a worsening of the diabetic state and diabetic complications (Williams and Mahan 1960; Sammalkorpi 1989; Yki-Jarvinen, Sammalkorpi et al. 1989; Miller, Manwell et al. 1992; Grossi, Skrepcinski et al. 1996; Taylor, Burt et al. 1996; Thorstensson, Kuylenstierna et al. 1996; Grossi, Skrepcinski et al. 1997), although the mechanism of how this occurs is still unclear.

Local and Systemic Response in Periodontal Disease and Diabetes

Gingival crevicular fluid (GCF), can be collected from the gingival sulcus surrounding the teeth, and exists as either a serum transudate or inflammatory exudate. The GCF contains substances from the host as well as from microorganism in the supra or subgingival plaque. A vast number of studies began to identify enzymes and host response in the crevicular fluid since Genco and Slots (1984) demonstrated the importance of the host response to the progression of periodontal disease, in different stages a) colonization, b) invasion, c) destruction and d) healing and placed into perspective the various host responses as they may affect each of these four stages.

Traditional methods to collect GCF use micropipettes. The most applicable method is the use of precut methylcellulose filter paper strips placed in the sulcus. The fluid is adsorbed and later analyzed. This is noninvasive, can be time consuming and very technique-sensitive method. Before collection of the GCF, plaque needs to be removed from the tooth, since plaque, saliva and blood can influence the volume of fluid

collected. The volume of GCF present at a given site may be directly related to inflammation and ulceration of the gingival crevicular epithelium. GCF can be analyzed to determine whether specific markers of systemic disease can be identified in the oral cavity. It has been shown that a greater volume of GCF is present in severe inflamed sites than in less inflamed sites. However, no studies have demonstrated that an increase of this volume is related to the risk for periodontal disease (Lamster and Ahlo 2007).

In looking specifically at diabetes influence on local inflammatory markers, Engebretson et al. (2004) found that there was a correlation between poor glycemic control and increased levels of IL-1 beta levels in GCF, emphasizing a plausible explanation for the increased incidence and severity of periodontal disease in patients with diabetes. Evidence exists suggesting that proinflammatory cytokines in particular IL-1 beta, may play an important role in the etiology of periodontal disease. IL-1 beta is biologically active in low concentrations and is a potent bone resorptive cytokine. Engebretson et al. (2002) compared GCF IL-1 beta expression in patients with different degrees of periodontal disease; baseline clinical parameters were significant associated with total IL-1 beta concentration in GCF and patients with severe periodontitis demonstrate increased of levels of IL-1 beta than those with mild/moderate disease.

Interleukin 6 (IL-6) is a major mediator of the host response to tissue injury, infection and bone resorption. Kurtis et al. (1999) measured IL-6 levels in GCF from twenty-four patients with type II diabetes with periodontitis, twenty-four adult periodontitis, and twenty-four healthy controls. GCF sampling was performed on the vestibular aspects of the maxillary incisors and canine teeth. Higher GCF IL-6 levels

were observed in the diabetic with periodontitis and adult with chronic periodontitis alone patients versus the healthy control ($p<0.05$). These findings suggested that GCF IL-6 levels were significantly higher in the area of inflammation and periodontal destruction locally.

Interleukin-8 (IL-8) is a potent chemokine with a distinct target for recruitment and activation of human granulocytes and mediation of inflammatory process. Monocytes/macrophages, lymphocytes, fibroblasts, endothelial cells and epithelial cells can secrete IL-8 and plays an important role in regulation of neutrophil function. Under normal conditions respond well to neutrophil infiltration, under inappropriate release the bacteria can't be eliminate by neutrophils and uncontrolled release of IL-8 can cause tissue damage by hyperactivity of neutrophils (Jin, Leung et al. 2002)

Regarding systemic markers, Manouchehr-Pour et al. (1981) demonstrated that diabetic patients with severe periodontitis have significant impairment of PMN chemotaxis. In contrast, diabetic patients with mild periodontal disease as well as non-diabetic subjects with either severe or mild periodontitis did not show such impairment. In addition, periodontitis and diabetes share a common pathogenesis involving an increased inflammatory response at the local and systemic level. Elevated serum levels of proinflammatory cytokines is often seen in patients with periodontal disease while patients with diabetes have hyper inflammatory immune cells that can exacerbate the elevated production of proinflammatory cytokines (Dag, Firat et al. 2009).

Salvi et al. (1997; 1998) studied 39 Insulin-dependent diabetes mellitus (IDDM) patients and 64 systemically healthy individuals. The patients were divided into groups A (gingivitis and mild periodontitis) and group B (moderate and severe periodontitis), 17

patients from the systemic healthy individuals were used as a control. These authors showed that diabetic patients had significant higher GCF levels of IL-1 beta as compared to non-diabetics. Within the diabetic population the GCF levels of this inflammatory mediator were higher in-group B than group A. Furthermore, diabetes as a group showed a higher systemic response of IL-1 beta upon stimulation by *Escherichia coli* and *Porphyromonas gingivalis* lipopolysaccharide (LPS) as compared to non-diabetic patients with adult periodontitis. They suggested that diabetes type II is a significant risk factor for more severe periodontal disease because they found that diabetic patients have exaggerated inflammatory responses when compared to non-diabetic controls.

Tumor necrosis factor (TNF-alpha) has been reported to play a key role in the pathogenesis of type II diabetes. TNF-α is associated with insulin resistance; the etiology of highest circulation levels in diabetics is not fully understood. Adipose tissue may be a major source of TNF-alpha secreting cells in type II diabetes but not all the studies have shown TNF-α to be associated with obesity. There are at least three main sources for increased circulating TNF-alpha levels in patients with diabetes, including the release of TNF-α from adipose tissues, from the stimulation of monocyte–macrophage cells/ leukocytes by advanced glycation end products, and from periodontal inflammation (Dag, Firat et al. 2009; Chen, Wei et al. 2010). Recent evidence suggests that inflammation influence the circulation TNF-α rather than obesity. Forty six patients with periodontitis and type II diabetes were evaluated in a cross-sectional study, the results demonstrated that chronic periodontitis is associated with plasma TNF-α levels in subjects with type II diabetes supporting the hypothesis that

periodontal infection and inflammation may contribute to insulin resistance (Engebretson, Chertog et al. 2007).

Correa et al. (2010) investigated the effect of periodontal therapy in diabetics, the sample was composed of 23 type II diabetes subjects, and higher levels of TNF-alpha, IL-4, IL-6, IL-8 and IL-10 were found at the baseline. The main finding of this prospective study was that the satisfactory clinical response to non-surgical periodontal therapy was followed by a reduction of circulation TNF-α concentration in type II diabetes. The other circulating cytokines investigated were also reduced but did reach statistical significance.

The literature suggests a two-way relationship between periodontal diseases and diabetes, although the mechanisms in which this relationship occurs is still not completely understood. It seems that diabetics are more susceptible to periodontal diseases due to both a hyper inflammatory component leading to more tissue destruction as well as an impaired immune response, which could delay/impair host's natural healing/regenerative capabilities during the disease course. On the other hand, periodontitis may aggravate the diabetic host inflammatory component both locally and systemically, leading to worsening of diabetes status/control.

The objective of the present investigation is to better understand the contribution of diabetes to the local and systemic inflammatory response in individuals with periodontitis.

CHAPTER 3
MATERIALS AND METHODS

Patient Selection

Two volunteer populations with periodontal disease were recruited for this study: Ten type II diabetic – DBT patients and ten non-diabetic-NDBT subjects. Diabetic patients were recruited from the Endocrinology clinic, at the Shands Medical Plaza. Non-Diabetic patients were recruited from the Graduate Periodontology clinic at the University of Florida college of Dentistry.

All subjects signed an informed consent in order to participate in the study according to the UF institutional Review Board approval (protocol #70-2007). These patients were included according to the following criteria:

Inclusion criteria for all patients (diabetics and non-diabetics). Subjects aged 40-75 years old; Presence of at least 20 teeth; Diagnosis of chronic periodontal disease defined by the presence of at least 4 sites with probing depth of 5mm or more and attachment loss of 3 mm or more with bleeding on probing.

Exclusion criteria for all patients (diabetics and non-diabetics). Subjects diagnosed with any forms of aggressive or necrotizing periodontal disease; any systemic diseases or conditions that could influence the course of either periodontal disease and/or glucose control; Subjects under any medications that could influence the characteristics or response to treatment of either diabetes and/or periodontal disease; Pregnant or lactating women.

Inclusion criteria for diabetic patients. Diagnosed with type II diabetes (HbA1c levels of ≥ 7%); Currently under standard treatment and physicians care for diabetes control; Have not altered their medication to treat diabetes in the past 3 months.

Exclusion criteria for diabetic patients. Type I diabetic patients, diabetic complications such as macrovascular diseases, kidney or liver failure, alteration of diabetes medications three months prior to study enrollment.

Periodontal Examination

All patients received a periodontal evaluation. All clinical parameters were recorded at six sites per tooth: probing depths, gingival marginal position, clinical attachment levels, plaque index and bleeding on probing. All parameters were measured and recorded by two calibrated examiners (LS & RM) with a periodontal electronic probe (Florida Probe, Gainesville, Fl) (Figure 3-1). Subject disease status was classified according to the 1999 Classification of Periodontal Disease established by the American Academy of Periodontology (Armitage 1999).

The two examiners' calibration and reproducibility were ensured in calibration sessions at the beginning of the study, obtaining duplicates measurements of pockets depths and gingival margin positions on different patients from the study. Calibration intra and inter-examiner was obtained once >80% of agreement (measurements within 1mm) was obtained between duplicate measurements of pocket depth and gingival margin position.

Collection of GCF (Local Response)

GCF samples were collected from 2 selected periodontal sites, one disease site (probing depths 5mm or greater, with bleeding on probing), and one healthy site (pocket depth < 3mm and no bleeding on probing) to serve as a control. Prior to GCF fluid sampling, supragingival plaque was removed using a sterile curette. These surfaces were gently air dried and isolated with cotton rolls. GCF fluid was collected using PerioPaper GCF collection strips (Oraflow Inc, Plainview, NY) by inserting the strip

roughly 1-2 mm into the gingival sulcus for ~10 sec each. The volume of GCF obtained was quantified with a Periotron 8000 (Oraflow Inc, Plainview, NY). The strips were placed into a sterile polypropylene eppendorf tube and then the samples were stored at -80°C until assayed (Figure 3-2).

Collection of Blood Samples (Systemic Response):

One vacutainer tube (4mL) of venous blood samples was collected; the plasma was used to measure systemic levels (stimulated and non stimulated) of cytokines and chemokines. The blood samples were obtained from each subject by a certified phlebotomist under the direct supervision of the examiners. The skin was wiped with 70% isopropyl alcohol and venipuncture was performed with an intravenous 22 gauge and vacuum tube (BD Biosciences, MD, USA)

Laboratory Procedures

Two ml of peripheral blood from the two groups (diabetics and non-diabetics) were diluted in 6 ml of RPMI 1640 (Fisher Scientific, PA), one ml of this diluted solution was placed into three wells of a 24–well plate. Five microliter of ultra-pure TLR-2 and TRL-4 agonist alone was added to separate wells. One well was not stimulated to serve as a control. Samples were incubated at 37°C in CO_2 for 24 hours (Figure 3-3). After 24 hours the blood from each well was transferred into the corresponding eppendorf tube. The labeled tubes containing the blood were placed into a small centrifuge and run for 5 minutes. After centrifuged, the supernatant from each of those tubes where placed into the corresponding eppendorf tube. Those tubes were stored in -80°C until assayed.

Luminex Analysis

Fluorescence detection kits (Milliplex® 22-plex cyto/chemokine detection kits, Millipore, St. Charles, MO) were used to detect and quantify 22 cyto/chemokines (IL1α, IL1β, IL2, IL4, IL5, IL6, IL7, IL8, IL10, IL12(p40), IL12(p70), IL13, IL15, Eotaxin, GM-CSF, IFNγ, IP10, MCP1, MIP1α, MIP1β, TNFα, VEGF). Briefly, 50µL of eluates and 25µL of a cyto/chemokine capture bead cocktail were placed in a 96-well filter plate and incubated overnight at 4^{o}C. Wells were washed 3 times with assay buffer, after which 25µL of a biotin labeled anti-cytokine cocktail and 75µL of assay buffer were plated and incubated for 1.5 hours. After which 25µL of SAV-PE was added and incubated for 30 minutes. Twenty-five µL of stop reagent was added for 5 minutes followed by 3 washes with assay buffer and beads resuspended in 125µL of sheath fluid. All incubations were performed at room temperature in the dark while gently shaking. Data were acquired using instrumentation (Luminex®100^{TM}, Millipore, St. Charles, MO) and analyzed using a software (Milliplex Analyst®, Viagene Tech, Carlisle, MA), standard curves and five-parameter logistics. Some samples were diluted 1:100 in assay buffer prior to running the above-described assay.

Statistical Analysis

Results were analyzed for each Cytokine / chemokine between diabetes and non-diabetes group using t-test, with a significance level of α=0.05.

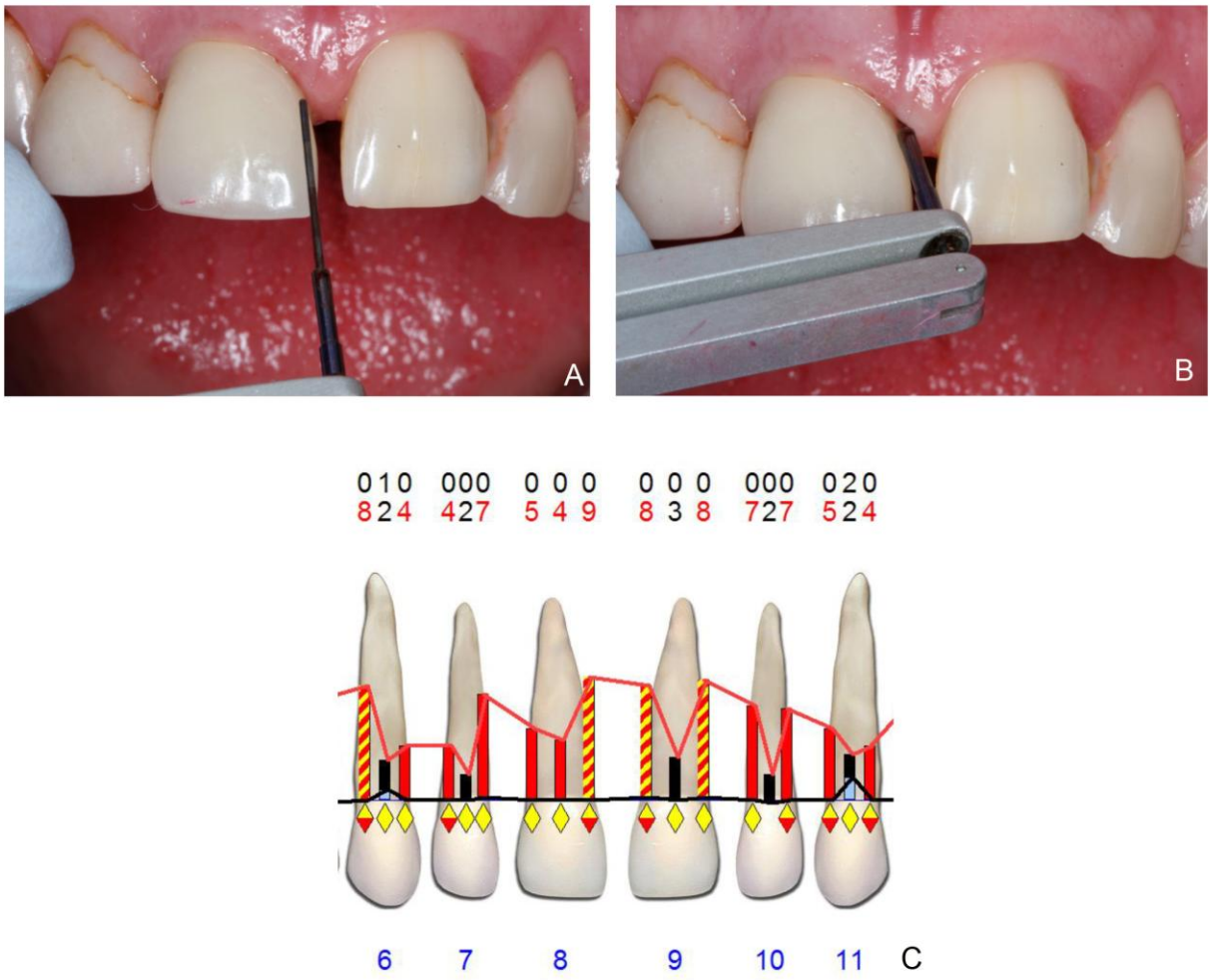

Figure 3-1. Periodontal clinical evaluation: All patients received a periodontal evaluation. Clinical parameters at six sites per tooth were taken (probing depths, gingival marginal positioning, attachment levels, plaque index and bleeding on probing). A) Florida probe before reading, B) Florida probe in the periodontal pocket. C) Florida probe software fraction's charting (Photo courtesy to Ruben Mesia).

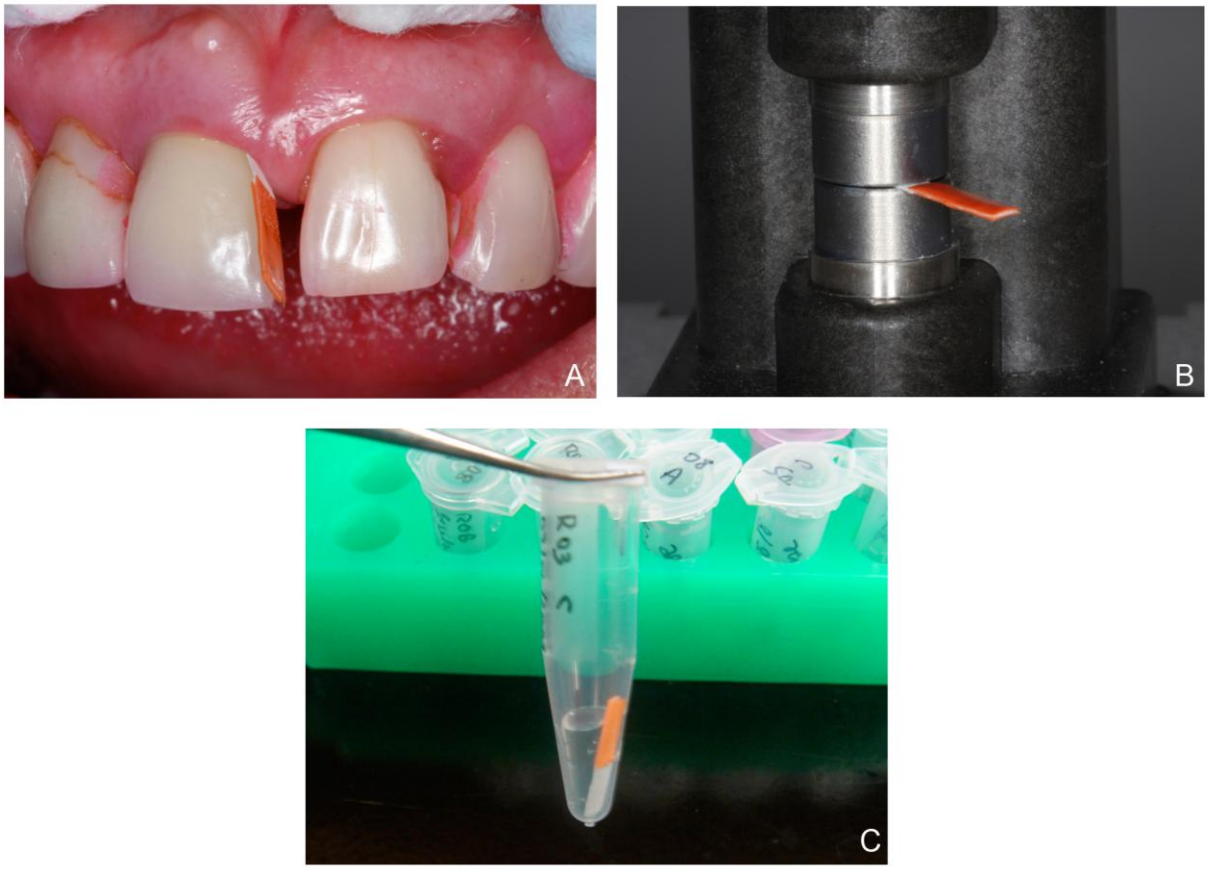

Figure 3-2. Gingival fluid collection: PerioPaper GCF strips were use to collect GCF from 2 selected periodontal sites, 1 disease and 1 healthy site. The volume obtained was quantified with a Periotron 8000. The samples were stored at -80oC until assayed. A) PerioPaper GCF strips in the gingival pocket, B) PerioPaper GCF strips quantify with periotron 8000. C) PerioPaper GCF strips ready to be assayed (Photo courtesy to Ruben Mesia).

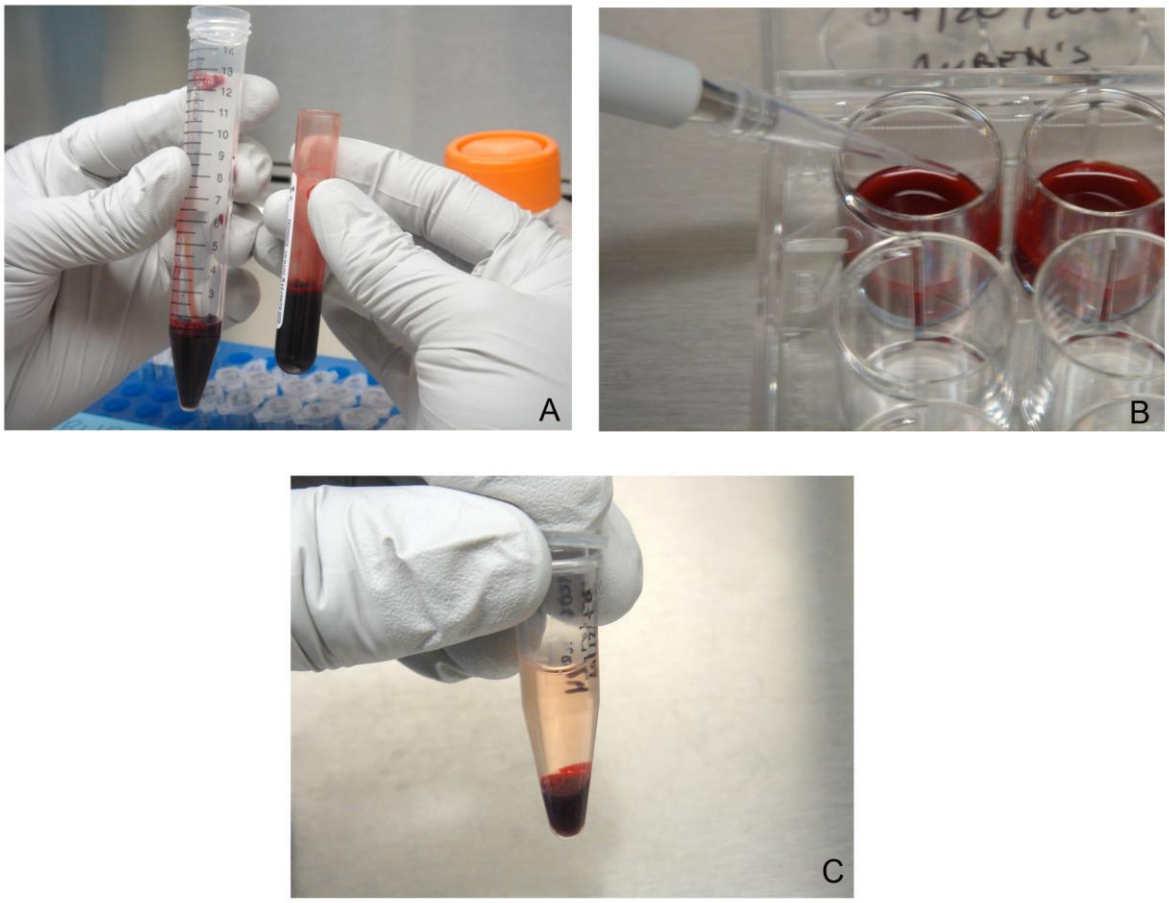

Figure 3-3. Laboratory procedures: 1mL of blood was stimulated with 1µL TRL-2 and TRL-4 agonists. One well was not stimulated to serve as a control. Samples were incubated at 37°C in CO2 for 24 hours. A) Blood samples, B) Stimulation with TRL-2, C) After 24 hours of incubation, each well was centrifuged and storage at -80°C until assayed (Photo courtesy to Ruben Mesia).

Figure 3-4. Luminex was used to analyze local (GCF) and systemic inflammatory (blood samples) markers (Photo courtesy to Ruben Mesia).

CHAPTER 4
RESULTS

The distribution of clinical results of these two populations is illustrated in Table 4-1. The ages of subjects in non-diabetics (N-DBT) and diabetics (DBT) range from 44 to 64 and 51 to 69 years old with mean ± standard deviation of 53.3 ± 6.4 and 61.3 ± 5.4, respectively. The ratios of female/male gender in groups N-DBT and DBT were 5/5 (1.0) and 4/6 (0.66) respectively. Among all subjects, the mean percentage of sites per subject with bleeding on probing (BOP) was 45.7 ± 22.4% and 25.4 ± 13.8% for N-DBT and DBT respectively. The mean percentages of plaque index were 57.3 ± 14.5% and 20.6 ± 18.1% for N-DBT and DBT respectively ($p<0.05$). The mean percentage of pockets depths greater than 4 mm were 41.8 ± 13.4% and 11.9 ± 8.4% for N-DBT and DBT respectively ($p<0.05$). The Periodontal probing depths was 5.22 ± 0.5 and 4.50 ± 0.3 for N-DBT and DBT respectively ($p<0.05$). Mean clinical attachment levels (CAL) in N-DBT and DBT were 5.64 ± 0.9 and 4.47 ± 1.2 respectively ($p<0.05$) and the mean HbA1c value for diabetic subjects was 8.13 ± 1.23.

The Analysis of systemic markers showed higher unstimulated levels of IL-8, TNFα, IL-10, MIP1α and MIP1β in the serum of diabetics (DBT) patients ($p<0.05$, Figures 4-1, 4-2, 4-3). After Lipopolysaccharides (LPS) stimulation with toll like receptors two and toll like receptors four (TRL-2 and TLR-4). DBT patients showed higher stimulated levels of IL-6, IL-8, IL-10, MIP1α and MIP1β ($p<0.05$, Figures 4-4, 4-5, 4-6). The DBT population also showed lower stimulated and unstimulated levels of GM-CSF than NDBT ($p<0.05$, Figure 4-7).

Regarding the local inflammatory response (local markers), DBT patients also showed higher levels of MIP1β in gingival crevicular fluid (GCF) from healthy sites

($p<0.001$) than NDBT, while NDBT showed higher levels of IL-7 in GCF from diseased sites ($p<0.05$) (Figure 4-8).

Table 4-1. Clinical results between diabetics and non-diabetics. All results shown by mean ± standard deviation.

	Non-Diabetic	Diabetic
% BOP	45.7 ± 22.4*	25.4 ± 13.8
% PLAQUE	57.3 ± 14.5*	20.6 ± 18.1
%PD>4 mm	41.8 ± 13.4*	11.9 ± 8.4
Mean PD	5.22 ± 0.5*	4.50 ± 0.3
Mean CAL	5.64 ± 0.9*	4.47 ± 1.2
% Female	50	40
Mean age	53.3 ± 6.4	61.3 ± 5.4*
Mean HbA1c	--	8.13 ± 1.23

* Denotes significant differences between groups ($p<0.05$).

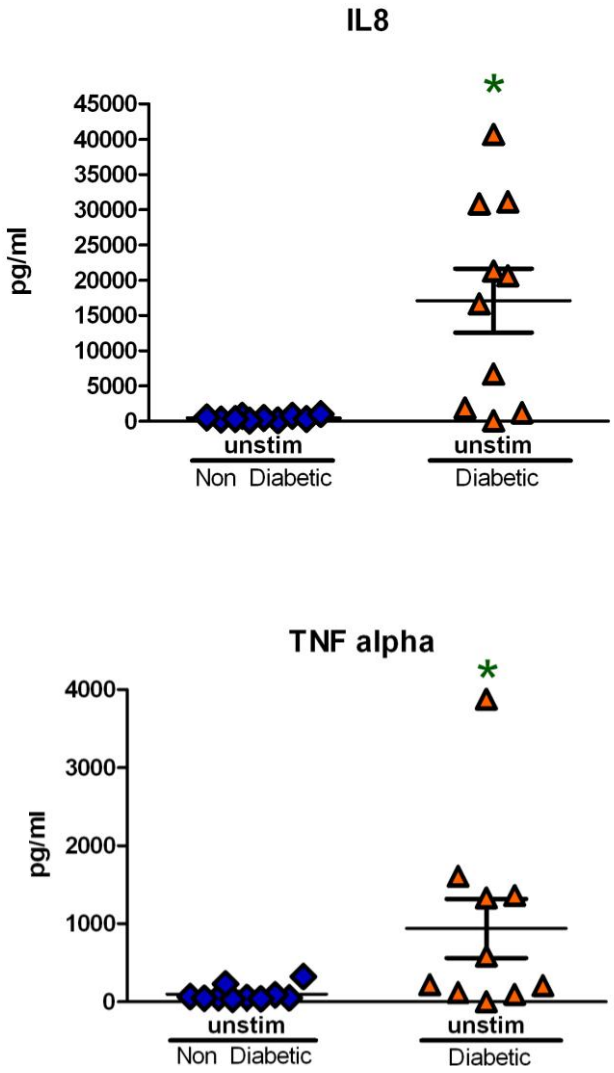

Figure 4-1. Systemic markers (Unstimulate): Diabetes patients present higher inflammatory markers in serum in IL- 8 and TNF alpha. * Denotes significant differences between diabetic and non-diabetic groups ($p<0.05$) (Photo courtesy to Ruben Mesia).

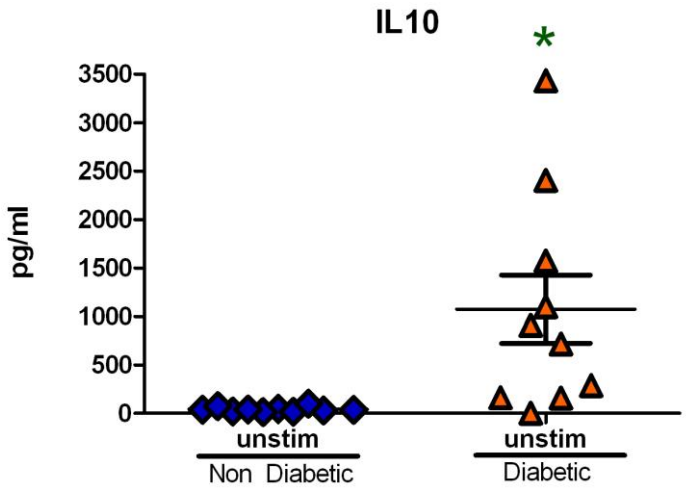

Figure 4-2. Systemic markers (Unstimulate): Diabetes patients present higher inflammatory markers in serum in IL- 10. * Denotes significant differences between diabetic and non-diabetic groups ($p<0.05$) (Photo courtesy to Ruben Mesia).

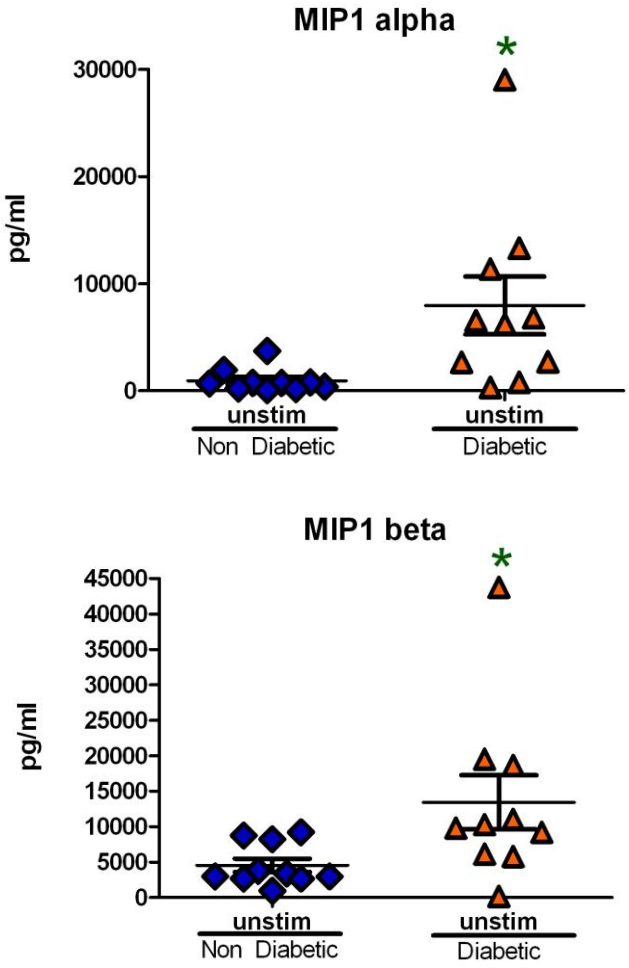

Figure 4-3. Systemic markers (Unstimulate): Diabetes patients present higher inflammatory markers in serum in MIP1 alpha and MIP1 beta. * Denotes significant differences between diabetic and non-diabetic groups ($p<0.05$) (Photo courtesy to Ruben Mesia).

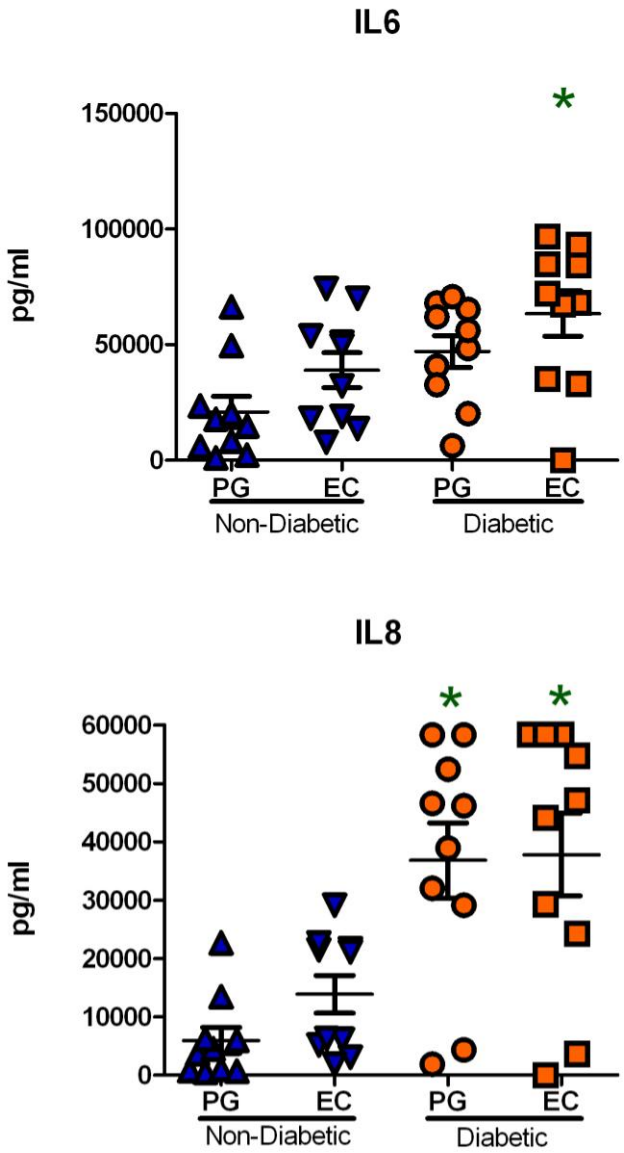

Figure 4-4. Systemic markers (Stimulate): Diabetes patients present higher inflammatory markers upon LPS stimulation in IL-6 and IL-8. * Denotes significant differences between diabetic and non-diabetic groups ($p<0.05$) (Photo courtesy to Ruben Mesia).

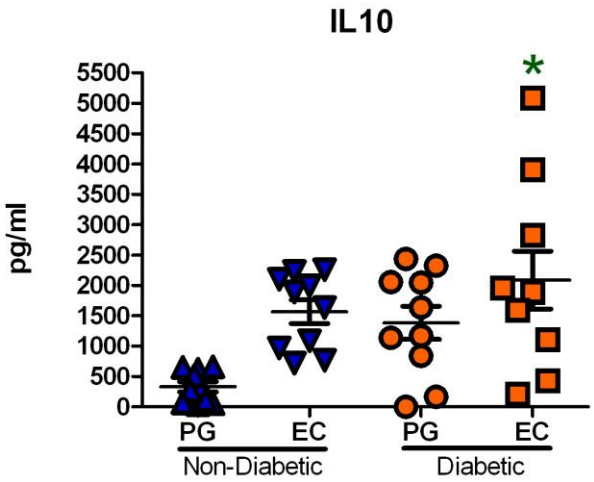

Figure 4-5. Systemic markers (Stimulate): Diabetes patients present higher inflammatory markers upon LPS stimulation in IL-10. * Denotes significant differences between diabetic and non-diabetic groups ($p<0.05$) (Photo courtesy to Ruben Mesia).

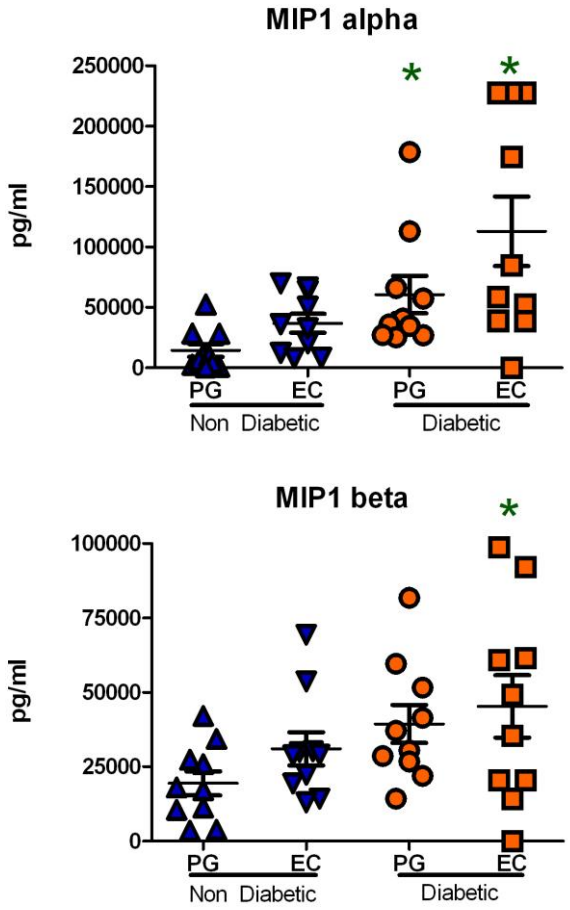

Figure 4-6. Systemic markers (Stimulate): Diabetes patients present higher inflammatory markers upon LPS stimulation in MIP1 alpha and MIP1 beta. * Denotes significant differences between diabetic and non-diabetic groups ($p<0.05$) (Photo courtesy to Ruben Mesia).

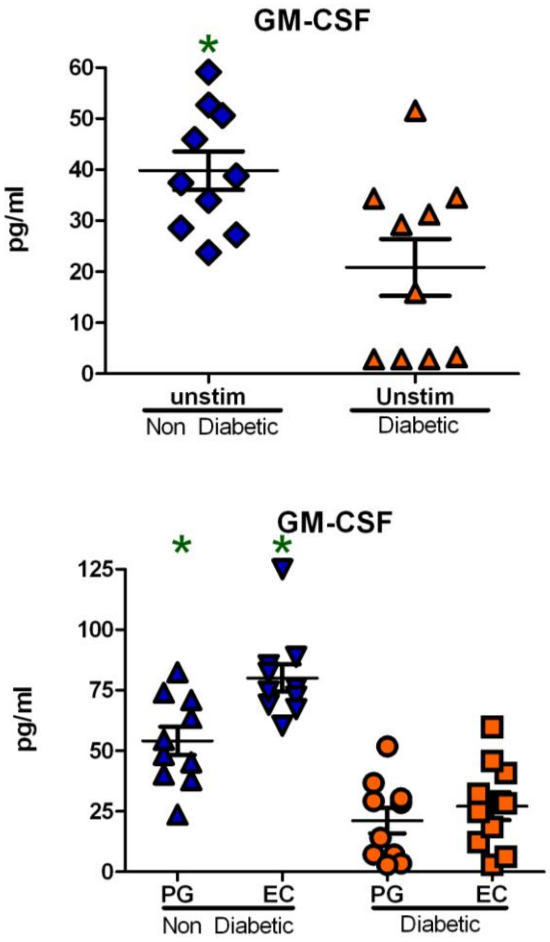

Figure 4-7. Systemic markers (Unstimulate and Stimulate): Diabetes patients present lower stimulated and unstimulated levels of GM-CSF than NDBT. * Denotes significant differences between diabetic and non-diabetic groups ($p<0.05$) (Photo courtesy to Ruben Mesia).

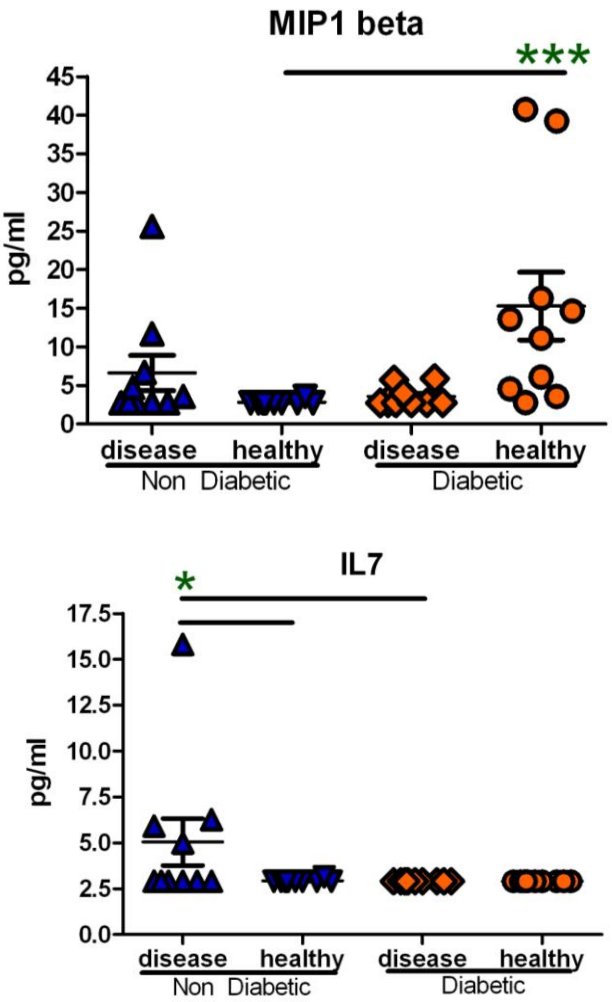

Figure 4-8. Local markers MIP1β and IL-7: Diabetes patients also showed higher levels of MIP1β in GCF from healthy sites than NDBT, while NDBT showed higher levels of IL-7 in GCF from diseased sites. Bars indicate differences between groups or sites, * ($p<0.05$), *** ($p<0.001$) (Photo courtesy to Ruben Mesia).

CHAPTER 5
DISCUSSION

The influence of diabetes on periodontal disease has been discussed in the literature, and there is substantial evidence indicating that diabetes is a risk factor for periodontal disease (Emrich, Shlossman et al. 1991; Taylor, Burt et al. 1998). The present study has reported, among patients with chronic gingival disease, those with diabetes seem to have more severe systemic inflammatory processes than those without diabetes, as measured by cytokine/chemokine expression. Those high levels of cytokines were previously highly correlated with active progressive periodontal lesions in non-diabetic healthy individuals (Tonetti, Imboden et al. 1994; Loos, Craandijk et al. 2000; Kikkert, Laine et al. 2007). However, the inflammatory response with regard to IL-6, IL-8, TNFα, IL-10, MIP1α and MIP1β in diabetes patients was exaggerate, we also reported lower levels of certain cytokines such as GM-CSF could indicate an impaired immune response in this patient population.

An interesting finding in this study was the observation of higher inflammatory response in the diabetic group even before lipopolysaccharides (LPS) stimulation. Those exaggerate responses may be a phenotypic marker for susceptibility in diabetics. In agreement with previous studies, we found elevated levels of TNF-α in serum in subject with diabetes with less severity of periodontal disease. This finding tend to support recent research by Chen et al. (2010), in which they failed to find a correlation between the severity of periodontal disease and serum TNF-α levels. One possible reason may be that the contribution of periodontal infection to circulating TNF-α was so minor that it was overwhelmed by others sources of TNF-α in patients with Type 2 diabetes. Other studies found no significant differences in serum levels of TNF-α at the

baseline between DBT and NDBT (Dag, Firat et al. 2009; Kardesler, Buduneli et al. 2010).

Previous investigations have demonstrated that chronic periodontitis severity is significantly associated with plasma TNF-α in Type II diabetes (Salvi, Beck et al. 1998; Crook 2004; Correa, Goncalves et al. 2010). However, Engebretson et al. (2007) measured plasma levels of TNF-α in subjects with type II diabetes and chronic periodontitis and found that TNF-α showed a significant positive correlation with attachment loss but not with probing depth, raising the question whether TNF levels influence diabetes severity, or indeed whether circulating TNF influences periodontitis severity. Salvi et al. (1998) also found a significant upregulated monocytic secretion of TNF-alpha (4.6-fold) when compared to non-diabetic controls, which is in agreement with the present findings.

According to the present findings, IL-6 levels of the groups with diabetes were higher than the NDBT after LPS stimulation. Loos et al. (Loos, Craandijk et al. 2000), reported that IL-6 can be detected in plasma of >50% of patients with severe periodontitis. Some studies show that elevated levels of IL-6 is found in diabetes Type II (Correa, Goncalves et al. 2010; Kardesler, Buduneli et al. 2010). We found higher levels of IL-6 in diabetics (DBT) than non-diabetics (NDBT) even though DBT did not have the same degree of periodontal severity. Ross et al. (2010) found that periodontal IL-6 expression increases when that disease status changes from ‘no disease’ to ‘one disease (periodontal disease)’ to ‘two diseases (periodontitis and diabetes)’, which may suggest that diabetes further aggravates systemic inflammation. The present study

findings may suggest that diabetes may play a more aggravating role to the systemic inflammatory response than the severity of periodontitis.

Interestingly, IL-8 was found to be elevated in DBT patients before and after stimulation in the present study. IL-8 is known to be involved in the recruitment of polymorphonuclear neutrophilic leukocytes (PMNs) and is highly expressed in the junctional epithelium adjacent to infected periodontal defects, where PMNs infiltrate. Therefore, IL-8 may be involved in the initial stages of periodontal breakdown (Tonetti, Imboden et al. 1994). The elevated levels of this cytokine in DBT patients in the present investigation may suggest that these patients are more susceptible to periodontal breakdown initiation than NDBT patients. In addition, the fact that the DBT patients in the present study had a more localized and milder form of periodontitis indicates that this disease status could be in its initial development, which further explains the over-expression of this cytokine. Similarly, MIP1-α, also found to be high in DBT patients, has been correlated to high levels of IL-8 in early mucositis lesions (Petkovic, Matic et al. 2010), which corroborates with the initial periodontal breakdown role of IL-8 previously mentioned in these patients.

DBT patients also expressed high systemic levels of IL-10 before and after stimulation with LPS. Since this cytokine is involved with inhibition of cytokine synthesis due to its inhibitory effect on macrophage-monocytes (Fiorentino, Zlotnik et al. 1991), high levels of this cytokine in this population could then be detrimental to the immune response activation and maintenance.

On the other hand, DBT patients showed lower levels of GM-CSF systemically when compared to NDBT patients. Abnormal maturation and other defects of dendritic

cells (DCs) have been associated with the development of diabetes. Evidence is accumulating that self-tolerance can be restored and maintained by semimature DCs induced by GM-CSF (Gaudreau, Guindi et al. 2007). Thus, a lower level of GM-CSF in poorly controlled diabetic patients is expected.

While previous studies (Salvi, Beck et al. 1998; Engebretson, Grbic et al. 2002; Engebretson, Hey-Hadavi et al. 2004) found higher levels of IL-1 β in DBT patients, our present findings did not show significant differences between IL-1 β locally and systemically between DBT and NDBT. The significant differences between periodontal parameters in our groups may have contributed to this finding.

One limitation of the present study was the significant difference in periodontal parameters between these two populations. The reason behind that difference was possible selection bias, since diabetic patients were recruited from a medical facility while non-diabetic patients were recruited from the graduate periodontal program, where periodontal disease status is expected to be more severe. This difference in periodontal severity between the two groups could have influenced the levels of certain cytokines, mainly locally, in the GCF. However, interestingly, this difference did not influence the higher systemic inflammatory response in DBT patients, which lead us to believe that non-controlled diabetes may have a stronger influence in systemic inflammation than periodontal disease alone. Other limitation was the absence of a healthy control group. The inclusion of a healthy control group would enable us to evaluate the role of periodontal disease alone in local and systemic inflammatory response. However, our objective in the present study was to evaluate the added role of diabetes into periodontal disease. Lastly, it is clear that a larger study population would

also be needed to draw any final conclusions regarding the biomarkers in diabetic patients with different levels of periodontitis and diabetic control.

In summary, significantly higher systemic pro-inflammatory response in DBT patients could explain the increased susceptibility to periodontal disease in these patients in general. Conversely, this increase in mediators could be a reflection of the negative effect periodontal disease has on diabetic metabolic control. In addition, the lower levels of certain defensive cytokines in DBT patients upon stimulation with TLRs could indicate an impaired immune response in this patient population. Further studies to assess the relationship between degree of inflammation, magnitude of diabetes control and extent/severity of periodontal disease are justified.

LIST OF REFERENCES

Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* **4,** 1-6.

Chen, L., Wei, B., Li, J., Liu, F., Xuan, D., Xie, B. & Zhang, J. (2010) Association of periodontal parameters with metabolic level and systemic inflammatory markers in patients with type 2 diabetes. *J Periodontol* **81,** 364-371.

Correa, F. O., Goncalves, D., Figueredo, C. M., Bastos, A. S., Gustafsson, A. & Orrico, S. R. (2010) Effect of periodontal treatment on metabolic control, systemic inflammation and cytokines in patients with type 2 diabetes. *J Clin Periodontol* **37,** 53-58.

Crook, M. (2004) Type 2 diabetes mellitus: a disease of the innate immune system? An update. *Diabet Med* **21,** 203-207.

Dag, A., Firat, E. T., Arikan, S., Kadiroglu, A. K. & Kaplan, A. (2009) The effect of periodontal therapy on serum TNF-alpha and HbA1c levels in type 2 diabetic patients. *Aust Dent J* **54,** 17-22.

DeFronzo, R. A. & Ferrannini, E. (1991) Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* **14,** 173-194.

Eke, P. I., Thornton-Evans, G. O., Wei, L., Borgnakke, W. S. & Dye, B. A. (2010) Accuracy of NHANES periodontal examination protocols. *J Dent Res* **89,** 1208-1213.

Emrich, L. J., Shlossman, M. & Genco, R. J. (1991) Periodontal disease in non-insulin-dependent diabetes mellitus. *J Periodontol* **62,** 123-131.

Engebretson, S., Chertog, R., Nichols, A., Hey-Hadavi, J., Celenti, R. & Grbic, J. (2007) Plasma levels of tumour necrosis factor-alpha in patients with chronic periodontitis and type 2 diabetes. *J Clin Periodontol* **34,** 18-24.

Engebretson, S. P., Grbic, J. T., Singer, R. & Lamster, I. B. (2002) GCF IL-1beta profiles in periodontal disease. *J Clin Periodontol* **29,** 48-53.

Engebretson, S. P., Hey-Hadavi, J., Ehrhardt, F. J., Hsu, D., Celenti, R. S., Grbic, J. T. & Lamster, I. B. (2004) Gingival crevicular fluid levels of interleukin-1beta and glycemic control in patients with chronic periodontitis and type 2 diabetes. *J Periodontol* **75,** 1203-1208.

Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. & O'Garra, A. (1991) IL-10 inhibits cytokine production by activated macrophages. *J Immunol* **147,** 3815-3822.

Gaudreau, S., Guindi, C., Menard, M., Besin, G., Dupuis, G. & Amrani, A. (2007) Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of CD4+CD25+ regulatory T cells. *J Immunol* **179,** 3638-3647.

Genco, R. J. & Slots, J. (1984) Host responses in periodontal diseases. *J Dent Res* **63,** 441-451.

Grossi, S. G., Skrepcinski, F. B., DeCaro, T., Robertson, D. C., Ho, A. W., Dunford, R. G. & Genco, R. J. (1997) Treatment of periodontal disease in diabetics reduces glycated hemoglobin. *J Periodontol* **68,** 713-719.

Grossi, S. G., Skrepcinski, F. B., DeCaro, T., Zambon, J. J., Cummins, D. & Genco, R. J. (1996) Response to periodontal therapy in diabetics and smokers. *J Periodontol* **67,** 1094-1102.

Harris, M. I., Hadden, W. C., Knowler, W. C. & Bennett, P. H. (1987) Prevalence of diabetes and impaired glucose tolerance and plasma glucose levels in U.S. population aged 20-74 yr. *Diabetes* **36,** 523-534.

Jin, L. J., Leung, W. K., Corbet, E. F. & Soder, B. (2002) Relationship of changes in interleukin-8 levels and granulocyte elastase activity in gingival crevicular fluid to subgingival periodontopathogens following non-surgical periodontal therapy in subjects with chronic periodontitis. *J Clin Periodontol* **29,** 604-614.

Kardesler, L., Buduneli, N., Cetinkalp, S. & Kinane, D. F. (2010) Adipokines and inflammatory mediators after initial periodontal treatment in patients with type 2 diabetes and chronic periodontitis. *J Periodontol* **81,** 24-33.

Kikkert, R., Laine, M. L., Aarden, L. A. & van Winkelhoff, A. J. (2007) Activation of toll-like receptors 2 and 4 by gram-negative periodontal bacteria. *Oral Microbiol Immunol* **22,** 145-151.

Kurtis, B., Develioglu, H., Taner, I. L., Balos, K. & Tekin, I. O. (1999) IL-6 levels in gingival crevicular fluid (GCF) from patients with non-insulin dependent diabetes mellitus (NIDDM), adult periodontitis and healthy subjects. *J Oral Sci* **41,** 163-167.

Lamster, I. B. & Ahlo, J. K. (2007) Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Ann N Y Acad Sci* **1098,** 216-229.

Loe, H. (1993) Periodontal disease. The sixth complication of diabetes mellitus. *Diabetes Care* **16,** 329-334.

Loos, B. G., Craandijk, J., Hoek, F. J., Wertheim-van Dillen, P. M. & van der Velden, U. (2000) Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. *J Periodontol* **71,** 1528-1534.

Lopez, N. J., Valenzuela, C. Y. & Jara, L. (2009) Interleukin-1 gene cluster polymorphisms associated with periodontal disease in type 2 diabetes. *J Periodontol* **80,** 1590-1598.

Manouchehr-Pour, M., Spagnuolo, P. J., Rodman, H. M. & Bissada, N. F. (1981) Comparison of neutrophil chemotactic response in diabetic patients with mild and severe periodontal disease. *J Periodontol* **52,** 410-415.

Mealey, B. L. & Oates, T. W. (2006) Diabetes mellitus and periodontal diseases. *J Periodontol* **77,** 1289-1303.

Miller, L. S., Manwell, M. A., Newbold, D., Reding, M. E., Rasheed, A., Blodgett, J. & Kornman, K. S. (1992) The relationship between reduction in periodontal inflammation and diabetes control: a report of 9 cases. *J Periodontol* **63,** 843-848.

Mokdad, A. H., Bowman, B. A., Ford, E. S., Vinicor, F., Marks, J. S. & Koplan, J. P. (2001) The continuing epidemics of obesity and diabetes in the United States. *JAMA* **286,** 1195-1200.

Natali, A., Toschi, E., Baldeweg, S., Ciociaro, D., Favilla, S., Sacca, L. & Ferrannini, E. (2006) Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes. *Diabetes* **55,** 1133-1140.

Nishimura, F., Takahashi, K., Kurihara, M., Takashiba, S. & Murayama, Y. (1998) Periodontal disease as a complication of diabetes mellitus. *Ann Periodontol* **3,** 20-29.

Novaes, A. B., Jr., Pereira, A. L., de Moraes, N. & Novaes, A. B. (1991) Manifestations of insulin-dependent diabetes mellitus in the periodontium of young Brazilian patients. *J Periodontol* **62,** 116-122.

Papapanou, P. N. (1996) Periodontal diseases: epidemiology. *Ann Periodontol* **1,** 1-36.

Petkovic, A. B., Matic, S. M., Stamatovic, N. V., Vojvodic, D. V., Todorovic, T. M., Lazic, Z. R. & Kozomara, R. J. (2010) Proinflammatory cytokines (IL-1beta and TNF-alpha) and chemokines (IL-8 and MIP-1alpha) as markers of peri-implant tissue condition. *Int J Oral Maxillofac Surg* **39,** 478-485.

Ross, J. H., Hardy, D. C., Schuyler, C. A., Slate, E. H., Mize, T. W. & Huang, Y. (2010) Expression of periodontal interleukin-6 protein is increased across patients with

neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases. *J Periodontal Res* **45,** 688-694.

Rylander, H., Ramberg, P., Blohme, G. & Lindhe, J. (1987) Prevalence of periodontal disease in young diabetics. *J Clin Periodontol* **14,** 38-43.

Salvi, G. E., Beck, J. D. & Offenbacher, S. (1998) PGE2, IL-1 beta, and TNF-alpha responses in diabetics as modifiers of periodontal disease expression. *Ann Periodontol* **3,** 40-50.

Salvi, G. E., Yalda, B., Collins, J. G., Jones, B. H., Smith, F. W., Arnold, R. R. & Offenbacher, S. (1997) Inflammatory mediator response as a potential risk marker for periodontal diseases in insulin-dependent diabetes mellitus patients. *J Periodontol* **68,** 127-135.

Sammalkorpi, K. (1989) Glucose intolerance in acute infections. *J Intern Med* **225,** 15-19.

Seppala, B., Seppala, M. & Ainamo, J. (1993) A longitudinal study on insulin-dependent diabetes mellitus and periodontal disease. *J Clin Periodontol* **20,** 161-165.

Shlossman, M., Knowler, W. C., Pettitt, D. J. & Genco, R. J. (1990) Type 2 diabetes mellitus and periodontal disease. *J Am Dent Assoc* **121,** 532-536.

Taylor, G. W., Burt, B. A., Becker, M. P., Genco, R. J., Shlossman, M., Knowler, W. C. & Pettitt, D. J. (1996) Severe periodontitis and risk for poor glycemic control in patients with non-insulin-dependent diabetes mellitus. *J Periodontol* **67,** 1085-1093.

Taylor, G. W., Burt, B. A., Becker, M. P., Genco, R. J., Shlossman, M., Knowler, W. C. & Pettitt, D. J. (1998) Non-insulin dependent diabetes mellitus and alveolar bone loss progression over 2 years. *J Periodontol* **69,** 76-83.

Thorstensson, H., Kuylenstierna, J. & Hugoson, A. (1996) Medical status and complications in relation to periodontal disease experience in insulin-dependent diabetics. *J Clin Periodontol* **23,** 194-202.

Tonetti, M. S., Imboden, M. A., Gerber, L., Lang, N. P., Laissue, J. & Mueller, C. (1994) Localized expression of mRNA for phagocyte-specific chemotactic cytokines in human periodontal infections. *Infect Immun* **62,** 4005-4014.

Williams, R. C., Jr. & Mahan, C. J. (1960) Periodontal disease and diabetes in young adults. *J Am Med Assoc* **172,** 776-778.

Yamaguchi, R., Yoshimura, A., Yoshioka, H., Kaneko, T. & Hara, Y. (2009) Ability of supragingival plaque to induce toll-like receptor 4-mediated stimulation is

associated with cytokine production by peripheral blood mononuclear cells. *J Periodontol* **80,** 512-520.

Yki-Jarvinen, H., Sammalkorpi, K., Koivisto, V. A. & Nikkila, E. A. (1989) Severity, duration, and mechanisms of insulin resistance during acute infections. *J Clin Endocrinol Metab* **69,** 317-323.

Yoshioka, H., Yoshimura, A., Kaneko, T., Golenbock, D. T. & Hara, Y. (2008) Analysis of the activity to induce toll-like receptor (TLR)2- and TLR4-mediated stimulation of supragingival plaque. *J Periodontol* **79,** 920-928.

BIOGRAPHICAL SKETCH

Ruben Fernando Mesia was born in Yurimaguas, Peru. He has got his dental degree in Cayetano Heredia Peruvian University in Lima, Peru. He completed further training in advanced education in general dentistry (AEGD) at the University of Connecticut School of Dental Medicine. Currently, Ruben Mesia is completing his post-doctoral residency in periodontics at the University of Florida College of Dentistry. Upon graduation in the spring of 2011, Ruben plans to practice clinical periodontics in Tampa, FL.