CHAPTER ONE

Introduction

Periodontal Disease and its Epidemiology

Periodontal infection, including gingivitis and chronic periodontitis, is found worldwide and is among the most prevalent human microbial diseases. Gingivitis is an inflammatory response to dental plaque accumulation elicited in the superficial periodontal tissues. Epidemiologically, gingivitis has been shown to affect more than 50% of the United States (U.S.) adults (Albandar and Kingman, 1999). It has been suggested (Scannapieco, 2004) that the prevalence of gingivitis might be understated because gingivitis can be clinically undetectable in its very early stages.

On the other hand, periodontitis is a more severe disease condition that is characterized by destruction of tooth-supporting structures. Periodontitis (when defined as active destruction of the periodontal supporting tissues as evidenced by the simultaneous presence of > 3 mm probing depth and > 3 mm periodontal attachment loss) was shown to affect at least 35% of the dentate U.S. adults aged 30 years or older with about two-thirds of the affected individuals having mild periodontitis and the remainder having moderate to severe periodontitis (Albandar *et al*., 1999).

History of the Periodontal Disease and Systemic Disease Connection

Over the past decade, there has been a resurgence of interest in the possible causal link between periodontal disease and systemic diseases. The concept that oral disease can adversely impact systemic health is not new. The history of this relationship has been

well described in two recent articles (Barnett, 2006; Williams, 2008) as follows. The belief that oral disease can cause systemic illness is attributed to W.D. Miller and William Hunter. Miller proposed a role for oral bacteria in the causation of numerous diseases in organs that are distant from the oral cavity. In the late 1800s, Miller published his book entitled “The Microorganisms of The Human Mouth: The Local and General Diseases Which Are Caused by Them”. Miller also published a frequently cited article entitled "The Human Mouth as a Focus of Infection". Both, his book and his article supported a causal relationship between oral and systemic diseases (Miller, 1891).

In 1900, William Hunter published an article that addressed the role of oral disease as a cause of systemic diseases (Hunter, 1900). In 1910, Hunter gave a keynote address at McGill University in Montreal, Canada, accusing oral sepsis of causing many systemic diseases. Moreover he described dental restorations as being "built in, on, and around diseased teeth which form a veritable mausoleum of gold over a mass of sepsis to which there is no parallel in the whole realm of medicine."

In 1912, the “theory of focal infection” was introduced by Frank Billings. The “focal infection” was defined as a localized or generalized infection caused by the dissemination of bacteria and/or their products through the circulating blood and lymphatics from local foci of infection including teeth. At that time, focal infections were declared to cause many systemic diseases such as arthritis, endocarditis, brain abscesses, pneumonia, diabetes, and many other diseases (Billings, 1914). At that time, because of the strong belief in the theory of focal infection, the extraction of all endodontically or periodontally

affected teeth was commonly practiced in an attempt to prevent or cure subsequent systemic problems.

Around 1930, the validity of the focal infection theory was questioned. Therefore, the focus of dental practice changed with the emphasis on restorative dentistry. However, with some advances in the scientific investigation methods, it was realized that there were situations such as infective endocarditis in which oral bacteria could cause disease in sites that are distant from the oral cavity.

For the past two decades, there has been a resurgence of interest in the possible causal relationship between oral and systemic diseases. A case-control study (Mattila *et al*., 1989) was conducted on patients who had experienced an acute myocardial infarction. These patients were compared to control subjects that were randomly selected from the community. A highly significant positive association between poor dental health and acute myocardial infarction was observed. This association was independent of the traditional risk factors for heart disease.

Following the investigation by Mattila *et al*., were multiple studies investigating the causal association between periodontal disease and systemic conditions, especially Coronary Heart Disease (CHD) (DeStefano *et al*., 1993; Offenbacher *et al*., 1996; Beck *et al*., 1996), reviving the historical interest in the impact of oral health on general health.

Current Status of the Periodontal and Systemic Diseases Connection

Currently with advances in scientific investigation methods and statistical analyses; and with improved understanding of periodontal diseases and associated systemic diseases, the field of the oral-systemic health connections has been a rapidly advancing area of research that has been gaining great interest not only by scientists and clinicians but also by the media and the public.

To date, findings from recent studies, mostly epidemiological studies, support an association between periodontal infection, with particular emphasis on chronic periodontitis, and a number of clinically important systemic diseases. These include cardiovascular disease (Beck and Offenbacher, 2005), respiratory disease (Scannapieco and Ho, 2001), diabetes (Taylor *et al*., 1996), adverse pregnancy outcomes (Xiong *et al*., 2006), pancreatic cancer (Michaud *et al*., 2007), Alzheimer's disease (Kamer *et al*., 2008), and other systemic conditions continue to appear in the literature. However, to date, a direct causal role of periodontal infection in the development and or/progression of systemic diseases is not established (Hujoel *et al*., 2001; Friedewald *et al*., 2009).

General Mechanisms Connecting Periodontal and Systemic Diseases

In spite of the rapid growth in the literature on the possible etiological role of periodontal disease in systemic diseases, the issue has not been completely resolved and the pathophysiological mechanisms underlying these associations remain unclear. To better understand this association, a number of hypotheses have been proposed (Paquette, 2002; Seymour *et al*., 2007) to explain such mechanisms including: (1) Common susceptibility

to both oral infection and systemic diseases through shared risk factors. This common susceptibility can be mediated through genetic or environmental factors such as age, smoking, and socioeconomic status. According to this hypothesis, periodontal disease is associated with systemic diseases, but the relationship is not causal. (2) Systemic inflammation with heightened levels of circulating inflammatory biomediators in response to the local infection or circulating bacteria. According to this hypothesis, periodontal infection induces systemic inflammation and immune responses that may play a causal role in systemic disease. (3) Systemic dissemination of oral bacteria (i.e., bacteremia) and/or their products. According to this hypothesis, periodontal infection causes bacteremias and endotoxemias which may subsequently play a causal role in systemic diseases. (4) Cross-reactivity between bacterial and host heat shock proteins. According to this hypothesis, the immune system does not discriminate between host and bacterial heat shock proteins and thus, results in an autoimmune response that may contribute to the progression or development of systemic diseases.

Strength of the Periodontal and Systemic Disease Causal Association

In spite of the large number of investigations that demonstrate an association between periodontal and systemic diseases, to date, most have provided insufficient evidence to support a causal relationship; and the currently available data, mostly from epidemiological studies, is not adequate in order to accurately estimate the strength of the association (Dietrich and Garcia, 2005). It has been argued (Dietrich and Garcia, 2005) that although randomized clinical trials are important in this field, such trials may not clearly answer whether or not periodontal disease is causally related to systemic diseases.

It has also been suggested (Dietrich and Garcia, 2005) that there is still a need for well-designed studies, in order to better understand the possible causal linkage between periodontal and systemic diseases.

The most recent systematic review and meta-analysis (Humphrey *et al*., 2008) has evaluated the epidemiologic literature assessing the possible etiological link between periodontal disease and CHD. The analysis has suggested that periodontal disease is an independent, relatively weak, risk factor for CHDand that different measures of periodontal disease explain approximately a 24–35% increase in risk of CHD.

When evaluating the strength of evidence in this public health area, it is important to pay attention to, and address the potential impact that publication bias, particularly positive results bias, might have on the validity and integrity of the related systematic reviews and meta-analyses.

Although periodontal disease has been associated with numerous systemic conditions, in our studies that are described in this dissertation, we focused on investigating the association between gingivitis and CHD risk (for reasons that will be explained in the following sections). However, many of the points made regarding the relationship between gingivitis and CHD can also be applied to other systemic diseases.

Coronary Heart Disease (CHD)

CHD is defined as a disease characterized by narrowing of the arteries that supply blood and oxygen to the heart (MedlinePlus Medical Encyclopedia). CHD is usually caused by atherosclerosis. According to the 'response-to-injury' model (Ross, 1999), the atherosclerotic process is a slow and complex disease process that starts as a protective response against insults to the endothelium and smooth muscle cells of the arterial walls, resulting in immune and inflammatory mediated tissue damage. Important clinical end-stage consequences of atherosclerosis include angina pectoris, myocardial ischemia, myocardial infarction, and sudden cardiac death.

The risk factors for atherosclerosis can be classified as either nonmodifiable or modifiable. Non-modifiable risk factors include family history, age, and gender with males being more susceptible than females (particularly before the age of menopause). Modifiable risk factors include diet, cigarette smoking, hypertension, high blood levels of low-density lipoprotein cholesterol, and low levels of high-density lipoprotein cholesterol, diabetes, obesity, and lack of physical activity. In addition, there is increasing evidence to support the role of infection and inflammation from a local focus in pathogenesis (Danesh *et al*., 1997).

We are particularly interested in CHD because of its importance as a public health problem in the U.S. and Worldwide. According to the Heart Disease and Stroke Statistics—2009 Update, CHD is the single largest killer of males and females in the U.S. (Lloyd-Jones *et al*., 2009). CHD comprises more than half of all cardiovascular events in

men and women aged less than 75 years old (Thom *et al*., 2001). The lifetime risk of developing CHD in adults aged 40 years or older is 49% for men and 32% for women (Lloyd-Jones *et al*., 1999). In 2005, the overall CHD death rate was 144.4 per 100,000 population. This rate varied among individuals of different genders and races. The death rates were 187.7 per 100,000 for white males and 213.9 per 100,000 for black males. In comparison, the death rates were 110.0 per 100,000 for white females and 140.9 per 100,000 for black females (American Heart Association, 2006). Worldwide, CHD causes death in more than 7 million people each year (WHO, 2004).

Economic Cost of CHD

The estimated total cost of CHD and stroke in the U.S. for 2009 is at about $165 billion (Lloyd-Jones *et al*., 2009). This number includes direct expenses including, the cost of physicians and other professionals, hospital and nursing home services, prescribed medications, home health care, and other medical durables; and on indirect expenses, which include disease related loss of productivity (Heart Disease and Stroke Statistics—2009 Update).

Periodontal Disease and CHD

Although multiple risk factors for CHD have been identified, a significant proportion of CHD is not explained by the traditional risk factors including elevated low density lipoprotein cholesterol, smoking, hypertension, obesity, and lack of physical activity (Whooley, 2006). Currently, there is increasing evidence to support the role of infection and inflammation in the pathogenesis of many systemic conditions. Infection and

inflammation are now known to play a critical role in diseases such as atherosclerosis and thus, cardiovascular disease. Because of the evidence attributing infection and inflammation to the etiology of CHD, the etiological role of oral disease such as periodontal infection has been addressed in relation to systemic diseases including, atherosclerosis and CHD (Mattila *et al.*, 1989; DeStefano *et al.*, 1993; Mattila *et al.*, 1995; Beck *et al*., 1996; Arbes *et al*., 1999; Buhlin *et al*., 2002).

There are two fundamental biological mechanisms by which periodontal disease might be causally associated with CHD. First, periodontal disease represents a chronic infection resulting in a chronic inflammatory state. This hypothesis is supported by several investigations demonstrating an association between periodontal disease status and elevated levels of systemic inflammatory biomarkers that possess potential atherogenic effects. Periodontal disease has been shown to be associated with elevations of several biomarkers of inflammation including pro-inflammatory cytokines (Loos *et al.*, 2000), acute phase reactants (Kweider *et al*., 1993; Ebersole *et al.* 1999; Loos *et al.*, 2000), white blood cell count (Kweider *et al.*, 1993), neutrophil count, and neutrophil activation state (Loos *et al.*, 2000; Matthews *et al*., 2007a). The second mechanism is related to the transient bacteremia and endotoxemia associated with periodontal disease and their possible role in producing a chronic inflammatory state or directly affecting endothelial cells.

The link between oral infection, with particular emphasis on chronic periodontitis, and systemic diseases has been addressed in many studies. However, while some of these

have been case-controlled and cohort studies, the majority have been cross-sectional in design. While results of these studies have generally supported an association, it has not been possible to elucidate whether oral infection plays a causal role in the systemic diseases. Longitudinal investigations are thus essential but are challenging ethically and logistically in the case of periodontitis, which is irreversible and has a long time course. The time course of periodontitis necessitates long-term studies to address its causal association with systemic diseases. These are time-consuming and expensive.

Gingivitis and CHD

Dental plaque accumulation and gingivitis are almost universal and thus, their potential contribution to systemic diseases could be immense. Unlike periodontitis, gingivitis develops over days/weeks, and is reversible. Since gingivitis is reversible and rapidly inducible, it could be an appropriate model for longitudinal investigation of the role of oral infection in systemic diseases.

Gingivitis can be induced experimentally using the so called "Experimental Gingivitis Model" (Löe *et al*., 1965). This is a controlled model that has long been used in the dental field, particularly preventive dentistry studies evaluating the efficacy of oral health care products. The classical model requires individuals to cease oral hygiene practices for 21 days, during which plaque accumulates resulting in experimental gingivitis. This is followed by a period of optimal oral hygiene performance and resumption of gingival health.

The possible role of gingivitis in systemic diseases has generally been ignored and has been addressed in only a few studies. The results of two cohort studies (DeStefano *et al*., 1993; Morrison *et al*., 1999), has suggested that an association exists between gingivitis and CHD among individuals with baseline gingivitis. Whether this was a causal association is unclear. In a recent study (Ylöstalo *et al*., 2008), self-reported gingivitis was weakly associated with elevated C reactive protein levels in 31-year old adults. The study could not confirm a causal association between gingivitis and heart disease because of limitations that are associated with the self-reported data.

Our lab has used the experimental gingivitis model in a pilot study (Kowolik *et al.*, 2001) conducted in Mexico and enrolling Hispanic subjects (N = 23). Utilizing the gingivitis model and following a three-week period of dental plaque accumulation, the study demonstrated an increase in peripheral blood white blood cell and neutrophil count, which is an independent risk factor for CHD (Brown *et al*., 2001; Margolis *et al*., 2005). Moreover, the study identified a subset of high-responders in terms of peripheral neutrophil count.

In our clinical studies, that are described in this dissertation, the experimental gingivitis model was employed because it permits longitudinal monitoring of clinical parameters from a baseline and in turn, these parameters can be correlated with a systemic response. Moreover, the experimental gingivitis model allows for minimization of confounder issues since each study participant acts as his/her own internal control. In addition, the experimental gingivitis model provides a well-controlled study framework to elucidate

whether the systemic response, if elicited by dental plaque accumulation, could be controlled by reducing dental plaque levels during the recovery phase.

Dissertation Outline

This dissertation is divided into five chapters. Chapter one (the current chapter) is a general introduction describing the periodontal and systemic disease connection. Chapters two and three describe human clinical studies that we have conducted in order to address the role of gingivitis in systemic disease risk (i.e., CHD). These chapters are followed by a description of a laboratory study addressing the interaction of one of the most numerically abundant organisms during gingivitis (i.e., *Fusobacterium nucleatum*) with systemic neutrophils, the primary cells of the acute inflammatory response (chapter four).

Chapter five concludes this dissertation and summarizes the collective significance of the data from our three studies; it also describes future directions for continuing research in this important area.

CHAPTER TWO

Dental Plaque Accumulation as a Risk Factor for Coronary Heart Disease

Abstract

Introduction: Interest is increasing in the possible causal link between oral infection and coronary heart disease (CHD). Objectives: We used the experimental gingivitis model to determine the effect of dental plaque accumulation on systemic markers of inflammation that are associated with CHD risk. Moreover, we addressed whether a gender/racial disparity in the systemic inflammatory responses to dental plaque accumulation exists. Methods: We recruited 156 healthy adults (aged 18-31 years), comprising black and white males and females. Participants brushed for 21 days (control phase), ceased brushing for 21 days (experimental phase), and resumed brushing for a further 21 days (recovery phase). Plaque levels and gingival inflammation were assessed. In addition, peripheral blood samples were collected at each visit to evaluate systemic markers of inflammation. Paired t-tests and Wilcoxon signed rank tests were used to test for significant changes during the experimental phase. Results: 128 participants completed the study. The correlation between the plaque index and gingival index changes during the experimental phase was 0.79 overall, and was similar across genders/races. During the experimental phase, participants had significant increases ($p<0.05$) in the plaque index, gingival index, mean corpuscular volume, mean platelet volume, endotoxin, and cortisol levels. In blacks, significant increases ($p<0.05$) were observed in the neutrophil oxidative activity, IL-1α, and mean corpuscular hemoglobin levels. In black males, the erythrocyte sedimentation rate increased ($p<0.05$). Fibrinogen levels increased ($p<0.05$) in white males and 8-isoprostane levels increased ($p<0.05$) in white females. Significant

decreases ($p<0.05$) occurred in total cholesterol, high density lipoprotein, and red blood cell count. Hematocrit and hemoglobin levels decreased ($p<0.05$) in blacks. In black males, decreases ($p<0.05$) were observed in the low density lipoprotein and 8-isoprostane levels. In addition, decreases ($p<0.05$) were observed in IL-1β levels for white males and in IL-6 levels for white females. Conclusions: In young healthy adults, the accumulation of dental plaque elicited systemic inflammatory responses, some of which have potential atherogenic consequences. These responses differed between individuals of different genders and races.

Introduction

Atherosclerosis is the underlying cause of CHD, which has an immense impact on world health. There is increasing evidence to suggest that infection and inflammation may be risk factors for CHD (Libby *et al*., 2002). A local focus of infection, or circulating bacteria and/or their products, may elicit a systemic inflammatory response with potential detrimental consequences on the arterial wall, lipid metabolism, coagulation and peripheral blood neutrophil response, all of which may promote atherogenesis.

Oral infection, in particular chronic periodontitis, is being increasingly associated with CHD risk. Results of studies support a relationship between periodontal disease and levels of systemic inflammatory biomarkers that possess potential atherogenic effects. However, it is not clear whether this is a systemic response to the bacteria that are the primary cause of periodontitis, or whether periodontitis is the result of an already-heightened host inflammatory response. The time course of periodontitis necessitates long-term studies to address this issue and these are both time-consuming and expensive. Gingivitis precedes periodontitis and is the clinical manifestation of the inflammatory response to dental plaque accumulation. In contrast to periodontitis, gingivitis develops over days/weeks, is reversible, and is universal.

The experimental gingivitis model, whereby the cessation of all oral hygiene practices for a 21-day period results in dental plaque accumulation and subsequent gingivitis, provides an appropriate model by which to study host responses to a localized bacterial challenge.

The long-term goal of this study was to identify individuals at increased risk of CHD. The objective of this investigation was to increase our understanding of the link between oral disease and CHD. The central hypothesis of this study was that the accumulation of dental plaque in healthy adults elicits both local and systemic inflammatory responses which are correlated but differ between individuals of different genders and races. The rationale for this study was that a definitive etiological role of oral disease in CHD risk must be established before steps can be taken in implementing successful CHD preventive strategies through oral health education.

The specific aims of this study were to use the experimental gingivitis model to determine:

1. The individual variation in dental plaque accumulation and subsequent local inflammatory response. We hypothesized that the rate and extent of dental plaque accumulation would vary between individuals, as would the resultant gingival inflammatory response. This variation would be dependent upon gender and race.

2. The systemic inflammatory response to dental plaque accumulation. We hypothesized that the accumulation of dental plaque or the resultant local inflammation would induce a host systemic response. This response would manifest as a change in the level of inflammatory biomarkers, hematologic factors, markers of lipid metabolism, markers of metabolic change and neutrophil response, all with potential atherogenic consequences.

We also hypothesized that this systemic response would differ between individuals of different genders and races.

3. Whether dental plaque accumulation results in circulating bacterial components (i.e., endotoxin). We hypothesized that dental plaque accumulation results in translocation of orally-derived bacterial products, such as endotoxin (LPS), into the systemic circulation.

Study Significance

CHD is the leading single cause of death of men and women in the U.S. (Heart Disease and Stroke Statistics—2009 Update). Infection and inflammation are being increasingly recognized as risk factors for CHD and, as described in chapter one, there is growing evidence for supporting an association between oral disease and CHD (Mattila *et al*., 1989; DeStefano *et al*., 1993; Mattila *et al.*, 1995; Beck *et al.*, 1996; Arbes *et al*., 1999; Buhlin *et al.*, 2002). However, it has not been established whether periodontal disease is a causal risk factor for CHD risk (Friedewald *et al*., 2009) and this signifies a critical gap in knowledge. Once a mechanism by which oral infection increases the risk of CHD has been identified, it will be indisputable that improving oral health will promote general health.

The experimental gingivitis model provides the ideal model with which to determine whether there is a direct systemic response to dental plaque accumulation. Our lab has previously used this model to demonstrate an increased peripheral blood neutrophil count (Kowolik *et al.* 2001), a recognized risk factor for CHD (Phillips *et al*., 1992; Sweetnam

et al., 1997). It is thus proposed that dental plaque accumulation and the resultant gingivitis can induce a systemic response with potential atherogenic effects. Since dental plaque and gingivitis are universal, the potential contribution to CHD risk, and thus to the burden of disease worldwide, could be immense.

It is important to note that this study has focused on potential systemic effects of gingivitis, rather than chronic periodontitis. Gingivitis is distinct from chronic adult periodontitis in terms of the microbial pathogenesis, the host response, the local tissue involvement and the temporal nature of the disease.

The results of this study may identify differences in the systemic response to plaque accumulation based on gender or race, which could have implications for preventive oral and general health care.

Literature Background

As described in chapter one, atherosclerosis is the basis for the development of cardiovascular disease including, CHD, stroke and peripheral vascular disease. There is increasing evidence to support the role of infection and inflammation from a local focus in pathogenesis (Danesh *et al*., 1997). Relevant to this, is the evidence for supporting the role of oral infection in CHD risk (Mattila *et al*., 1989; DeStefano *et al*., 1993; Mattila *et al*., 1995; Beck *et al*., 1996; Arbes *et al*., 1999; Buhlin *et al*., 2002).

As discussed earlier in this dissertation, local infection may promote atherosclerosis via two central mechanisms, 1) the direct involvement of circulating bacteria or their components from the focus of infection (Haraszthy *et al*., 2000); or 2) a systemic inflammatory response to the circulating bacteria and/or their components, or to the contained local infection (Danesh *et al*., 1997). In this study, systemic inflammatory response factors to the local oral infection were investigated. In addition, LPS which is a cell-wall component of Gram-negative bacteria, was also assessed.

Pro-inflammatory Cytokines

During the acute inflammatory response to an infectious agent, pro-inflammatory cytokines, including interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, may initiate or enhance a cascade of events, both at the local site of infection and systemically (Gabay and Kushner, 1999). These cytokines are released locally in response to dental plaque accumulation (Ebersole *et al*., 1993; Heasman *et al*., 1993; Ebersole *et al*., 1999) and there is potential for systemic dissemination (Salvi *et al*., 1998; Ebersole *et al*., 1999).

Recently, it has been shown that periodontitis is associated with increased blood levels of IL-6 (Loos *et al*., 2000). Potential proatherogenic effects of these cytokines include stimulation of hepatic production of acute phase reactants (Baumann and Gauldie, 1994), leukocyte recruitment (Yoshimura *et al*., 1987), upregulation of leukocyte adhesion factor expression on endothelial cells (Munro, 1993), enhanced mitogenic activity of smooth muscle cells (Ikeda *et* al., 1990), induction of procoagulation activity (Bevilacqua

et al., 1986; Nachman *et al*., 1986) and increased levels of serum triglycerides and cholesterol (Memon *et al*., 1997). Accordingly, elevated cytokine levels have been associated with increased risk of CHD (Ridker *et al*., 2000a; Ridker *et al*., 2000b).

Acute phase reactants

An increase in serum C reactive protein (CRP) and fibrinogen levels occurs in response to infection (Kiechl *et al*., 2001). Increased systemic CRP levels are associated with oral disease (Kweider *et al.* 1993; Ebersole *et al.* 1999; Wu *et* al., 2000) and, more specifically, chronic periodontitis (Ebersole *et* al., 1997; Slade *et* al., 2000; Loos *et* al., 2000).

CRP has prothrombotic effects (Cermak *et al*., 1993) and can activate complement (Volanakis, 1982). Results of prospective studies suggest that baseline levels of serum CRP are predictive of risk for coronary events (Koenig *et al*., 1999; Danesh *et al*., 2000; Pearson *et al*., 2003). Fibrinogen may mediate atherosclerosis by increasing blood viscosity (Yarnell *et al*., 1991; Lowe *et al*., 1997), promoting platelet aggregation (Cook and Ubben, 1990), and stimulating smooth muscle proliferation (Ishida and Tanaka, 1982). The results of prospective studies have demonstrated that an increased plasma fibrinogen level is a risk factor for atherosclerotic disease (Meade *et al*., 1986; Yarnell *et al*., 1991; Danesh *et al*., 1998).

Albumin is a 'negative' acute phase reactant. Serum levels of albumin decline during acute inflammation (Gabay and Kushner, 1999). CHD risk has been shown to decrease with increasing levels of serum albumin (Corti *et al*., 1996; Danesh *et al*., 1998).

White Blood Cells

Local infection results in an increase in the counts of circulating white blood cells (WBC), particularly neutrophils, the primary cellular component of the acute inflammatory response. Periodontal disease is associated with increased numbers of peripheral blood WBCs (Kweider *et al*., 1993; Wakai *et al*., 1999; Herrera *et al*., 2000) and, more specifically, neutrophils (Loos *et al*., 2000). Dental plaque accumulation in periodontally-healthy subjects was shown to elicit a peripheral neutrophil response, in terms of neutrophil count (Kowolik *et al*., 2001).

WBCs may contribute to atherogenesis by causing microvascular injury (Ernst *et al*., 1987). Prospective studies have consistently identified systemic WBC count (Phillips *et al*., 1992; Danesh *et al*., 1998), and more specifically neutrophil count (Kawaguchi *et al*., 1996; Sweetnam *et al*., 1997) as a risk factor for CHD. Bacterial infection may also result in priming of peripheral neutrophils so that they circulate in a state of partial-activation (Barbour *et al*., 1980; McCarthy *et al*., 1980).

Neutrophil priming is characterized by migration of some of the cytoplasmic granules to the cell surface, and fusion of these granules with the plasma membrane. Thus, priming results in an increase in the number of receptors and neutrophil's surface proteins

(Edwards, 1994). Further challenge of primed neutrophils results in full scale neutrophil activation, which includes the release of reactive oxygen species and proteolytic enzymes, with subsequent detrimental effects on the endothelium (Dallegri and Ottonello, 1997). Chronic periodontitis may also result in the priming of peripheral neutrophils (Kimura *et al*., 1993; Fredriksson *et al*., 1998; Matthews *et al*., 2007a; Matthews *et al.,* 2007b).

Serum lipid profile

Elevated levels LDL, triglycerides and total cholesterol, and lower levels of high-density lipoprotein cholesterol (HDL) are risk factors for CHD (National Heart, Lung, and Blood Institute, 2001). Chronic infection results in a shift of the serum lipid profile in favor of atherogenesis by increasing levels of LDL, triglycerides, and total cholesterol and decreasing levels of HDL (Sammalkorpi *et al*., 1988). On the other hand, acute infection has been commonly associated with decreased levels of blood cholesterol (Fraunberger *et al*., 1999; Khovidhunkit *et al*., 2000). A positive correlation between the severity of periodontitis and serum levels of LDL, triglycerides, and total cholesterol has been shown in the non-human primate model (Ebersole *et al*., 1999) and in humans (Cutler *et al*., 1999; Rufail *et al*., 2007). On the other hand, serum levels of HDL have not been associated with oral health status (Ebersole *et al*., 1999; Wu *et al*., 2000). The concentration of plasma lipoprotein (a), Lp(a), a proatherogenic particle, increases during the acute phase response (Min *et al*., 1997). Increased levels of Lp(a) are also associated with increased risk of CHD (Scanu and Fless, 1990).

LPS

Circulating levels of LPS increase with ligature-induced periodontitis in the non-human primate model (Ebersole *et al*., 1999). In the experimental gingivitis model, plaque maturation is associated with a shift towards an increasingly Gram-negative flora (Theilade *et al*., 1966). However, the possible systemic translocation of orally-derived LPS has not been previously investigated using this model. LPS may enhance the systemic inflammatory response by stimulating the production of pro-inflammatory cytokines (Lindemann *et al*., 1988), and causing hypertriglyceridemia (Feingold *et al*., 1992) and endothelial cell dysfunction (Moncada *et al*., 1991). The results of a prospective study have identified elevated baseline levels of plasma LPS as a risk factor for atherosclerosis (Wiedermann *et al*., 1999).

Other biomarkers of inflammation

Oxidation of LDL plays a central role in the development of atherosclerosis (Steinberg, 1997). Increasing evidence suggests that free radicals may cause oxidation of LDL (Darley-Usmar and Halliwell, 1996). 8-isoprostane, a prostaglandin isomer, is a marker of lipid peroxidation and oxidative stress that has been recently identified. Blood levels of 8-isoprostane have been shown to correlate with CHD status (Vassalle *et al*., 2003). Measurement of 8-isoprostane levels has emerged as an approach to examine the oxidative stress status in vivo (Lubrano *et al*., 2002).

In the context of periodontal disease, salivary 8-isoprostane levels were shown to increase with dental plaque accumulation and development of periodontal disease

(Wolfram *et al*., 2006). However, to our knowledge, there are no published data on the effect of dental plaque accumulation on blood levels of 8-isoprostane.

Metabolic changes

Insulin resistance leads to increased risk for developing diabetes mellitus (Haffner *et al*., 1992), a known risk for CHD. Inflammation, as evidenced through an increased peripheral blood leukocyte count, is associated with increased insulin resistance and may predict the development of type II diabetes (Vozarova *et al*., 2002). Stress hormones, including cortisol, are also known to play a role in insulin resistance and their levels increase during the development of inflammation. To our knowledge, there are no published data on the effect of dental plaque accumulation on these biomarkers of metabolic change.

Gingivitis and CHD Risk

The link between oral infection and systemic diseases has been addressed in many studies. As discussed in chapter one, the majority of these studies have been cross-sectional in design and it has not been possible to elucidate whether oral infection plays a causal role in atherogenesis. Longitudinal investigations are essential to address this issue but are ethically and logistically challenging in the case of chronic periodontitis, given the long time course of the disease and its irreversible nature.

Gingivitis, on the other hand, is reversible and can be experimentally induced rapidly. Thus, the gingivitis model is a more appropriate model for determining whether oral

disease plays a causal role in increased risk of CHD. Moreover, individual variation in the host inflammatory response, based on gender and race, can be addressed using this model.

Individual Variation in Susceptibility to Gingivitis

Disparities exist in periodontital disease prevalence between blacks and whites, with blacks exhibiting higher prevalence of periodontitis than whites (Albandar *et al*., 1999; Borrell *et al*., 2002). Disparity in periodontitis risk between individuals appears to be dependant upon host factors, including factors influencing the magnitude of the inflammatory response (Hart *et al*., 1994; Kornman *et al*., 1997). A hyper-inflammatory response in the periodontal tissues could have implications for CHD risk (Kornman *et al*., 1999). In the case of gingivitis, the variation in the host response to dental plaque accumulation has been addressed using the experimental gingivitis model, and high and low responders have been identified based on the severity of gingivitis in relation to dental plaque level (Abbas *et al*., 1986; Lie *et al*., 1998). The results of these studies have addressed the importance of dental plaque composition in determining the inflammatory outcome in the experimental gingivitis model. The general consensus is that the composition of dental plaque appears to be of minimal significance, suggesting that the host response plays a greater role.

Cigarette smoking is known to influence the clinical expression of gingivitis through its effect on the host response and this has also been demonstrated using the experimental gingivitis model (Bergstrom and Preber, 1986; Bergstrom *et al*., 1988). Thus smoking

history should be considered when attempting to understand the basis for individual variation in the host response to dental plaque accumulation.

The influence of gender and race on the inflammatory response to dental plaque accumulation within the experimental gingivitis model has not been previously addressed. While gingival bleeding has been reported to be greater in blacks as compared to whites and in males as compared to females (Albandar and Kingman, 1999), it has not been established whether this disparity is the result of variation in dental plaque control or in host susceptibility among these groups. Hormonal changes in women may also alter gingival response to dental plaque accumulation. Menstruation and the use of oral contraceptives have been implicated in this respect. However results of studies have been inconsistent in their conclusions (Holm-Pedersen and Löe, 1967; Preshaw *et al*., 2001).

Individual Variation in Levels of Hematological Parameters

Diurnal variations in some host response factors have been previously demonstrated (Bain and England, 1975a; Petrovsky *et al*., 1998), thus establishing the rationale for temporal standardization of blood samples collection in our study.

Some of these systemic factors are also influenced by genetic and environmental factors. For example, circulating neutrophil counts are affected by gender (Bain and England, 1975a), race (Freedman *et al*., 1997), smoking history (Petitti and Kipp, 1986) and female sex hormones (Bain and England, 1975b), and circulating fibrinogen levels are similarly

influenced by gender (Meade *et al*., 1979), race (Cook *et al*., 2001), smoking history (Meade *et al.* 1987) and female sex hormones (Cederblad *et al*., 1977).

Individual Variation in Susceptibility to CHD

Gender and racial differences exist in the incidence, morbidity, and mortality associated with complex diseases that possess an inflammatory component, including rheumatoid arthritis, diabetes mellitus, and cardiovascular disease (Cooper and Stroehla, 2003; Albert, 2007). Identification of individuals exhibiting an exaggerated inflammatory response to dental plaque accumulation, whether due to gender, race, or other factors, is important when designing preventive oral health care programs, and could provide information on the susceptibility of individuals to inflammatory diseases. Heart disease risk is greatest among black males living in poor neighborhoods (Polednak, 1998). In 2005, the overall CHD death rate was higher in black males as compared to white males and in black females as compared to white females (American Heart Association, 2006).

This disparity in CHD risk can not be fully explained by traditional risk factors (Escobedo *et al*., 1997), but may depend on hematological and inflammatory variables as discussed above. To date, disparity in levels of the systemic factors described above, in response to induced oral infection (i.e., gingivitis) based on gender or race has not been previously investigated.

Materials and Methods

Study Design

This study was conducted in compliance with the Institutional Review Board (IRB) and the U.S. Federal Regulations governing protection, privacy and informed consent.

Prior to initiation of the study, approval by the IRB of Indiana University Purdue University Indianapolis/Clarian Health (approval number 0405-50) was obtained for the study protocol, consent form, and advertising for subject recruitment (Appendix 1).

The study enrolled 156 subjects (Figure 2.1), aged 18-31 years. Due to the well-recognized effect of tobacco (smoking and chewing) on the local and systemic inflammatory response, only non-users of tobacco were eligible for study participation. The study was conducted in three phases: In Cohort I, 59 subjects were enrolled. In Cohort II, 60 subjects were enrolled, and finally, in Cohort III, 37 subjects were enrolled. The final goal was to obtain complete data sets for a minimum of 100 subjects, including 25 white males, 25 white females, 25 black males, and 25 black females.

Subjects provided written informed consent (Appendix 2) for study participation and completed a medical history and demographic questionnaire. Current tobacco use status was self-reported through questionning, and reported non-smoker status was further confirmed through measurement of expired air carbon monoxide level. Those satisfying the non-user criteria received an oral soft tissue examination, and screening examination to determine periodontal health status. Based on the inclusion/exclusion criteria, those

subjects who qualified for participation in the study received a thorough professional dental prophylaxis and oral hygiene instruction (OHI). Subjects then entered the three study phases in chronological order. In the control phase, subjects were asked to perform optimal oral hygiene (OH) practices for a period of 21 days with an ADA-accepted toothbrush, conventional fluoride dentifrice and dental floss. Subjects returned to the clinic on days 7, 14 and 21 of the control phase for examination of gingival inflammation and dental plaque levels, OHI reinforcement, and peripheral venous blood sample collection.

Subjects then entered the experimental phase of the study. During this phase, subjects were asked to refrain from all OH practices (including gum chewing) for a period of 21 days. On days 7, 14 and 21, subjects were asked to return for peripheral blood sample collection and examination of gingival inflammation and dental plaque levels. At the end of the experimental phase, subjects received a thorough dental prophylaxis, fluoride treatment, and OHI.

Finally, subjects entered the recovery phase. During this phase, subjects resumed optimal mechanical dental plaque control measures (i.e., toothbrushing and flossing). On days 14 and 21 of the recovery phase, peripheral blood samples were collected and again, subjects were examined for gingival inflammation and dental plaque levels. This concluded the clinical phase of the study.

Study Population

Subjects met the following inclusion/exclusion criteria.

Inclusion criteria

Each subject was (Appendix 3):

- Between 18 and 30 years of age, of either gender, and was either non-Hispanic black or white.
- Willing to provide written informed consent and complete a medical history (Appendix 6), including information regarding tobacco use and, for females, current menstrual cycle and demographic questionnaire.
- In good general health and had no factors in their medical history, which would indicate that they would be adversely affected by their participation in this study.
- Available for the duration of the study and was willing to adhere to the procedures required for the study.
- Willing to refrain from the use of dentifrices, mouthrinses, dental floss, or any other dental product during the experimental phase.
- Available for appointments.
- In possession of a minimum of 20 natural teeth.

Exclusion criteria

No subject was (Appendix 4):

- A current user of tobacco products.
- Participating simultaneously in any other clinical study.

- Showing evidence of periodontal disease (periodontal probing depths ≥ 4 mm), or gross neglect, caries or other conditions necessitating immediate care.
- Having factors which would pose a risk to themselves, to study personnel or to other participants. This included hepatitis, HIV-positive status, active tuberculosis, diabetes, and conditions requiring antibiotic pre-medication prior to dental treatment e.g. rheumatic fever, cardiac regulating devices, heart murmur, and prosthetic joint replacement. All decisions of medical acceptability were made at the discretion of the examining dentist.
- Using a medication that is known to affect the oral soft tissues or local/systemic inflammatory response during the course of the study.
- Using antimicrobial drug therapy within 3 months of Day 0 of the control phase.
- Having latex allergy.

No subject was excluded on the basis of having a perceived high plaque or gingivitis level at screening. Continuance criteria are summarized in Appendix 5.

Clinical Study Procedures

The clinical procedures of the study are summarized in Table 2.1 and are described in more detail in the following section of this chapter. The clinical component of the study was conducted at The General Clinical Research Center (GCRC), Indiana University School of Medicine, and The Oral Health Research Institute (OHRI), Indiana University School of Dentistry.

Clinical Procedures at Each Visit

Monitoring episodes of Infection

At each visit, subjects were asked to report any episodes of clinical infection, for example a common cold, gastro-intestinal upset, sinusitis, skin infection, and whether or not any treatment was administered.

Oral examination procedure

At each visit, an oral soft tissue examination was performed to ascertain the presence of any mucosal pathology. Dental plaque levels (non-disclosed) were assessed according to the Plaque Index (PI) (Löe and Silness, 1963):

Score	Description
0	No plaque in the gingival area
1	A film adhering to the free gingival margin and adjacent area of the tooth
2	Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/or adjacent tooth surface which can be seen by the naked eye
3	Abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface

Gingivitis severity was evaluated using the Gingival Index (GI) (Silness & Löe 1964):

Score	Description
0	No inflammation – normal gingivae
1	Mild inflammation – slight change in color and little change in texture
2	Moderate inflammation – moderate glazing, redness, edema and hypertrophy. Bleeding on pressure
3	Severe inflammation – marked redness and hypertrophy, tendency to spontaneous bleeding, ulceration

All measurements were made on six sites per tooth (distobuccal, buccal, mesiobuccal, mesiolingual, lingual and distolingual) on all the teeth present, with the exception of third molars. Periodontal examinations (Appendix 7) were performed by a single trained and calibrated examiner, using a Hu-Friedy UNC probe (Chicago, IL, USA).

Screening visit (visit 1)

During this visit, subjects provided written informed consent for study participation and completed a medical history form and demographic questionnaire. Those individuals who reported to be current non-smokers were subjected to an expired air carbon monoxide test (Smokerlyzer®, Bedfont Scientific Ltd, Medford, NJ, USA). A carbon monoxide level of 8 ppm or less verified non-smoking status (Hughes *et al*., 1987), and only those fulfilling both non-smoking criteria (self-reported and CO level ≤ 8 ppm) were enrolled in the study**.** As a measure of obesity and body fat distribution, the body mass index (BMI) was

calculated as body weight (Kg) divided by (height in meters)^2. Both height and weight were measured by an experienced member of the clinical team.

The subject's medical history was reviewed, and an oral soft tissue examination was performed. A whole-mouth periodontal screening examination was performed to determine that the subject had no periodontal pockets equal to or greater than 4 mm. Subjects who met all inclusion/exclusion criteria were then appointed for their next visit.

Dental cleaning visit (visit 2)

During this visit, subjects attended OHRI and received a thorough professional dental prophylaxis and detailed OHI, and were appointed for their next visit.

Control phase (visits 3-6)

For each visit of the control phase, subjects attended GCRC before 12 noon for phlebotomy following an overnight fast. A total of 60 ml of peripheral venous blood was collected for laboratory analysis. Subjects then received a dental examination for gingival inflammation and for non-disclosed dental plaque levels, as described earlier. On days 0, 7 and 14 of the control phase, subjects received OHI reinforcement from a dental hygienist. Subjects then entered the experimental phase of the study.

Experimental phase (visits 6-9)

During this phase, subjects refrained from all OH measures. At each visit subjects attended GCRC before 12 noon for phlebotomy following an overnight fast. A further 60

ml of peripheral venous blood was collected for laboratory analysis. Subjects then received a dental examination for gingival inflammation and for non-disclosed dental plaque levels. On visit 9 (Day 21 of the experimental phase), subjects received OHI from a dental hygienist and were asked to resume their normal oral hygiene practices. In addition, subjects were scheduled for a complete dental prophylaxis and fluoride treatment at OHRI within 1-2 days of this visit. At the conclusion of the experimental phase, subjects entered the recovery phase.

Recovery phase (visits 10 and 11)

Following 14 days of optimal OH, subjects attended GCRC for phlebotomy and dental examination of gingival inflammation and undisclosed dental plaque levels. This was repeated one week later (Day 21 of recovery phase).

Subject Compensation

Panelists were compensated $20 for attending the screening visit, whether or not they were subsequently enrolled in the study. Those who entered the study received $25 at the dental cleaning visit, $50 per visit during the control phase, $125 for visits on each of days 7, 14 and 21 of the experimental phase, $75 for each of the two visits in the recovery phase, and a final payment of $100 for completing all study visits (total compensation, $870).

Laboratory Analysis

At each blood draw, performed at GCRC, a total of 60 ml of blood was collected from each subject in order to perform the laboratory analysis described below.

Standard Hematology

A total and differential WBC count was measured using an automated cell counter (Coulter Stack S, Coulter Electronics Inc, Hialeah, FL, USA). Platelet counts, red blood cell (RBC) counts, hemoglobin (Hb) levels, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean platelet volume (MPV) were also assessed as part of the normal laboratory protocol. The erythrocyte sedimentation rate (ESR), which depends largely on the plasma concentration of fibrinogen, was determined using the Westergren method. This provides a simple yet indirect measure of the acute phase response. Standard hematology was performed in the laboratories of Clarian Health Partners Inc and the Department of Pathology and Laboratory Medicine at Indiana University (IU) School of Medicine (referred to as Clarian Health Laboratories).

Neutrophil Function Assays

Neutophils were isolated from heparinized peripheral blood samples by density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) (Sabroe, 2003) at 400 g for 25 minutes at 25^{o}C. Neutrophils were washed twice with isotonic phosphate buffered saline solution (PBS) (Sigma) and once with RPMI-1640 (Sigma) by centrifugation at 250 g for 10 minutes. Isolated cells were suspended in RPMI-1640 and

neutrophils were counted using a hemacytometer, and viability was assessed using trypan blue exclusion. Cells were primed for 30 minutes at 37^{o}C with 10^{-10} M formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) and resuspended to a concentration of 1 x 10^{6} cells/ml in RPMI-1640.

Chemiluminescence, the light energy produced during the neutrophil interaction with a stimulant, was used to quantify the level of peripheral neutrophil metabolic oxidative activity. Chemiluminescence is a sensitive, non-invasive technique that measures the amount of produced and released reactive oxygen species that are generated by the activity of NADPH oxidase (Edwards, 1994). Luminol-enhanced chemiluminescence was measured in this study using a 1251 Bio-Orbit Luminometer (Bio-Orbit, Turku, Finland) for 90 minutes. Into each cuvette, 100 µl of 10^{-5} M Luminol (Sigma) was dispensed at baseline, followed by cell activation with 100 µl of 10^{-5} M fMLP at 30 minutes.

Chemiluminescence values were expressed as peak chemiluminescence (millivolt) which is the maximum amount of light energy produced at a single point in time or (the peak of the chemiluminescence response curve) and total chemiluminescence (millivolt.min) which is the total integrated energy output over the reaction time (the area under the chemiluminescence response curve). Samples were run in triplicate and means were measured. Negative controls included the reaction solution without cells.

DNA Extraction

DNA was extracted from blood cells using standard methodology (Davis *et al*., 1980; Miller *et al*., 1988). Briefly, cells were lysed, protein removed, and alcohol-precipitated DNA was washed, air-dried and stored in Tris-EDTA buffer at -80^{o} C for future analysis.

Cytokine Assays

Serum cytokine levels were assayed using commercial immunoassay kits. Briefly, serum was separated from the blood samples and stored at -20^{o}C until assayed. All serum samples were processed at one time to minimize the effects of inter-assay variation, and analyzed in duplicate. The LINCO plex™ multiplex immunoassays kit (Linco Research Inc., MO, USA) was used for the quantitative determination of cytokines IL-1β, IL-6, IL-8 and TNF-α. A sandwich enzyme immunoassay (Quantikine®, R&D Systems Inc, MN, USA) was employed for the quantitative determination of IL-1α. All cytokine assays were performed in the GCRC Laboratory at the IU School of Medicine.

Acute Phase Protein Assays

Plasma fibrinogen levels were measured using an automated clot detection system in the Clarian Health Laboratories. Serum albumin levels were determined by using colorimetric methods by Clarian Health. A commercial kit, the Active™ C-reactive protein ELISA (Diagnostic Systems Laboratories Inc, TX), was used for the quantitative measurement of CRP, which was performed in the GCRC Laboratory. All samples were processed at one time and in duplicate.

Lipid Assays

Serum concentrations of total cholesterol and HDL were measured in the Clarian Health Laboratories using colorimetric methods, and the LDL level was mathematically derived from these parameters. Triglyceride levels were similarly measured using colorimetric methods. Serum Lp(a) was assayed in the GCRC Laboratory using a commercially available kit. Briefly, serum was separated and stored. The SPQ™ II test system (DiaSorin Inc, MN, USA) was used for the quantitative determination of Lp(a) by immunoprecipitin analysis. All samples were processed at one time and in duplicate.

LPS Assay

As a surrogate marker of Gram-negative bacteremia, plasma LPS levels were assayed, using a Limulus Amebocyte Lysate commercial kit utilizing chromogenic methods. Briefly, serum was separated and stored. The ENDOSAFE® Endochrome-K™ test (Charles River Laboratories, SC, USA), a kinetic colorimetric assay, was used for the quantitative determination of LPS levels in samples. All samples were processed at one time and in duplicate. This assay was performed in the Charles River Laboratories.

Isoprostane Assay

Serum levels of 8-isoprostane were quantified using a commercial specific enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) based on competition between 8-isoprostane and an 8-isoprostane/acetylcholinesterase conjugate for a limited number of 8-isoprostane-specific rabbit antiserum binding sites. Again, all samples were processed at one time and in duplicate**.**

Fasting Glucose, Insulin and Cortisol Assays

To determine whether changes in insulin resistance occurred, fasting blood glucose was measured using a standard spectrophotometric method (Sigma) and serum insulin levels were measured using a radioimmunoassay (Linco Research Inc, MO, USA) in the GCRC Laboratory. Insulin resistance was calculated as the Homeostatis Model Assessment (HOMA) score: fasting insulin (μU/ml) multiplied by fasting glucose (mmol) divided by 22.5 (Matthews *et al.* 1985). A HOMA score of more than 3.5 is consistent with insulin resistance (Mather *et al.* 2001). Serum cortisol levels were measured by radioimmunoassay (DPC, CA, USA), in the GCRC Laboratory.

Statistical Analyses

Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA) statistical software. Spearman correlation coefficients (r) were calculated to assess the relationships between measurements. Natural logarithms of most measurements (PI, GI, WBC counts, neutrophil counts, chemiluminescence, IL-1β, IL-1α, IL-6, IL-8, TNF-α, CRP, cortisol, insulin, isoprostane, endotoxin) were used for analyses since the distributions of the outcomes were log-normal. Means and confidence intervals (CI) reported for these measurements were transformed to the original measurement scale to aid in interpretation of the results. Repeated measures analysis of variance (ANOVA) was used for these outcome to test for changes between the ends of the control, experimental, and recovery phases and to compare genders and races. ANOVAs included gender, race, and time effects as well as interactions of time with gender and race. The ANOVAs allowed each phase to have a different variance and allowed different

correlations between phases. Endotoxin was also analyzed as a binary variable (≥ 0.1 or < 0.1) using chi-square tests. For the remaining measurements, comparisons to test for significant changes between the beginning and the end of the experimental phase were performed using nonparametric Wilcoxon Signed Rank tests and comparisons between groups were performed using nonparametric Wilcoxon Rank Sum and Kruskal-Wallis tests. For these measurements the comparisons between the measurements at the end of control and end of experimental phases against the end of recovery phase were performed using repeated measures ANOVA using the ranks of the measurements. Additional analyses for the effects of age and BMI on all measurements were analyzed using methods similar to those described above.

Results

Demographics

Of the 156 subjects enrolled, 128 subjects completed the study (32 black males, 30 black females, 31 white males, and 35 white females) (Figure 2.1). 48 subjects completed Cohort I, 49 subjects completed Cohort II, and 31 subjects completed Cohort III. Only data for the 128 who completed the study were included in the final analyses. The cohort comprised healthy young adults (mean age 24 years; range, 18-31 years), with a relatively high mean BMI of 30 (range, 18-54).

Plaque and Gingivitis

Baseline values and change in PI and GI are shown in Tables 2.2-2.10 and Figures 2.2 and 2.3. PI was marginally higher in blacks versus (vs.) whites (0.35 vs. 0.29, $p = 0.06$)

and in males vs. females (0.35 vs. 0.29, p = 0.08) whereas GI was not significantly different by race (p = 0.12) or gender (p = 0.40).

PI increased on average by 1.61 (95% CI, 1.46 – 1.78) during the experimental phase (p < 0.0001). The increase in PI in blacks was significantly less than in whites (p = 0.0003), while there was no significant difference in the increase for males vs. females (p = 0.49). GI also increased during the experimental phase (p<0.0001), on average by 0.62. GI increases were not significantly different between races (p = 0.82) or genders (p = 0.67).

During the experimental phase, there was a strong positive correlation between changes in PI and GI (r = 0.79, p<0.0001). This correlation was similar across gender/race groups (range for r, 0.77 - 0.81). PI and GI were lower at the end of the recovery phase compared to the end of the experimental phase (p<0.0001) but were still elevated at the end of the recovery phase compared to the end of the control phase (p<0.0001).

Standard Hematology

Baseline values and change in WBC and neutrophil counts are shown in Tables 2.2-2.10 and Figures 2.4 and 2.5. Baseline WBC counts (x 10^9 cells/L) were lower in blacks vs. whites (5.3 vs. 6.1, p = 0.0094) and in males vs. females (5.3 vs. 6.1, p = 0.0090). More specifically, neutrophil counts (x 10^9 cells/L) were lower in blacks vs. whites (2.8 vs. 3.5, p = 0.0021) and in males vs. females (2.9 vs. 3.4, p = 0.0111).

Total WBC counts and neutrophil counts did not change overall from the beginning to the end of the experimental phase ($p = 0.98$ and $p = 0.81$, respectively) or within any subgroup. There were no differences between races or genders for change in WBC or neutrophil counts during this time period ($p>0.27$). No changes in WBC or neutrophil counts were found between the control and recovery phases ($p = 0.29$ for WBC counts and $p = 0.15$ for neutrophil counts).

Baseline values and change in RBC count, Hb levels, Hct, MCV, MPV, MCH, MCHC, and ESR are shown in Tables 2.2-2.10 and Figures 2.6-2.12. Blacks were higher than whites for baseline eosinophils % (2.50 vs. 1.80, $p = 0.0291$), baseline lymphocytes % (35.00 vs. 31.86, $p = 0.0442$), and baseline RDW (13.54 vs. 12.96, $p = 0.0001$).

On the other hand, blacks were lower than whites for baseline MCH (pg/cell) (28.92 vs. 30.11, $p = 0.0013$), MCHC (gm/dL) (33.47 vs. 34.18, $p<0.0001$), and MCV (femtoliters/cell) (86.23 vs. 88.08, $p = 0.0366$). Males were higher than females for baseline RBC count (x 10^{12} cells/L) (5.12 vs. 4.53, $p<0.0001$), baseline Hct (%) (44.32 vs. 38.75, $p<0.0001$), baseline Hb (gm/dL) (15.42 vs. 13.41, $p<0.0001$), baseline MCHC (gm/dL) (34.00 vs. 33.69, $p = 0.0226$, difference larger in whites), and baseline monocytes % (8.67 vs. 6.88, $p<0.0001$). Whereas males were lower than females for baseline ESR (mm/hr) (2.92 vs. 12.52, $p<0.0001$), baseline lymphocytes count (x 10^9 cells/L) (1.33 vs. 1.57, $p = 0.0336$, difference larger in whites), and baseline platelet count (x 10^9 cells/L) (254.90 vs. 299.40, $p = 0.0004$).

From the beginning to the end of the experimental phase, the RBC count (x 10^{12} cells/L) decreased significantly on average by -0.04 ($p = 0.0321$); this decrease in the RBC count was greater in blacks vs. whites (-0.09 vs. 0.00, $p = 0.0103$) and greater in females vs. males (-0.05 vs. -0.04, $p = 0.0095$). At the recovery phase, RBC count was not significantly different ($p>0.05$) than the experimental phase for any of the study groups.

Also, during the experimental phase, there was a decrease in Hb levels that was greater in blacks vs. whites ($p = 0.0125$) and in females vs. males ($p = 0.0011$). For blacks, there was a decrease in the Hct levels by 0.59 % ($p = 0.0184$). A larger decrease in Hct was observed in blacks vs. whites ($p = 0.0113$) and in females vs. males ($p = 0.0049$). Hb levels decreased by 0.19 gm/dL ($p = 0.0216$), and RBC count decreased by 0.09 (x 10^{12} / L) ($p = 0.0018$). For black males, Hb levels decreased by 0.23 gm/dL ($p = 0.0391$), and RBC count decreased by 0.10 ($p = 0.0110$) during the experimental phase.

During the experimental phase, MCV increased by 0.21 femtoliters/cell ($p = 0.0156$), and MPV increased by 0.09 femtoliters/cell ($p = 0.0048$). For blacks, MCH increased by 0.17 pg/cell ($p = 0.0476$), MCV increased by 0.22 femtoliters/cell ($p = 0.048$), and MPV increased by 0.16 ($p = 0.0007$). For males, MCV increased by 0.33 femtoliters/cell ($p = 0.0160$), and MPV increased by 0.12 ($p = 0.0103$). For black males, ESR increased by 1.22 mm/hr ($p = 0.0215$), and MPV increased by 0.18 ($p = 0.0061$). ESR levels in black males decreased significantly during the recovery phase ($p = 0.0387$) to levels similar to those during the control phase.

Neutrophil Activity Assay

Baseline values and change in total and peak chemiluminescence during the course of the study are shown in Tables 2.2-2.10 and Figures 2.13 and 2.14. Baseline total chemiluminescence was not significantly different between blacks and whites ($p = 0.99$) or between males and females ($p = 0.12$). For blacks, total chemiluminescence increased on average by 1.7 millivolt.min (95% CI, 0.2 – 3.7; $p = 0.0269$). Total chemiluminescence did not change significantly for whites ($p = 0.94$) during the experimental phase, and changes were not significantly different between races ($p = 0.13$) or genders ($p = 0.32$). Total chemiluminescence was not significantly different between the control and recovery phases overall ($p = 0.35$). Similar results were observed for peak chemiluminescence.

Cytokine Assays

Baseline IL-6 levels (pg/ml) were significantly lower in blacks vs. whites (9.4 vs. 51.6, $p = 0.0006$) and a similar trend was observed for baseline IL-8 levels (pg/ml) (20.5 vs. 45.7, $p = 0.0029$). During the experimental phase, a significant increase was observed for IL-1α in blacks by 6.1 pg/ml ($p = 0.0426$). On the other hand, a significant decrease was observed for IL-1β in white males by 2.0 pg/ml ($p = 0.0151$) and in IL-6 in white females ($p = 0.0453$) whereas no significant changes were observed in levels of TNF-α or IL-8 for any of the study groups during the experimental period of dental plaque accumulation ($p>0.05$). Levels of IL-6 and IL-1α were not different at the end of the recovery phase as compared to the end of the experimental phase ($p = 0.8505$ and $p = 0.1038$, respectively). However, levels of IL-1β were higher at the end of the recovery phase compared to the

end of the experimental phase in males (4.0 vs. 2.5, p = 0.0269) and white males (5.8 vs. 2.9, p = 0.0192) and were not different from their levels at the end of the control phase (p = 0.1489). Baseline values and changes in levels of cytokines are shown in Tables 2.2-2.10 and Figures 2.15-2.17.

Acute Phase Protein Assays

Baseline values and changes in levels of fibrinogen over the course of the study are summarized in Tables 2.2-2.10 and Figure 2.18. Baseline CRP levels (mg/dL) were lower in males vs. females (0.8 vs. 2.1, p<0.0001), and in individuals with a lower BMI (<25) vs. individuals with a higher BMI (> 30) (0.6 vs. 2.6, p<0.0001). Baseline fibrinogen levels (mg/dL) were significantly lower in males vs. females (275.39 vs. 358.60, p<0001).

For white males, fibrinogen levels increased significantly on average by 15.67 mg/dL from the beginning to the end of the experimental phase (p = 0.0106). Levels of CRP and albumin did not change during the study course (p>0.05) for any of the study groups.

The levels of fibrinogen at the end of the recovery phase, were not different than fibrinogen levels at the end of the experimental phase (p>0.05) but were higher than fibrinogen levels at the end of the control phase overall (p = 0.0348) and for males (p = 0.0452) and white males (p = 0.0211).

Lipid Assays

Baseline values and changes in levels of serum lipids over the course of the study are shown in Tables 2.2-2.10 and in Figures 2.19-2.21. Baseline triglycerides levels (mg/dL) were significantly lower in blacks vs. whites (68.75 vs. 91.48, $p = 0.0085$). In addition, baseline HDL (mg/dL) levels were significantly lower in males vs. females (51.55 vs. 58.11, $p = 0.0072$). From the beginning to the end of the experimental phase, total cholesterol decreased on average by 5.13 mg/dL ($p = 0.0010$); the decrease in the total cholesterol in blacks was significantly greater than in whites ($p = 0.0221$). In addition, the HDL decreased on average by 1.20 mg/dL from the beginning to the end of the experimental phase ($p = 0.0288$); the decrease in the HDL was significantly greater in blacks than in whites ($p = 0.0145$).

For blacks, there was a decrease in the total cholesterol by 9.03 mg/dL ($p = 0.0003$), LDL by 5.20 ($p = 0.0137$), and HDL by 2.38 mg/dL ($p = 0.0007$). For females, there was a significant decrease in total cholesterol by 6.14 mg/dL ($p = 0.0062$), and HDL by 1.52 mg/dL ($p = 0.0232$). For black females, total cholesterol decreased by 9.10 mg/dL ($p = 0.0166$) during the experimental phase. For black males, total cholesterol decreased by 8.97 mg/dL ($p = 0.0084$), LDL decreased by 5.97 mg/dL ($p = 0.0446$), and HDL decreased by 3.34 mg/dL ($p = 0.0037$) during the experimental phase. The decrease in HDL was greater for black males than white males ($p = 0.0026$).

The interaction between race and sex was significant ($p<0.10$) only for HDL ($p = 0.0197$).

Levels of total cholesterol, LDL, and HDL did not change significantly at the end of the recovery phase compared to the end of the experimental phase for any of the study groups ($p>0.05$).

LPS Assay

Baseline values and change in levels of LPS over the course of the study are shown in Tables 2.2-2.10 and Figure 2.22. In brief, no statistically significant differences were observed for baseline plasma LPS levels for any of the study groups or subgroups, with overall levels around 0.08 Endotoxin Unit (EU)/ml. During the experimental phase, blacks were less likely than whites to have plasma LPS measurements ≥ 0.1 EU/ml ($p = 0.0138$), mostly due to only a small percentage of black males with LPS levels that are above the cutoff. When analyzing the LPS measurements as a continuous variable, LPS levels increased significantly ($p = 0.0001$) during the experimental phase by 0.04 EU/ml, but increased less in blacks vs. whites (0.02 vs. 0.06, $p = 0.0369$). At the end of the recovery phase, plasma LPS levels returned back to levels similar to those at the end of the control phase (0.08 EU/ml, $p = 0.25$).

8-Isoprostane Assay

Baseline values and change in blood levels of 8-isoprostane over the study course are shown in Tables 2.2-2.10 and Figure 2.23. Baseline plasma 8-isoprostane levels (pg/ml) were greater in blacks vs. whites (24.2 vs. 10.7, $p = 0.0017$). During the experimental phase, 8-isoprostane levels decreased significantly in black males by 9.9 pg/ml ($p = 0.0169$) and increased significantly in white females by 7.4 pg/ml ($p = 0.0149$), leading to

sex ($p = 0.0058$) and race ($p = 0.0493$) significant differences in the change in levels of 8-isoprostane during the experimental phase. Levels of 8-isoprostane were still significantly lower at the end of the recovery phase when compared to the end of the control phase for males (20.8 vs. 27.3, $p = 0.016$) and for black males (19.2 vs. 31.3, $p = 0.0024$) and were still significantly higher at the end of the recovery phase when compared to the end of the control phase for females (23.3 vs. 18.1, $p = 0.0316$) and for white females (24.7 vs. 16.5, $p = 0.0085$).

Fasting Glucose, Insulin and Cortisol Assays

Baseline values and change in levels of fasting blood glucose, insulin, and cortisol over the study course are shown in Tables 2.2-2.10 and Figure 2.24 and 2.25. Baseline insulin level (μU/ml) was higher in the younger age participants (< 25 years) vs. the older age participants (>25 years) (15.0 vs. 12.2, $p = 0.0384$) and in those with a lower BMI (< 25) vs. those with a higher BMI (> 30) (10.2 vs. 18.2, $p<0.0001$). Fasting glucose and insulin levels did not change significantly during the experimental phase of the study ($p>0.05$).

Overall, from the beginning to the end of the experimental phase, cortisol levels increased significantly on average by 1.3 μg/dL ($p = 0.0065$). Cortisol levels at the end of the recovery phase were lower compared to the end of the experimental phase (9.9 vs. 10.9, $p = 0.0211$) and were similar to cortisol levels at the end of the control phase (9.9 vs. 9.7, $p = 0.6263$). Insulin levels were significantly higher at the end of the recovery phase than at the end of the control phase (15.3 vs. 14.1, $p = 0.0472$).

A summary of all the statistically significant changes that occurred from the beginning to the end of the experimental phase of this study is presented in Table 2.11.

Discussion

This investigation was innovative in several aspects. First, this is the largest, and one of the only, studies published to utilize the experimental gingivitis model to monitor systemic inflammation. Second, this is the first study to use the experimental gingivitis model to address racial differences between blacks and whites in terms of the local and systemic response to dental plaque accumulation. By virtue of its design, this study permitted a longitudinal investigation of the systemic effects of dental plaque accumulation within a short time span. Moreover, confounder issues were minimized since each subject acted as his/her own internal control. As a further control, all venous blood samples were collected prior to 12 noon and following an overnight fast to minimize the effect of diurnal variation on the inflammatory responses to dental plaque accumulation.

The experimental gingivitis model requires individuals to cease all oral hygiene practices for 21 days. This can be an unpleasant experience and thus, subjects' retention and compliance are critical for success of the model. In our study, with the help of the skilled clinical team, the retention rate was high (128 subjects completed out of 156 enrollees); only one of the subjects was excluded due to non-compliance with the model. This high retention rate supports the use of the experimental gingivitis model to study inflammation in a large cohort of subjects.

In this study, subjects' compliance was verified by demonstration of a strong positive correlation between changes in mean PI and mean GI ($r = 0.79$); and as anticipated, a significant increase in both mean PI and mean GI from days 0 to 21 during the experimental phase of the study, a significant decrease in mean PI and mean GI during the recovery phase, and no significant changes in mean PI and mean GI during the control phase.

The findings from this study provide some support to the hypothesis that dental plaque accumulation and the resultant gingivitis can elicit systemic inflammatory responses, and that these responses may differ among different race/gender groups. In this experimental gingivitis study, several parameters that have been associated with systemic inflammation and systemic diseases, including atherosclerosis and CHD, were expressed.

At baseline, the mean PI was higher in blacks vs. whites and in males vs. females, whereas, the mean GI was not significantly different between blacks and whites**.** There are no published epidemiological data in the U.S. comparing detailed PI and GI for individuals of similar age/gender/ethnic background as those included in our study. However, previous surveys in the U.S. have reported that blacks suffer from poorer periodontal health as compared to whites; Blacks were previously shown to have a higher prevalence and extent of gingival recession than whites, and males were shown to have more severe gingival recession and gingival bleeding as compared to females (Albandar and Kingman, 1999; Albandar *et al*., 1999; Borrell *et al*., 2002).

The baseline differences in WBC and neutrophil counts among the race/gender groups in this study are of interest. The lower baseline neutrophil count in blacks as compared to whites and in males as compared to females is consistent with published data from the 1999 to 2004 National Health and Nutrition Survey (NHANES) (Hsieh *et al*., 2007). The NHANES survey found that in the U.S., the total WBC and neutrophil counts are lower in blacks vs. whites and in males vs. females. This disparity in the WBC and neutrophil counts has also been observed in other surveys (Reed and Diehl, 1991; Freedman *et al*., 1997). The cause for these differences in WBC and neutrophil counts between different race/gender groups is unclear. However, it has previously been postulated (Bain *et al*., 2000) that the lower WBC and neutrophil counts in blacks are due to either a reduced bone marrow reserve of granulocytes or due to altered distribution of neutrophils between the circulating and marginated cell pools.

In addition to the baseline WBC and neutrophil count, other baseline parameters in this study are in agreement with previous population-based studies, including the higher baseline CRP levels in females vs. males (Khera *et al*., 2005; Lakoski *et al*., 2006), the higher baseline CRP levels in individuals with high BMI vs. those with low BMI, which has been previously suggested to indicate a state of low-grade systemic inflammation in overweight and obese persons (Visser *et al*., 1999), the higher baseline RBC count and subsequently, the higher Hb and Hct in males vs. females (Yip *et al*., 1984), the lower MCV in blacks vs. whites and the higher lymphocytes in blacks vs. whites (Beutler and west, 2005), the higher baseline triglyceride levels in blacks vs. whites (Haffner *et al*., 1999), and the higher levels of HDL in females vs. males (NHANES III, 1998).

In this study, several systemic factors changed during the experimental phase, providing evidence for the ability of the experimental gingivitis model to induce a systemic inflammatory response. During the experimental phase of this study, a decrease in the RBC count was observed. The decrease in the RBC count was previously reported in the literature as a host response to infection and inflammation, a phenomenon that has been described as "anemia of chronic disease" (Lee, 1983). In addition, previous studies demonstrated changes in several RBC parameters in individuals with periodontitis (Lainson *et al*., 1968; Hutter *et al*., 2001). In these studies, when compared to healthy individuals, individuals with periodontitis were shown to have a lower Hct, lower RBC counts and consequently, lower Hb levels. Similarly, we observed in this study a decrease in the RBC count, Hct, and Hb levels in black subjects in general and black males in particular following dental plaque accumulation and development of gingivitis during the experimental phase.

During the experimental phase of the current study, an increase in the MCV was also observed. MCV is a measurement of the average size of the RBCs. The MCV is elevated when the RBCs are larger than normal. Additionally, the MCH increased significantly in blacks during the experimental phase of this study. MCH is a calculation of the average amount of hemoglobin inside a red blood cell and thus, macrocytic RBCs (with increased MCV) tend to have a higher MCH.

The ESR increased in black males during the experimental phase of this study. ESR is a non-specific inflammatory biomarker that has been associated with vascular injury,

atherosclerosis, and ultimately CHD (Lowe, 2001; Abou-Raya *et al*., 2007). Similarly, elevated levels of ESR were associated with periodontal disease in previous studies (Mercado *et al*., 2001).

During the experimental phase of this study, we demonstrated an increase in the MPV, particularly in black and male subjects. MPV is a marker of platelet size, function, and activation. Increased MPV is an indicator of active and large platelets (Thompson et al., 1982). Little is reported in the literature regarding the clinical significance of this factor. However, it is speculated that increased MPV indicates the expression of an inflammatory proatherogenic condition (Coban et al., 2005). Recently, it has been reported that MPV plays an important role in the pathophysiology of atherosclerosis and CHD (Sen et al., 2009).

The mean total WBC and neutrophil counts did not change significantly for any of the race/gender groups over the course of the study. The results of this study differ from the findings of our pilot study, conducted in Mexico (Kowolik *et al*., 2001). In the Mexico study, total WBC and neutrophil counts increased from beginning to end of the experimental phase (6.47 vs. 6.80). However, this increase was not statistically significant ($p>0.05$). The neutrophil counts were significantly higher during the experimental phase of the gingivitis model on both days 7 (6.53 vs. 5.99, $p = 0.0301$) and 21 (6.80 vs. 6.18, $p = 0.009$) as compared to the corresponding days of the control phase.

Regarding total WBC count, this was significantly higher on day 21 of the experimental phase when compared to the corresponding day of the control phase. It is important to note that there were differences in the study design between this study and the Mexico study. The Mexico study was a 4 phase study that consisted of a 21-day oral hygiene phase, followed by a 21-day experimental phase that, in turn, was followed by a 28-day recovery phase and finally, a 21-day control phase that mirrored the experimental phase but with subjects performing normal oral hygiene practices. Furthermore, it can be speculated that differences in ethnicity of the study populations might have played a role in the difference in findings between the two studies.

In the present study, although dental plaque accumulation and the resulting gingivitis did not result in a change in the total and differential WBC counts, an increase in the activity of peripheral blood neutrophils was observed in the black individuals, predominantly in black males. This neutrophil hyperactivity was evidenced by a statistically significant increase in both peak and total chemiluminescence in blacks during the experimental phase of the study. While ethnic background has previously been shown to influence the metabolic oxidative activity in neutrophils (Siddiqi *et al*., 2001), in that study fMLP-stimulated oxidative activity was significantly lower in neutrophils obtained from African Americans compared with neutrophils obtained from Caucasians (Siddiqi *et al*., 2001). In contrast to our study, Siddiqi *et al*. (2001) evaluated neutrophil metabolic oxidative activity in the absence of induced inflammation.

We postulate that the occurrence of neutrophil hyperactivity in the absence of an increase in total and differential WBC counts may be due to the fact that gingivitis is an inflammation of the superficial periodontal tissues. As a mild insult to the host, it may not be strong enough to induce an increase in the number of circulating neutrophils, but may be sufficient to cause hyperactivity of the existing peripheral blood neutrophils. In agreement with this postulation, it was previously suggested that neutrophil hyperactivity may be an earlier and more sensitive response by circulating neutrophils to a bacterial challenge than leukocytosis (Hill *et al*., 1974).

In this study, the demonstration of a heightened neutrophil inflammatory response to dental plaque accumulation in black individuals may be of clinical interest. Neutrophil hyperactivity in terms of oxidative burst is known to play an important role in endothelial injury (Lentsch and Ward, 2000) and subsequent tissue damage and organ dysfunction in several disease processes (Di Filippo *et al*., 2007; Wittkowski *et al*., 2007).

Reactive oxygen species may exert their cytotoxic effects by inducing changes in the metabolic pathways of host cells. These changes may include damage to cellular DNA, peroxidation of polyunsaturated fatty acids, inhibition of mitochondrial enzymes, inactivation of membrane sodium channels, and altering some other proteins that are involved in crucial cellular functions. These changes may lead to cellular hypertrophy, altered cellular growth, and apoptosis (Kowaltowski and Vercesi, 1999; Droge, 2002; Martindale and Holbrook, 2002; Martinet *et al*., 2002). Accordingly, oxidative stress

plays a central role in pathogenesis and development of many chronic and degenerative diseases (Droge, 2002) including atherosclerosis (Martinet *et al*., 2002).

Another systemic factor that changed during this study, following dental plaque accumulation, was serum fibrinogen. In white males, plasma fibrinogen levels increased during the experimental phase. Fibrinogen levels are often increased as an acute response of the host to infection and inflammation. Previous studies have shown elevated serum levels of fibrinogen in individuals with periodontal infection including severe gingivitis as compared to periodontally healthy individuals (Kweider *et al*., 1993; Wu *et al*., 2000; Sahingur *et al*., 2003). Elevated serum levels of fibrinogen may mediate atherosclerosis by increasing blood viscosity, promoting platelet aggregation, and stimulating smooth muscle proliferation (Wu *et al*., 2000; Lowe, 2001).

In addition, levels of serum cortisol increased during the experimental phase of our study. During acute illness and inflammation, serum cortisol levels are reported to be elevated (Vogeser *et al*., 2002). Prolonged exposure to elevated cortisol levels may have effects that could promote CHD (Sher, 2005). Sustained high levels of serum cortisol are shown to further enhance inflammatory responses, lead to dyslipidemia and insulin resistance, and may be associated with atherosclerosis and ultimately, CHD (Sher, 2005; Nijm *et al*., 2007). In the context of periodontal disease, serum cortisol levels were previously shown to increase in association with necrotizing ulcerative gingivitis (Enwonwu *et al*., 2005).

During the experimental phase of this study, significant decreases were reported in total cholesterol and HDL levels. In addition, LDL levels decreased in blacks and more specifically in black males during the experimental phase. Changes in serum lipoproteins have previously been shown to occur during the host acute-phase response to infection (Khovidhunkit *et al*., 2000). The mechanisms that modify lipoproteins during acute illness are mediated by host cytokines and bacterial products, such as LPS (Kitchens *et al*., 2003).

A decrease in levels of blood total cholesterol has been commonly found during infection (Khovidhunkit *et al*., 2000; Fraunberger *et al*., 1999). Moreover, studies have shown a strong positive association between low levels of blood total cholesterol and mortality in critically ill patients (Fraunberger *et al*., 1999; Windler *et al*., 1994). Although hypercholesterolemia is a traditional risk factor for the development of atherosclerosis and CHD, interestingly, low levels of blood total cholesterol, LDL, and HDL have been found to predict adverse outcomes in many critical illnesses, including heart failure (Horwich *et al*., 2002). HDL and LDL play an important role in regulating the host response to LPS (Feingold *et al*., 1995) since they bind LPS and prevent it from inducing toxic inflammatory effects to the host. HDL and LDL levels were shown to decrease sharply in septic patients and thus, impairing the ability of the host to neutralize LPS (Fraunberger *et al*., 1999, Windler *et al*., 1994).

HDL is the most abundant lipoprotein (Van Leeuwen *et al*., 2001). When LPS enters the circulation, most of the lipoprotein-bound LPS is in the HDL fraction, whereas the rest is

bound to LDL (Vesy *et al*., 1999). HDL levels were previously shown to decrease in response to infection. Low levels of HDL are associated with atherosclerosis and thus, CHD. HDL plays a fundamental role against atherogenesis by promotion of the "reverse cholesterol transport" process. This is the process by which cholesterol is effluxed from peripheral cells onto circulating HDL particles and transported to the liver for excretion into bile and feces (Rader, 20003; Cuchel and Rader, 2006). It has been proposed that inflammation adversely affects the reverse cholesterol transport process by altering the composition and metabolism of HDL (McGillicuddy *et al*., 2009). This is suggested (McGillicuddy *et al*., 2009) to be of clinical significance and can contribute to atherosclerosis during inflammation.

During the experimental phase of this study, blood levels of 8-isoprostane increased significantly in white females and decreased significantly in black males. 8-isoprostane levels are important determinants of LDL peroxidation (Lubrano *et al*., 2002; Liu *et al*., 2004). The increase in 8-isoprostane levels in white females during the period of dental plaque accumulation confirms our hypothesis that dental plaque accumulation and the resultant gingivitis can result in increased levels of systemic oxidative stress. On the other hand, the decrease in systemic levels of 8-isoprostane in black males during the experimental phase of this study may be attributed to the decrease in serum levels of LDL in black males since the level of 8-isoprostane is suggested (Liu *et al*., 2004) to be affected by multiple factors including susceptibility of LDL particles to oxidation, size of LDL particles, number of LDL particles in the circulation, composition of LDL particles, local oxidative stress in the arterial wall, or a combination of these factors.

Peroxidation of LDL particles has been associated with subclinical atherosclerosis in healthy individuals (Hulthe and Fagerberg, 2002) and has been implicated in multiple processes that are related to atherosclerosis including, inhibition antithrombin activity, induction of procoagulation, stimulation of platelet aggregation, and alteration of vascular responses (Gray and Barrowcliffe, 1985; Barrowcliffe *et al*., 1987; Minuz *et al*., 1998; Morrow and Roberts, 1997).

Interestingly, systemic levels of LPS increased significantly during the experimental phase of this study. The increase in levels of LPS was more in whites compared to blacks. We anticipated that accumulation of dental plaque and its inherent transition to an increasingly Gram-negative microbial composition of dental plaque would result in an increase in circulating endotoxin levels. Endotoxemia occurs in individuals with periodontal disease and is a risk factor for atherosclerosis (Kallio *et al*., 2008). LPS is a potent immune activator that is known to induce a systemic inflammatory response. LPS stimulates the cellular activity of neutrophils, including secretion of lysosomal enzymes (Fittschen *et al*., 1988; Ottonello *et al*., 1997; Haslett *et al*., 1985), production of reactive oxygen radicals (Guthrie *et al*., 1984), and phagocytosis (Cohn and Morse, 1960). It has been suggested (Lopes-Virella, 1993) that LDL and LPS can mediate a direct link between Gram-negative bacterial infections and atherosclerosis. LPS can form an immune complex with oxidized LDL particles, enhancing the release of proinflammatory cytokines, and inducing further endothelial cell damage (Renaud *et al*., 1970; Lopes-Virella, 1993).

Regarding serum cytokine levels, an increase in blood levels of IL-1α in blacks but a decrease in blood levels of IL-1β in white males and a decrease in blood levels of IL-6 in white females during the experimental phase of this study was observed. These cytokines may increase blood levels of acute-phase proteins and induce changes in the number of circulating RBCs and WBCs (Ramadori *et al*., 1985; Marinkovic *et al*., 1989; Ulich *et al*., 1989). Serum IL-6 levels are elevated during acute bacterial infections (Helfgott *et al*., 1989), including periodontal infection (Loos *et al*., 2000; Forner *et al*., 2006b). IL-1α plays an important role during the host inflammatory response. IL-1α enhances the expression of endothelial adhesion molecules in order to enable transmigration of WBCs to the site of infection (Edwards, 1994).

The reasons behind the decrease in blood levels of IL-1β in white males and IL-6 in white females in this study are unclear. It has been proposed (Di Padova *et al*., 1991) that the circulating levels of these cytokines may depend upon multiple factors including the type and duration of the stimuling factor. It is important to note that in the current study, cytokine levels in only the systemic circulation and not in the local gingival area were measured.

During this study, while levels of some of the systemic factors that changed during the experimental phase did, at the end of the recovery phase, return to levels similar to those reported at the end of the control phase, levels of other systemic factors did not. Explanations can be provided to this finding. Subjects started this study with varying amounts of dental plaque accumulation. Those who started the study with high levels of

dental plaque accumulation may, according to the study hypotheses, already have had increased levels of systemic factors associated with CHD risk, which may not have decreased significantly by the end of the control phase. In addition, following the end of the experimental phase, the monitoring period of 21 days during the recovery phase might have been insufficient to show a return to baseline values of these levels. An additional explanation is that dental plaque control methods that were performed during the recovery phase of this study may have been ineffective in reversing altered levels of these systemic factors in the specific group of subjects studied. Although this may be perceived as a potential problem, such a finding may be of importance in designing future studies to further understand the temporal aspects of the systemic consequences of dental plaque accumulation.

It is important to note that in this study, systemic factors that changed during the course of the study, whether increasingly or decreasingly, remained within normal physiological levels. We did not anticipate, following three weeks of dental plaque accumulation, that systemic levels of the factors that were measured in this study would reach pathological values. However, as suggested in other studies, "LITTLE and OFTEN" systemic challenge in some individuals can be of significant importance (Roberts, 1999).

Our study has limitations. Although well-controlled, the conditions of the experimental gingivitis model differ from those of clinical gingivitis. A recent trial (Deinzer *et al*., 2007) revealed that the experimental model may result in local inflammatory differences when compared to persistent gingivitis. It is important to note, however, that in our study,

we evaluated the effects of dental plaque on systemic, rather than local, inflammatory markers.

With regards to the neutrophil function assay, multiple assays including those assessing granular enzyme activities would have been ideal. However, we focused in this study on the neutrophil oxidative function assay which is of great relevance to inflammation and tissue destruction.

Our cytokine assays have limitations. Immunological quantification of cytokines is not as specific as mass spectrographic methods. Blood cytokine assays are not activity assays and concentration may not correlate with active infection. However, as discussed earlier in this chapter, the literature supports a quantitative cytokine response to infection and inflammation.

Hormonal fluctuations in women, which were not measured during this study, may alter gingival and/or systemic responses to dental plaque (Holm-Pedersen and Löe, 1967; Preshaw *et al*., 2001).

In this study, multiple comparisons were performed. However, no adjustment was made to correct for the multiple comparisons problem. This can be associated with a higher probability of type I error. There are several arguments against doing a multiple comparisons adjustment in this study. First, the analyses were all pre-specified prior to conduction of the study and thus, there was no 'fishing' for statistical significance. In

addition, the study was powered to detect pre-specified differences between race/sex groups and between days within each group for the study outcomes. Finally, the study was exploratory in nature rather than confirmatory.

Although the results from this study did not conclusively show a systemic response to dental plaque accumulation across all subject groups studied, the experimental gingivitis model in this study manifested systemically as a classical acute infectious challenge and several factors that are associated with systemic inflammation and systemic diseases (including CHD) have been expressed in this experimental gingivitis study.

The findings from this study in black individuals including the heightened systemic neutrophil activity, the changes in hematological factors, and the altered lipid profiles following dental plaque accumulation, in addition to the understanding that black race is associated with higher prevalence of periodontal disease (Borrell *et al*., 2002) and higher levels of inflammatory parameters necessitates further research in this at-risk population.

In conclusion, as anticipated, the accumulation of dental plaque elicited a systemic response as evidenced by change in levels of systemic factors, which have been linked to CHD risk. These responses differed between individuals of different genders/races. The findings from this study provide some evidence to support the hypothesis for a causal role of oral disease in CHD, thus creating a powerful argument for the importance of oral health care in CHD prevention.

Further studies, including those that utilize the experimental gingivitis model, could help in identifying individuals with an innately increased inflammatory response to a bacterial challenge. A more complete knowledge of the role of dental plaque accumulation in eliciting systemic inflammation and a better understanding of gender/ethnic differences in the systemic inflammatory responses could provide clinicians with new therapeutic targets for the prevention or reduction of tissue injury through improving oral hygiene measures in individuals exhibiting an exaggerated inflammatory response.

Table 2.1. Overview of the clinical procedures performed during each study visit (Wahaidi *et al*., 2009).

	Screening Visit	Cleaning	Control Phase				Experimental Phase				Recovery Phase			
Day of Phase			0	7	14	21	0	7	14	21	0	7	14	21
Day of Study	0	7	14	21	28	35[a]	35[a]	42	49	56[b]	56[b]	63	70	77
Visit Number	1	2	3	4	5	6	6	7	8	9			10	11
Blood Draw			X	X	X	X	X	X	X	X			X	X
Plaque and Gingivitis Assessment			X	X	X	X	X	X	X	X			X	X
Dental Prophylaxis		X								X[c]				
Oral Hygiene Instruction		X	X	X	X					X				

a, b: Same day of study.

c: Dental cleaning scheduled no more than 2 days after Day 21 of the experimental phase

Table 2.2.A. Mean and 95% confidence interval for baseline study outcomes and changes during the experimental phase for study subjects (N = 128).

	Baseline Mean (95% CI)	Change during experimental phase	p-value
Gingival Index	0.53 (0.5, 0.56)	0.63 (0.6, 0.66)	0.0000
Plaque Index	0.32 (0.29, 0.35)	1.63 (1.56, 1.69)	0.0000
Total CL	6.7 (5.7, 7.7)	0.5 (-1.2, 2.2)	0.5443
Peak CL	11.4 (9.6, 13.4)	1.5 (-1.5, 4.5)	0.3295
Neutrophil count	3.2 (2.9, 3.4)	0 (-0.2, 0.2)	0.7079
WBC count	5.7 (5.4, 6)	0 (-0.2, 0.2)	0.8800
Total Cholesterol	184 (183, 185)	-5.1 (-5.9, -4.4)	0.0010
LDL Cholesterol	114 (113, 115)	-2.4 (-3.1, -1.7)	0.0917
HDL Cholesterol	54.9 (54.3, 55.6)	-1.2 (-1.6, -0.7)	0.0288
Triglycerides	80.7 (79.4, 81.9)	-2.2 (-3.7, -0.8)	0.7325
Fasting Blood Glucose	87.6 (87.1, 88.1)	-0.1 (-0.5, 0.4)	0.4756
Basophils %	0.62 (0.49, 0.74)	0.02 (-0.11, 0.14)	0.7216
ESR	7.9 (7.3, 8.5)	0.6 (0.2, 1)	0.0572
Eosinophils %	2.2 (1.9, 2.4)	0 (-0.2, 0.1)	0.6799
Fibrinogen	319 (317, 321)	8.3 (6.9, 9.7)	0.2007
Hct	41.4 (41.1, 41.8)	-0.2 (-0.5, 0)	0.2286
Hb	14.4 (14.2, 14.6)	-0.09 (-0.23, 0.04)	0.1284
Lymphocytes count	1.46 (1.32, 1.59)	-0.05 (-0.18, 0.08)	0.2968
Lymphocytes %	33.4 (32.9, 33.9)	-0.4 (-0.8, 0.1)	0.6243

Table 2.2.B. Mean and 95% confidence interval for baseline study outcomes and changes during the experimental phase for study subjects (N = 128).

	Baseline Mean (95% CI)	Change during experimental phase	p-value
MCHC	33.84 (33.68, 34)	0.02 (-0.12, 0.17)	0.6854
MCV	87.2 (86.8, 87.6)	0.21 (0.04, 0.38)	0.0156
MPV	8.9 (8.7, 9)	0.09 (-0.02, 0.19)	0.0048
Monocytes %	7.7 (7.5, 8)	0 (-0.3, 0.2)	0.6542
Neutrophils %	56.4 (55.9, 56.9)	0.4 (-0.1, 0.9)	0.5332
Platelet count	278 (277, 279)	0.9 (-0.1, 1.8)	0.7921
RBC count	4.8 (4.7, 4.9)	-0.04 (-0.12, 0.04)	0.0321
RDW	13.2 (13.1, 13.4)	-0.02 (-0.13, 0.09)	0.7112
IL-1β	3 (2, 5)	-0.5 (-1, 0)	0.0591
IL-1α	29 (14, 59)	4.5 (-1.2, 11.9)	0.1336
IL-6	23 (14, 38)	-2 (-5.4, 2.3)	0.3377
IL-8	31 (24, 41)	1.6 (-2.2, 5.9)	0.4272
TNF-α	8 (6, 12)	0.3 (-0.8, 1.6)	0.6173
CRP	1.3 (1.0, 1.6)	-0.1 (-0.3, 0.2)	0.5116
Cortisol	9.7 (8.8, 10.6)	1.3 (0.3, 2.3)	0.0065
Insulin	13.5 (12.2, 14.9)	0.5 (-0.4, 1.5)	0.2596
Isoprostane	15.8 (12.2, 20.5)	0.2 (-3.0, 4.0)	0.9086
Endotoxin	0.08 (0.08, 0.08)	0.04 (0.03, 0.06)	0.0000

Table 2.3.A. Mean and 95% confidence interval for baseline study outcomes for race and gender subgroups.

	Baseline Mean (95% CI)				p-value	
	BF	BM	WF	WM	Race	Sex
Age	25.5 (24.1, 26.9)	22.2 (20.9, 23.4)	24.5 (23.3, 25.8)	24.7 (23.6, 25.8)	0.2132	0.0156
BMI	32.1 (28.5, 35.6)	28.4 (25.5, 31.3)	32 (28.7, 35.3)	27.2 (25.2, 29.2)	0.7789	0.0047
Gingival Index	0.50 (0.45, 0.56)	0.51 (0.47, 0.56)	0.58 (0.53, 0.64)	0.52 (0.45, 0.6)	0.1159	0.4004
Plaque Index	0.35 (0.3, 0.41)	0.35 (0.28, 0.43)	0.25 (0.21, 0.31)	0.34 (0.29, 0.41)	0.0604	0.0810
Total CL	7.1 (5.3, 9.5)	6.2 (4.3, 9)	7.8 (6, 10.1)	5.4 (3.9, 7.5)	0.9952	0.1211
Peak CL	10.8 (7.6, 15.4)	10 (6.8, 14.7)	14.8 (11.3, 19.3)	10.1 (6.9, 14.8)	0.2820	0.1640
Neutrophil Count	2.9 (2.4, 3.5)	2.7 (2.3, 3.2)	4 (3.5, 4.5)	3 (2.8, 3.3)	0.0021	0.0111
WBC Count	5.4 (4.8, 6.2)	5.2 (4.7, 5.7)	6.6 (6.1, 7.3)	5.4 (5.1, 5.8)	0.0094	0.0090
Total Cholesterol	184 (182, 186)	172 (170, 174)	185 (184, 187)	194 (191, 196)	0.1612	0.5284
LDL Cholesterol	113 (111, 115)	106 (104, 108)	113 (111, 114)	123 (120, 126)	0.3862	0.8321
HDL Cholesterol	59.4 (57.9, 60.9)	52.3 (51, 53.5)	57 (55.8, 58.2)	50.9 (49.7, 52)	0.9146	0.0072
Triglycerides	67 (64.2, 69.7)	70.5 (68.4, 72.7)	82.3 (79.9, 84.7)	101.8 (98.9, 104.7)	0.0085	0.0821
Fasting Blood Glucose	87.3 (86.2, 88.3)	89.3 (88.3, 90.3)	85.5 (84.6, 86.5)	88.6 (87.5, 89.7)	0.3007	0.0636
Basophils %	0.67 (0.39, 0.94)	0.7 (0.43, 0.97)	0.51 (0.27, 0.76)	0.6 (0.34, 0.86)	0.2016	0.4767
ESR	17 (15.5, 18.5)	3.7 (2.6, 4.7)	8.8 (7.8, 9.8)	2.2 (1.7, 2.6)	0.4634	0.0000
Eosinophils %	2.3 (1.8, 2.8)	2.8 (2.3, 3.3)	1.5 (1.3, 1.8)	2.1 (1.6, 2.6)	0.0291	0.1103
Fibrinogen	372 (369, 376)	283 (280, 286)	347 (344, 350)	268 (265, 270)	0.3692	0.0000
Hct	37.7 (37.1, 38.4)	44.4 (43.7, 45)	39.6 (39.1, 40.2)	44.3 (43.7, 44.9)	0.3159	0.0000
Hb	12.9 (12.6, 13.3)	15.3 (14.9, 15.6)	13.8 (13.5, 14.1)	15.6 (15.2, 15.9)	0.0513	0.0000

Table 2.3.B. Mean and 95% confidence interval for baseline study outcomes for race and gender subgroups.

	Baseline Mean (95% CI)				p-value	
	BF	BM	WF	WM	Race	Sex
Age	25.5 (24.1, 26.9)	22.2 (20.9, 23.4)	24.5 (23.3, 25.8)	24.7 (23.6, 25.8)	0.2132	0.0156
BMI	32.1 (28.5, 35.6)	28.4 (25.5, 31.3)	32 (28.7, 35.3)	27.2 (25.2, 29.2)	0.7789	0.0047
Gingival Index	0.50 (0.45, 0.56)	0.51 (0.47, 0.56)	0.58 (0.53, 0.64)	0.52 (0.45, 0.6)	0.1159	0.4004
Plaque Index	0.35 (0.3, 0.41)	0.35 (0.28, 0.43)	0.25 (0.21, 0.31)	0.34 (0.29, 0.41)	0.0604	0.0810
Total CL	7.1 (5.3, 9.5)	6.2 (4.3, 9)	7.8 (6, 10.1)	5.4 (3.9, 7.5)	0.9952	0.1211
Peak CL	10.8 (7.6, 15.4)	10 (6.8, 14.7)	14.8 (11.3, 19.3)	10.1 (6.9, 14.8)	0.2820	0.1640
Neutrophil Count	2.9 (2.4, 3.5)	2.7 (2.3, 3.2)	4 (3.5, 4.5)	3 (2.8, 3.3)	0.0021	0.0111
WBC Count	5.4 (4.8, 6.2)	5.2 (4.7, 5.7)	6.6 (6.1, 7.3)	5.4 (5.1, 5.8)	0.0094	0.0090
Total Cholesterol	184 (182, 186)	172 (170, 174)	185 (184, 187)	194 (191, 196)	0.1612	0.5284
LDL Cholesterol	113 (111, 115)	106 (104, 108)	113 (111, 114)	123 (120, 126)	0.3862	0.8321
HDL Cholesterol	59.4 (57.9, 60.9)	52.3 (51, 53.5)	57 (55.8, 58.2)	50.9 (49.7, 52)	0.9146	0.0072
Triglycerides	67 (64.2, 69.7)	70.5 (68.4, 72.7)	82.3 (79.9, 84.7)	101.8 (98.9, 104.7)	0.0085	0.0821
Fasting Blood Glucose	87.3 (86.2, 88.3)	89.3 (88.3, 90.3)	85.5 (84.6, 86.5)	88.6 (87.5, 89.7)	0.3007	0.0636
Basophils %	0.67 (0.39, 0.94)	0.7 (0.43, 0.97)	0.51 (0.27, 0.76)	0.6 (0.34, 0.86)	0.2016	0.4767
ESR	17 (15.5, 18.5)	3.7 (2.6, 4.7)	8.8 (7.8, 9.8)	2.2 (1.7, 2.6)	0.4634	0.0000
Eosinophils %	2.3 (1.8, 2.8)	2.8 (2.3, 3.3)	1.5 (1.3, 1.8)	2.1 (1.6, 2.6)	0.0291	0.1103
Fibrinogen	372 (369, 376)	283 (280, 286)	347 (344, 350)	268 (265, 270)	0.3692	0.0000
Hct	37.7 (37.1, 38.4)	44.4 (43.7, 45)	39.6 (39.1, 40.2)	44.3 (43.7, 44.9)	0.3159	0.0000
Hb	12.9 (12.6, 13.3)	15.3 (14.9, 15.6)	13.8 (13.5, 14.1)	15.6 (15.2, 15.9)	0.0513	0.0000

Table 2.4.A. Mean and 95% confidence interval (CI) for baseline study outcomes by race and gender.

	Baseline Mean (95% CI)				p-value	
	BF	BM	WF	WM	Race	Sex
Age	25.5 (24.1, 26.9)	22.2 (20.9, 23.4)	24.5 (23.3, 25.8)	24.7 (23.6, 25.8)	0.2132	0.0156
BMI	32.1 (28.5, 35.6)	28.4 (25.5, 31.3)	32 (28.7, 35.3)	27.2 (25.2, 29.2)	0.7789	0.0047
Gingival Index	0.50 (0.45, 0.56)	0.51 (0.47, 0.56)	0.58 (0.53, 0.64)	0.52 (0.45, 0.6)	0.1159	0.4004
Plaque Index	0.35 (0.3, 0.41)	0.35 (0.28, 0.43)	0.25 (0.21, 0.31)	0.34 (0.29, 0.41)	0.0604	0.0810
Total CL	7.1 (5.3, 9.5)	6.2 (4.3, 9)	7.8 (6, 10.1)	5.4 (3.9, 7.5)	0.9952	0.1211
Peak CL	10.8 (7.6, 15.4)	10 (6.8, 14.7)	14.8 (11.3, 19.3)	10.1 (6.9, 14.8)	0.2820	0.1640
Neutrophil Count	2.9 (2.4, 3.5)	2.7 (2.3, 3.2)	4 (3.5, 4.5)	3 (2.8, 3.3)	0.0021	0.0111
WBC Count	5.4 (4.8, 6.2)	5.2 (4.7, 5.7)	6.6 (6.1, 7.3)	5.4 (5.1, 5.8)	0.0094	0.0090
Total Cholesterol	184 (182, 186)	172 (170, 174)	185 (184, 187)	194 (191, 196)	0.1612	0.5284
LDL Cholesterol	113 (111, 115)	106 (104, 108)	113 (111, 114)	123 (120, 126)	0.3862	0.8321
HDL Cholesterol	59.4 (57.9, 60.9)	52.3 (51, 53.5)	57 (55.8, 58.2)	50.9 (49.7, 52)	0.9146	0.0072
Triglycerides	67 (64.2, 69.7)	70.5 (68.4, 72.7)	82.3 (79.9, 84.7)	101.8 (98.9, 104.7)	0.0085	0.0821
Fasting Blood Glucose	87.3 (86.2, 88.3)	89.3 (88.3, 90.3)	85.5 (84.6, 86.5)	88.6 (87.5, 89.7)	0.3007	0.0636
Basophils %	0.67 (0.39, 0.94)	0.7 (0.43, 0.97)	0.51 (0.27, 0.76)	0.6 (0.34, 0.86)	0.2016	0.4767
ESR	17 (15.5, 18.5)	3.7 (2.6, 4.7)	8.8 (7.8, 9.8)	2.2 (1.7, 2.6)	0.4634	0.0000
Eosinophils %	2.3 (1.8, 2.8)	2.8 (2.3, 3.3)	1.5 (1.3, 1.8)	2.1 (1.6, 2.6)	0.0291	0.1103
Fibrinogen	372 (369, 376)	283 (280, 286)	347 (344, 350)	268 (265, 270)	0.3692	0.0000
Hct	37.7 (37.1, 38.4)	44.4 (43.7, 45)	39.6 (39.1, 40.2)	44.3 (43.7, 44.9)	0.3159	0.0000
Hb	12.9 (12.6, 13.3)	15.3 (14.9, 15.6)	13.8 (13.5, 14.1)	15.6 (15.2, 15.9)	0.0513	0.0000

Table 2.4.B. Mean and 95% confidence interval (CI) for baseline study outcomes by race and gender.

	Baseline Mean (95% CI)				p-value	
	Blacks	Whites	Males	Females	Race	Sex
Lymphocytes count	1.38 (1.2, 1.57)	1.52 (1.32, 1.72)	1.33 (1.14, 1.52)	1.57 (1.38, 1.76)	0.2066	0.0336
Lymphocytes %	35 (34.2, 35.8)	31.9 (31.2, 32.5)	33.5 (32.8, 34.3)	33.2 (32.5, 33.9)	0.0442	0.9272
MCH	28.9 (28.5, 29.3)	30.1 (29.8, 30.4)	29.8 (29.4, 30.2)	29.3 (28.9, 29.6)	0.0013	0.1371
MCHC	33.47 (33.24,	34.18 (33.99,	34 (33.77, 34.23)	33.69 (33.48, 33.9)	0.0000	0.0226
MCV	86.2 (85.6, 86.8)	88.1 (87.6, 88.6)	87.7 (87.1, 88.3)	86.7 (86.2, 87.3)	0.0366	0.2934
MPV	9 (8.8, 9.3)	8.7 (8.5, 8.9)	8.9 (8.7, 9.2)	8.8 (8.6, 9.1)	0.1708	0.9980
Monocytes %	7.8 (7.4, 8.2)	7.7 (7.3, 8)	8.7 (8.3, 9)	6.9 (6.5, 7.2)	0.7269	0.0000
Neutrophils %	54.3 (53.5, 55.1)	58.4 (57.7, 59)	54.9 (54.2, 55.7)	57.8 (57, 58.5)	0.0149	0.1314
Platelet count	277 (274, 279)	279 (277, 281)	255 (253, 257)	299 (297, 301)	0.9391	0.0004
RBC count	4.8 (4.6, 5)	4.8 (4.6, 5)	5.1 (5, 5.3)	4.5 (4.4, 4.7)	0.9410	0.0000
RDW	13.5 (13.3, 13.8)	13 (12.7, 13.2)	13.1 (12.8, 13.3)	13.4 (13.1, 13.7)	0.0001	0.1817
IL-1β	3 (2, 6)	3 (1, 6)	4 (2, 8)	2 (1, 5)	0.8956	0.3068
IL-1α	15 (5, 45)	54 (22, 132)	42 (15, 114)	21 (8, 57)	0.0725	0.3378
IL-6	9 (4, 22)	52 (32, 84)	27 (13, 55)	20 (10, 40)	0.0006	0.5314
IL-8	20 (13, 33)	46 (35, 60)	36 (25, 52)	27 (18, 40)	0.0029	0.2933
TNF-α	7 (4, 11)	10 (6, 16)	11 (7, 18)	7 (4, 11)	0.2558	0.1430
CRP	1.2 (0.9, 1.8)	1.3 (1.0, 1.9)	0.8 (0.6, 1.0)	2.1 (1.5, 3.0)	0.7357	0.0000
Cortisol	8.9 (7.8, 10.2)	10.5 (9.2, 11.9)	9.9 (8.8, 11.3)	9.5 (8.2, 10.9)	0.0889	0.6239
Insulin	14.0 (12.3, 16.0)	13.0 (11.2, 15.1)	12.5 (10.9, 14.3)	14.4 (12.5, 16.7)	0.4430	0.1494
Isoprostane	24.2 (18.2, 32.3)	10.7 (7.1, 16.1)	19.3 (13.9, 26.7)	13.1 (8.7, 19.7)	0.0017	0.1411
Endotoxin	0.08 (0.08, 0.08)	0.08 (0.08, 0.09)	0.08 (0.08, 0.08)	0.08 (0.08, 0.09)	0.2453	0.4769

Table 2.5.A. Mean and 95% confidence interval (CI) for baseline study outcomes by age.

	Baseline Mean (95% CI)		p-value
	Age < 25 (years)	Age > 25 (years)	
Age (years)	21 (20.5, 21.6)	27.3 (26.8, 27.7)	0.0000
BMI	29.9 (27.7, 32)	30 (27.8, 32.2)	0.9184
Gingival Index	0.54 (0.5, 0.58)	0.52 (0.48, 0.57)	0.7221
Plaque Index	0.34 (0.3, 0.38)	0.3 (0.26, 0.34)	0.1993
Total CL	7 (5.6, 8.8)	6.3 (5.1, 7.7)	0.5058
Peak CL	12 (9.3, 15.3)	10.8 (8.5, 13.6)	0.5396
Neutrophil Count	3.2 (2.9, 3.6)	3.1 (2.8, 3.4)	0.6927
WBC Count	5.7 (5.3, 6.2)	5.6 (5.2, 6)	0.6503
Total Cholesterol	178 (176, 179)	190 (189, 192)	0.0296
LDL Cholesterol	110 (109, 112)	117 (116, 118)	0.1417
HDL Cholesterol	53.3 (52.4, 54.2)	56.5 (55.6, 57.5)	0.1560
Triglycerides	77.7 (75.9, 79.5)	83.5 (81.7, 85.3)	0.5974
Fasting Blood Glucose	86.8 (86.2, 87.5)	88.4 (87.6, 89.1)	0.4092
Basophils %	0.6 (0.42, 0.79)	0.63 (0.45, 0.81)	0.7785
ESR	6.7 (5.9, 7.6)	9 (8.2, 9.9)	0.0045
Eosinophils %	1.9 (1.6, 2.2)	2.4 (2.1, 2.8)	0.0279
Fibrinogen	312 (309, 314)	327 (325, 329)	0.1757
Hct	42.5 (42, 43)	40.3 (39.8, 40.8)	0.0007
Hb	14.8 (14.5, 15.1)	14 (13.7, 14.3)	0.0012

Table 2.5.B. Mean and 95% confidence interval (CI) for baseline study outcomes by age.

	Baseline Mean (95% CI)		p-value
	Age < 25	Age > 25	
Lymphocytes Count	1.46 (1.26, 1.66)	1.45 (1.27, 1.64)	0.9078
Lymphocytes %	33.2 (32.5, 33.9)	33.5 (32.8, 34.2)	0.6607
MCH	29.7 (29.3, 30)	29.4 (29, 29.8)	0.8026
MCHC	33.84 (33.63, 34.06)	33.84 (33.61, 34.07)	0.6618
MCV	87.7 (87.1, 88.2)	86.7 (86.1, 87.3)	0.5770
MPV	8.9 (8.7, 9.1)	8.9 (8.6, 9.1)	0.8824
Monocytes %	8 (7.7, 8.4)	7.4 (7.1, 7.8)	0.1239
Neutrophils %	56.5 (55.7, 57.2)	56.3 (55.5, 57.1)	0.7262
Platelet Count	274 (272, 276)	282 (280, 284)	0.5463
RBC Count	4.9 (4.7, 5.1)	4.7 (4.5, 4.9)	0.0072
RDW	13.2 (13, 13.5)	13.3 (13, 13.5)	0.9116
IL-1β	4 (2, 8)	3 (1, 5)	0.4288
IL-1α	21 (7, 62)	40 (16, 102)	0.3662
IL-6	15 (7, 35)	34 (19, 61)	0.1263
IL-8	29 (19, 45)	33 (24, 46)	0.6345
TNF-α	9 (6, 15)	8 (5, 13)	0.7093
CRP	1.2 (0.8, 1.7)	1.4 (1.0, 2.0)	0.5308
Cortisol	10.2 (9.0, 11.6)	9.2 (8.0, 10.5)	0.2586
Insulin	15.0 (13.1, 17.1)	12.2 (10.6, 14.1)	0.0384
Isoprostane	16.2 (10.5, 24.8)	15.4 (11.3, 21.1)	0.8572
Endotoxin	0.08 (0.08, 0.08)	0.08 (0.08, 0.09)	0.4569

Table 2.6.A. Mean and 95% confidence interval (CI) for baseline study outcomes by BMI.

	Baseline Mean (95% CI)			p-value
	BMI < 25	BMI 25-30	BMI > 30	
Age	23.7 (22.6, 24.8)	24.2 (23, 25.4)	24.6 (23.5, 25.8)	0.4528
BMI	22.4 (21.9, 23)	27.3 (26.7, 27.9)	38.3 (36.3, 40.3)	0.0000
Gingival Index	0.51 (0.47, 0.56)	0.5 (0.44, 0.56)	0.57 (0.53, 0.62)	0.0806
Plaque Index	0.33 (0.28, 0.38)	0.28 (0.24, 0.33)	0.33 (0.28, 0.39)	0.3529
Total Chemiluminescence	7 (5.5, 8.9)	6 (4.3, 8.3)	6.7 (5.2, 8.7)	0.7459
Peak Chemiluminescence	11.3 (8.6, 14.8)	10.7 (7.3, 15.8)	11.9 (9, 15.7)	0.9060
Neutrophil Count	2.7 (2.4, 3)	3.2 (2.8, 3.7)	3.6 (3.2, 4)	0.0033
WBC Count	5 (4.7, 5.4)	5.7 (5.2, 6.3)	6.3 (5.8, 6.9)	0.0005
Total Cholesterol	176 (174, 177)	183 (180, 185)	192 (190, 194)	0.2559
LDL Cholesterol	102 (100, 103)	114 (111, 116)	125 (123, 127)	0.0065
HDL Cholesterol	58 (56.9, 59.1)	53.1 (51.8, 54.4)	53.3 (52.3, 54.3)	0.1479
Triglycerides	73 (70.9, 75.1)	84.1 (81.6, 86.7)	85.4 (83.2, 87.6)	0.7962
Fasting Blood Glucose	86.7 (85.9, 87.5)	86.4 (85.3, 87.5)	89.2 (88.4, 90)	0.1099
Basophils %	0.73 (0.52, 0.94)	0.67 (0.41, 0.92)	0.48 (0.27, 0.69)	0.0407
ESR	3.5 (2.9, 4.2)	5.1 (4.2, 5.9)	13.6 (12.5, 14.7)	0.0000
Eosinophils %	2.5 (2.1, 3)	2.3 (1.8, 2.8)	1.8 (1.5, 2.1)	0.1717
Fibrinogen	263 (261, 266)	310 (306, 313)	376 (373, 379)	0.0000
Hct	42.1 (41.6, 42.7)	41.6 (40.9, 42.4)	40.7 (40.1, 41.2)	0.1940
Hb	14.7 (14.3, 15)	14.5 (14.1, 15)	14 (13.7, 14.4)	0.0762

Table 2.6.B.Mean and 95% confidence interval (CI) for baseline study outcomes by BMI.

	Baseline Mean (95% CI)			p-value
	BMI < 25	BMI 25-30	BMI > 30	
Lymphocytes Count	1.31 (1.11, 1.52)	1.4 (1.11, 1.69)	1.62 (1.39, 1.85)	0.0466
Lymphocytes %	34.2 (33.3, 35)	33 (32, 34)	32.9 (32.1, 33.7)	0.7212
MCH	30.3 (29.9, 30.7)	29.6 (29.1, 30.1)	28.8 (28.4, 29.3)	0.0031
MCHC	33.96 (33.7, 34.21)	34.03 (33.74, 34.33)	33.62 (33.35, 33.89)	0.0761
MCV	89 (88.4, 89.6)	87.2 (86.4, 88.1)	85.5 (84.9, 86.2)	0.0025
MPV	8.9 (8.6, 9.2)	8.8 (8.5, 9.1)	8.9 (8.6, 9.2)	0.7724
Monocytes %	8.2 (7.7, 8.6)	7.6 (7.1, 8.1)	7.4 (7, 7.9)	0.2477
Neutrophils %	54.8 (53.9, 55.7)	56.8 (55.7, 57.9)	57.6 (56.8, 58.5)	0.2774
Platelet Count	266 (264, 268)	263 (260, 266)	298 (296, 300)	0.0124
RBC Count	4.8 (4.6, 5)	4.8 (4.6, 5.1)	4.8 (4.6, 5)	0.9863
RDW	12.9 (12.6, 13.1)	13.1 (12.8, 13.5)	13.6 (13.3, 13.9)	0.0002
IL-1β	4 (2, 9)	1 (0, 4)	4 (2, 9)	0.1853
IL-1α	23 (7, 77)	55 (14, 213)	24 (7, 81)	0.6010
IL-6	20 (9, 48)	35 (14, 85)	20 (8, 47)	0.6515
IL-8	31 (21, 46)	39 (25, 62)	27 (16, 46)	0.5806
TNF-α	7 (4, 13)	8 (5, 15)	10 (6, 18)	0.6292
CRP	0.6 (0.4, 0.9)	1.2 (0.7, 1.9)	2.6 (1.8, 3.6)	0.0000
Cortisol	10.4 (8.8, 12.3)	9.4 (7.8, 11.3)	9.3 (8.0, 10.7)	0.5233
Insulin	10.2 (8.9, 11.8)	12.3 (10.2, 14.8)	18.2 (15.7, 21.1)	0.0000
Isoprostane	22.4 (15.6, 32.2)	13.6 (7.8, 23.9)	12.8 (8.1, 20.3)	0.1523
Endotoxin	0.08 (0.08, 0.08)	0.08 (0.08, 0.08)	0.08 (0.08, 0.09)	0.3363

Table 2.7.A. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in black females (BF) and black males (BM).

	Changes during experimental phase		p-value	
	BF	BM	BF	BM
Gingival Index	0.62 (0.55, 0.69)	0.61 (0.55, 0.66)	0.0000	0.0000
Plaque Index	1.64 (1.51, 1.77)	1.54 (1.42, 1.67)	0.0000	0.0000
Total Chemiluminescence	1.3 (-2.3, 4.9)	2.9 (-0.4, 6.2)	0.4573	0.0790
Peak Chemiluminescence	2 (-4.1, 8.1)	6.4 (0, 12.7)	0.5009	0.0492
Neutrophil Count	0.1 (-0.2, 0.4)	0.1 (-0.2, 0.4)	0.4628	0.3750
WBC Count	0 (-0.3, 0.4)	0.1 (-0.2, 0.5)	0.8034	0.5005
Total Cholesterol	-9.1 (-10.8, -7.4)	-9 (-10.6, -7.4)	0.0166	0.0084
LDL Cholesterol	-4.3 (-5.8, -2.9)	-6 (-7.4, -4.6)	0.1636	0.0446
HDL Cholesterol	-1.3 (-2.1, -0.5)	-3.3 (-4.2, -2.5)	0.0987	0.0037
Triglycerides	5 (3.4, 6.6)	6.6 (4, 9.2)	0.2406	0.5154
Fasting Blood Glucose	-1.7 (-2.7, -0.7)	0.7 (-0.4, 1.7)	0.0747	0.9313
Basophils %	0 (-0.25, 0.25)	-0.06 (-0.33, 0.21)	1.0000	0.7539
ESR Bld Qn	0.1 (-0.9, 1)	1.2 (0.6, 1.8)	0.5695	0.0215
Eosinophils %	-0.2 (-0.6, 0.2)	-0.2 (-0.6, 0.3)	0.4465	0.3828
Fibrinogen	-2.9 (-5.8, 0.1)	14 (11.2, 16.9)	0.8346	0.8766
Hct	-0.5 (-1.1, 0.1)	-0.7 (-1.2, -0.2)	0.1990	0.0528
Hb	-0.13 (-0.47, 0.2)	-0.23 (-0.51, 0.04)	0.3063	0.0391
Lymphocytes Count	-0.15 (-0.44, 0.14)	0 (-0.26, 0.26)	0.3125	1.0000
Lymphocytes %	-1 (-2, 0)	-0.9 (-1.8, -0.1)	0.6473	0.3828
MCH	0.22 (-0.09, 0.54)	0.13 (-0.17, 0.42)	0.1460	0.4240
MCHC	0.11 (-0.17, 0.39)	0.13 (-0.19, 0.44)	0.4531	0.4850

Table 2.7.B. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in black females (BF) and black males (BM).

	Changes during experimental phase		p-value	
	BF	BM	BF	BM
MCV	0.15 (-0.19, 0.48)	0.28 (-0.07, 0.63)	0.4240	0.0949
MPV	0.14 (-0.09, 0.37)	0.18 (-0.04, 0.4)	0.0501	0.0061
Monocytes %	-0.2 (-0.8, 0.4)	0 (-0.5, 0.4)	0.3753	0.7298
Neutrophils %	1.4 (0.3, 2.4)	1.1 (0.3, 2)	0.3130	0.3155
Platelet Count	0.3 (-1.8, 2.3)	-3.4 (-5.4, -1.4)	0.8793	0.0772
RBC Count	-0.07 (-0.26, 0.12)	-0.1 (-0.26, 0.06)	0.0726	0.0110
RDW	0 (-0.25, 0.25)	-0.03 (-0.22, 0.15)	0.9557	0.6015
IL-1β	-0.8 (-1.7, 0.8)	0.1 (-0.6, 1.3)	0.2733	0.7847
IL-1α	3.8 (-1.9, 13.1)	7.7 (-0.9, 21.3)	0.2318	0.0882
IL-6	-0.2 (-2.9, 3.8)	-2.6 (-6.1, 2.6)	0.8902	0.2848
IL-8	3.9 (-0.9, 10)	2.2 (-4, 10.1)	0.1178	0.5216
TNF-α	-0.3 (-1.5, 1.5)	0.7 (-1.8, 4.1)	0.7222	0.6203
CRP	-0.7 (-1.4, 0.3)	0 (-0.2, 0.3)	0.1548	0.7163
Cortisol	1.2 (-0.7, 3.5)	0.3 (-1.3, 2.2)	0.2170	0.6991
Insulin	0.6 (-1.5, 3)	0.8 (-1.1, 2.9)	0.6046	0.4391
Isoprostane	1.9 (-4.3, 10.4)	-9.9 (-15.6, -2.1)	0.5951	0.0169
Endotoxin	0.04 (0.01, 0.08)	0.01 (-0.01, 0.04)	0.0168	0.3951

Table 2.8.A. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in white females (WF) and white males (WM).

	Changes during experimental phase		p-value	
	WF	WM	WF	WM
Gingival Index	0.63 (0.57, 0.69)	0.66 (0.59, 0.74)	0.0000	0.0000
Plaque Index	1.63 (1.5, 1.76)	1.69 (1.55, 1.83)	0.0000	0.0000
Total Chemiluminescence	-0.7 (-5, 3.5)	-1.3 (-3.7, 1.1)	0.7314	0.2654
Peak Chemiluminescence	-0.7 (-8.1, 6.8)	-1.7 (-5.8, 2.3)	0.8584	0.3820
Neutrophil Count	-0.1 (-0.7, 0.5)	0 (-0.1, 0.2)	0.7252	0.7074
WBC Count	-0.1 (-0.7, 0.5)	0 (-0.3, 0.3)	0.7359	0.9807
Total Cholesterol	-3.6 (-5, -2.2)	0.9 (-0.7, 2.5)	0.1613	0.9920
LDL Cholesterol	-2.7 (-4, -1.5)	3.5 (2.1, 4.8)	0.2765	0.2456
HDL Cholesterol	-1.7 (-2.6, -0.7)	1.7 (0.7, 2.6)	0.1331	0.0739
Triglycerides	-0.9 (-3.2, 1.5)	-19.6 (-23.4, -15.8)	0.6756	0.6954
Fasting Blood Glucose	-0.5 (-1.3, 0.3)	1.1 (0.2, 2.1)	0.4931	0.4861
Basophils %	0.15 (-0.11, 0.41)	-0.03 (-0.27, 0.2)	0.2266	1.0000
ESR	0.8 (-0.1, 1.8)	0.1 (-0.3, 0.6)	0.5671	0.6270
Eosinophils %	0.1 (-0.2, 0.4)	0.1 (-0.3, 0.4)	0.3954	0.6536
Fibrinogen	5.3 (2.4, 8.2)	15.7 (13.6, 17.8)	0.6695	0.0106
Hct	-0.2 (-0.6, 0.3)	0.4 (0, 0.9)	0.7162	0.1308
Hb	-0.11 (-0.38, 0.16)	0.1 (-0.16, 0.36)	0.4595	0.2717
Lymphocytes Count	0 (-0.23, 0.23)	-0.06 (-0.34, 0.21)	1.0000	0.7539
Lymphocytes %	1.3 (0.4, 2.2)	-1.1 (-1.9, -0.3)	0.2533	0.2666

Table 2.8.B. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in white females (WF) and white males (WM).

	Changes during experimental phase		p-value	
	WF	WM	WF	WM
MCH	0.03 (-0.22, 0.28)	0.03 (-0.25, 0.32)	1.0000	1.0000
MCHC	0 (-0.26, 0.26)	-0.13 (-0.45, 0.19)	1.0000	0.4850
MCV	0.03 (-0.3, 0.35)	0.39 (-0.01, 0.79)	0.8734	0.0946
MPV	-0.01 (-0.2, 0.17)	0.06 (-0.16, 0.27)	0.9597	0.3943
Monocytes %	0.1 (-0.3, 0.6)	0 (-0.5, 0.5)	0.3966	0.6463
Neutrophils %	-1.5 (-2.5, -0.5)	0.9 (0.1, 1.8)	0.2262	0.4086
Platelet Count	3.1 (1.3, 4.9)	3.3 (1.5, 5)	0.3677	0.2041
RBC Count	-0.03 (-0.19, 0.12)	0.03 (-0.11, 0.17)	0.4887	0.1856
RDW	-0.01 (-0.23, 0.21)	-0.03 (-0.26, 0.2)	0.8156	0.6944
IL-1β	-0.2 (-0.9, 0.8)	-2 (-3, -0.5)	0.6342	0.0151
IL-1α	-3.7 (-13.2, 11.3)	9.1 (-15.7, 48.8)	0.5627	0.5343
IL-6	-12.8 (-21.4, -0.3)	13.1 (-6.7, 42.2)	0.0453	0.2237
IL-8	-6.8 (-14.4, 2.8)	4.4 (-7.3, 19.3)	0.1498	0.4919
TNF-α	0.1 (-1.7, 2.3)	1.5 (-1.7, 5.7)	0.9591	0.3983
CRP	-0.2 (-0.8, 0.6)	0.1 (-0.2, 0.5)	0.5656	0.6963
Cortisol	2.1 (0.4, 4.1)	1.5 (-0.4, 3.7)	0.0160	0.1341
Insulin	0.3 (-1.5, 2.3)	0.6 (-1, 2.3)	0.7694	0.5017
Isoprostane	7.4 (1.3, 15.7)	-1.0 (-7.1, 7.4)	0.0149	0.7935
Endotoxin	0.08 (0.04, 0.13)	0.05 (0.02, 0.10)	0.0000	0.0014

Table 2.9.A. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in black (B) and white (W) subjects.

	Changes during experimental phase		p-value	
	B	W	B	W
Gingival Index	0.61 (0.57, 0.66)	0.65 (0.6, 0.69)	0.0000	0.0000
Plaque Index	1.59 (1.5, 1.68)	1.66 (1.57, 1.75)	0.0000	0.0000
Total Chemiluminescence Peak	2.2 (-0.1, 4.6)	-1 (-3.4, 1.4)	0.0639	0.3980
Chemiluminescence	4.5 (0.1, 8.8)	-1.2 (-5.4, 3)	0.0442	0.5681
Neutrophil Count	0.1 (-0.1, 0.3)	0 (-0.4, 0.3)	0.2453	0.8065
WBC Count	0.1 (-0.2, 0.4)	-0.1 (-0.4, 0.3)	0.4972	0.7596
Total Cholesterol	-9 (-10.2, -7.9)	-1.5 (-2.5, -0.4)	0.0003	0.3115
LDL Cholesterol	-5.2 (-6.2, -4.2)	0.2 (-0.7, 1.1)	0.0137	0.9973
HDL Cholesterol	-2.4 (-3, -1.8)	-0.1 (-0.8, 0.6)	0.0007	0.9184
Triglycerides	5.8 (4.2, 7.5)	-9.8 (-12, -7.6)	0.2221	0.5828
Fasting Blood Glucose	-0.4 (-1.2, 0.3)	0.3 (-0.4, 0.9)	0.2627	0.9567
Basophils %	-0.03 (-0.22, 0.15)	0.06 (-0.11, 0.24)	0.7905	0.4545
ESR	0.7 (0.1, 1.2)	0.5 (-0.1, 1.1)	0.0580	0.4503
Eosinophils %	-0.2 (-0.5, 0.1)	0.1 (-0.1, 0.3)	0.1851	0.2932
Fibrinogen	6.3 (4.3, 8.3)	10.1 (8.3, 12)	0.8140	0.1176
Hct	-0.6 (-1, -0.2)	0.1 (-0.2, 0.4)	0.0184	0.4676
Hb	-0.19 (-0.4, 0.02)	-0.01 (-0.19, 0.18)	0.0216	0.8815
Lymphocytes %	-1 (-1.6, -0.3)	0.2 (-0.4, 0.8)	0.3459	0.8384

Table 2.9.B. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in black (B) and white (W) subjects.

	Changes during experimental phase		p-value	
	B	W	B	W
MCH	0.17 (-0.04, 0.38)	0.03 (-0.15, 0.22)	0.0476	0.8238
MCHC	0.12 (-0.09, 0.33)	-0.06 (-0.26, 0.14)	0.1665	0.4610
MCV	0.22 (-0.02, 0.46)	0.2 (-0.05, 0.45)	0.0480	0.1383
MPV	0.16 (0.01, 0.32)	0.02 (-0.12, 0.16)	0.0007	0.4345
Monocytes %	-0.1 (-0.5, 0.2)	0.1 (-0.2, 0.4)	0.3741	0.7600
Neutrophils %	1.2 (0.6, 1.9)	-0.4 (-1, 0.3)	0.1515	0.6746
Platelet Count	-1.7 (-3.1, -0.3)	3.2 (2, 4.4)	0.2981	0.1243
RBC Count	-0.09 (-0.21, 0.03)	0 (-0.11, 0.1)	0.0018	0.7274
RDW	-0.02 (-0.17, 0.13)	-0.02 (-0.17, 0.14)	0.6740	0.9234
IL-1β	-0.2 (-0.8, 0.7)	-0.9 (-1.5, 0)	0.6077	0.0425
IL-1α	5.4 (0.2, 12.7)	0.2 (-11.2, 16.1)	0.0426	0.9731
IL-6	-1.3 (-3.6, 1.7)	-3.3 (-13.1, 9.5)	0.3569	0.5705
IL-8	3.1 (-0.9, 7.8)	-2.1 (-9, 6.1)	0.1351	0.5862
TNF-α	0.1 (-1.3, 1.7)	0.6 (-1.1, 2.7)	0.9323	0.5186
CRP	-0.1 (-0.4, 0.2)	0 (-0.3, 0.4)	0.4418	0.9168
Cortisol	0.8 (-0.5, 2.2)	1.8 (0.5, 3.2)	0.2485	0.0061
Insulin	0.7 (-0.8, 2.2)	0.4 (-0.8, 1.8)	0.3692	0.4908
Isoprostane	-3.4 (-7.8, 2.0)	3.5 (-1.0, 9.1)	0.1955	0.1328
Endotoxin	0.02 (0.00, 0.05)	0.06 (0.04, 0.10)	0.0203	0.0000

Table 2.10.A. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in male and female subjects.

	Changes during experimental phase		p-value	
	Males	Females	Males	Females
Gingival Index	0.63 (0.59, 0.68)	0.63 (0.58, 0.67)	0.0000	0.0000
Plaque Index	1.61 (1.52, 1.71)	1.64 (1.55, 1.73)	0.0000	0.0000
Total Chemiluminescence	0.8 (-1.2, 2.9)	0.2 (-2.6, 2.9)	0.4190	0.9075
Peak Chemiluminescence	2.4 (-1.4, 6.2)	0.5 (-4.3, 5.3)	0.2186	0.8370
Neutrophil Count	0.1 (-0.1, 0.2)	0 (-0.4, 0.4)	0.3363	0.9725
WBC Count	0.1 (-0.2, 0.3)	0 (-0.4, 0.3)	0.5669	0.8517
Total Cholesterol	-4.1 (-5.2, -3)	-6.1 (-7.2, -5.1)	0.0567	0.0062
LDL Cholesterol	-1.3 (-2.3, -0.3)	-3.5 (-4.4, -2.5)	0.5908	0.0649
HDL Cholesterol	-0.9 (-1.5, -0.2)	-1.5 (-2.1, -0.9)	0.4026	0.0232
Triglycerides	-6.3 (-8.6, -4)	1.8 (0.3, 3.3)	0.8980	0.7478
Fasting Blood Glucose	0.9 (0.2, 1.6)	-1 (-1.7, -0.4)	0.5452	0.0913
Basophils %	-0.05 (-0.22, 0.13)	0.08 (-0.1, 0.26)	0.6072	0.3018
ESR	0.7 (0.3, 1.1)	0.5 (-0.2, 1.2)	0.0307	0.3748
Eosinophils %	0 (-0.3, 0.2)	0 (-0.3, 0.2)	0.6012	0.9910
Fibrinogen	14.8 (13, 16.6)	1.7 (-0.4, 3.7)	0.1065	0.8727
Hct	-0.1 (-0.5, 0.2)	-0.3 (-0.7, 0)	0.6566	0.2249
Hb	-0.07 (-0.26, 0.12)	-0.12 (-0.33, 0.09)	0.4593	0.1773
Lymphocytes Count	-0.03 (-0.22, 0.15)	-0.07 (-0.24, 0.11)	0.8145	0.4316

Table 2.10.B. Mean and 95% confidence interval for changes in study outcomes during the experimental phase of the study in male and female subjects.

	Changes during experimental phase		p-value	
	Males	Females	Males	Females
Lymphocytes %	-1 (-1.6, -0.4)	0.3 (-0.4, 1)	0.1554	0.5655
MCH	0.08 (-0.12, 0.28)	0.11 (-0.08, 0.31)	0.3273	0.1295
MCHC	0 (-0.22, 0.22)	0.05 (-0.14, 0.24)	1.0000	0.6291
MCV	0.33 (0.07, 0.59)	0.08 (-0.15, 0.31)	0.0160	0.4312
MPV	0.12 (-0.03, 0.27)	0.05 (-0.09, 0.2)	0.0103	0.1651
Monocytes %	0 (-0.3, 0.3)	0 (-0.4, 0.3)	0.5375	0.9953
Neutrophils %	1 (0.4, 1.6)	-0.2 (-1, 0.5)	0.1917	0.7391
Platelet Count	-0.1 (-1.4, 1.2)	1.9 (0.5, 3.2)	0.7159	0.4393
RBC Count	-0.04 (-0.15, 0.07)	-0.05 (-0.17, 0.07)	0.2180	0.0703
RDW	-0.03 (-0.18, 0.11)	0 (-0.17, 0.16)	0.4830	0.8926
IL-1β	-0.7 (-1.3, 0.2)	-0.4 (-1, 0.4)	0.1278	0.3170
IL-1α	9.4 (-1.6, 24.6)	1.5 (-4, 9.2)	0.1012	0.6318
IL-6	0.3 (-5.9, 8.6)	-3.7 (-7.3, 1.1)	0.9251	0.1168
IL-8	3 (-3.2, 10.5)	0.2 (-4.2, 5.5)	0.3592	0.9303
TNF-α	1.1 (-1, 3.6)	-0.1 (-1.2, 1.2)	0.3405	0.8244
CRP	0.1 (-0.1, 0.3)	-0.4 (-0.9, 0.2)	0.5892	0.1593
Cortisol	0.9 (-0.4, 2.3)	1.6 (0.4, 3.1)	0.1794	0.0117
Insulin	0.6 (-0.6, 2)	0.4 (-1, 1.9)	0.3059	0.5676
Isoprostane	-5.2 (-9.5, 0.2)	4.7 (0.2, 10.3)	0.0591	0.0411
Endotoxin	0.03 (0.01, 0.06)	0.06 (0.03, 0.09)	0.0039	0.0000

Table 2.11. Summary of statistically significant changes ($p<0.05$) from the beginning to the end of the experimental phase of the study.

Significant Increases*
• Study subjects collectively: Endotoxin (LPS), cortisol, mean corpuscular volume, mean platelet volume • Blacks: total and peak chemiluminescence, mean corpuscular hematocrit, mean corpuscular volume, mean platelet volume, Interleukin (IL)-1α • Males: mean corpuscular volume, mean platelet volume • Black males: erythrocyte sedimentation rate, mean platelet volume • White males: fibrinogen • White females: 8-isoprostane
Significant Decreases
• Study subjects collectively: total cholesterol, high density lipoprotein, red blood cell count • Blacks: total cholesterol, low density lipoprotein, high density lipoprotein, hematocrit, hemoglobin, red blood cell count • Females: total cholesterol, high density lipoprotein • Black females: total cholesterol • Black males: low density lipoprotein, high density lipoprotein, hemoglobin, red blood cell count, 8-isoprostane • White males: IL-1β • White females: IL-6

* Plaque index and gingival index increased significantly ($p<0.05$) from the beginning to the end of the experimental phase in all study groups.

Figure 2.1. Flow chart of the study entitled "Dental Plaque Accumulation as a Risk Factor for CHD"

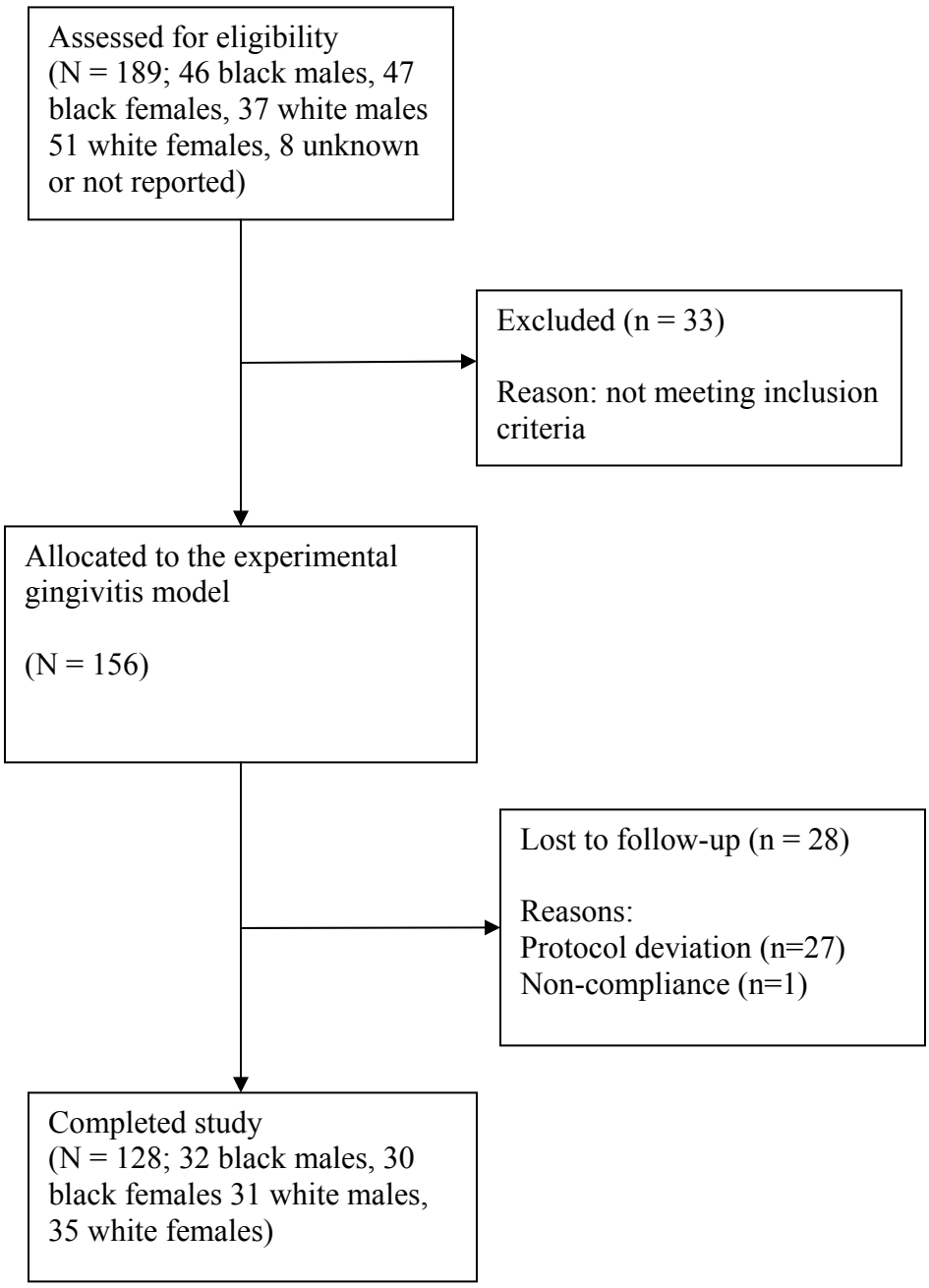


Figure 2.2. Mean (± 95% confidence interval) plaque index over the course of the study (N = 128).

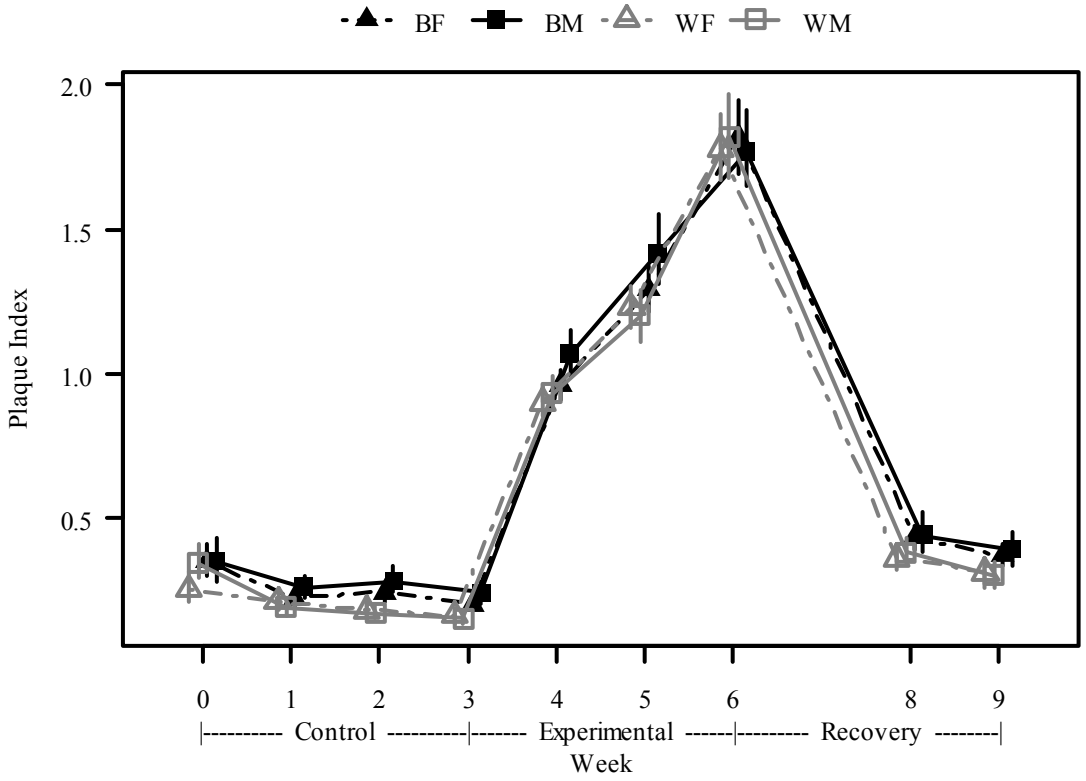


Figure 2.3. Mean (± 95% confidence interval) gingival index over the course of the study (N = 128).

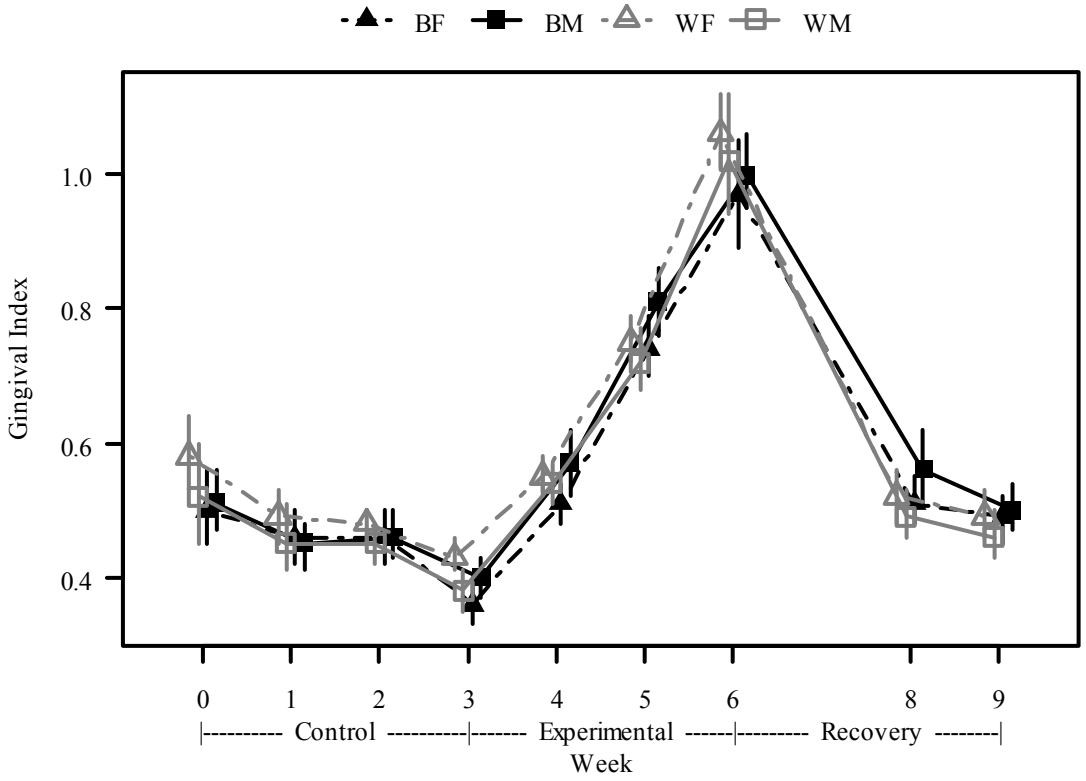


Figure 2.4. Mean (± 95% confidence interval) white blood cell count (x10^9 cells/L) over the course of the study (N = 128).

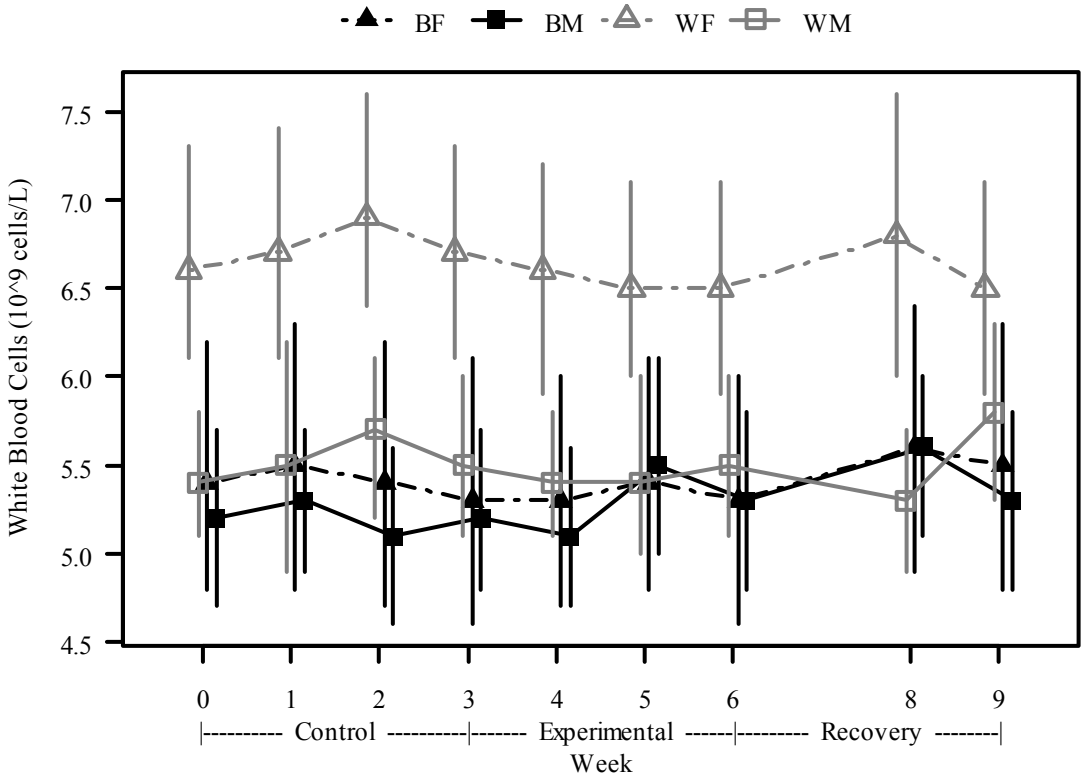


Figure 2.5. Mean (± 95% confidence interval) peripheral blood neutrophil count (x10^9 cells/L) over the course of the study (N = 128).

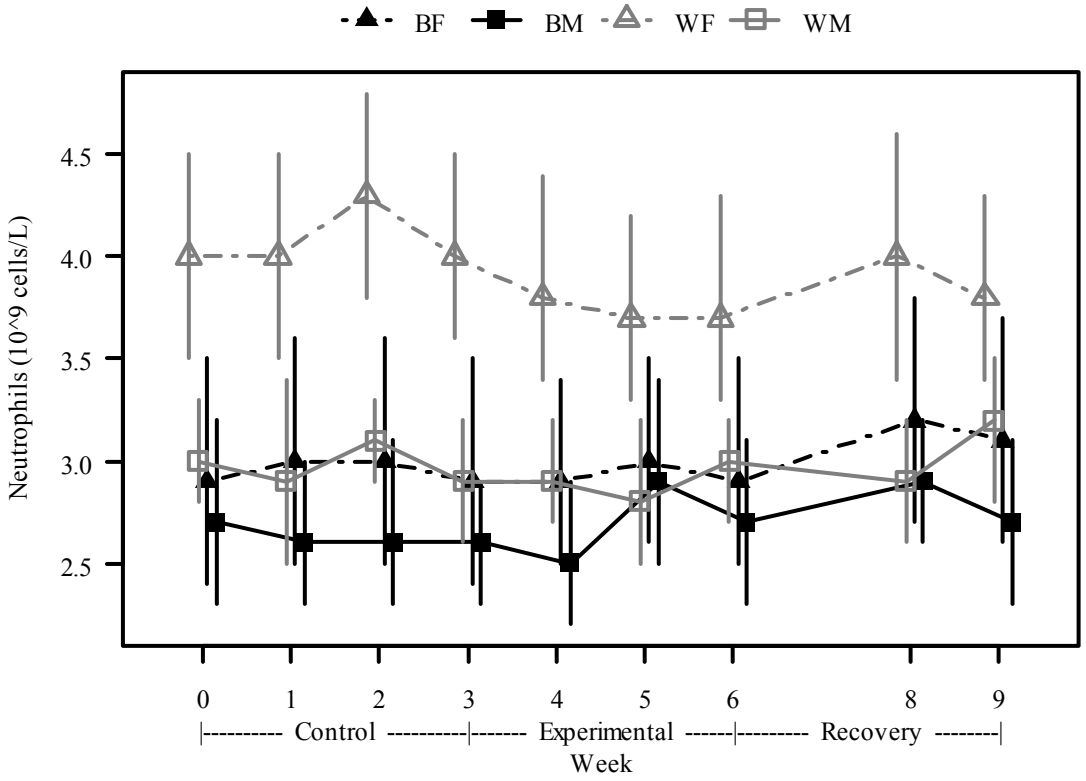


Figure 2.6. Mean (± 95% confidence interval) red blood cell count (x10^{12} cells/L) over the course of the study (N = 128).

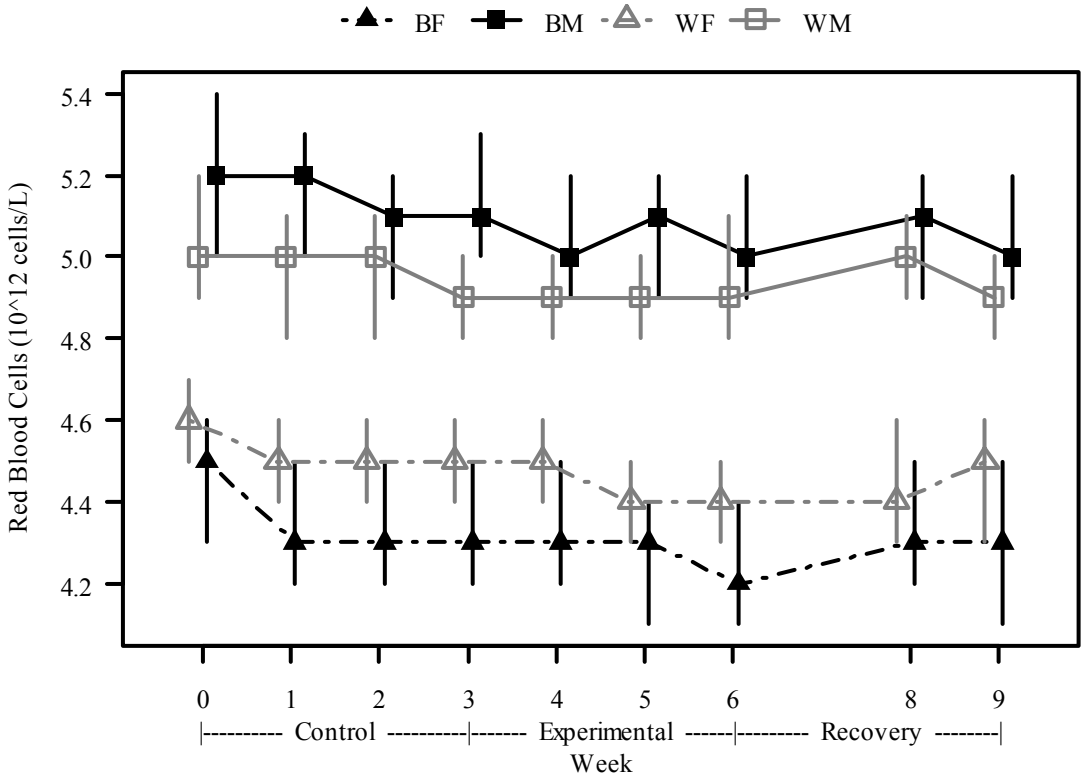


Figure 2.7. Mean (± 95% confidence interval) blood hemoglobin level (g/dl) over the course of the study (N = 128).

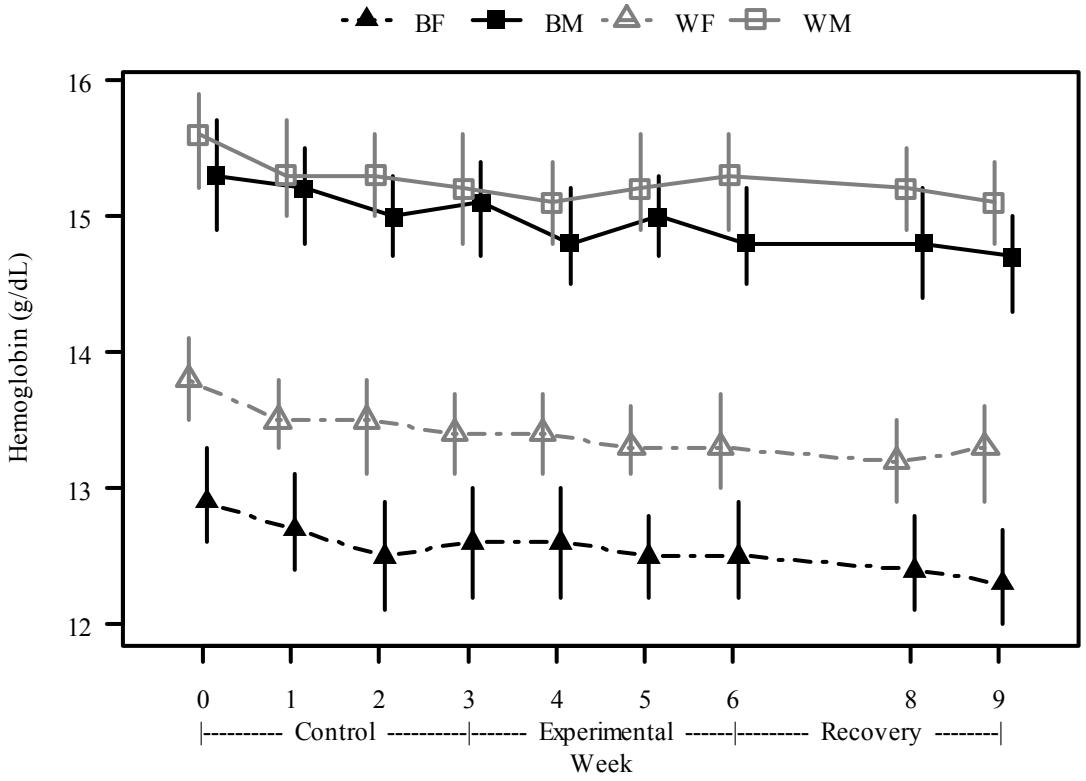


Figure 2.8. Mean (± 95% confidence interval) blood hematocrit (%) over the course of the study (N = 128).

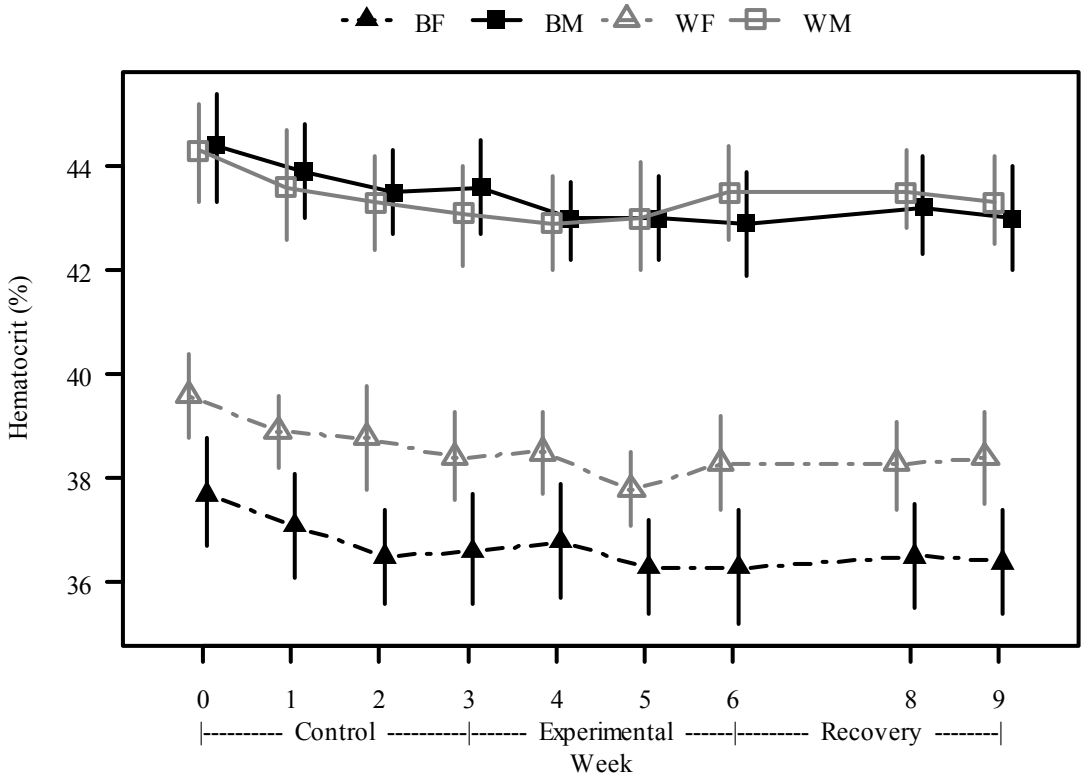


Figure 2.9. Mean (± 95% confidence interval) mean corpuscular volume (MCV) (femtoliters/cell) over the course of the study (N = 128).

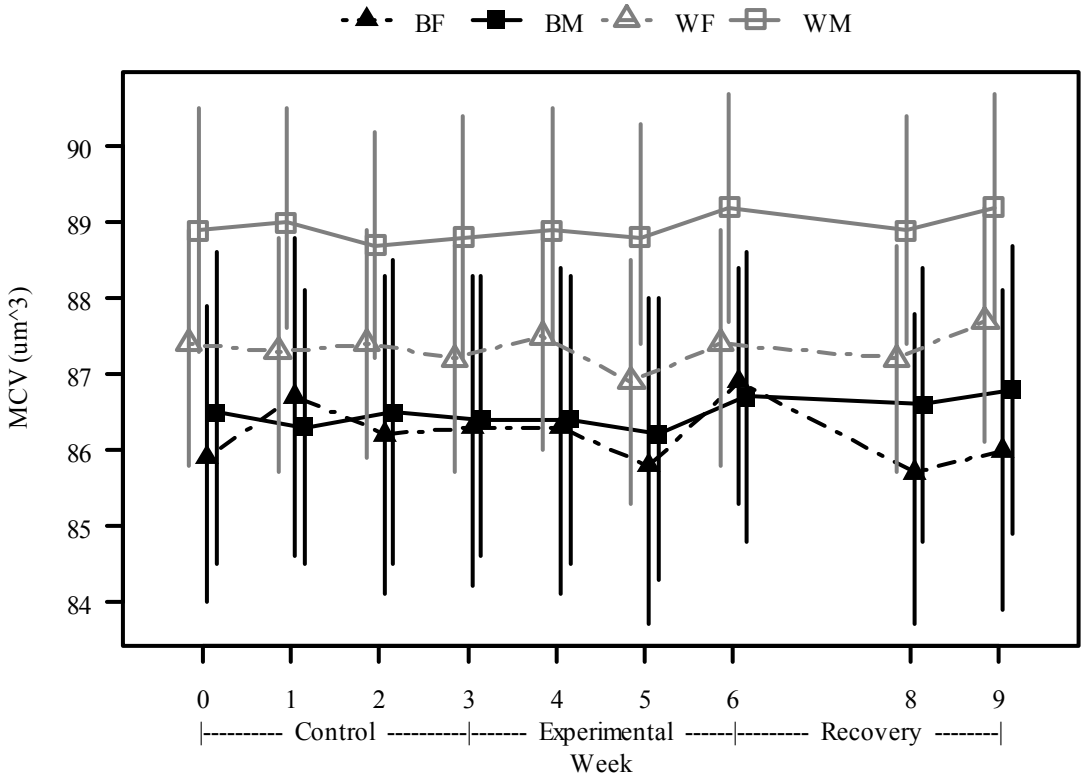


Figure 2.10. Mean (± 95% confidence interval) mean platelet volume (MPV) (femtoliers/cell) over the course of the study (N = 128).

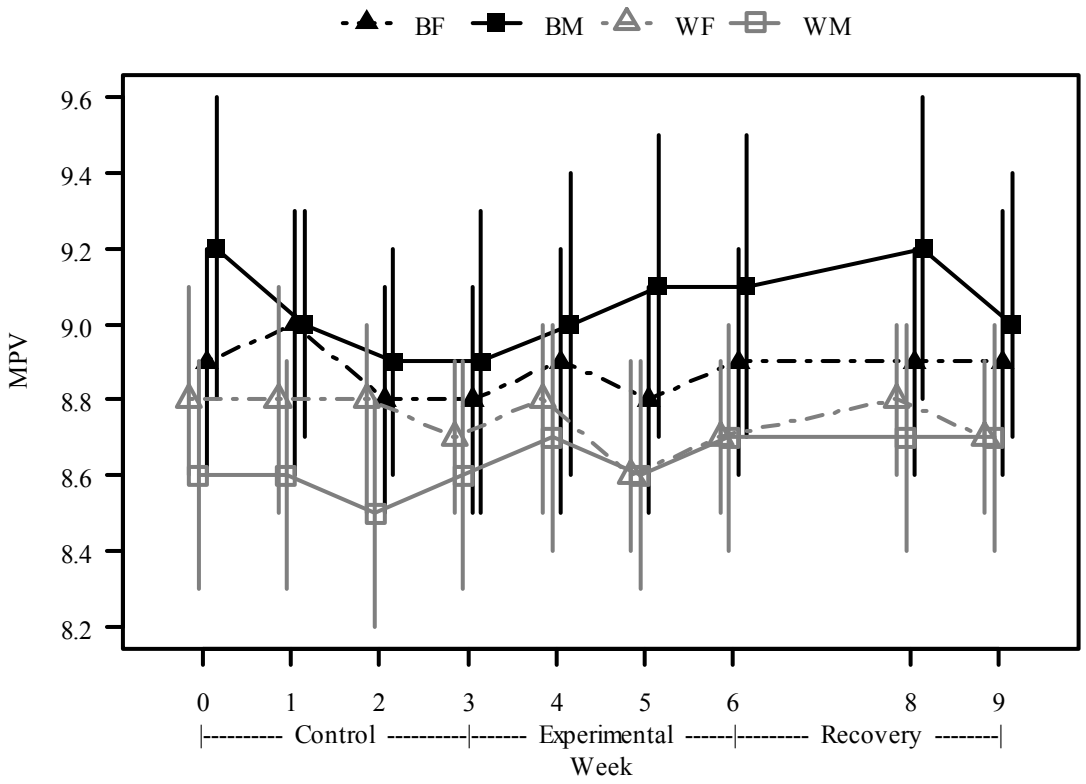


Figure 2.11. Mean (± 95% confidence interval) mean corpuscular hemoglobin (MCH) (pg/cell) over the course of the study (N = 128).

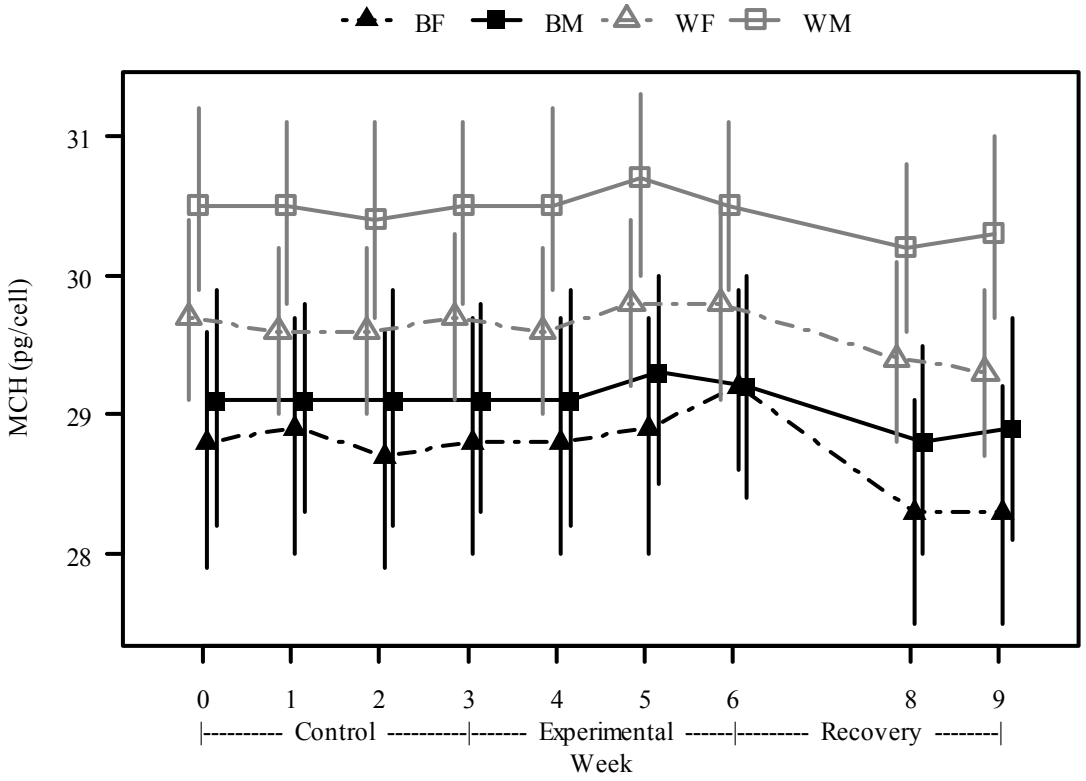


Figure 2.12. Mean (± 95% confidence interval) erythrocyte sedimentation rate (ESR) (mm/hr) over the course of the study (N = 128).

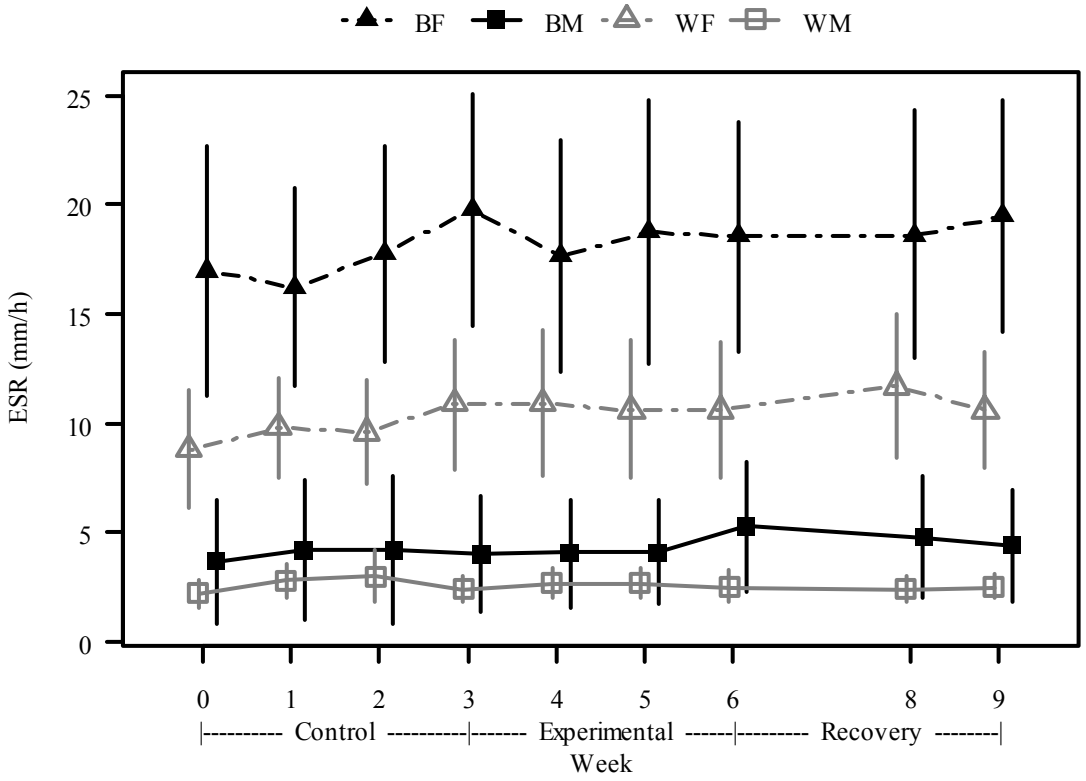


Figure 2.13. Mean (± 95% confidence interval) peripheral blood neutrophil total chemiluminescence (millivoltage.min) over the course of the study (N = 128).

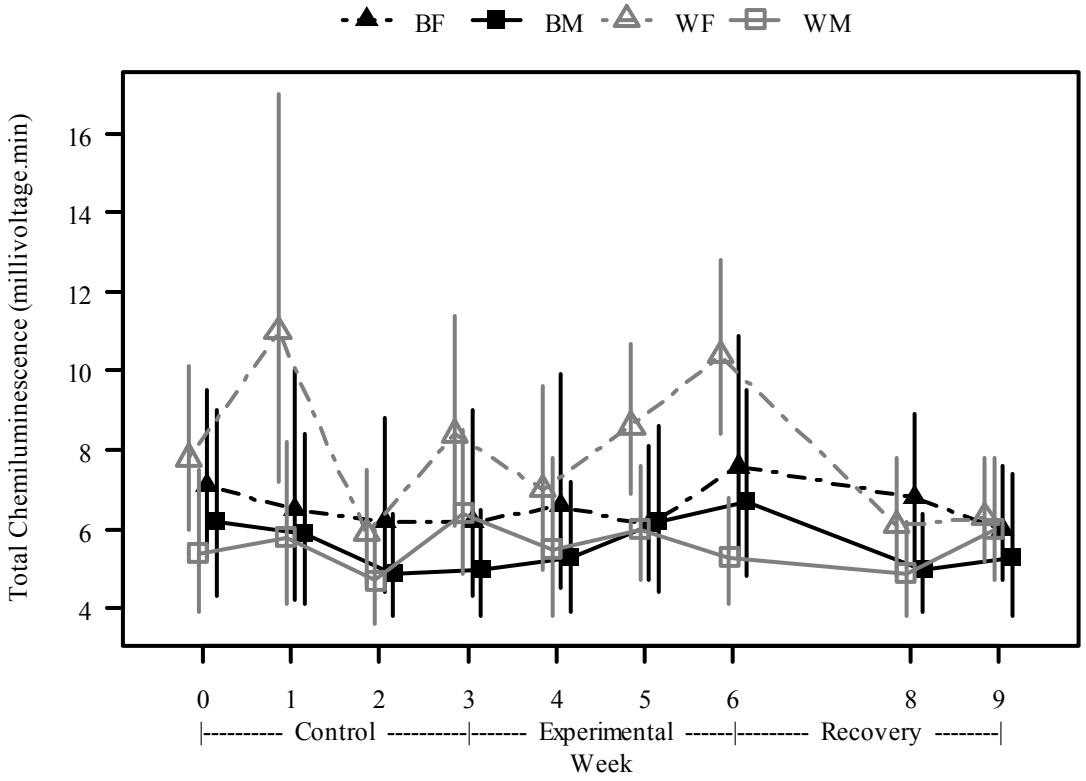


Figure 2.14. Mean (± 95% confidence interval) peripheral blood neutrophil peak chemiluminescence (millivoltage) over the course of the study (N = 128).

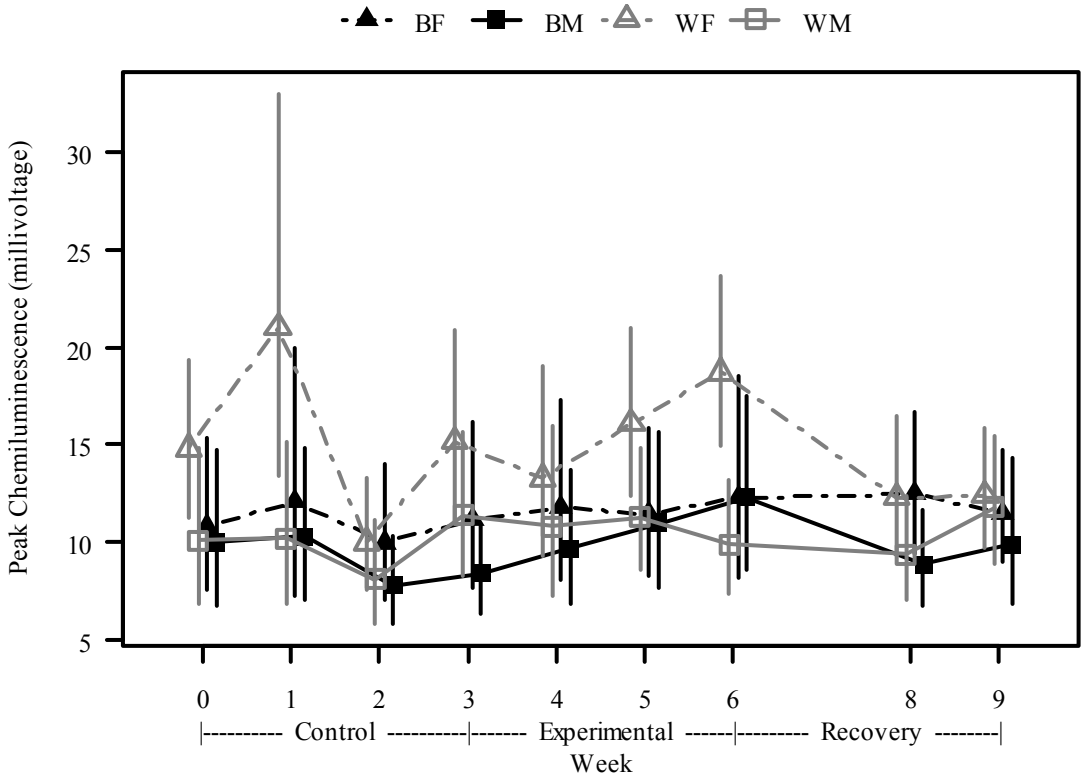


Figure 2.15. Mean (± 95% confidence interval) blood interleukin-1 alpha (IL-1 alpha) level (pg/ml) over the course of the study (N = 128).

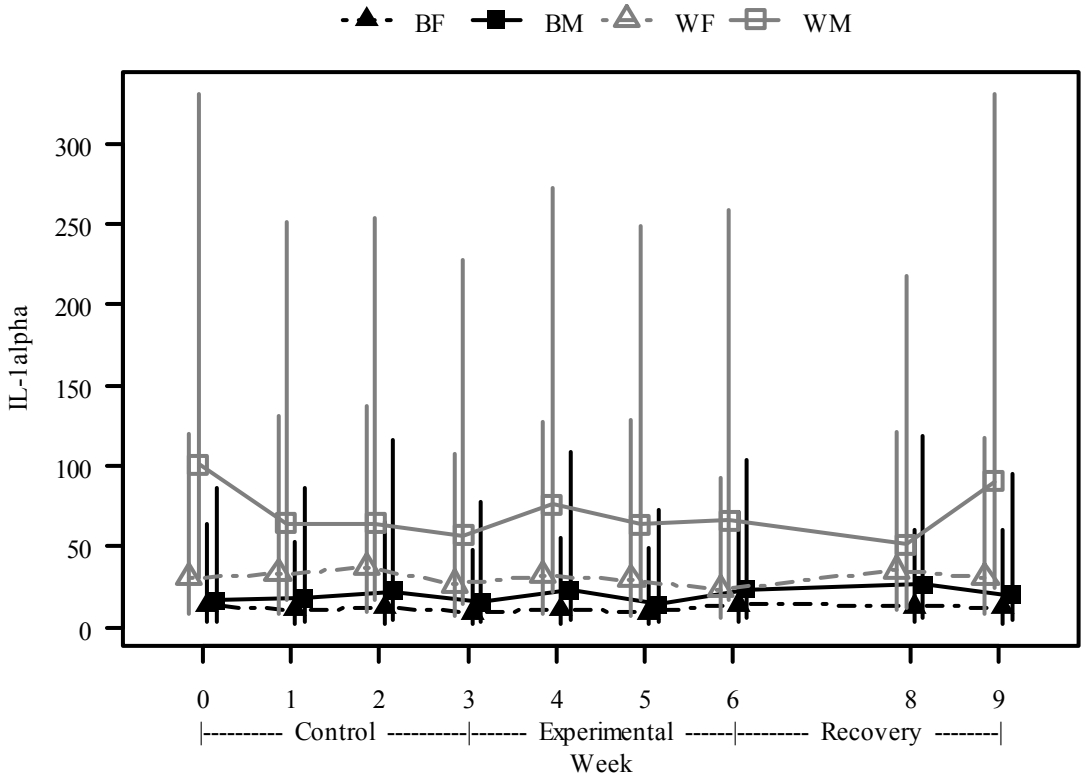


Figure 2.16. Mean (± 95% confidence interval) blood interleukin-1 beta (IL-1 beta) level (pg/ml) over the course of the study (N = 128).

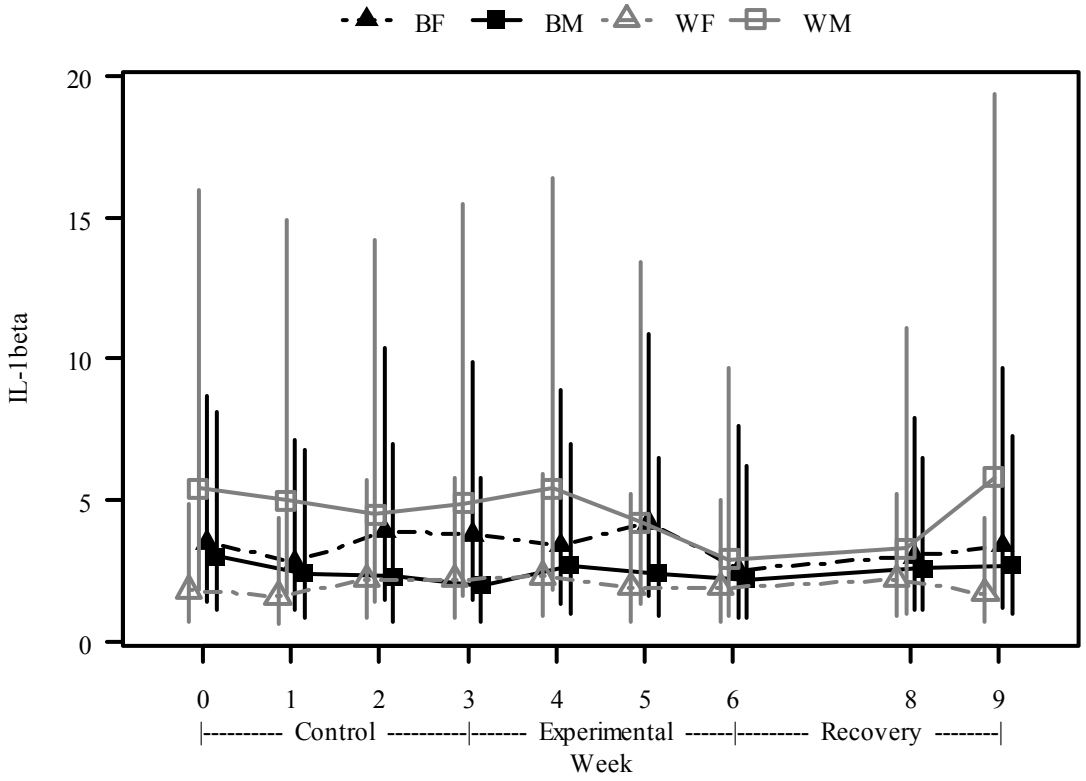


Figure 2.17. Mean (± 95% confidence interval) blood interleukin-6 (IL-6) level (pg/ml) over the course of the study (N = 128).

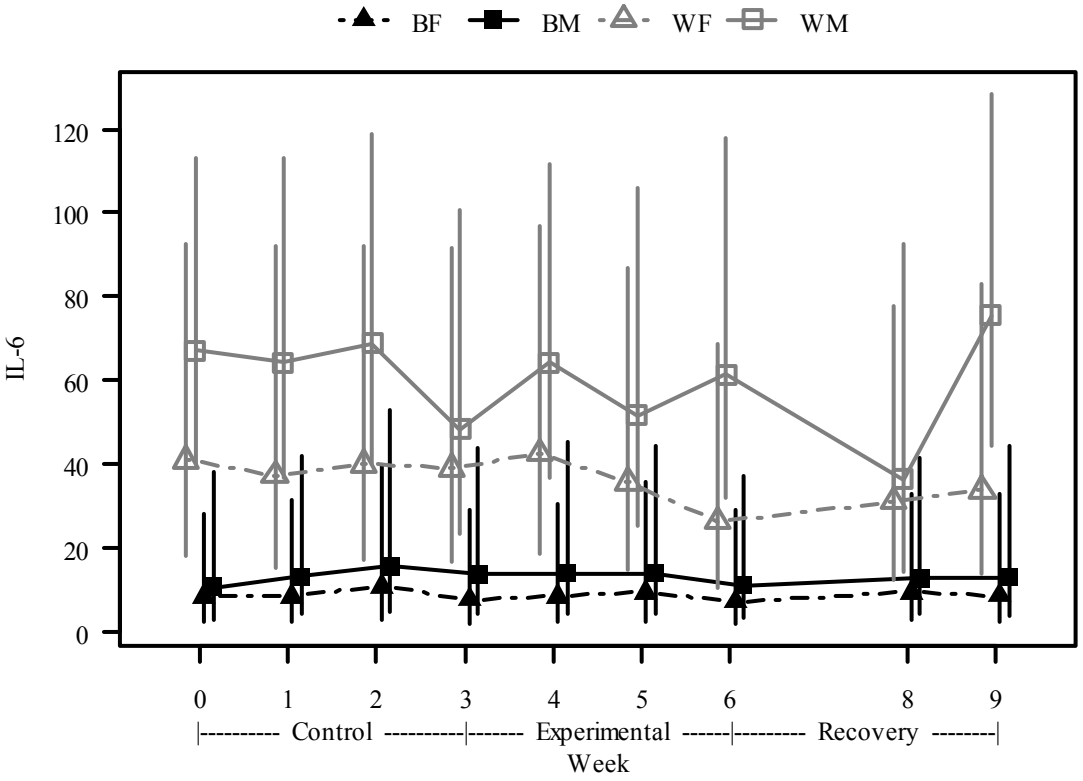


Figure 2.18. Mean (± 95% confidence interval) blood fibrinogen level (mg/dl) over the course of the study (N = 128).

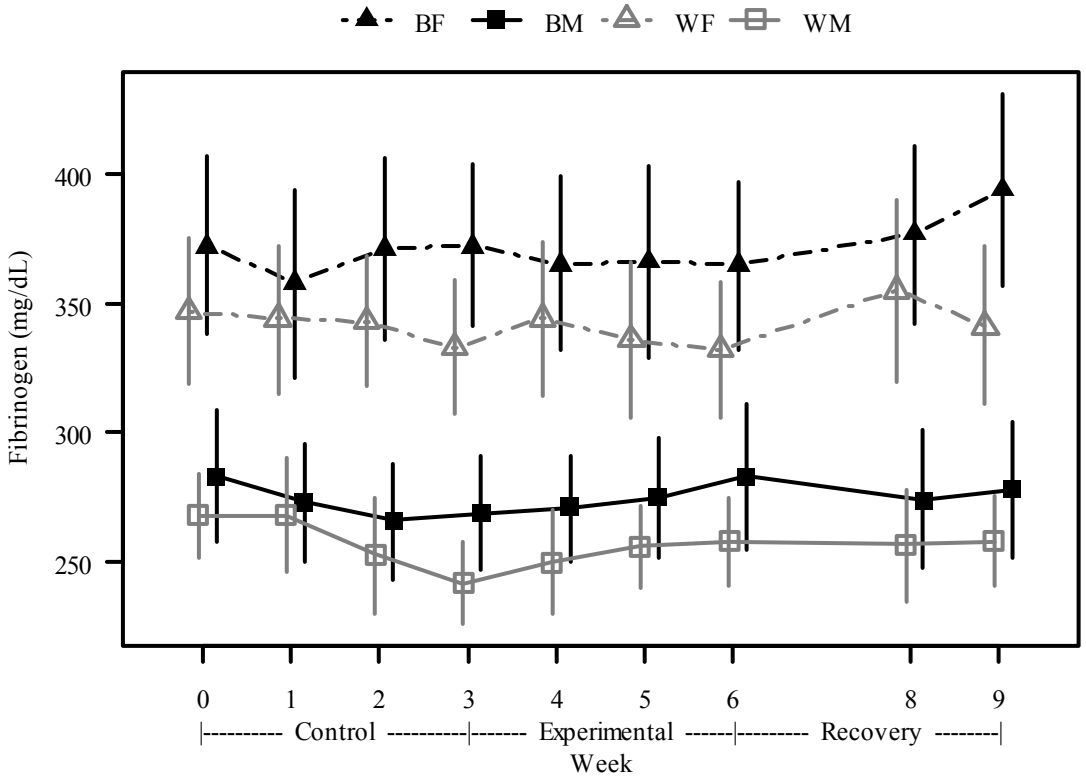


Figure 2.19. Mean (± 95% confidence interval) blood total cholesterol level (mg/dl) over the course of the study (N = 128).

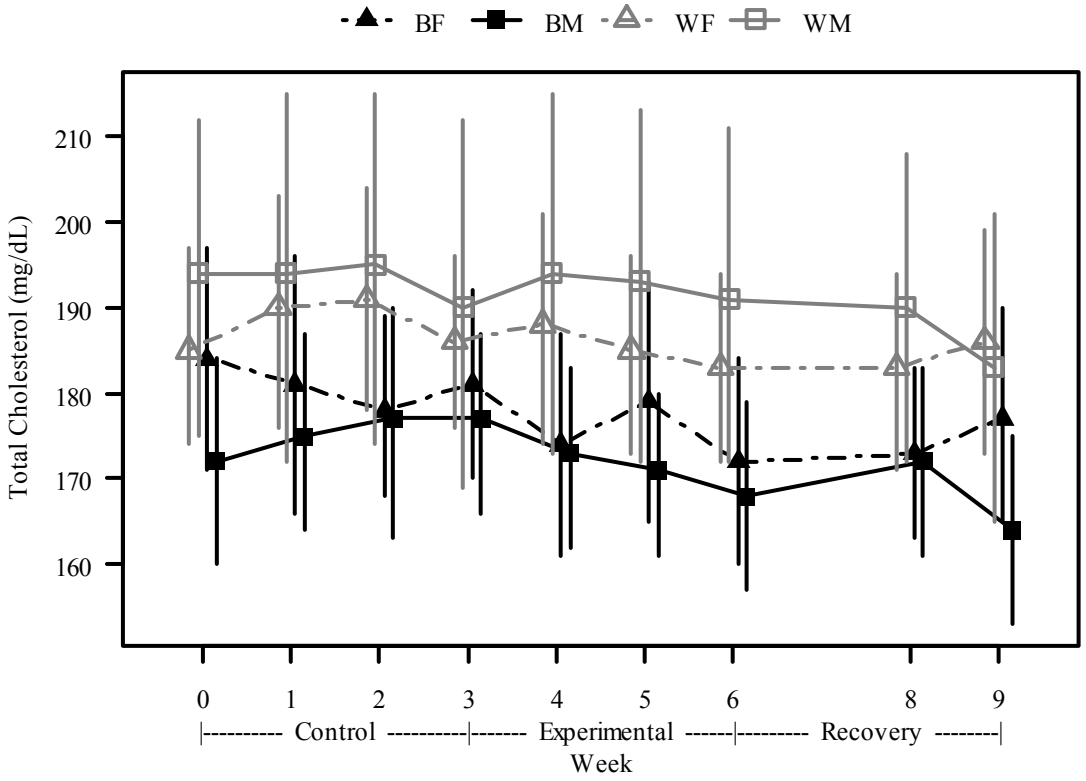


Figure 2.20. Mean (± 95% confidence interval) blood HDL-cholesterol level (mg/dl) over the course of the study (N = 128).

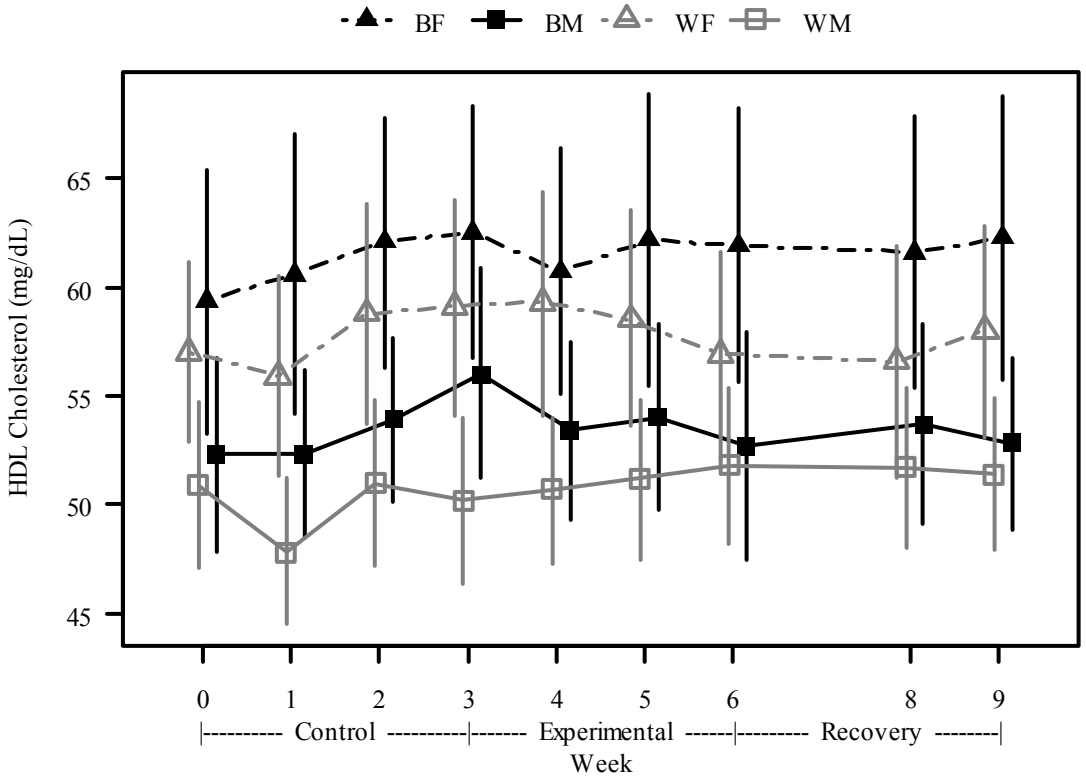


Figure 2.21. Mean (± 95% confidence interval) blood LDL-cholesterol level (mg/dl) over the course of the study (N = 128).

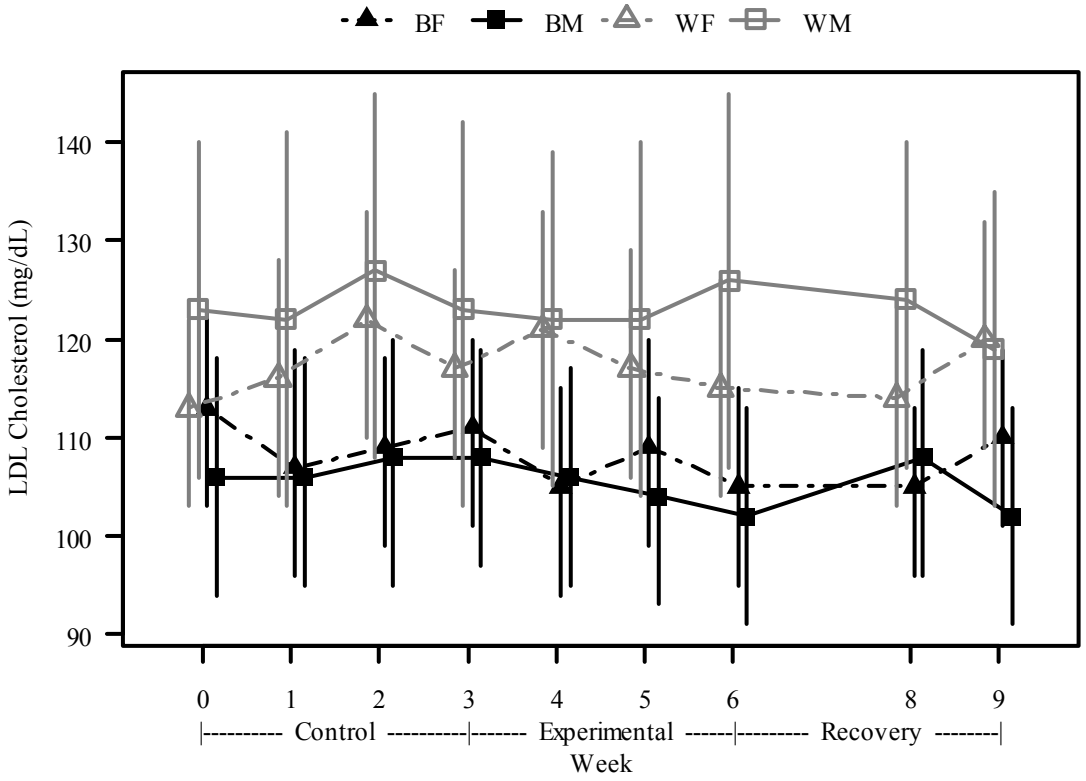


Figure 2.22. Mean (± 95% confidence interval) blood endotoxin level (EU/ml) over the course of the study (N = 128).

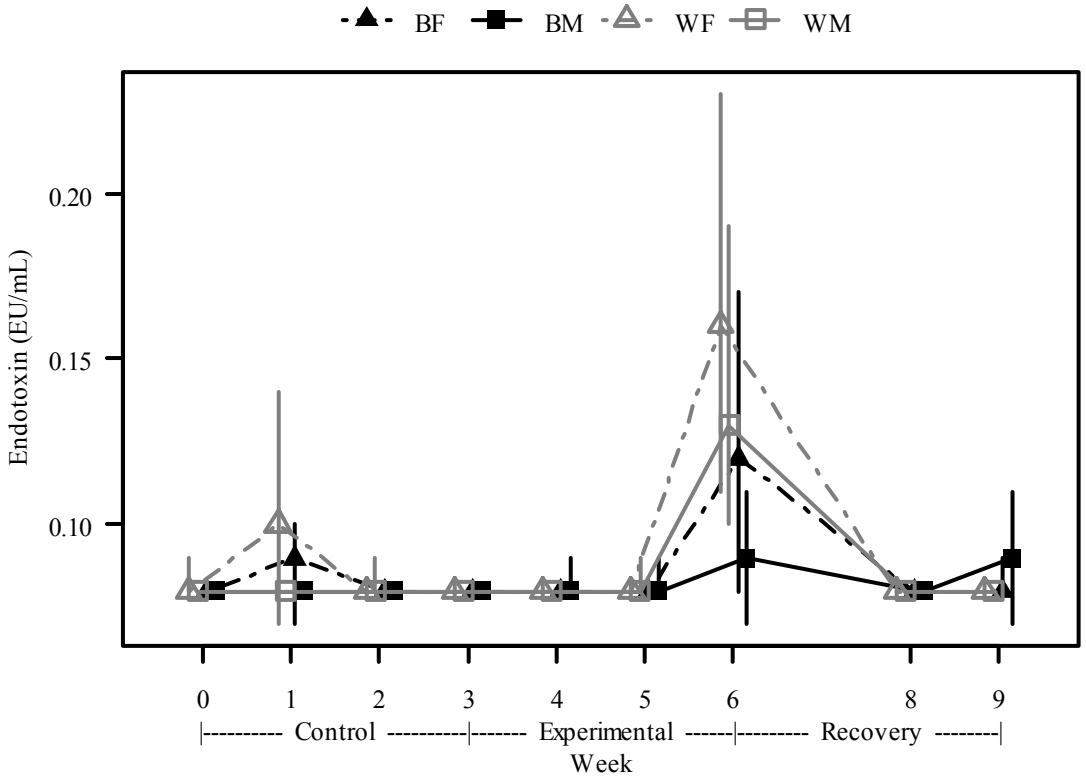


Figure 2.23. Mean (± 95% confidence interval) blood 8-isoprostane level (pg/ml) over the course of the study (N = 128).

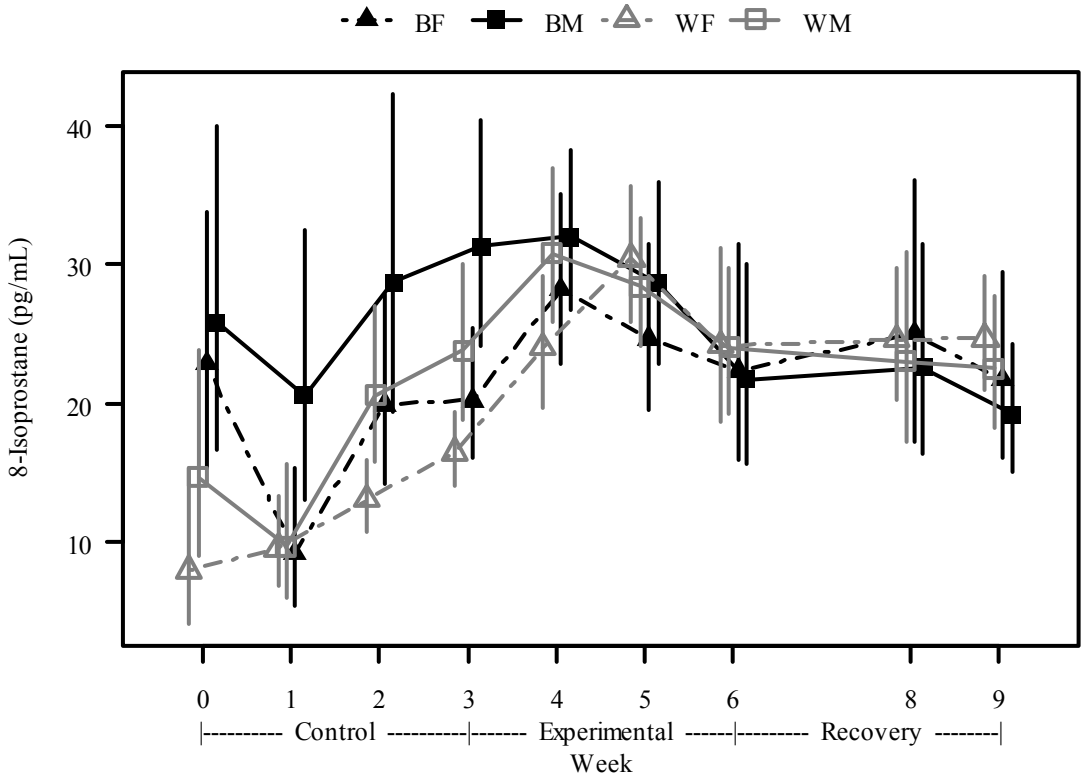


Figure 2.24. Mean (± 95% confidence interval) blood cortisol level (μg/dl) over the course of the study (N = 128).

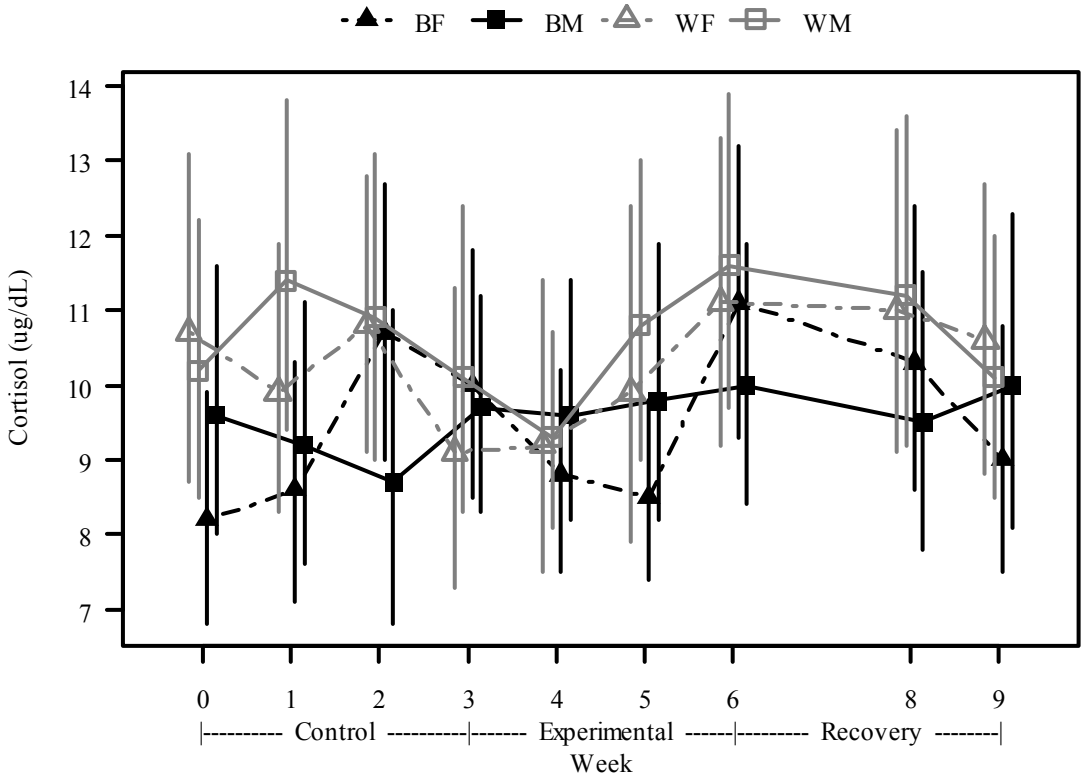
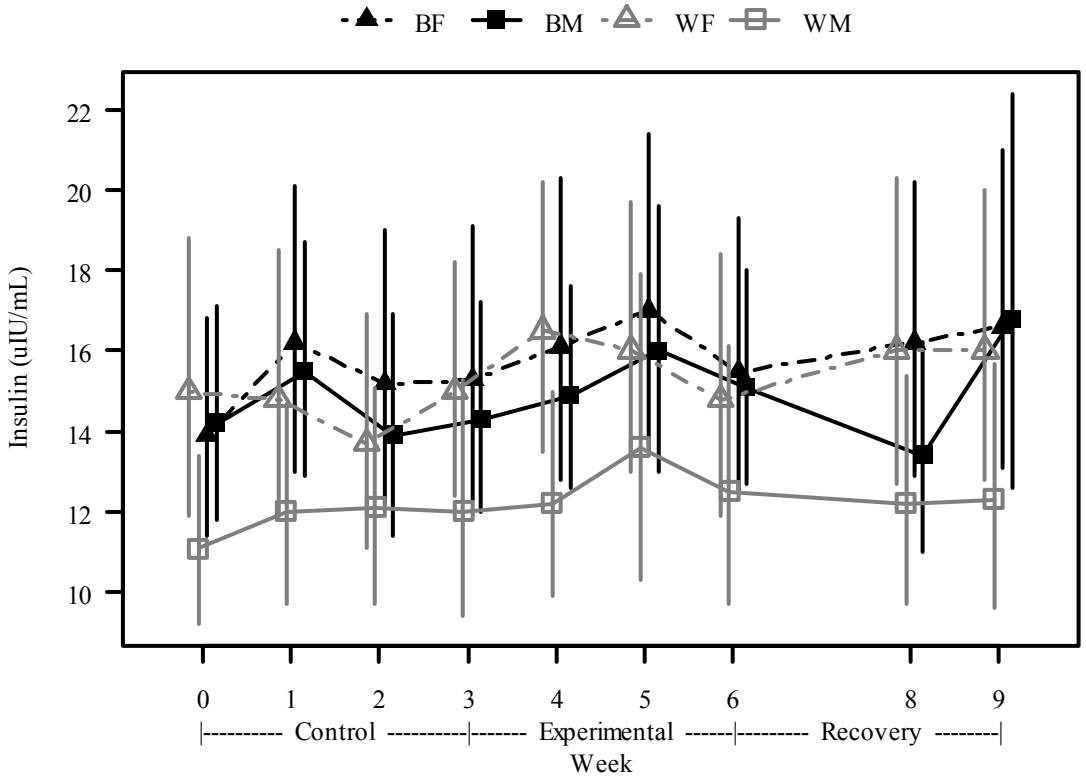


Figure 2.25. Mean (± 95% confidence interval) blood insulin level (µIU/ml) over the course of the study (N = 128).



CHAPTER THREE

Bacteremia and the Syatemic Host Response Following Toothbrushing in Healthy Adults

Abstract

Bacteremia and the systemic inflammatory response are central mechanisms linking oral and systemic diseases. Objective: to use a modified experimental gingivitis model to determine whether dental plaque accumulation results in bacteremia and subsequent systemic inflammatory responses following toothbrushing. Methods: Following IRB approval, 24 healthy adults, 18-30 years provided written informed consent to participate. After dental prophylaxis and oral hygiene (OH) instructions, subjects performed OH for 7 days (control phase), ceased OH for 7 days (experimental phase), and resumed OH for further 7 days (recovery phase). Plaque Index (PI) and Gingival Index (GI) were recorded. Peripheral venous blood samples were collected at each visit to evaluate systemic markers of infection and inflammation including bacteremia, complete blood count, and neutrophil oxidative activity. At the end of the experimental phase, subjects performed a supervised toothbrushing. Peripheral blood samples were collected immediately before (baseline), 0.5, 5, and 30 minutes after toothbrushing. Additional blood samples were collected 4 hours after toothbrushing to assess serum IL-6 and IL-8 levels. Changes in values were analyzed using repeated measures ANOVAs. Results: 21 subjects completed the study. The correlation between changes in PI and GI was 0.86. During the experimental phase, PI and GI increased as compared to the control and recovery phases ($p<0.05$). Three subjects were bacteremic 0.5 minutes following toothbrushing and one subject was bacteremic 30 minutes following toothbrushing.

Bacterial isolates comprised *Veillonella*, *Corynebacterium*, and *Fusobacterium* species. Immediately following toothbrushing, total white blood cell and neutrophil counts increased significantly as compared to baseline ($p<0.05$). These counts decreased significantly during the recovery phase when compared to the experimental phase immediately following toothbrushing ($p<0.05$). Conclusions: Toothbrushing, when performed where there was already dental plaque accumulation, elicited bacteremic episodes and systemic inflammatory responses that may be of importance in understanding mechanistic pathways mediating oral and systemic diseases.

Introduction

Recently, there has been a resurgence of interest in the possible causal association between periodontal disease and CHD. However, there is still a gap in knowledge regarding the mechanistic pathways mediating such an association. Bacteremia involving oral bacteria and the series of the host systemic inflammatory responses that it elicits are fundamental mechanisms suggested to causally link both diseases.

Evidence is available to support the occurrence of bacteremia involving oral organisms, including periodontal pathogens. Several studies have demonstrated the presence of periodontal pathogens in atherosclerotic plaques and abdominal aneurysms as an indication of bacteremia (Haraszthy *et al*., 2000; Ishihara *et al*., 2004; Kurihara *et al*., 2004).

Periodontal pathogens including *Porphyromonas gingivalis* have been detected in human atherosclerotic plaques using polymerase chain reaction (PCR) techniques (Haraszthy *et al*., 2000; Ishihara *et al*., 2004; Kozarov *et al*., 2005). It was reported, using PCR, that about 45% of examined carotid endarterectomy specimens were positive for oral bacteria including *Bacteroides forsythus*, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans,* and *Prevotella intermedia* (Haraszthy *et al*., 2000).

Another PCR-based study (Taylor-Robinson *et al*., 2002), reported that 31% of specimens from the aorta and from the iliac, internal mammary, and coronary arteries

were positive for DNA from oral bacteria. Whether the bacteria were present persistently, intermittently, or transiently was unclear.

In addition, it has been suggested that within atheromas, bacteria may be involved in the development and progression of the atherosclerotic lesion (Chiu, 1999; Lalla *et al*., 2003; Gibson *et al*., 2004). Although DNA of oral and periodontal bacteria has been commonly found in atherosclerotic plaques, evidence is lacking as to whether bacteria are actively involved in the disease process. Thus, attempts have been made to isolate viable bacteria from atherosclerotic lesions.

In 2005, Fiehn *et al*. attempted to determine firstly, if viable oral bacteria, occurring in subgingival plaque of individuals with periodontitis, could be isolated from atheromas and secondly, the presence of DNA from periodontal pathogens in atheromas using PCR. The study failed to isolate viable bacteria from atheromas. However, DNA from *P. intermedia* was found consistently, re-confirming that DNA of periodontal pathogens can be found in atherosclerotic plaques.

Invasion of host cells by *P. gingivalis* has been proposed as a possible mechanism of pathogenesis in periodontal and cardiovascular diseases (Lamont *et al*., 1995; Deshpande *et al*., 1998). Recently, invasion of host cells by viable periodontal pathogens including, *P. gingivalis* and *A. actinomycetemcomitans*, has been successfully shown in atherosclerotic plaque lesions (Kozarov *et al*., 2005). The presence of these bacteria was confirmed by PCR. The study (Kozarov *et al*., 2005) provided evidence not only for the

presence of periodontal bacteria at the sites of atherosclerotic lesions but also for their viability.

Suggesting an active role of oral bacteria in the atherosclerosis process, *P. gingivalis* has been shown to induce a proatherogenic response in endothelial cells. This response is manifested as an increase in leukocyte adhesion, enhanced production of proinflammatory cytokines, and induction of prothrombotic properties (Kang and Kuramitsu, 2002; Roth *et al*., 2007b). Interestingly, it has been suggested that these effects on endothelial cells cannot be induced by bacterial components only, but require the invasion of host cells by viable bacteria (Darveau *et al*., 2002; Roth *et al*., 2007a).

Transient bacteremia, caused by oral bacteria, occurs commonly in association with dental procedures or with routine daily activities including toothbrushing and chewing. The frequency and intensity of the bacteremias are believed to be related to the nature and magnitude of the tissue trauma, the density of the microbial flora, and the degree of periodontal inflammation at the site of trauma (Wilson *et al*., 2007). Whereas, the nature of the microbial species entering the systemic circulation depends upon the composition of the microflora at the traumatized site (Wilson *et al*., 2007).

The majority of the existing literature has focused on transient bacteremias following dental procedures. It used to be generally believed that the bleeding associated with oral procedures is a requirement for bacteremia to occur and thus, the previous AHA guidelines recommended antibiotic prophylaxis only for dental procedures in which

bleeding was anticipated (Dajani *et al*., 1997). However, there is no evidence to show that bleeding during a dental procedure is a reliable predictor of bacteremia and currently, it is generally believed that bleeding is not a prerequisite for bacteremia to occur. It is proposed that oral bacteria can be aspirated into the gingival blood vessels and eventually the systemic circulation by negative pressure (Roberts, 1999). Moreover, it has been suggested that daily events such as chewing and toothbrushing contribute more significantly to the cumulative exposure of the vascular system to oral bacteria and thus, may be of greater systemic importance than a single isolated clinical procedure (Roberts, 1999).

Toothbrushing has been shown to induce bacteremia in subjects with gingivitis (Lucas and Roberts, 2000). Toothbrushing, professional polishing, and scaling have been compared with regard to the prevalence and intensity of bacteremia that they may elicit. The results have found no significant differences in the prevalence or intensity of bacteremia when comparing the three groups. Additionally, the isolated bacterial species were similar in all three groups and included *Streptococcus mitis*, *Streptococcus sanguis*, and Coagulase-negative staphylococci, all of which may play a role in bacterial infective endocarditis. The findings of this study (Lucas and Roberts, 2000) suggested that, in susceptible individuals, the risk of developing bacteremia from toothbrushing is similar to that from scaling and professional polishing, an issue that has given rise to the dilemma of using antibiotic prophylaxis to prevent bacterial infective endocarditis in susceptible individuals.

Recently, the British Society for Antimicrobial Chemotherapy (Gould *et al*., 2006) and the AHA (Wilson *et al*., 2007) have revised their guidelines related to the use of antibiotic prophylaxis for the prevention of bacterial infective endocarditis. One of the main reasons behind this revision was the belief that frequent exposures to bacteremias associated with daily activities are much more likely to cause infective endocarditis than the exposure to bacteremia caused by an isolated dental procedure (Wilson *et al*., 2007). Accordingly, the routine administration of prophylactic antibiotics is no longer recommended. The new recommendation is to implement effective oral hygiene measures and to prevent oral disease in order to reduce the potential for cumulative bacteremia, especially in patients at risk for distant site infection and particularly for those at risk of infective endocarditis (Lockhart *et al*., 2008).

Regarding the intensity of bacteremia that is required for the development of infective endocarditis, findings from animal studies have suggested that this intensity is about 10^6 to 10^8 cfu/ml blood (Fowler *et al*., 2005; Osler, 1885). In contrast, findings from human studies have reported that the intensity of bacteremia in humans ranges from 1 to 240 cfu/ml blood (Lockhart and Durack., 1999). This contradiction has led to supporting the cumulative bacteremia concept as a cause of infective endocarditis and possibly other systemic diseases (Jones *et al*., 1955; Strom *et al*., 1998; Lockhart *et al*., 2002).

The cumulative bacteremia concept has led to the generation of two theories concerning infective endocarditis (Lucas *et al*., 2008): the ‘General Theory’ and the ‘Special Theory’. Toothbrushing plays a central role in both theories. According to the ‘General

Theory', daily toothbrushing is the cause of bacteremia especially in individuals with frequently practiced but inadequate OH. On the other hand, according to the 'Special Theory', the thorough toothbrushing that is practiced by individuals before their visit to the dental clinic is the most likely cause of bacteremia.

In addition to producing bacteremia, dental plaque accumulation and gingivitis may promote a systemic inflammatory host response, either in reaction to the local plaque accumulation or secondary to a bacteremia. Both, peripheral blood neutrophils and inflammatory cytokines play a central role in this response.

Peripheral blood neutrophils comprise the primary cellular component of acute inflammation. Circulating WBCs, particularly neutrophils, increase in their count following periodontal infection including gingivitis (Loos *et al*., 2000; Kowolik *et al*., 2001). Periodontal infection may also prime circulating neutrophils (Fredriksson *et al*, 1998) so that they circulate in a pre-active state. Further activated neutrophils can release reactive oxygen species and proteolytic enzymes such as elastase and myeloperoxidase that have a potential for tissue destruction (Naruko *et al*., 2002).

In addition to neutrophils, cytokines are prominent components of the inflammatory response to infection. Cytokines that are associated with inflammation, including IL-6 and IL-8, can initiate or enhance a cascade of events, both at the local site of infection and systemically (Gabay and Kushner, 1999). Accordingly, they are released locally in response to dental plaque accumulation (Ebersole *et al*. 1993; Ebersole *et al*. 1999) and

there is potential for systemic dissemination (Salvi *et al*., 1998; Ebersole *et al*., 1999). IL-6 is an important inflammatory mediator; it is the main inducer of hepatic production of acute phase reactants (Gauldie *et al*., 1987). Studies have shown that periodontitis is associated with increased blood levels of IL-6 (Loos *et al*., 2000; Mengel *et al*., 2002; Forner *et al*., 2006a; Forner *et al*., 2006b). IL-8 is a potent chemoattractant for neutrophils. It induces degranulation and reactive oxygen metabolite production in neutrophils. Studies have been controversial regarding the changes in circulating levels of IL-8 with periodontitis (Sugano *et al*, 2004; Forner *et al*, 2006b).

Although the occurrence of bacteremia with oral bacteria has been recognized for more than a century, to date, there is lack of investigations evaluating the immediate systemic host response to bacteremia with an oral origin following toothbrushing.

Study Significance

There is currently considerable interest in the possible causal association between periodontal infection and CHD but, to date, there is still a knowledge gap regarding the mechanisms that could mediate such an association. Since gingivitis due to plaque accumulation is the most prevalent form of periodontal infection, it is relevant to not only investigate the possible role that plaque accumulation and subsequent gingivitis play in CHD risk, but moreover to evaluate the biological plausibility linking both diseases.

Bacteremia involving oral organisms, including periodontal pathogens, and the systemic host responses to plaque accumulation, are two factors strongly suggested to play a role in CHD pathogenesis and thus, are worthy of further investigation.

Study Hypothesis

We hypothesized that daily OH practices (i.e., toothbrushing) when performed where there is already dental plaque accumulation and resulting gingivitis would cause a transient bacteremia which would subsequently elicit a systemic host inflammatory response.

Study Objective

The objective of the current study was to use a 7-day experimental gingivitis model to examine whether dental plaque accumulation results in bacteremic episodes and subsequent systemic inflammatory responses following toothbrushing in a sample (N = 24) of young, healthy, male and female, white Caucasian adults.

Specific Aims

The specific aims of this study were to use a 7-day experimental gingivitis model to:

1. Determine whether dental plaque accumulation results in the occurrence of bacteremia and a subsequent systemic inflammatory response following toothbrushing. The working hypothesis was that tooth brushing following the accumulation of dental plaque could induce a bacteremia and in turn, this bacteremia could elicit a host systemic response in

terms of increased total and differential WBC counts, heightened peripheral blood neutrophil oxidative activity, and increased serum cytokine levels.

2. Evaluate the systemic inflammatory response to dental plaque accumulation. The working hypothesis was that dental plaque accumulation would induce a host systemic inflammatory response. This response would manifest as an increase in the total and differential WBC counts and an elevated peripheral blood neutrophil oxidative activity.

Materials and Methods

Study Design

The study protocol was approved by the IRB of Indiana University Purdue University Indianapolis/Clarian Health (approval number 0712-67) (Appendix 8). All subjects provided written informed consent (Appendix 9) for study participation and completed a medical history form.

During the screening visit, subjects received an oral examination to determine their periodontal health status. Subjects who qualified to be enrolled in the study attended the clinic for a cleaning visit and received a thorough professional dental cleaning and OH instructions.

Following the cleaning visit, subjects entered the three study phases in chronological order. In the control phase, subjects performed optimal OH for 7 days. In the experimental phase, subjects refrained from OH measures including brushing, flossing,

mouth rinsing, and gum chewing for 7 days. Finally, in the recovery phase, subjects received a professional oral prophylaxis and OH instructions and resumed their normal OH practices for further 7 days.

Subjects visited the clinic once/week during each of the study phases. During each visit, oral examinations were conducted and peripheral blood samples were collected. At the end of the experimental phase visit, subjects performed a supervised and standardized 2-minute toothbrushing procedure using a soft-bristle tooth brush. During this visit, peripheral venous blood samples were collected at baseline (immediately before the commencement of toothbrushing), as well as 0.5, 5, and 30 minutes after completion of toothbrushing.

All blood samples were evaluated for the occurrence of bacteremia, complete blood cell counts, and peripheral blood neutrophil metabolic oxidative activity. An additional blood sample was collected 4 hours after toothbrushing to evaluate changes in serum IL-6 and IL-8 levels as compared to baseline levels.

The study protocol incorporated the use of the experimental gingivitis model. Initially, subjects were provided with a toothbrush, floss and toothpaste and were asked to perform optimal OH for 7 days (control phase). On Day 7 of the control phase (Day 14 of the study), plaque levels and gingival health status were measured and peripheral venous blood samples were collected to assess neutrophil count and function and any ensuing bacteremia.

Subjects then refrained from all OH practices for 7 days (experimental phase**).** On Day 7 of the experimental phase (Day 21 of the study), subjects performed a supervised and standardized 2-minute toothbrushing procedure using a soft bristle tooth brush. Venous blood samples were collected at baseline, just before the commencement of toothbrushing, as well as 0.5, 5, and 30 minutes after completion of the toothbrushing procedure. These samples were collected to evaluate and compare the occurrence of bacteremia and the resulting systemic neutrophil response. An additional blood sample was collected 4 hours after toothbrushing to evaluate changes in plasma IL-6 and IL-8 levels as compared to baseline levels.

Subjects then received a thorough dental prophylaxis and entered the recovery phase during which they resumed OH practices in order to allow gingival tissues to return to normal health. This was confirmed by both clinical examination and blood sample analyses after a further 7 days.

Study Population

The study enrolled healthy adult volunteers (N = 24; 10 females and 14 males) (Figure 3.1).

Inclusion Criteria

The enrolled participants were (Appendix 10):

- Between the ages of 18 and 30 years, of either gender.

- In good general health and have no factors in their medical history, which would indicate that they would be adversely affected by their participation in this study.
- Willing to refrain from the use of dentifrices, mouth rinses, dental floss, or any other dental products during the experimental phase.
- Possessing a minimum of 20 natural teeth.

Exclusion Criteria

None of the participants were (Appendix 11):

- Current users of tobacco products as determined by self reports and measurement of expired CO level using a CO test (Smokerlyzer®, Bedfont Scientific Ltd, Medford, NJ). A CO level of 8ppm or less verified non-smoking status (Hughes *et al*., 1987).
- Showing evidence of periodontal disease (probing depth not > 4 mm), or gross neglect, active caries or other conditions necessitating immediate care as evaluated by the examining dentist.
- Having factors which would pose a risk to themselves, to study personnel or to other subjects. This includes pregnancy, hepatitis, HIV-positive status, active tuberculosis, diabetes, and conditions requiring antibiotic pre-medication prior to dental treatment e.g. rheumatic fever, cardiac regulating devices, heart murmur, prosthetic joint replacement. All decisions of medical acceptability were at the discretion of the examining dentist.
- Using a medication that is known to affect the oral soft tissues or local/systemic inflammatory response.

- Receiving antimicrobial drug therapy within 3 months prior to commencement of control phase of the study.

The continuance criteria are summarized in Appendix 12.

Clinical Procedures

The clinical procedures (Table 3.1) of this study were conducted at the OHRI at the Indiana University School of Dentistry and included the following:

1. CO Test: At each visit, an expired air CO test was performed to determine the smoking status of the participants. As mentioned earlier, a CO level of 8 ppm or less was used to verify non-smoking status.

2. Oral Examinations: Oral examinations were performed at each visit, including examination of the oral soft tissues by a visual examination of the oral cavity and perioral area using a standard dental light, dental mirror, and gauze. The structures examined included the gingiva, hard and soft palate, oropharynx/uvula, buccal mucosa, tongue, floor of the mouth, labial mucosa, mucobuccal/mucolabial folds, lips, and perioral area.

Observations were determined to be "Normal" or "Abnormal". Plaque levels and gingival inflammation were assessed by a single trained and calibrated examiner using the PI (Silness and Löe, 1964) and GI (Löe and Silness, 1963), respectively. A Hu-Friedy UNC probe (Hu-Friedy, Chicago Illinois) was used during the oral exams. Measurements were

made on six sites per tooth (distobuccal, buccal, mesiobuccal, mesiolingual, lingual and distolingual) on all teeth present, with the exception of third molars (Appendix 14).

3. Venipuncture Blood Sampling: Blood sampling was performed using a sterile technique. The skin at the venipuncture site was disinfected with isopropyl alcohol and chlorhexidine. To obtain blood samples, a 21gauge butterfly device with first a 20cc syringe and followed with a vacutainer tube was placed into the median cubital vein. At the experimental phase visit, prior to collecting the baseline blood sample, both arms were disinfected as described above.

The venipuncture site of the arm not having the baseline-blood sample collection was covered with a large sterile Telfa pad to prevent contamination. Immediately following toothbrushing the Telfa pad was removed and the 0.5-minute blood sample was collected. Venipuncture sites of each arm were again disinfected immediately prior to collection of the 5-minute and 30-minute blood samples. The timing of collection of the blood samples was determined based on previous related studies (Roberts *et al*., 1992; Forner *et al*., 2006a). Prior to commencement of the actual study, proper collection of blood samples, in a timely fashion, was practiced.

Laboratory Analyses

Bacteremia Analyses

10 ml of each blood draw was inoculated directly into an aerobic blood culture bottle and a 10 ml aliquot was inoculated directly into an anaerobic blood culture bottle. When all of

the samples were collected, they were transported immediately to the laboratory for processing and microbiological analysis.

In the Clarian Pathology Clinical Microbiology Laboratory, blood samples were cultured in a blood culture instrument (BACTEC 9240; Becton and Dickinson Microbiology Systems, Sparks, MD) using established procedures (Winn, 2006). Initially, a Gram stain was performed on each positive blood culture. In accordance with the laboratory's established procedures, all positive blood culture bottles were subcultured on blood agar and chocolate agar; MacConkey agar was inoculated if gram-negative rods were observed or if the blood culture was mixed. Colistin-naladixic agar was inoculated if the culture was mixed. All positive blood culture bottles were also subcultured on anaerobe blood agar. If fungi (i.e., yeast) were present, Sabauraud's dextrose agar was inoculated.

Following inoculation, the subculture plates were incubated in an atmosphere appropriate for isolating aerobic, capnophilic, microaerophilic and anaerobic microorganisms as described elsewhere (Winn, 2006). The instrumented blood culture system monitored the aerobic and anaerobic blood culture bottles for a minimum of 7 days before results were finalized. The microbial isolates from the subculture plates were isolated in pure culture, characterized and identified by established methods (Winn, 2006).

Standard Hematology

3 ml/sample of collected whole blood was used for standard hematology. Total and differential white WBC counts were measured using an automated cell counter (Coulter

Stack S, Coulter Electronics Inc, Hialeah, FL, USA) in the laboratories of Clarian Health Partners Inc. RBC counts, Platelet counts, Hct, and Hb levels were also assessed as part of the normal laboratory protocol.

Neutrophil Metabolic Oxidative Assay

10 ml/sample of collected whole blood was used for this assay. To determine the level of peripheral blood neutrophil metabolic oxidative activity, neutrophils were isolated from heparinized peripheral venous blood samples by a density gradient centrifugation method on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) at 400 x *g* for 25 minutes at 25^{o}C (Sabroe, 2003).

The cells were washed twice with isotonic PBS and once with RPMI-1640 (Sigma) by centrifugation at 250 x *g* for 10 minutes. Isolated cells were suspended in RPMI-1640. Neutrophils were counted using a hemacytometer, and viability was assessed using trypan blue dye exclusion. Cells were primed for 30 minutes at 37^{o}C with 10^{-10} M fMLP peptide and resuspended to a concentration of 1 x 10^{6} cells/ml in RPMI-1640.

A luminol-dependent chemiluminescence assay was used to quantify the production of reactive oxygen species by the neutrophils using a 1251 Bio-Orbit Luminometer (Bio-Orbit, Turku, Finland) for 90 minutes. Into each cuvette, 100 µl of 10^{-5} M Luminol was dispensed at baseline, followed by cell activation with 100 µl of 10^{-5} M fMLP at 30 minutes. The final volume of the reaction solution was 1 ml/cuvette.

Chemiluminescence values were expressed as peak (millivolt) and total (millivolt.min) integrated energy output over the fixed experimental time period. Samples were run in triplicate and mean values were calculated. Negative controls included the reaction solution without cells.

Cytokine analysis

10 ml/sample of collected whole blood was used for this assay. Serum was separated from the collected blood samples and aliquoted and stored at -40°C until required for analysis. Cytokine analysis was conducted at the GCRC of the Indiana University School of Medicine.

Evaluation of serum IL-6 and IL-8 levels was determined using a Linco multiplex cytokine assay kit (Linco Research St. Louis, MO, USA) and a Luminex 100 multiplex reader (Luminex, Austin, TX).

All serum samples were analyzed in duplicate by the MasterPlex™ QT software. During the experimental phase, serum levels of IL-6 and IL-8 were compared at baseline before brushing and 4 hours after brushing for each subject.

Statistical Analysis

Natural logarithms of the measurements were used for all analyses since the distributions of all outcomes were non-normal. Means and confidence intervals reported were transformed to the original measurement scale to aid in interpretation of the results.

Correlation coefficients were calculated to assess the association between PI and GI to validate the experimental gingivitis model. Repeated measures ANOVA was performed to evaluate the changes in each outcome during the study. Comparisons between females and males for differences in the responses were also evaluated using repeated measures ANOVA.

Subjects Remuneration

Subjects were compensated $20 for attending the screening visit (visit 1), whether or not they were subsequently enrolled in the study. Subjects who were enrolled in the study received $50 at the dental cleaning visit (visit 2), $50 at the control phase visit (visit 3), $250 at the experimental phase visit (visit 4), $50 at the recovery phase visit (visit 5), and a final payment of $100 for completing all study visits (total compensation, $520).

Results

Of the 24 subjects enrolled, 21 completed the study (8 females and 13 males). Of the three subjects not completing the study, two were lost due to failure to follow up and one was lost because of fainting during blood sample collection (Figure 3.1). The mean age of the subjects completing the study was 26.4 ± 2.06 years.

Mean and 95% confidence intervals for study outcomes over the course of the study are summarized in Table 3.2. The average PI (Figure 3.2) at the end of the control phase was 0.59 (95% CI 0.53 - 0.65) and the average GI (Figure 3.3) was 0.62 (0.55 - 0.71). Increases in PI (1.29, 95% CI 1.12 - 1.47) and GI (1.43, 95% CI 1.24 - 1.65) during the

experimental phase of the study were significant ($p<0.0001$), as compared to the control phase. There were no significant differences in the changes in PI or GI between genders during the experimental phase ($p\geq0.39$).

During the experimental phase, there was a strong positive correlation between changes in PI and GI ($r = 0.87$ overall, $r = 0.88$ for females, $r = 0.88$ for males). The average PI (0.27, 95% CI 0.15 - 0.48) and average GI (0.47, 95% CI 0.40 - 0.57) during the recovery phase returned to levels slightly lower than those seen during the control phase ($p\leq0.01$).

Bacteremia was not detected in any of the subjects during the control and recovery phases. In addition, none of the subjects were bacteremic at baseline (before toothbrushing) during the experimental phase. Following toothbrushing during the experimental phase visit, four out of 21 subjects were bacteremic. Each of the four subjects had a single positive blood culture, collected within 0.5 minutes to 30 minutes following toothbrushing, which yielded one organism. At 0.5 minutes post-toothbrushing, a blood culture from one subject yielded *Veillonella* spp., and blood cultures from two other subjects yielded *Corynebacterium* spp. At 30 minutes post-toothbrushing, one blood culture from a fourth subject yielded *F. nucleatum*.

Total WBC counts (Figure 3.4), neutrophil counts (Figure 3.5), RBC counts (Figure 3.6), platelet counts (Figure 3.7), Hb levels (Figure 3.8), and Hct (Figure 3.9) did not change significantly from the end of the control phase to experimental phase before brushing ($p>0.25$, Table 3.2). However, immediately (0.5 minutes) following toothbrushing, all

levels increased significantly ($p<0.05$) as compared to pre-brushing counts. Neutrophil count was still significantly increased 30 minutes after toothbrushing as compared to pre-brushing ($p = 0.01$).

RBC counts, total WBC counts, neutrophil counts, Hct, and Hb levels decreased significantly ($p<0.05$) during the recovery phase when compared to the experimental phase immediately (0.5 minutes) following toothbrushing.

No significant changes were observed in neutrophil oxidative function (Figures 3.10 and 3.11). Cytokine levels during the experimental phase before brushing (IL-6 average 130 pg/mL, 95% CI 70 - 240 pg/mL; IL-8 average 87 pg/mL, 95% CI 56 - 134 pg/mL) and 4 hours after brushing (IL-6 average 85 pg/mL, 95% CI 48 - 150 pg/mL; IL-8 average 50 pg/mL, 95% CI 29 - 88 pg/mL) were not significantly different ($p = 0.24$ for IL-6, $p = 0.07$ for IL-8). Females and males responded similarly during this study for all study outcomes.

Discussion

This is the first study to address and demonstrate an immediate systemic host inflammatory response to bacteremia involving oral bacteria. By virtue of its design, this study permitted a short-term, longitudinal investigation of the systemic effects of dental plaque accumulation and the host systemic inflammatory responses to bacteremia following toothbrushing in systemically healthy individuals with gingivitis.

In spite of the inconveniences associated with the procedures conducted in this study (abstaining from OH practices for 7 days, and providing multiple blood samples during the experimental phase visit), the subject retention rate was high; 21 subjects out of 24 enrolled, completed the study. In this study, subject compliance was verified by the significant increase in PI and GI over the course of the experimental phase as compared to the control and recovery phases and the strong positive correlation between changes in PI and GI ($r = 0.87$).

In the current study, toothbrushing induced bacteremic episodes in four out of the 21 gingivitis subjects who completed the study. The incidence of bacteremia detected in this study is within the incidence range that is reported in the literature. The incidence of bacteremia in adults, following toothbrushing, has been reported to be between 0 and 57% (Cobe, 1954; Sconyers *et al*., 1973; Berger *et al*., 1974; Silver *et al*., 1977; Forner *et al*., 2006a; Hartzell *et al*., 2005). The variation between studies is suggested (Forner *et al*., 2006a) to be the result of differences in the particular procedures performed to induce bacteremia, the methods used to analyze bacteremia, the oral and periodontal health stauts, and the individual characteristics of the study subjects.

In the current study and as anticipated, no bacteremic episodes were detected during the control and recovery phase visits, and neither were any episodes of bacteremia detected at baseline before toothbrushing during the experimental phase visit. In the experimental phase visit, four bacteremic episodes were detected following toothbrushing. Three of the four episodes occurred 0.5 minutes following toothbrushing, which is reported to be the

optimal time for occurrence of bacteremia (Roberts *et al*., 1992). In addition, one bacteremic episode occurred 30 minutes following toothbrushing, which is in agreement with the time limit reported for detection of bacteremia.

With regard to the bacterial species isolated in this study*, Veillonella* are Gram-negative anaerobic cocci that are part of the normal flora of the oral cavity. This species has been reported to be a cause of endocarditis (Loughrey and Chew, 1990). *Corynebacterium* are Gram-positive aerobic or facultatively anaerobic rods. *Corynebacterium* can be of an oral origin or part of the commensal human skin flora. *F. nucleatum* is a Gram-negative anaerobic bacillus and is one of the most numerically abundant bacterial species found in subgingival plaque samples from healthy and diseased sites (Moore and Moore, 1994). This organism has been previously associated with bacteremia (Edson *et al*., 1982) and moreover, with pericarditis (Mangan *et al*., 1991).

The oral cavity was the most likely source of the *F. nucleatum* and the *Veillonella* spp.; transient bacteremia involving each of these isolates was interpreted to have occurred. On the other hand, the source of the two *Corynebacterium* spp. isolates is not clear. As mentioned earlier, *Corynebacterium* spp. not only are members of the oral microbiota, but also are found on skin. Thus, either *Corynebacterium* isolate could have seeded a volunteer's blood during toothbrushing, or could have contaminated the specimen at the time of collection.

The interaction between bacterial species and the host defense system is a central mechanism mediating the possible causal link between oral and systemic diseases. A

healthy host has no immediate problem with bacteremia occurrences and can spontaneously clear it within 30-45 minutes by the elicited immune response and hence, bacteremia is usually transient. However, in the long term, these repeatedly induced transient bacteremic episodes and the subsequent systemic inflammatory host responses may have detrimental systemic effects and therefore, elevate systemic disease risk. If this is indeed the case, then prevention of dental plaque accumulation by practicing and maintaining a high standard of OH would play an important role in prevention of systemic diseases.

In this study, an increase in the WBC and neutrophil counts was observed 0.5 minutes following toothbrushing during the experimental phase visit. These counts remained significantly increased 30 minutes following toothbrushing. The time points for the increase in WBC and neutrophil counts during the study coincide with those for the occurrence of bacteremic episodes. The rapid increase in the WBC counts in general and the neutrophil counts in particular is most likely due to demargination of neutrophils from the marginal to the circulating pool. Traditionally, the measures of WBC and neutrophil counts have been used as a measure for inflammation. Clinically, these counts are screening tools for occult bacteremia (Gombos *et al*., 1998).

The demonstration of an elevated WBC and neutrophil count immediately following toothbrushing in subjects with dental plaque accumulation may be of clinical relevance. Increased WBC and neutrophil counts have been described as risk factors for

cardiovascular disease and may enhance atherogenesis (Ernst *et al*., 1987; Phillips et al., 1992; Sweetnam *et al*., 1997; Lee *et al*., 2001).

In addition to the change in WBC and neutrophil counts, we observed an increase in the RBC counts, platelet counts, Hct, and Hb levels immediately following toothbrushing. The reason for such findings is unclear. There is no previous data in the existing literature that reports or explains the elevation in these hematological measures in relation to bacteremia and toothbrushing. However, spuriously elevated platelet counts following bacteremia have been previously reported in few cases (Gloster *et al*., 1985). In addition, psychological stress during the experimental phase visit due to the multiple injections may have contributed to this observation since acute psychological stress can cause hemoconcentration (Patterson *et al*., 1995).

A change in neutrophil metabolic oxidative activity was not detected in this study. Additionally, we could not detect a cytokine response following toothbrushing in subjects with dental plaque accumulation. This might be related to the small size of this study and to the methodological difficulties associated with detection of cytokines. For example, it might be that the time point that we chose to detect the cytokine response (4 hours after toothbrushing) was not optimal. Previous studies detected serum cytokine responses for both IL-6 and IL-8 in subjects with bacteremia 8 hours following induction (Forner *et al*., 2006b).

This study had some limitations. One of the important limitations is the relatively small subject sample size. In addition, although a well-controlled model, the conditions of the experimental gingivitis model may differ from those of clinical gingivitis (Deinzer *et al*., 2007). Moreover, there are limitations associated with the use of blood culture to detect bacteremia episodes. Clinically, blood culture is considered the gold standard for diagnosis of bacteremia, and the Bactec 9240 is sensitive in detecting bacterial growth (Shaoul *et al*., 2008). Previous studies have reported that a wider range of bacterial species and low levels of bacteremia can be detected by using the BACTEC system as compared to the traditional lysis filtration technique (Lucas *et al*., 2002; Bahrani-Mougeot *et al*., 2008). However, blood culture procedures overall, can't grow all bacteria from bacteremic blood samples. The oral microbiota is highly diverse and recently it has been estimated that more than 19,000 species contribute to the ultimate diversity of oral species (Keijser *et al*., 2008). Many oral bacteria are uncultivable and moreover, it is not easy to satisfy the growth requirements for all cultivable oral bacteria (Aas *et al*., 2005; Paster *et al*., 2006). Therefore, the results of this study might represent only a fraction of the bacteremic episodes that could have developed after toothbrushing (false negative). On the other hand, bacterial growth as detected by blood culture may represent contamination (false positive cultures) in some instances.

In this study we did not evaluate the intesnity of the bacteremic episodes that occurred following toothbrushing. The available literature lacks human studies that address the intensity of bacteremia whether after dental procedures or from daily activities and there

are no published data to demonstrate that a higher intensity of bacteremia is more likely to cause distant infection including infective endocarditis (Wilson *et al*., 2007).

In conclusion, the results from this study generally support the hypothesis that toothbrushing, when performed where there is already dental plaque accumulation, can elicit bacteremic episodes and induce systemic inflammatory responses (i.e., heightened WBC and neutrophil counts) but with certain exceptions (i.e., absence of changes in the neutrophil oxidative activity and cytokine levels). The bacteremic episodes and the systemic inflammatory responses that were observed in this study may be of importance in understanding mechanistic pathways mediating oral and systemic diseases (e.g. CHD).

This investigation provides some in vivo evidence to support a possible causal association between oral and systemic diseases. In agreement with the current recommendations of the AHA, this study also provides a rationale for an emphasis on maintaining good OH and avoiding dental disease in order to decrease the frequency of bacteremia from routine daily activities.

Table 3.1. Overview of the clinical procedures performed during each study visit.

	Screening Visit	Control Phase		Experimental Phase		Recovery Phase	
Day of Phase		0	7	0	7	0	7
Day of Study	0	7	14 [a]	14 [a]	21 [b]	21 [b]	28
Visit Number	1	2	3	3	4	4	5
CO Test	X	X	X		X		X
Blood Draw			X		X		X
Plaque & Gingivitis Assessment	X		X		X		X
Dental Prophylaxis		X			X		
Oral Hygiene Instruction		X			X		X

a, b: same day of study

Table 3.2. Mean and 95% confidence intervals (CI) for study outcomes over the course of the study (N = 21).

	End of Control Phase	End of Experimental Phase			End of Recovery Phase
		Pre-Brushing	Post-Brushing		
			30 seconds	30 minutes	
White Blood Cells ($x10^9$ cells / L)	5.8 (5.3, 6.4)	5.6 (5.1, 6.1)	6.0 (5.5, 6.5)	5.7 (5.2, 6.3)	5.4 (4.9, 6.0)
Red Blood Cells ($x10^{12}$ cells /L)	4.65 (4.48, 4.84)	4.65 (4.47, 4.84)	4.76 (4.57, 4.96)	4.66 (4.45, 4.89)	4.56 (4.37, 4.75)
Hemoglobin (g/dL)	14.5 (14.0, 15.0)	14.4 (14.0, 14.9)	14.8 (14.3, 15.3)	14.5 (14.0, 15.0)	14.2 (13.8, 14.7)
Hematocrit (%)	42.0 (40.7, 43.4)	41.9 (40.6, 43.2)	42.9 (41.5, 44.3)	42.0 (40.5, 43.5)	41.0 (39.7, 42.3)
Platelets ($x10^9$ cells / L)	237 (215, 261)	239 (216, 264)	244 (221, 270)	240 (216, 267)	247 (223, 274)
Neutrophils ($x10^9$ cells / L)	3.1 (2.7, 3.5)	3.1 (2.7, 3.5)	3.3 (2.9, 3.8)	3.2 (2.8, 3.8)	2.9 (2.5, 3.3)
Total CL[a] (millivolt.min)	5.2 (3.7, 7.1)	3.6 (2.4, 5.6)	4.2 (2.7, 6.4)	3.3 (2.4, 4.4)	4.0 (2.8, 5.6)
Peak CL[a] (millivolt)	9.3 (6.7, 12.9)	5.3 (3.3, 8.4)	6.0 (3.7, 9.6)	4.6 (3.2, 6.5)	7.3 (5.0, 10.8)

a: CL = Chemiluminescence

Figure 3.1. Flow chart of the study entitled "Bacteremia and the Systemic Host Response Following Toothbrushing in Healthy Adults"

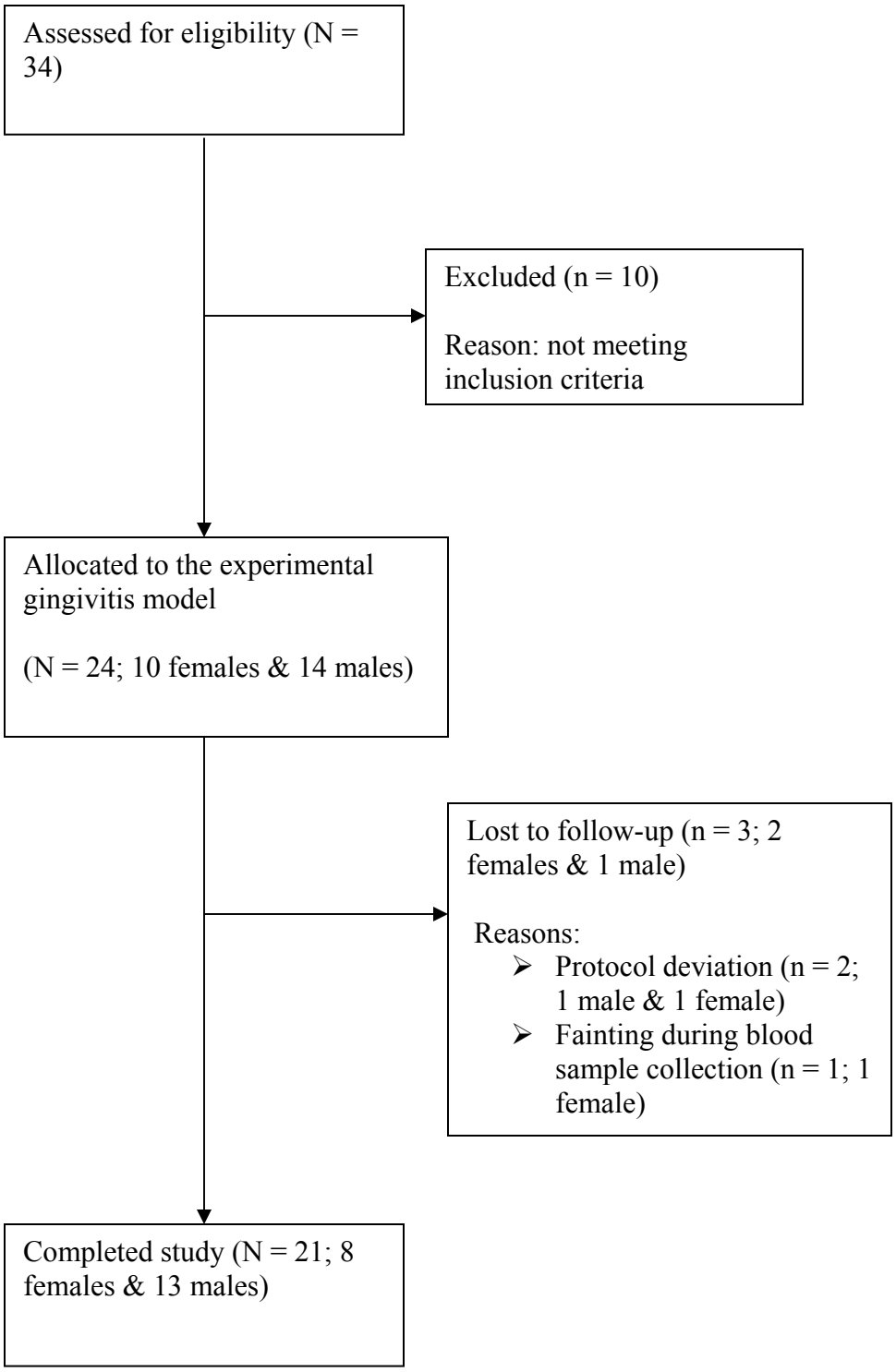


Figure 3.2. Mean (± 95% confidence interval) plaque index over the course of the study (N = 21).

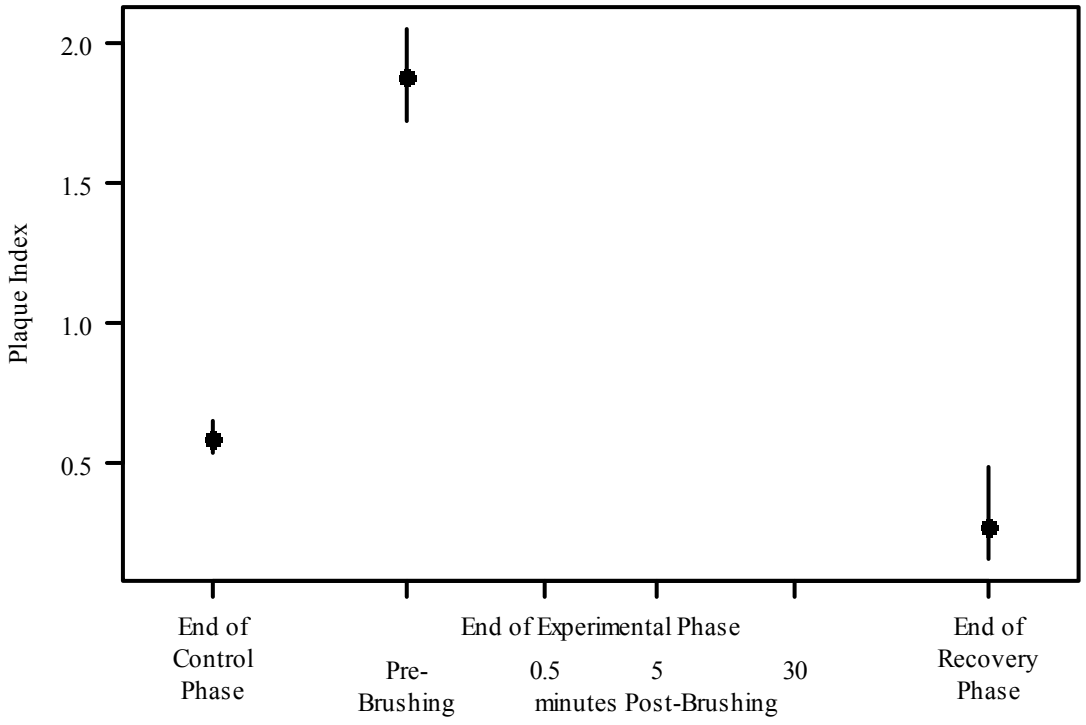


Figure 3.3. Mean (± 95% confidence interval) gingival index over the course of the study (N = 21).

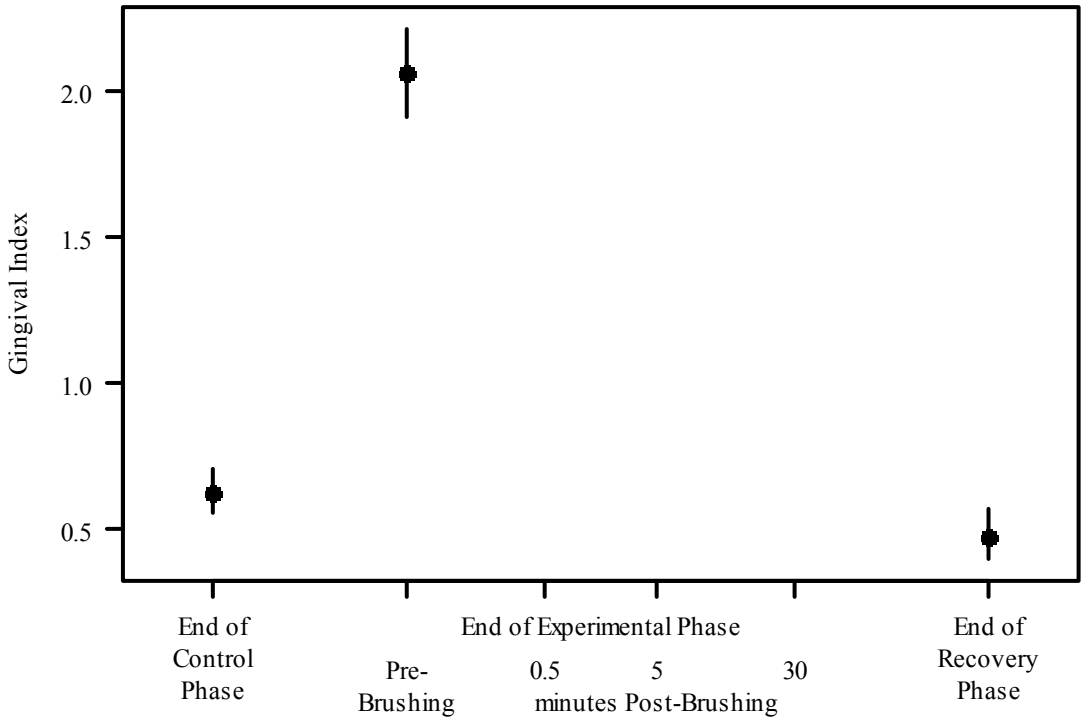


Figure 3.4. Mean (± 95% confidence interval) white blood cell count (x 10^9 cells/L) over the course of the study (N = 21).

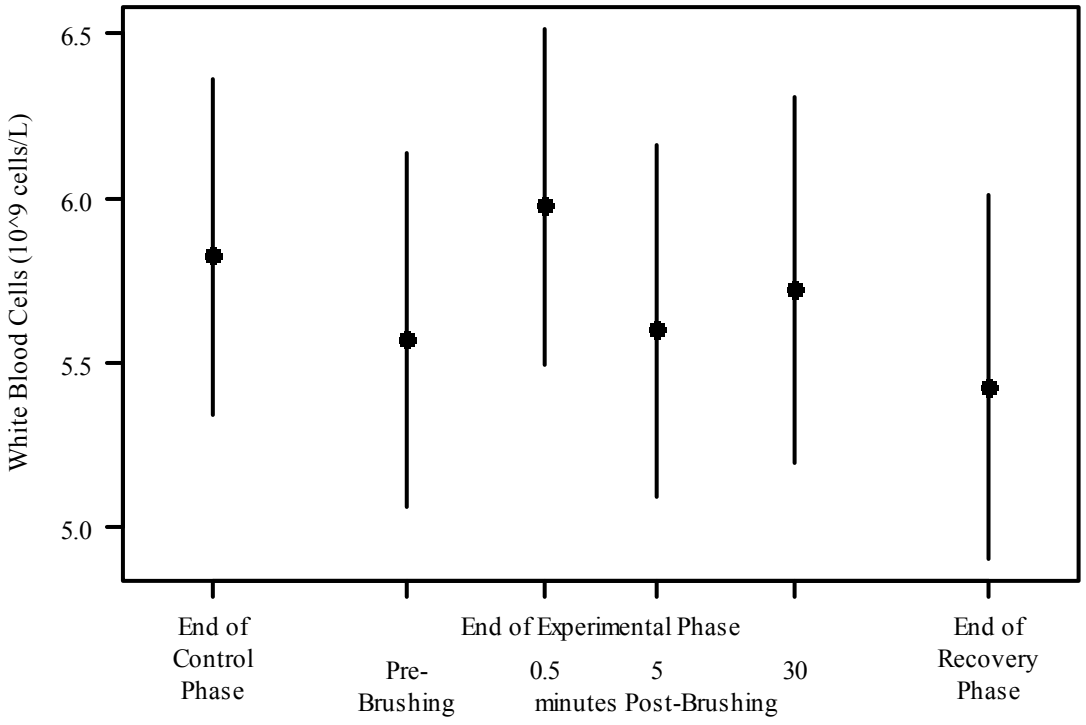


Figure 3.5. Mean (± 95% confidence interval) neutrophil count (x10^9 cells/L) over the course of the study (N = 21).

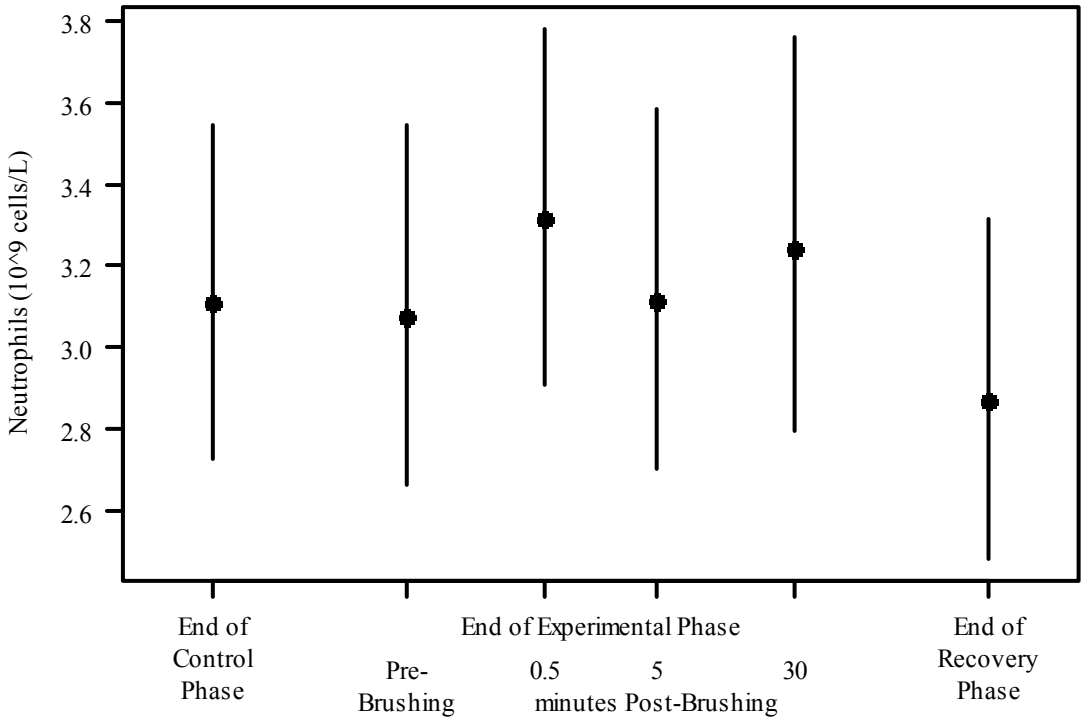


Figure 3.6. Mean (± 95% confidence interval) red blood cell count (x10^{12} cells/L) over the course of the study (N = 21).

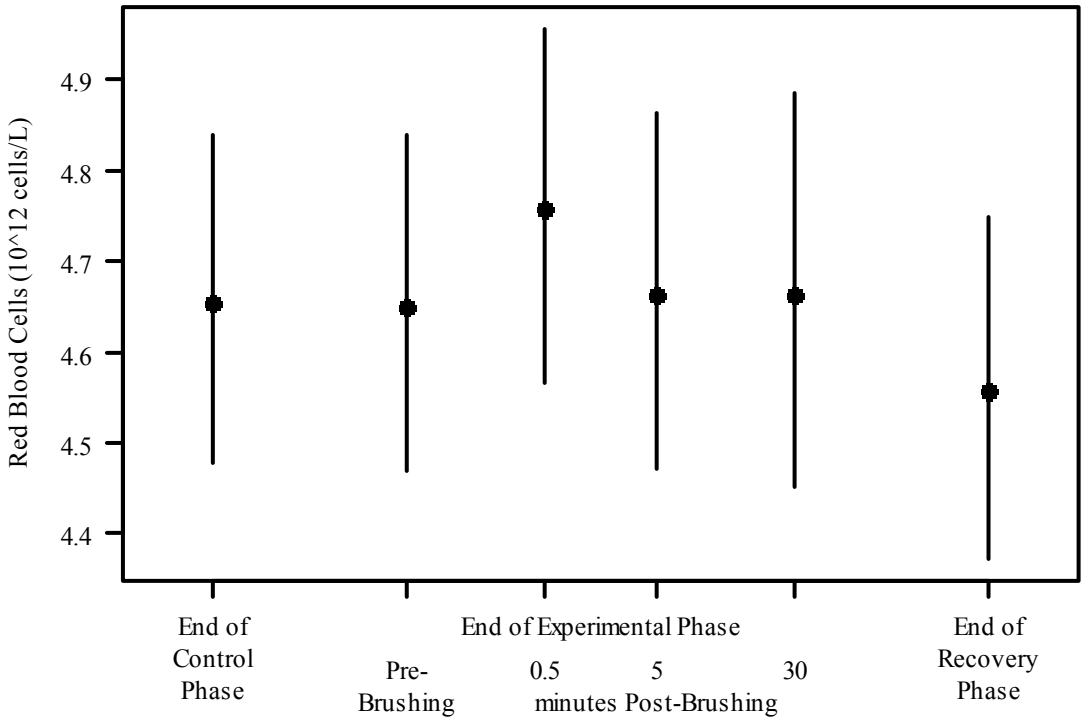


Figure 3.7. Mean (± 95% confidence interval) platelet count (x10^9 cells/L) over the course of the study (N = 21).

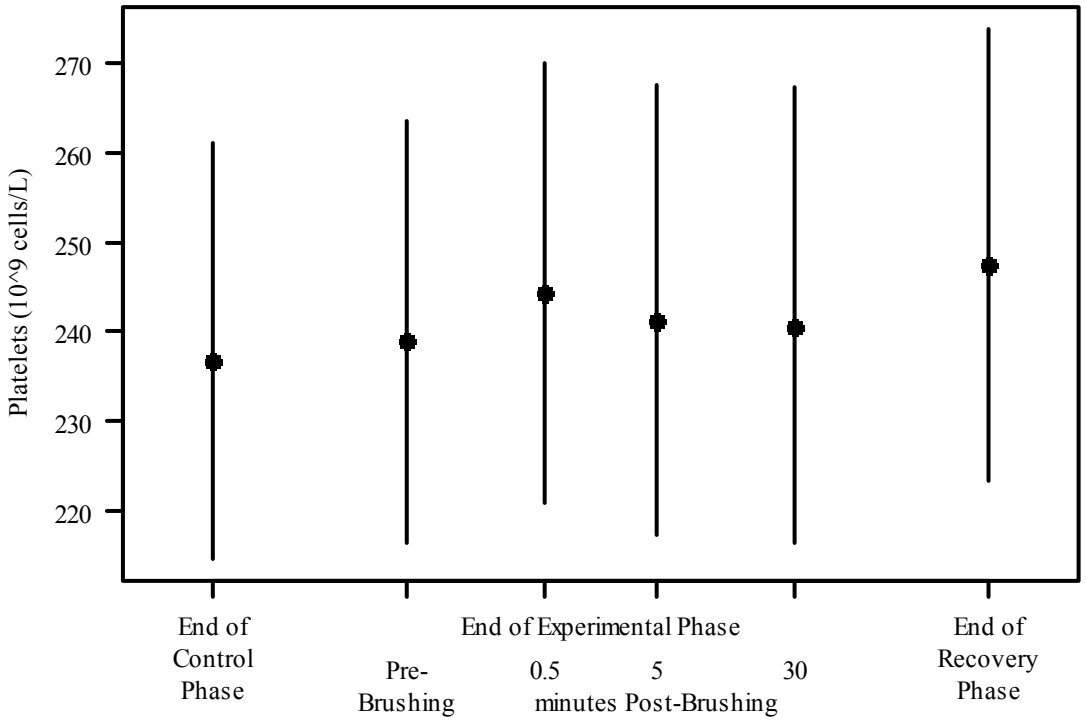


Figure 3.8. Mean (± 95% confidence interval) hemoglobin (g/dL) over the course of the study (N = 21).

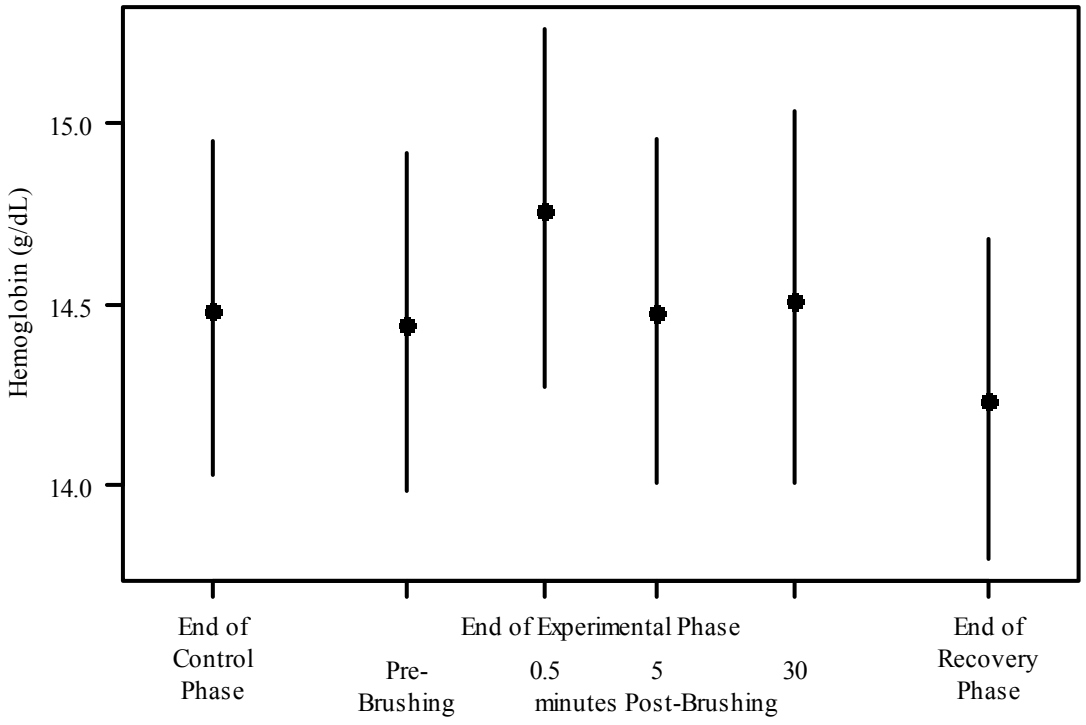


Figure 3.9. Mean (± 95% confidence interval) hematocrit (%) over the course of the study (N = 21).

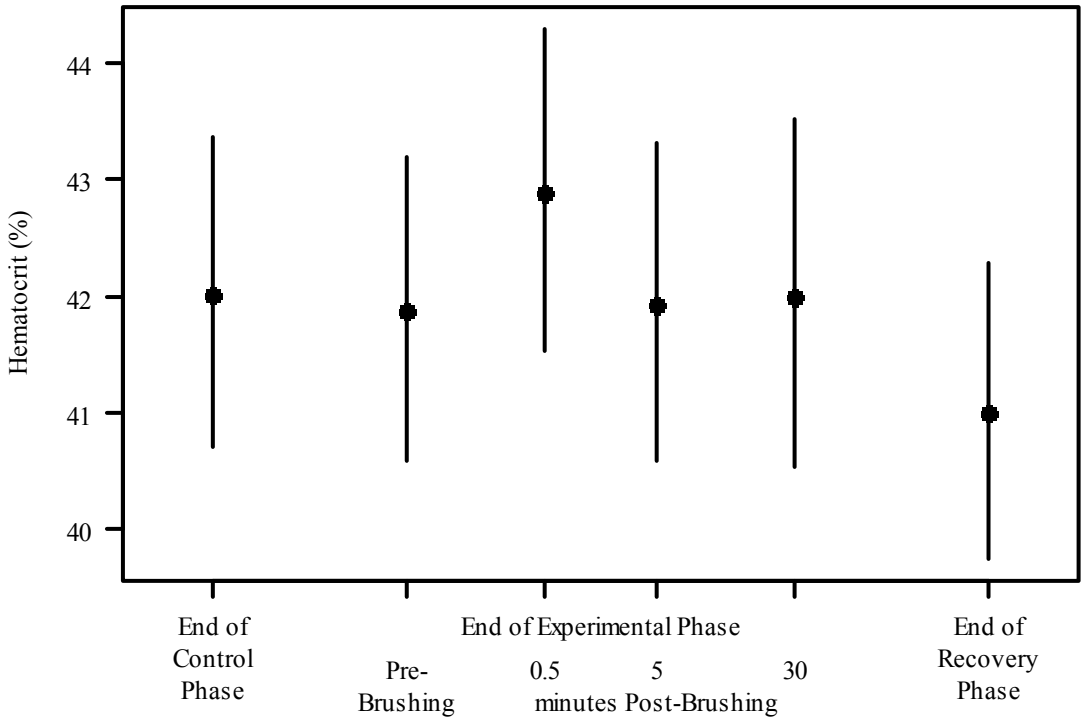


Fig 3.10. Mean (± 95% confidence interval) total chemiluminescence (millivoltage.min) over the course of the study (N = 21).

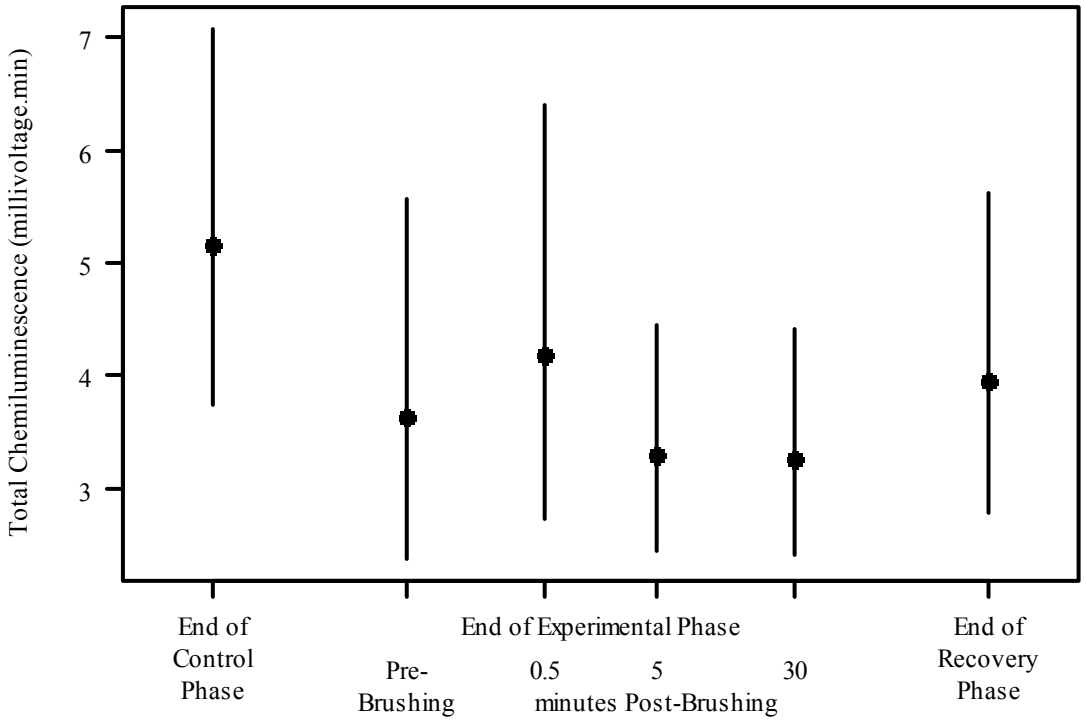
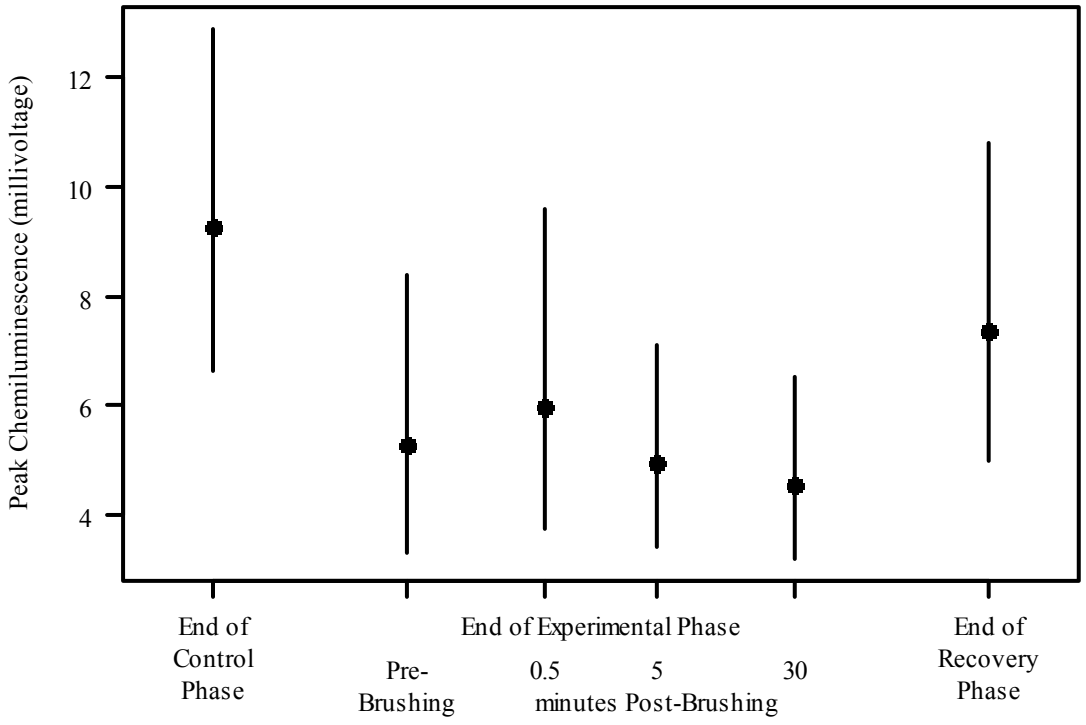


Figure 3.11. Mean (± 95% confidence interval) peak chemiluminescence (millivoltage) over the course of the study (N = 21).



CHAPTER FOUR

The In Vitro Response of Human Peripheral Blood Neutrophils to *Fusobacterium Nucleatum*

Abstract

F. nucleatum is one of the most numerically prevalent bacteria associated with gingivitis, and is associated with bacteremia of oral origin. The interaction between oral bacteria and host defense is a central mechanism linking oral and systemic diseases. We hypothesized that the metabolic oxidative activity of peripheral blood neutrophils is stimulated by exposure to *F. nucleatum*. Objective: To evaluate the oxidative response of human peripheral blood neutrophils, in terms of priming and activation, to *F. nucleatum*. Materials & Methods: The oxidative activity of peripheral blood neutrophils was evaluated by lumiol-enhanced chemiluminescence. Assays were conducted using *F. nucleatum* ATCC 10953. In addition, *Staphylococcus aureus* ATCC 9144 and *Aggregatibacter actinomycetemcomitans* ATCC 33384 were used as bacterial controls. All bacteria were killed by exposure to UV light prior to use in the assays. Comparisons among the groups were performed using ANOVA. Results: Activation of neutrophils with *F. nucleatum* resulted in significantly higher total chemiluminescence ($p<0.05$) as compared to activation with *A. actinomycetemcomitans* and *S. aureus*. In turn, the total chemiluminescence induced by activation with *A. actinomycetemcomitans* was higher than that produced by activation with *S. aureus* ($p<0.05$). Not priming the neutrophils or priming the neutrophils with N-Formyl-L-methionyl-L-leucyl-L-phenylalanine or *F. nucleatum* prior to activation with *F. nucleatum*, did not significantly affect the levels of total chemiluminescence. Conclusions: The study could not demonstrate a priming effect

of *F. nucleatum*. However, activation of human peripheral blood neutrophils with *F. nucleatum* induced the highest levels of total chemiluminescence whether neutrophils were primed or unprimed. The findings of this study may shed light on the mechanistic pathways by which oral infection may impose risk for systemic diseases.

Introduction

F. nucleatum is a filamentous gram-negative anaerobic organism that has been associated with periodontal disease, halitosis, and dental pulp infection (Bolstad *et al*., 1996). In addition, this organism has been associated with bacteremia (Edson *et al*., 1982) and pericarditis (Mangan *et al*., 1991).

F. nucleatum, a member of the so called "orange complex" of periodontal organisms (Socransky *et al*., 1998), is one of the most numerically prevalent bacterial species found in subgingival dental plaque samples that are obtained from both healthy and diseased sites (Moore and Moore, 1994). A shift from mainly streptococci and actinomyces in healthy sites to primarily fusobacteria in gingivitis sites has been previously identified (Kolenbrander *et al*., 2006).

Fusobacteria can reach their numerical abundance by metabolic communication and coaggregation with oral bacteria that populate the echo niche in health and in disease conditions (Kolenbrander *et al*., 1989). *F. nucleatum* has been proposed as a key organism in bridging between early and late colonizers by coaggregating with both (Kolenbrander *et al*., 1989) and thus, *F. nucleatum* is suggested (Kolenbrander *et al*., 1989) to play a central role in facilitating an ordered microbial succession during the dental plaque formation process.

The interaction between bacterial species and the host defense system is a central mechanism that underlies the link between oral and systemic diseases and thus, it is essential to investigate interactions between different periodontal bacteria and host cells.

F. nucleatum has been reported to be generally immunosuppressive in nature (Shenker *et al*., 1984; Jewett *et al*., 2000). This oral species has been shown to inhibit functions of both B and T lymphocytes, by inducing changes in cellular DNA, RNA, and protein synthesis (Shenker *et al*., 1984). *F. nucleatum* has also been shown to induce apoptosis in peripheral blood monocytes and granulocytes (Jewett *et al*., 2000). This induction of apoptosis might be important in the pathogenesis of periodontal disease.

It has been proposed (Jewett *et al*., 2000) that *F. nucleatum* may induce periodontal disease by initially colonizing the tissues, increasing its counts, suppressing lymphocytes, and eventually, depleting immune cells. This depression of the immune system may allow for the recruitment and the binding of other periodontally pathogenic bacteria to *F. nucleatum*. Binding of other bacteria to *F. nucleatum*,in turn, competes with the sites on *F. nucleatum* that induce apoptosis of lymphocytes and thus, immune activation is reinitiated, resulting in inflammatory injury to periodontal tissues and development of periodontal disease (Jewett *et al*., 2000).

Recently, it has been demonstrated that peripheral blood neutrophils from individuals with chronic periodontitis are more activate in response to stimulation with *F. nucleatum* as compared to peripheral blood neutrophils from healthy individuals (Mathews *et al*., 2007). These peripheral blood neutrophils from individuals with chronic periodontitis have also been reported (Mathews *et al*., 2007) to be primed as evidenced by the release of high levels of reactive oxygen radicals in the absence of an exogenous stimulant.

F. nucleatum has been implicated in the occurrence of bacteremia, a feasible mechanism by which *F. nucleatum* may impose risk to the development of systemic disease. In this regard, it was suggested that about 12% of bacteremias may be caused by anaerobes including *F. nucleatum* (Washington, 1975; Edson *et al*., 1982).

Several studies have confirmed the presence of oral and periodontal bacteria in atherosclerotic plaques, as an indication of bacteremia. In a recent study (Ford *et al*., 2005), real-time PCR has demonstrated the presence of *F. nucleatum* in approximately 80% of atherosclerotic plaques. Findings from the study (Ford *et al*., 2005) provide evidence to support the hypothesis that oral bacteria, including *F. nucleatum*, can invade blood vessels and disseminate systemically. However, the study could not determine if *F. nucleatum* plays an active role in atherosclerosis.

F. nucleatum is also one of the most prevalent oral species found in infections of the placenta and amniotic fluid (Chaim and Mazor, 1992; Hill, 1998). The prevalence of *F. nucleatum* infection in amniotic fluid was reported to be around 10 to 30% in women who are in preterm labor with intact membranes and 10% in women with preterm premature rupture of membranes. This prevalence was reported (Chaim and Mazor, 1992; Hill, 1998) to be higher than for most other single oral species that may cause amniotic fluid infection.

It is hypothesized that *F. nucleatum* infection of the placenta and amniotic fluid occurs via bacteremia from the oral cavity. Related to this, *F. nucleatum* was shown to induce

preterm labor and fetal death when injected into the bloodstream of pregnant mice (Han *et al*., 2004). The findings from this study (Han *et al*., 2004) shed light on the pathophysiological mechanisms underlying the possible causal link between periodontal disease and adverse pregnancy outcomes.

Interestingly, a recent publication (Saito *et al*., 2008) has demonstrated that *F. nucleatum* can enhance the ability of periodontal pathogens (i.e., *Porphyromonas. gingivalis*) to invade human cells including, gingival epithelial and endothelial cells. The study suggested that this invasion of host cells by periodontal pathogens may be a mechanism that mediates the progression of not only periodontal disease but also systemic diseases such as cardiovascular disease.

Because of *F. nucleatum*'s involvement in bacteremia, its numerical abundance in dental plaque samples during gingivitis and our interest in the possible link between gingivitis and systemic diseases, we believe that *F. nucleatum* may serve as the organism of choice to further explore the possible mechanisms underlying the connection between gingivitis and systemic diseases.

During gingivitis, the oral mucosa is inflamed and the number of *F. nucleatum* increases dramatically. As a result, transient bacteremias comprised of *F. nucleatum* may occur; and it is proposed that circulating blood neutrophils will respond as the primary acute host defense cells.

The activation of peripheral blood neutrophils occurs as a two-stage process consisting of priming and full scale activation. This two-stage activity protects the host from non-specific activation of neutrophils and thus, aids in avoiding indiscriminate destruction of host tissues (Edwards, 1994). Priming occurs in vitro and in vivo and can be achieved by sub-stimulatory concentrations of many types of neutrophil agonists. Primed neutrophils produce higher levels of reactive oxidants and are of greater phagocytic activity. Thus, primed neutrophils are much more potent in killing bacterial pathogens and, at the same time, are more powerful in producing inflammatory tissue damage (Edwards, 1994).

Study Objective

To evaluate the response of human peripheral blood neutrophils, in terms of priming and activation, to a bacterial challenge of *F. nucleatum*. The broad goal of this study was to increase our understanding of possible mechanistic pathways linking oral and systemic inflammatory diseases.

Specific Aims

The specific aims of this study were to use a luminol-dependent chemiluminescence assay to determine:

1. The effect of *F. nucleatum* on priming of peripheral blood neutrophils. The working hypothesis was that neutrophils could be primed in response to a challenge with a substimulatory dose of *F. nucleatum*.

2. The effect of *F. nucleatum* on activation of peripheral blood neutrophils. The working hypothesis was that neutrophils could be activated when challenged with a stimulatory dose of *F. nucleatum*.

3. The effect of *F. nucleatum* on both priming and activation of peripheral blood neutrophils. The working hypothesis was that neutrophils could be both primed and activated in response to *F. nucleatum*.

Materials and Methods

Since the study involved the use of human blood cells, the study protocol was submitted to the IRB of Indiana University Purdue University Indianapolis (IUPUI)/Clarian and approved (approval # 0712-67) (Appendix 15) prior to commencement of the study.

Bacterial Strains and Cultures

In addition to *F. nucleatum* which was the experimental bacterium in this study, the study included the use of *S. aureus* and *A. actinomycetemcomitans* as bacterial controls. *S. aureus* has been widely used as a control bacterium in studies that investigated bacterial-neutrophil interactions (Matthews *et al*., 2007a; Matthews *et al.,* 2007b). In the current study we also used *A. actinomycetemcomitans* as an additional bacterial control because both *A. actinomycetemcomitans* and *F. nucleatum* are Gram-negative bacteria that are involved in the pathogenesis of human periodontal disease.

The bacterial strains that were used in the current study were all from the American Type Culture Collection (ATCC, Rockville, MD). The study involved the use of *F. nucleatum* ATCC 10953 (type strain, obtained from inflamed gingiva of an adult male), *S. aureus* ATCC 9144 (the laboratory reference strain), and *A. actinomycetemcomitans* ATCC 33384 (type strain; was obtained from Dr. Galli's lab). These strains were chosen because they are all type-strains that have been more fully characterized and widely used in studies as compared to other strains.

S. aureus ATCC 9144 and *A. actinomycetemcomitans* ATCC 33384 were grown in TSBYE (3% tryticase soy broth and 0.6% yeast extract) agar plates and inoculated into TSBYE broth at 37°C in 10% CO_2 (Permpanich, 2003).

F. nucleatum ATCC 10953 was initially grown in modified chopped meat medium (Remel, Lenexa, KS, USA) and then in thioglycollate medium (Remel) and on CDC anaerobic blood agar plates (Fisher Scientific) at 37°C under anaerobic conditions that were generated by using an anaerobic gas chamber (Clarian Pathology Clinical Microbiology Laboratory) during the initial transfer of the bacteria from the ATCC vial containing the lyophilized bacteria and followed by using GasPak anaerobic jars (GasPak; BBL Microbiology Systems,Cockeysville, Md, USA) to create anaerobic conditions during the subsequent experiments.

Growth curves were plotted for each of the bacterial species by reading the turbidity of bacterial cultures using a spectrophotometer at optical density $(OD)_{600nm}$. The number of

viable cells at different timepoints of growth was determined by serial plating the bacterial cultures. Bacterial cells were grown and harvested at mid-late log growth phase. Purity of bacterial cultures was confirmed by checking the colonies' morphology on agar plates and by periododically performing gram staining and catalase testing for *F. nucleatum* bacterial suspensions.

Bacteria were isolated from broth cultures by centrifugation, washed twice and resuspended in sterile PBS.

All three bacterial species were killed by either heat, using a water bath at 75°C for 1 hour or by exposure to Ultra Violet (UV) radiation by pipetting 1 ml of the liquid bacterial suspension in PBS into petri plates and exposing the plates that contain the bacterial suspensions to UV radiation for 1 hour in a laboratory fume hood (Dr. Fontana's lab).

Effectiveness of both methods that were used to kill the bacteria (heat vs. UV) was assessed by serial plating of both undiluted and diluted killed bacterial suspensions using the three bacterial species. Both bacterial killing methods were then compared for their effects on neutrophil metabolic oxidative activity by performing a chemiluminescence assay to evaluate the neutrophil metabolic oxidative activity generated by interaction of neutrophils with each of the three bacteria following killing by both heat and UV radiation. Stocks of the killed bacteria were stored at −70°C in aliquots to avoid freezing/thawing variability.

Pilot experiments, similar to the experiments described later in this chapter, were conducted using different bacterial/neutrophil ratios in order to determine the optimal bacteria/neutrophil ratio to be used in neutrophil priming and activation experiments for each of the study organisms. Based on findings of these pilot experiments, we selected a ratio of 100:1 bacteria/neutrophil to be used in this study for the three bacterial species.

Isolation of Human Neutrophils

Buffy coats were purchased from the Central Indiana Regional Blood Center (CIRBC) in Indianapolis, Indiana following separation from freshly collected whole blood from healthy adult donors. Once in the laboratory, the buffy coats were diluted 1:1 with Roswell Park Memorial Institute (RPMI)-1640 cell media (Sigma) to maximize efficiency of separation. This protocol for obtaining human neutrophils has been previously used in other studies and approved by the IUPUI IRB (IRB number 0309-56) (Appendix 16). The neutrophils were separated by the Double Dextran Gradient Method (Boyum, 1968) as follows:

- Add 3 ml room temperature HISTOPAQUE-1119 (Sigma).
- Carefully layer on 3 ml HISTOPAQUE-1077 (Sigma).
- Carefully layer on 6 ml buffy coat/RPMI-1640 mixture.
- Centrifuge at 1700 RPM for 35 minutes (18-26◦C).
- Draw off neutrophil layer with a pipette.
- Wash twice with 10 ml of PBS at room temperature. Centrifuge at 950 RPM for 10 minutes.

- Repeat wash with 10 ml RPMI-1640.
- Resuspend in 10 ml of RPMI-1640, stain a sample of cells with Trypan Blue (Sigma), count cells, and determine cell concentration by hemocytometer.

The neutrophils obtained from the buffy coat separation process were stored at 25◦C for up to 18 hours post separation based upon findings of previous investigations (Gaydos, 1999).

Chemiluminescence Assays

The chemiluminescence assays were performed according to established protocols previously conducted for neutrophils (Gaydos, 1999; Permpanich *et al*., 2006) in the Model 1251 BioOrbit Luminometer (BioOrbit, Turku, Finland) located in the Host Defense Laboratory.

For each run of the experiment, there were 25 reaction cuvettes (as described in the following section). To each reaction cuvette, the following was added: 500 µl of neutrophil suspension in RPMI-1640 (containing 1 x 10^6 cells), 300 µl of PBS, and 100 µl luminol which was used as a chemiluminescent probe for signal augmentation.

100 µl of the appropriate stimulant including fMLP, *A. actimomycetemcomitans*, *S. aureus*, or *F. nucleatum* was added to activate the neutrophils. The reaction was followed for a total of 90 minutes. Neutrophil activation was recorded in terma of total

chemiluminescence (millivolts.min) and peak chemiluminescence (millivolt). Data analyses were performed on the mean values of triplicate experiments.

Study Groups and Controls

As described earlier, for each run of the experiment, there were 25 reaction cuvettes: The study included negative and positive control groups for both priming and activation, as described below.

To investigate the role of *F. nucleatum* in neutrophil priming, activation, or both, the neutrophil respiratory burst, as measured by the chemiluminescence assay, was compared for the following groups:

- Blank (reagents only)
- (1 replicate)
- Unprimed neutrophils + reagents
- (1 replicate)
- Neutrophils primed with 10^{-11} M fMLP + reagents
- (1 replicate)
- Neutrophils primed with *F. nucleatum* + reagents
- (1 replicate)
- Neutrophils primed with 10^{-11} M fMLP + *S. aureus* (stimulant) + reagents
- (3 replicates)

- Neutrophils primed with 10^{-11} M fMLP + *A. actinomycetemcomitans* (stimulant) + reagents
- (3 replicates)
- Neutrophils primed with 10^{-11} M fMLP + *F. nucleatum* (stimulant) + reagents
- (3 replicates)
- Neutrophils primed with 10^{-11} M fMLP + 10^{-6} M fMLP (stimulant) + reagents
- (3 replicates)
- Neutrophils primed with *F. nucleatum* + 10^{-6} M fMLP (stimulant) + reagents
- (3 replicates)
- Neutrophils primed with *F. nucleatum* + *F. nucleatum* (stimulant) + reagents
- (3 replicates)
- Unprimed neutrophils + *F. nucleatum* (stimulant) + reagents
- (3 replicates)

The experiment was repeated five times using buffy coats from five different blood donors in order to account for inter-subject variability.

Statistical Analysis

The chemiluminescence measurements for each group were divided by the chemiluminescence measurement for the control within each blood sample to standardize the data from each run of the experiment. Comparisons among the groups were performed using ANOVA. The ANOVA included a random effect for blood sample to correlate the data within each experimental run. Pair-wise comparisons were performed

using Tukey's multiple comparisons procedure to control the overall significance level of the tests at 5%. The natural logarithm of the chemiluminescence measurements was used in the analysis to satisfy the distribution assumption of the ANOVA.

Results

The results of the neutrophil chemiluminescence assays are presented in tables 4.1 and 4.2 and in Figures 4.1 and 4.2, each representing the mean results for total and peak chemiluminescence from five similar experiments using five different blood samples.

Activation of peripheral blood neutrophils with *F. nucleatum* produced significantly higher total chemiluminescence as compared to fMLP, *A. actimomycetemcomitans* and *S. aureus* ($p<0.05$). The total chemiluminescence induced by activation of neutrophils with *A. actimomycetemcomitans* was significantly higher than that induced by activation with *S. aureus* ($p<0.05$) (Figure 4.2).

Total chemiluminescence: (*S. aureus* < *A. actimomycetemcomitans* < *F. nucleatum*)

Regarding peak chemiluminescence (Figure 4.2), activation of peripheral neutrophils with *F. nucleatum* produced significantly higher peak chemiluminescence as compared to activation with fMLP, *A. actimomycetemcomitans*, and *S. aureus*. In turn, activation of peripheral blood neutrophils with fMLP produced higher peak chemiluminescence as compared to activation with *A. actimomycetemcomitans* and *S. aureus*.

Peak chemiluminescence: (*A. actimomycetemcomitans* and *S. aureus* < FMLP < *F. nucleatum*)

In this study, not priming the neutrophils or priming the neutrophils with fMLP or *F. nucleatum* prior to activation with *F. nucleatum* or, did not significantly affect the levels of total or peak chemiluminescence ($p>0.05$) (Figure 4.1).

Discussion

The results of this study did not show a neutrophil priming effect of *F. nucleatum*. However, the study confirmed that *F. nucleatum* is a potent activator of circulating neutrophils.

The data from this study demonstrate that *F. nucleatum*, when compared with fMLP, *A. actimomycetemcomitans*, and *S. aureus*, produced the greatest amount of peripheral blood neutrophil metabolic oxidative activity as measured by both, total and peak chemiluminescence. The demonstration of highest luminol-dependent chemiluminescence generation by neutrophils following stimulation with *F. nucleatum* as compared to other bacteria is in agreement with findings from previous related studies. For example, in a previous related study (Passo *et al*., 1982), when compared to Bacteroides, Capnocytophaga, Selenomonas, and Treponema species, *F. nucleatum* (heat-killed) was shown to be a strong stimulator of neutrophil chemiluminescence.

In agreement with our investigation, it was demonstrated (Katsuragi *et al*., 2003) that both the intracellular and extracellular oxygen radical production by neutrophils phagocytosing *F. nucleatum* is significantly greater than that produced by neutrophils

when phagocytosing other periodontal pathogens including *P. gingivalis* and *A. actinomycetemcomitans*.

It has been postulated (Katsuragi *et al*., 2003) that the differences in production of oxygen radicals by neutrophils when interacting with different periodontal bacteria may be explained by differences in the size of these bacteria. *F. nucleatum* is about 5-8 μm long. In comparison, *P. gingivalis* and *A. actinomycetemcomitans* are only about 1 μm in length. In addition, it was suggested (Katsuragi *et al*., 2003) that bacterial pathogens such as *P. gingivalis* and *A. actinomycetemcomitans* possess some resistance factors (i.e., capsules) that might have contributed to the reduced generation of reactive oxygen species following stimulation of neutrophils by these bacteria. Moreover, some live strains of *A. actinomycetemcomitans* produce leukotoxin that kills neutrophils and enhances bacterial pathogenicity (Rabie *et al*., 1988).

In this context, it is also important to note that LPS from various bacteria may differ in its ability to activate neutrophils. LPS from *F. nucleatum* and *A. actinomycetemcomitans* was reported to be 10 times more potent in priming neutrophils and in producing oxygen species when compared to LPS from other periodontal pathogens including *P. gingivalis* and *Prevotella denticola* (Jean-Pierre *et al*., 2006).

Regarding the mechanism of interactions between *F. nucleatum* and neutrophils, previous studies (Mangan *et al*., 1989) reported that the cell wall of *F. nucleatum* can adhere to human neutrophils by lectin-like adherence factors that are present in the bacterial cell

envelope. In addition to binding neutrophils, these factors can mediate binding of *F. nucleatum* to other human cells and thus, these adherence factors may play an important role in bacterial colonization and in mediating damage to host cells (Falkler and Hawley, 1977; Mangan *et al*., 1989).

F. nucleatum binds avidly to neutrophils (Mangan *et al*., 1989; Sheikhi *et al*., 2000). This binding was shown to be associated with neutrophil aggregation, membrane depolarization, increased levels of intracellular Ca^{2+}, release of lysozyme, and enhanced production of reactive oxygen species (Passo *et al*., 1982) which are capable of causing lipid peroxidation (Sheikhi *et al*., 2001) and oxidative damage to host cells (Katsuragi *et al*., 2003).

In our current study, activation of neutrophils by *F. neucleatum* was compared to activation by *A. actinomycetemcomitans*, *S. aureus*, and fMLP. *A. actinomycetemcomitans* is a gram negative facultative non motile rod. The strain used in this study, ATCC 33384, is a type strain that is charactarized by low levels of leukotoxin. In the context of oral and systemic disease connection, studies have demonstrated that *A. actinomycetemcomitans* is associated with increased risk for CHD and cardiovascular disease (Desvarieux *et al*., 2005; Pussinen *et al*., 2005; Pussinen *et al*., 2007).

A recent animal study (Tuomainen *et al*., 2008) suggested that the periodontal pathogen *A. actinomycetemcomitans* may contribute to atherosclerosis by inducing inflammatory

responses and causing increases in serum levels of CRP, triglyceride, and blood total cholesterol.

As mentioned earlier, *A. actinomycetemcomitans* was chosen as a control bacterium in this study because of the resemblance of this organism to *F. nucleatum*; both are gram negative organisms that are involved in periodontal disease pathogenesis.

The other control bacterium used in this study was *S. aureus*. *S. aureus* is a facultative anaerobic, gram positive coccus. *S. aureus* may occur as a commensal organism on human skin, nose, and throat. *S. aureus* has been commonly used as a control bacterium in previous related studies (Matthews *et al*., 2007a; Matthews *et al*., 2007b).

Another control stimulant used in this study was fMLP. FMLP is a synthetic peptide that resembles the structure of bacterial peptides with formylated N-terminal methionine groups. FMLP activates neutrophils, resulting in enhanced chemotaxis, degranulation, and superoxide generation. FMLP has been, and still is, one of the most commonly used in vitro activators of neutrophils (Edwards, 1994).

In this study, *F. nucleatum* and the other tested bacteria were killed by using UV radiation rather than heat. To our knowledge, this is the first study to use UV radiation to treat *F. nucleatum* and the control bacteria prior to investigating their interaction with peripheral blood neutrophils.

Most previous studies, related to this study, used heat to kill bacteria. It was reported that both live and heat killed ATCC strains of *F. nucleatum* are capable of inducing the production of reactive oxygen species by neutrophils (Sheikhi *et al*., 2000). However, previous data demonstrated that pretreatment of *F. nucleatum* with heat may inhibit neutrophil binding and activation. Heating is known to denature bacterial proteins. Denatured proteins were shown to activate neutrophils and stimulate superoxide production (Wilkinson, 1988; Mangan *et al*., 1989). However, heating was reported to inhibit neutrophil chemiluminescence (Passo *et al*., 1982).

The bactericidal properties of UV radiation have been recognized for more than a century. Bacterial death may be induced by short wavelength (< 280 nm) UV radiation (Coohill and Sagripanti, 2008). Short wavelength UV radiation kills bacteria by damaging the chemical bonds in DNA molecules and producing thymine dimmers that adversely affect bacterial DNA replication (Hambidge, 2001). By utilizing UV radiation, rather than heating, as a method of killing bacteria in this study, the concern related to denaturation of bacterial proteins was avoided.

The study has limitations. In this study, we examined the interaction of killed bacteria with circulating neutrophils. Although killed bacteria are commonly used in similar investigations in the literature, live bacteria may affect priming and/or activation of human neutrophils in a different manner and thus, the effect that killed bacteria exerts on neutrophils may not fully reflect the situation with live bacteria (DeChatelet *et al*., 1974). In addition, in this study, we investigated only one aspect of the neutrophil activation

response (i.e., the production of oxygen radicals); while this might be the most important aspect of neutrophil activation for bacterial killing, other components of neutrophil activation upon interaction with oral bacteria should also be considered. A significant release of proteases such as elastase was observed following activation of neutrophils with *F. nucleatum* in previous studies (Sheikhi *et al*., 2000).

The demonstration of high oxidative metabolic activity by peripheral blood neutrophils when activated with *F. nucleatum* may be of clinical importance. Oxygen radicals can result in tissue destruction by causing DNA damage, lipid peroxidation (Sheikhi *et al*., 2001), and oxidation of important proteins and enzymes (Chapple, 1997). As described earlier, numerous inflammatory and systemic conditions are mediated by neutrophil oxidant injury, including rheumatoid arthritis, diabetes, CHD, stroke, and inflammatory lung disease (Malech and Gallin, 1987; Morel *et al*., 1991; Noguera *et al*., 2001).

We hypothesized that sub-stimulatory concentrations of *F. nucleatum* would result in priming of peripheral blood neutrophils. However, the data from current study failed to support this part of our hypothesis; the reason behind this is unclear. Since peripheral blood neutrophils can be primed, but not fully active, in the vascular circulation, depending on the clinical status of the blood donors, it might be that the neutrophils used in this study were already in a primed state when the blood samples were obtained. Both priming and full scale activation of circulating neutrophils are of clinical importance. It has been suggested that priming can be an important etiological risk factor whereas full scale activation is important in disease progression (Matthews *et al*., 2007a).

In conclusion, this study did not demonstrate a neutrophil priming effect by *F. nucleatum*. However, this does not obviate a possible role of *F. nucleatum* in systemic disease risk since activation of peripheral blood neutrophils with *F. nucleatum* induced significantly high levels of total and peak chemiluminescence compared to the other bacteria tested, whether neutrophils were primed or unprimed. The results of this study may shed light on a feasible mechanistic pathway by which oral infection may impose risk for the development and/or progression of systemic diseases.

Table 4.1. Mean (N = 5) total and peak neutrophil chemiluminescence response induced by each of the study groups.

	Group	N	Mean	SD	SE	Min	Max
Total CL (millivolt.min)	AA	15	2.79	2.56	0.66	1.21	7.78
	FMLP	15	1.97	1.44	0.37	1.09	4.76
	FN	15	38.51	54.73	14.13	3.91	144.69
	No PSFN	15	38.63	53.65	13.85	3.74	146.63
	PFN	15	2.24	1.84	0.48	1.19	6.23
	PSFN	15	42.23	57.97	14.97	3.74	163.50
	SA	15	1.38	0.66	0.17	0.99	2.73
Peak CL (millivolt)	AA	15	4.33	5.17	1.33	1.32	14.58
	FMLP	15	16.85	27.11	7.00	1.54	70.27
	FN	15	72.15	112.51	29.05	5.33	295.72
	No PSFN	15	75.39	114.49	29.56	4.93	304.11
	PFN	15	19.23	30.77	7.95	2.07	84.61
	PSFN	15	72.48	106.77	27.57	4.94	288.87
	SA	15	5.90	9.99	2.58	0.99	28.53

CL = chemiluminescence

SA: neutrophils were primed with fMLP and stimulated with *S. aureus*

AA: neutrophils were primed with fMLP and stimulated with *A. actinomycetemcomitans*

FN: neutrophils were primed with fMLP and stimulated with *F. nucleatum*

fMLP: neutrophils were primed and stimulated with fMLP

NoPSFN: neutrophils were not primed but were stimulated with *F. nucleatum*

PSFN: neutrophils were primed and stimulated with *F. Nucleatum*

PFN: neutrophils were primed with *F. nucleatum* and stimulated with fMLP

Table 4.2. P-values for mean (N = 5) neutrophil chemiluminescence comparisons between study groups.

Group comparison			p-values for total chemiluminescence	p-values for peak chemiluminescence
AA	vs.	FMLP	0.7259	0.0001
AA	vs.	FN	0.0001	0.0001
AA	vs.	No PSFN	0.0001	0.0001
AA	vs.	PFN	0.9555	0.0001
AA	vs.	PSFN	0.0001	0.0001
AA	vs.	SA	0.0377	0.2931
FMLP	vs.	FN	0.0001	0.0001
FMLP	vs.	No PSFN	0.0001	0.0001
FMLP	vs.	PFN	0.9981	0.6662
FMLP	vs.	PSFN	0.0001	0.0001
FMLP	vs.	SA	0.6914	0.0001
FN	vs.	No PSFN	1.0000	1.0000
FN	vs.	PFN	0.0001	0.0001
FN	vs.	PSFN	0.9993	0.9999
FN	vs.	SA	0.0001	0.0001
No PSFN	vs.	PFN	0.0001	0.0001
No PSFN	vs.	PSFN	0.9990	1.0000
No PSFN	vs.	SA	0.0001	0.0001
PFN	vs.	PSFN	0.0001	0.0001
PFN	vs.	SA	0.3448	0.0001
PSFN	vs.	SA	0.0001	0.0001

SA: neutrophils were primed with fMLP and stimulated with *S. aureus*

AA: neutrophils were primed with fMLP and stimulated with *A. actinomycetemcomitans*

FN: neutrophils were primed with fMLP and stimulated with *F. nucleatum*

fMLP: neutrophils were primed and stimulated with fMLP

NoPSFN: neutrophils were not primed but were stimulated with *F. nucleatum*

PSFN: neutrophils were primed and stimulated with *F. nucleatum*

PFN: neutrophils were primed with *F. nucleatum* and stimulated with fMLP

Figure 4.1 Mean (+ SE) total and peak chemiluminescence for each of the priming study groups.

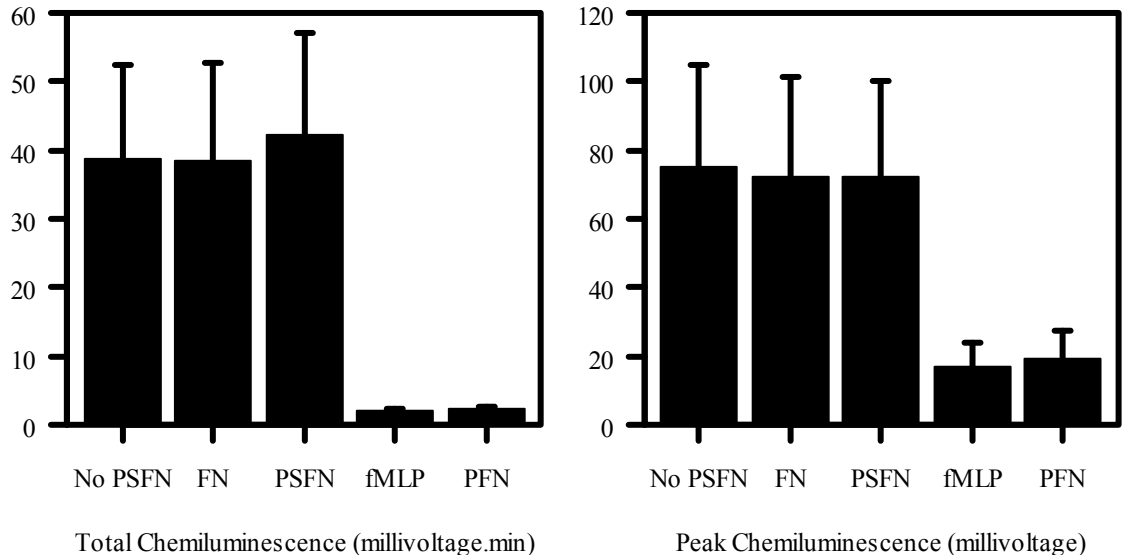
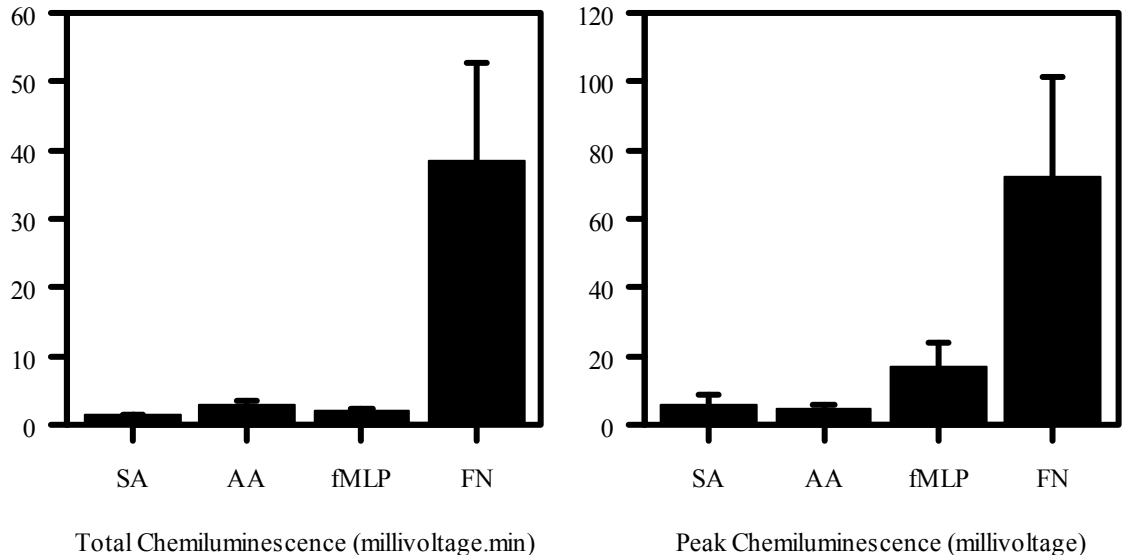


Figure 4.2. Mean (+ SE) total and peak chemiluminescence for each of the activation study groups.



Note: Neutrophils were primed with fMLP prior to activation in all activation experiments

CHAPTER FIVE

General Summary and Conclusions

This chapter provides a general summary and conclusions from the three studies that were described in the previous chapters (two-four) of this dissertation. This chapter also discusses the significance of our study results in general terms and suggests future directions for continuing the research.

The broad objective of our investigations was to increase our understanding of the causal association between oral disease and CHD, a systemic disease with significant public health implications. The rationale for our research studies was that a definitive etiological role of oral disease in CHD must be established before steps can be taken in implementing successful CHD preventive strategies through maintaining an optimal oral health status.

Our studies collectively, and our clinical studies in particular, aimed at exploring pathophysiological mechanisms underlying the possible etiological link between gingivitis and CHD. Bacteremia involving oral bacteria and the host systemic response to the local oral infection and/or to circulating bacteria are fundamental mechanisms that could causally link oral and systemic diseases.

In general, we hypothesized that dental plaque accumulation and the resultant gingivitis induces bacteremia and or/endotoxemia, and elicits systemic host responses (Figure 5.1).

We explored the possible cauasal relationship between induced oral infection (i.e., gingivitis) and systemic diseases (i.e., CHD) in two prospective clinical studies and a laboratory investigation. Both clinical studies investigated the possible mechanistic pathways that can causally relate gingivitis to CHD, in young healthy adults. These pathways included the systemic dissemination of oral bacteria (i.e., bacteremia) and or the systemic dissemination of LPS following a period of dental plaque accumulation, and the systemic inflammatory responses to the disseminating bacteria or bacterial products and/or to the local dental plaque accumulation and gingivitis. Both of our clinical studies utilized the experimental gingivitis model to induce gingivitis. We anticipated that these systemic responses would manifest as a change in the level of inflammatory biomarkers, hematological factors, serum lipids, biomarkers of metabolic change, and neutrophil response, some of which may be with potential atherogenic consequences. Moreover, we anticipated that these responses would differ between individuals and may be dependant on gender and race.

The findings from our first study did not conclusively show obvious systemic responses across all groups. However, several systemic factors that have been associated with systemic inflammation and systemic disease (including CHD) were expressed in this experimental gingivitis study. The expression of these factors differed between individuals of different genders/races. In brief, significant increases were observed in the neutrophil oxidative activity, IL-1α, and MCH levels in blacks. In black males, the ESR increased. Fibrinogen levels increased in white males and 8-isoprostane levels increased in white females. Overall, significant decreases occurred in total cholesterol, HDL, and

RBC count. Hct and Hb levels decreased in blacks. In black males, decreases were observed in the LDL and 8-isoprostane levels. In addition, decreases were observed in IL-1β levels for white males and in IL-6 levels for white females.

The results of the second study support the hypothesis that dental plaque accumulation results in bacteremic episodes and induces systemic inflammatory responses. This investigation provides some evidence to support a possible causal association between oral and systemic diseases.

In both studies, we employed the experimental gingivitis model. While not biologically identical to chronic gingivitis, the findings from both studies may add weight to the hypothesis that repeatedly and in the long-term, low grade infectious challenges may be of greater systemic importance than isolated, clinically obvious events (Roberts, 1999).

Based on findings from our two clinical studies, we concluded that the experimental gingivitis model can be used to induce a classical systemic acute infectious/inflammatory challenge. The findings provide some in vivo evidence to support a causal association between oral and systemic diseases, thus creating a powerful argument for the importance of oral health care in CHD prevention.

In our third study, we used *F. nucleatum*, which is one of the most numerically abundant oral microorganisms in health and gingivitis, as an example of a dental plaque-forming microorganism, to explore the interaction of oral bacteria with systemically circulating

neutrophils, in terms of priming and stimulation. The study did not support a neutrophil priming effect of *F. nucleatum*. However, the study confirmed that *F. nucleatum* is a potent stimulator of circulating neutrophils, a pathophysiological mechanism by which oral microorganisms may impose risk to development/progression of systemic disease.

The cumulative evidence from our three studies supports the existence of pathophysiological mechanisms that could plausibly explain and possibly underlie an etiological relationship between gingivitis and systemic diseases (e.g. CHD), and contribute to risk in susceptible individuals. These biological mechanisms mediating such a relationship are still not fully understood. However, scientists are successfully putting together the pieces of this puzzle. Further large and well controlled clinical trials are necessary to identify and adequately understand a definitive causal relationship between oral and systemic diseases.

Significance of Study Results

As previously described in this chapter, the results of the studies described in chapters two-four provide further knowledge concerning the causal association between periodontal infection, gingivitis in particular, and the risk of developing systemic disease (i.e., CHD).

As mentioned earlier, CHD continues to be the leading cause of death in the U.S. and worldwide, and its impact on global health is immense. Traditional modifiable risk factors do not completely explain future cardiovascular events and there is increasing

interest in emerging risk factors including infection and inflammation. In this respect, there is an association between CHD risk and oral infection, but it is difficult to establish whether oral infection is the cause or effect since this necessitates longitudinal studies.

The results of our research studies can be considered as a step forward in determining whether there is a definitive etiological link between oral infection and risk of CHD via atherogenesis. In addition, since dental plaque accumulation and the resultant gingivitis are almost universal, these results could be significant in providing new targets for the prevention of CHD through the reduction of risk factors. Moreover, our studies have highlighted the innovative application and the successful implementation of the experimental gingivitis model to monitor systemic inflammatory responses.

Future Directions

Our research studies described in this dissertation can be considered as general preliminary or screening investigations that possess a great potential to further generate subsequent investigations. The fundamental objective of our studies was to assess the systemic responses to dental plaque accumulation generally and broadly. Thus, our findings can direct future, and more specific and focused investigations.

Our three research studies assessed the neutrophil metabolic oxidative activity, which is of clinical relevance to inflammation and tissue destruction, using a luminol-enhanced chemiluminescence assay. Although the oxidative burst might be the most important neutrophil activity assay in the context of our studies, multiple assays examining

additional aspects of the neutrophil activity would be ideal and thus, additional assays including those monitoring proteases and granular enzyme activity could be employed in future studies.

In our laboratory investigation (described in chapter four) we utilized UV-killed bacteria to study their interaction with human circulating neutrophils. Future studies, similar to ours, could be conducted utilizing and comparing UV-treated and live bacteria in order to better understand bacterial interactions with human peripheral blood neutrophils. In addition, further studies, similar to ours, can be conducted to determine the role of strain specificity by examining the effects of different *F. nucleatum* strains on priming and activation of peripheral blood neutrophils.

Our second clinical study (described in chapter three) was of a relatively small sample size and thus, included only white Caucasian adults. Based on findings from our first clinical study, described in chapter two, demonstrating a racial variation in the host response to dental plaque accumulation, future studies investigating bacteremic episodes can be conducted with a larger sample of subjects and including individuals from different races (i.e., African-Americans) in order to examine the disparity in the bacteremic episodes and the subsequent inflammatory responses in individuals of different races.

In addition, our second study examined bacteremic episodes based on blood culture techniques. As described earlier in this dissertation, traditional culture techniques for

detection of bacteremic episodes possess some limitations including low sensitivity. Further studies, similar to our investigation, can be conducted using the appropriate molecular diagnostic techniques for detection of bacteria in blood samples.

Since both of our clinical studies employed the experimental gingivitis model, which is short-term in nature, these studies focused on assessing acute inflammatory biomarkers that are most likely to appear within a short time frame, future studies can explore additional long-term inflammatory biomarkers. Similarly, the relatively short-term monitoring period during the recovery phase of our studies might have been inadequate to reverse altered levels of some of biomarkers. Future studies can be conducted in order to further understand the temporal nature of the systemic effects of dental plaque accumulation.

Our clinical studies did not monitor the microbial composition of dental plaque during the experimental gingivitis and thus, future studies could be designed to examine whether the microbial composition of dental plaque is associated with levels of systemic factors. Related to this, for general screening purposes, we utilized a non specific Limulus Amebocyte Lysate assay to detect endotoxemia in our first clinical study. Future studies could possibly consider focusing on the detection of LPS originating from specific oral bacterial species.

The dental plaque control methods that were performed during the recovery phase of our first study (toothbrushing and flossing) might have been ineffective in reversing altered

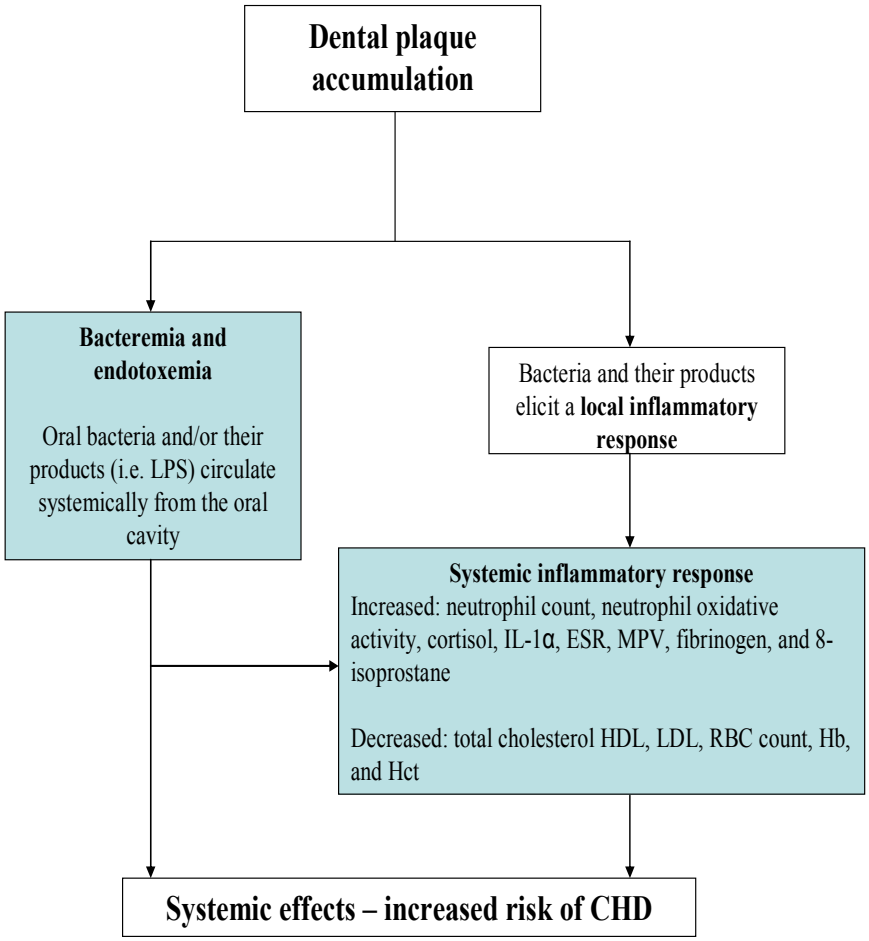
levels of some systemic factors in certain groups of subjects. Thus, future intervention studies can investigate the role of alternative methods for modification of dental plaque and their role in reducing levels of systemic inflammatory biomarkers associated with systemic disease risk. In this context, future studies may also explore the effectiveness of antioxidants and their role in modification of subsequent potential detrimental effects developed by dental plaque accumulation or in response to bacteremia.

In our first study, the response to dental plaque accumulation was addressed at the phenotypic level. Evidence exists to support the role of genetic factors in susceptibility to infection and in the inflammatory response evoked. During our first study (described in chapter two) we had an opportunity to collect and archive genomic DNA from the study participants. The extracted DNA may be important for designing future studies to explore and identify candidate genes contributing to the observed inflammatory response to dental plaque accumulation. Examples of some candidate genes that may be targeted in future studies include those associated with fibrinogen, the neutrophil metabolic oxidative activity, and the production of 8-isoprostane.

Our clinical investigations were conducted in a group of young and healthy adults. Findings from these studies can not be generalized to the population. Similar future studies may be conducted in individuals of different age groups and in individuals with systemic conditions that may affect the inflammatory profile in order to further understand the host systemic effects of dental plaque accumulation in those populations. For example, the experimental gingivitis model could be applied to a population of older

individuals who have already experienced CHD events and to a matched control group. Such an investigation would be of great significance especially with the growing number of elderly individuals and given the fact that the prevalence of periodontal disease increases with increasing age.

Figure 5.1. Summary of hypothetical mechanisms by which dental plaque accumulation may increase the risk of CHD.



Appendix 1. Final Institutional Review Board approval document for the study entitled "Dental Plaque Accumulation as a Risk Factor for CHD"

INTERDEPARTMENTAL COMMUNICATION

Research Compliance Administration
Indiana University - Purdue University Indianapolis

DATE: May 24, 2004

TO: Michael J. Kowolik
Periodontology/Oral Biology
DS 260
IUPUI

FROM: Regina Wininger
Research Compliance Administration

SUBJECT: Final Approval

Study Number: 0405-50
Study Title: Dental Plaque Accumulation as a Risk Factor for CHD (04-G-011)
Sponsor: NIH

The study listed above has received final approval from the Institutional Review Board (IRB-04). IMPORTANT NOTICE: The Institutional Review Board (IRB) requires that the consent statement given to subjects have the IRB approval stamp on the last page. As the principal investigator of this study, you assume the following reporting responsibilities:

1. CONTINUING REVIEW - A status report must be filed with the Board. The Research Compliance staff will generate these reports for your completion; however, you must request and complete these forms if the study is terminated for any reason in the interim. This study is approved from **May 24, 2004** to **May 24, 2005.**

2. STUDY AMENDMENTS - Investigators are required to report on these forms ANY changes to the research study including protocol design, dosages, timing or type of test performed, population of the study, and informed consent statement. An amendment form can be obtained on our website. See link http://www.iupui.edu/~resgrad/spon/amendment-irb.htm.

3. ADVERSE EVENTS - If this is a medical study, all side effects or adverse reactions which are serious and unexpected and associated with the study intervention must be reported immediately to the Board as they occur. See link http://www.iupui.edu/~resgrad/spon/adverse-report-notice.htm for adverse reaction reporting requirements and to obtain an Adverse Reaction Reporting Form. If the study involves gene therapy, all serious adverse events must be reported to both the IRB and the Institutional Biosafety Committee (IBC).

4. UPDATED INVESTIGATIONAL BROCHURES, PROGRESS REPORTS and FINAL REPORTS - If this is an investigational drug or device study, updated clinical investigational brochures must be submitted as they occur. See link http://www.iupui.edu/%7Eresgrad/spon/cibrequire.htm for requirements. Three copies of progress or final reports must be provided to the Board with the investigator's written assessment of the report, briefly summarizing any changes and their significance to the study.

5. ADVERTISEMENTS - If you will be advertising to recruit study participants for a drug or device study regulated under FDA requirements, i.e., investigational drugs or devices will be used, and the advertisement was not submitted to the Board at the time your study was reviewed, a copy of the information contained in the advertisement and the mode of its communication must be submitted to the reviewing board as an amendment to the study. These advertisements must be reviewed and approved by the Board PRIOR to their use.

6. LEAVING THE UNIVERSITY - If the principal investigator leaves the Institution, the Board must be notified as to the disposition of EACH study.

PLEASE REFER TO THE ASSIGNED STUDY NUMBER AND THE EXACT TITLE IN ANY FUTURE CORRESPONDENCE WITH OUR OFFICE. All documentation related to this study must be neatly typed and must also be maintained in your files for audit purposes for at least three years after termination of the research (seven years if you are subject to HIPAA). If you have any questions, please call Research Compliance Administration at 274-8289.

Enclosures:
- ☒ Documentation of Review and Approval
- ☐ Expedited Review Checklist
- ☒ Informed Consent Statement
- ☒ Advertisement
- ☒ Authorization form
- ☒ Other: Waiver of authorization for recruitment

Appendix 2. Informed consent statement form for the study entitled "Dental Plaque Accumulation as a Risk Factor for CHD"

IUPUI and Clarian Informed Consent Statement for: "Dental Plaque Accumulation as a Risk Factor for CHD (04-G-011)"

You are being invited to participate in a research project that is described as follows:

Purpose
The purpose of this project is to 1) determine the amount of plaque (sticky build up on teeth) and gingivitis (gum disease) that occurs under optimal (best) oral hygiene conditions and no oral hygiene conditions, 2) determine how this plaque build up relates to different markers in the blood related to heart disease, and 3) determine how plaque relates to the circulation of bacteria in the blood that could lead to heart disease.

Number of People Taking Part in the Study
If you agree to take part, you will be one of 300 adult subjects, 18 to 30 years of age, screened to be in this study. If you qualify, you will be one of about 140 subjects participating in the study.

Products and Methods
There will be no experimental products in this study. You will be given a marketed (sold in stores) toothbrush, floss and fluoridated toothpaste to use at home during the study. There will be a three-week no brushing period during which you will not be able to brush your teeth or use any dental products or chew gum. You will not be able to use any tobacco products throughout the study. You will be given a carbon monoxide reading (breath test that checks the air you blow out for signs of smoking) at each visit.

This study will require you to have a fasting blood sample drawn at 9 visits. A fasting blood sample means you cannot eat or drink anything but water on the morning of your blood draws. Up to four tablespoons of blood will be drawn intravenous (from a vein in your arm) at each visit. You will need to report to the General Clinical Research Center (GCRC) at Indiana University (IU) Hospital by noon each visit morning for your blood draw.

You will have a dental exam of your mouth and an exam for plaque and gingivitis after each blood draw visit. These exams will also occur at the GCRC. A light snack and juice will be provided after the exams. You will have your teeth cleaned twice, once at the beginning of the study and again at the end of the no brushing phase. The teeth cleanings will occur at the Oral Health Research Institute (OHRI).

Procedures
This study has three phases that require 11visits over an11-week period. The phases and visits are described as follows:

Screening Visit: Visit 1 – 45 to 60 minutes
During today's visit, you will be asked to read and sign this informed consent letter and an authorization for the release of health information for research letter. The information in these letters will be explained to you and you will have the opportunity to ask questions and to determine if you would like to be in the study. If you decide to participate, you will be asked to complete a medical history form and asked questions about your medical and dental history that will help the dentist determine if you qualify to participate. You will be asked if you use tobacco products and a carbon monoxide reading will be performed. Our study dentist will perform an exam to determine how healthy your gum tissue is and your height and weight will be measured. If you qualify, you will be scheduled for a dental cleaning appointment.

Phase 1 – Control Phase: Visits 2 – 6
Visit 2 – 60 minutes (OHRI): You will receive a thorough dental cleaning, including the possible use of sonicating cleaner (dental cleaning instrument that help to remove tartar build-up through vibration) and a review of good oral hygiene instructions including brushing, flossing etc.

 Subject's Initials: _____

Visits 3-6 – 30-45 minutes (GCRC): You will attend a weekly visit every seven days (Visit 3, 4, 5 and 6) to have a fasting blood sample drawn and receive a dental exam for gum disease and plaque build-up. You will receive a review of the good oral hygiene instructions during Visits 3-5. Visit 6 will end the Phase 1 and begin the no oral hygiene phase (Phase 2).

Phase 2 – No Oral Hygiene Phase: Visits 6 - 9 (3 weeks):

Visits 6-9 – 30- 45 minutes (GCRC): You will not be able to use any oral hygiene products or procedures (brushing, flossing, mouthrinsing, chewing gum etc) for three weeks following Visit 6. You will attend a weekly visit every seven days (Visit 7, 8, and 9) to have a fasting blood sample drawn and receive a dental exam for gum disease and plaque build-up. During Visit 9 you will again receive instructions on good oral hygiene and can begin your normal brushing routine following this visit. You will receive a dental cleaning (60 minutes) at OHRI either immediately following your visit #9 appointment or within a few days of this appointment.

Phase 3 – Recovery Phase: Visits 10 and 11 (3 weeks)

Visit 10 & 11- 30-45 minutes (GCRC): At two and three weeks following your dental cleaning you will have a fasting blood sample drawn and receive a dental exam for gum disease and plaque build-up. Your participation will end at the conclusion of Visit 11 unless the dentist feels it is in your best interest that you receive an additional dental cleaning. This cleaning appointment will be at OHRI within a few days of 11.

Digital Images

Digital images of changes in your gum tissue may be taken throughout the study for the purpose of using as a dental teaching aid. These images will be taken of your gum tissues only and your facial features and name will not be identified.

Risks:

The development of mild to moderate gingivitis (gum disease) is expected during Phase 2 due to not brushing your teeth for 21 days. At the end of Phase 2 you will receive a dental cleaning and can begin your normal brushing habits. These steps should help your gum tissue recover back to normal. If it does not, you will have another dental cleaning and more follow up exams to make sure your gum tissue recovers back to normal.

The exam for gum disease involves rubbing a dental instrument over the gum tissue and may cause mild bleeding. The amount of bleeding is scored to determine how healthy the gum tissue is. This bleeding is like bleeding that may occur while brushing your teeth, if your gums are not as healthy as they could be.

You could experience bruising, swelling or pain in the area of the blood draw. You could feel nauseous or lightheaded in association with not eating and getting your blood drawn. To help prevent this, an experienced person trained at drawing blood will take your blood sample following strict infection control procedures. A light snack and juice will be provided at the end of the appointment. We will try to schedule your appointment to best meet your needs.

As with any dental procedure, irritation of oral soft tissues (cheek linings, and gums) is a possibility. The risk for the spread of germs and accidental loss of confidentiality of dental records (someone else seeing your records) is also possible. These risks will be reduced through the use of experienced personnel using strict, infection control (germ fighting) procedures (sterilized instruments, etc) as outlined by the Infection Control Committee of the IU School of Dentistry. Further, all records and data made by this study will be stored in locked cabinets that only study personnel can access. With the exception of the forms you completed with your name on them (like this consent), a study number and your initials will be your record's only identifiers.

Benefits

You will receive at least two dental cleanings during the study. No other health benefits should be anticipated from participating in this study.

Costs/Compensation

There are no costs to you for participating in this study. If you attend the first visit of the study, you will receive a $20 check whether you qualify or not, which will be sent to you in the mail following the visit. If you qualify to continue, you will receive $25 for completing cleaning visit (visit 2) and $50 per completed visit for Phase 1 of the study (Visits 3 through 6). You will receive these payments in the form of one check for $225 at the completion of Visit 6, either by mail or at the end of the appointment.

You will receive $125 per visit for Phase 2 during the non-brushing period (Visits 7, 8 and 9) plus $25 for the cleaning at Visit 9. You will receive these payments in the form of one check for $400 at the completion of Visit 9, either by mail or at the end of the appointment. You will receive $75 each for completing Visits 10 and 11 during Phase 3 of the study. You will receive these payments in the form of one check for $150 at the completion of Visit 11, either by mail or at the end of the appointment. If you are required to attend an additional cleaning visit after Visit 11, you will receive an additional $25. Therefore, the total compensation for completing the screening and study will be $795 ($820 if extra cleaning is required). You will receive a $100 bonus check at the end of the study if you attend every visit of the study. Partial payment may be given in cases of family emergency or illness. You will only be paid for visits completed.

In the event of physical injury resulting from your participation in this research, necessary medical treatment will be provided to you and billed as part of your medical expenses. Costs not covered by your health care insurer will be your responsibility. Also, it is your responsibility to determine the extent of your health care coverage. There is no program in place for other monetary compensation for such injuries. However, you are not giving up any legal rights or benefits to which you are otherwise entitled. In case of questions, please call Dr. Michael Kowolik at (317) 278-0223 or his staff at (317) 274-8822. If you have a study related emergency after hours, you may page the dentist on call by dialing (317) 562–8447and entering your call back number after the prompt.

Alternatives to Participating in the Study

You are not being treated for a dental condition; hence, there is no alternative therapy. An alternative to participating in this study is to choose not to participate.

Confidentiality

Efforts will be made to keep your personal information confidential (private). We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. While the results of this research study may be published in a scientific journal at a future date, the identity of the participants as well as their records will be kept in strict confidence. Copies of records may be submitted to the Sponsor, the General Clinical Research Clinic at the Indiana University Hospital, the IUPUI/Clarian Institutional Review Board or its designees and to state or federal agencies such as the United States Food and Drug Administration for the purpose of quality assurance and data analysis. Your contact information (name, address, telephone number) will be kept on record at Oral Health Research Institute in the event you need to be contacted for information relating to this project.

Contacts for Questions or Problems

If you have any questions or wish further information, please feel free to call Dr. Kowolik at (317)

278-0223. If you cannot reach the researcher during regular business hours (i.e. 8:00AM-5:00PM), please call the IUPUI/Clarian Research Compliance Administration office at 317/278-3458 or 800/696-2949. For questions about your rights as a research participant or to discuss problems, complaints or concerns about a research study, or to obtain information, or offer input, contact the IUPUI/Clarian Research Compliance Administration office at 317/278-3458 or 800/696-2949.

Voluntary Nature and Withdrawal From The Study

Your participation in this study is purely voluntary. You may withdraw from this study at any time without penalty or loss of benefits to which you are entitled. There will be no change in your medical care or participation in future studies. If you withdraw from this study, the information about you may still be processed and used in submissions to regulatory agencies. You will be informed of any significant new findings developed during the course of the research that may affect your willingness to continue participation. Your participation may end before the study is completed if the investigator feels it is in your best interest for safety concerns or if he feels you are not properly following the study instructions.

If you are willing to participate in this study, we ask that you initial the first two pages of this consent and sign and date the following Subject's Consent.

****

Subject's Consent

In consideration of the above, I give my consent to participate in this research study. I may drop out of or be withdrawn from the study without jeopardizing my participation in future studies. I acknowledge receipt of a copy of this informed consent statement.

_____

Subject's Name (Please Print)

_____

Subject's Signature Date

_____

Signature of Study Personnel Obtaining Informed Consent Date

Sincerely,

Dr. Michael Kowolik, BDS, PhD
Principal Investigator

Appendix 3. Inclusion criteria form for subject enrollment in the study entitled "Dental Plaque Accumulation as a Risk Factor for CHD"

STUDY # 04-G-011
INCLUSION CRITERIA FORM

Screening #_____ Subject Initials_____

Visit 1- Screening Date_____ Mar 2005
(dd mmm yyyy)

INCLUSION CRITERIA:
To be eligible for study participation, ALL of the following questions must be marked ☒ "YES".

1. Has the subject provided written informed consent and a complete medical history form prior to their participation?	☐Yes ☐No
2. Is the subject between 18 and 30 years of age at the time of recruitment?	☐Yes ☐No
3. Is the subject either non-Hispanic, black or white?	☐Yes ☐No
4. Is the subject in good general health with no factors in the medical history, which would indicate that subject would be adversely affected by his/her participation in the study?	☐Yes ☐No
5. Does the subject possess a minimum of 20 natural teeth?	☐Yes ☐No
6. Is the subject available for all study appointments and agree to comply with all subjects' responsibilities as stated in the protocol?	☐Yes ☐No
7. Is the subject willing to refrain from the use of dentifrices, mouthrinses, dental floss, or any other dental product during the Experimental phase?	☐Yes ☐No

RECORDER'S SIGNATURE_____ DATE_____ 2005
(dd mmm yyyy)

Appendix 4. Exclusion Criteria form criteria form for the study entitled "Dental Plaque Accumulation as a Risk Factor for CHD"

STUDY # 04-G-011
EXCLUSION CRITERIA FORM

Screening #_____ Subject Initials_____

Visit 1- Screening Date_____ Mar 2005
(dd mmm yyyy)

EXCLUSION CRITERIA:
To be eligible for study participation, ALL of the following questions must be marked ☒ "NO".

1. Does the subject currently use tobacco products?	☐Yes ☐No
2. Is the subject participating simultaneously in any other clinical study?	☐Yes ☐No
3. Does the subject show evidence of periodontal disease(no pocket greater than 3mm), or gross neglect caries, or other conditions necessitating immediate care?	☐Yes ☐No
4. Does the subject have factors which would pose a risk to themselves, to study personnel or other participants such as hepatitis, HIV-positive status, active tuberculosis, diabetes?	☐Yes ☐No
5. Does the subject have any medical conditions requiring antibiotic pre-medication prior to dental treatment (rheumatic fever, cardiac regulating devices, heart murmur, prosthetic joint replacement)?	☐Yes ☐No
6. Is the subject currently taking a medication, which is known to affect the oral soft tissues or local/systemic inflammatory response?	☐Yes ☐No
7. Has the subject received an antimicrobial drug therapy within 3 months of Day 0 of the Control phase?	☐Yes ☐No
8. Does the subject have a latex allergy?	☐Yes ☐No
9. Is the subject's CO reading ≥ 8ppm?	☐Yes ☐No

RECORDER'S SIGNATURE_____ DATE_____ 2005
(dd mmm yyyy)

Appendix 5. Continuance criteria form for the study entitled “Dental Plaque Accumulation as a Risk Factor for CHD”

STUDY # 04-G-011
CONTINUANCE CRITERIA

Subject #_____ Subject Initials_____

Visit _____ Date_____ 2005
(dd mmm yyyy)

If the answer is "YES" to questions 1, documentation should be written on General Comments.
If the answer is "YES" to questions 2, documentation should be written on Concomitant Medication Log.
After study enrollment in order to continue questions 3, 4 and 5 of the following continuance criteria must be answered "NO".

1. Has anything changed in the subject's medical history since the last visit?	☐Yes ☐No
2. Has the subject added or deleted any medications since the last visit	☐Yes ☐No
3. Has the subject developed an inability to comply with study procedures?	☐Yes ☐No
4. Has the subject started using antibiotics or antimicrobial medicines?	☐Yes ☐No
5. Is the subject currently using a tobacco product?	☐Yes ☐No

RECORDER'S SIGNATURE_____ DATE_____ 2005
(dd mmm yyyy)

Appendix 6. Medical history form for the study entitled "Dental Plaque Accumulation as a Risk Factor for CHD"

STUDY # 04-G-011
OHRI MEDICAL HISTORY INFORMATION

Screening #_____ **Subject Initials**_____

Visit 1- Screening **Date**_____ Mar 2005
(dd mmm yyyy)

Your answers are for our records only and will be considered confidential. It is important that you answer these questions correctly and to the best of your knowledge. During your visit you may be asked some questions about your responses to this questionnaire and there may be additional questions concerning your health. If you have any changes in your health or medications, please inform the dentist at your next appointment.

1. Please place an "X" in the box next to any of the conditions that you have had or have at present:

- ☐ Alcoholism
- ☐ Allergies (seasonal)
- ☐ Allergies (environmental)
- ☐ Anxiety
- ☐ Anemia or Hemophilia
- ☐ Arthritis
- ☐ Artificial Heart Valve
- ☐ Artificial Joint
- ☐ Asthma
- ☐ Cancer or Tumor
- ☐ Chest Pain (Angina)
- ☐ Coronary Heart Disease
- ☐ Depression
- ☐ Diabetes
- ☐ Emphysema
- ☐ Epilepsy or Seizures
- ☐ Fainting or Dizzy Spells
- ☐ Gastric Reflux
- ☐ Glaucoma
- ☐ Heart Attack
- ☐ Heart Murmur
- ☐ Hepatitis
- ☐ Headaches
- ☐ High Blood Pressure
- ☐ High Cholesterol
- ☐ Hyperthyroidism
- ☐ Hypothyroidism
- ☐ Hypoglycemia
- ☐ Insomnia
- ☐ Jaundice
- ☐ Kidney Disease_____
- ☐ Liver Disease_____
- ☐ Lung Disease_____
- ☐ Menstrual Cramps
- ☐ Migraines
- ☐ Mitral Valve Prolapse
- ☐ Rheumatic Fever
- ☐ Stroke
- ☐ Tuberculosis

Other conditions not listed: Please specify below **OR** ☐ None of the above

_____

_____

2. Have you ever had a serious illness, operation or hospitalization? ☐Yes ☐No
If yes, please describe:_____

_____

3. Are you under the care of a physician (other than routine care)? ☐Yes ☐No
If yes, please explain:_____

_____

4. Have you been ill (e.g., cold, other infection) in the past 2 weeks? ☐Yes ☐No

5. Have you been in an accident (car wreck, fell on ice, etc) in the past 2 weeks? ☐Yes ☐No

If yes, please describe: _____

_____

6. Have you had a vaccination(e.g., flu shot) in the past 2 weeks? ☐Yes ☐No
If yes, please describe: _____

_____

7. Have you taken up any vigorous exercise (e.g., intense training for the Mini) in the past 2 weeks? ☐Yes ☐No

8. Have you ever taken any prescription diet medications such as Phen-Fen, Redux (Dexfenfluramine), or Pondimin (Fenfluramine)? ☐Yes ☐No

10. Are you presently taking any ***prescription*** medications/drugs? ☐Yes ☐No
If yes, please list on attached concomitant medication log.

11. Are you presently taking any ***nonprescription*** medications/ drugs? ☐Yes ☐No
If yes, please list on attached concomitant medication log.

12. Are you allergic to any medications/drugs? ☐Yes ☐No

If yes, please list: _____

_____

13. Are you allergic to any foods (i.e., milk, peanuts)? ☐Yes ☐No

If yes, please list: _____

14. Are you allergic to any latex or rubber products (i.e., balloons, condoms, rubber gloves)? ☐Yes ☐No

10. Do you have dental implants? ☐Yes ☐No

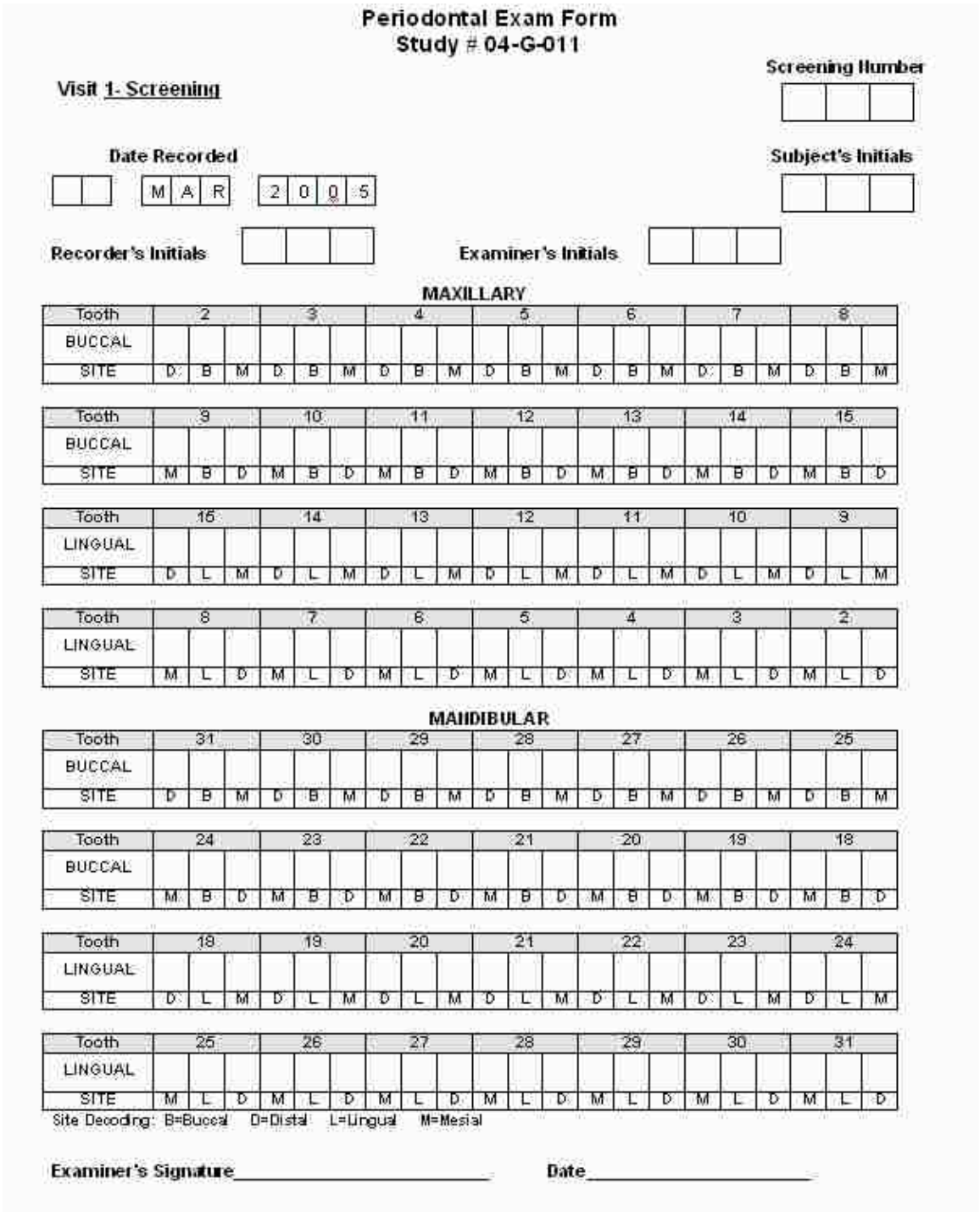
11. Do you smoke or use any tobacco products? ☐Yes ☐No

12. *Women only:* Are you pregnant? ☐Yes ☐No
Are you nursing? ☐Yes ☐No
Do you anticipate becoming pregnant during the study? ☐Yes ☐No
Date of your last menstrual cycle_____

☐**ACCEPTED** ☐**REJECTED**

REVIEWER_____ **DATE**_____**2005**_
(dd mmm yyyy)

Appendix 7. Periodontal exam form.



Periodontal Exam Form
Study # 04-G-011

Screening Number

Visit 1- Screening

Date Recorded: ___ MAR 2005

Subject's Initials

Recorder's Initials

Examiner's Initials

MAXILLARY

Tooth	2			3			4			5			6			7			8		
BUCCAL																					
SITE	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M

Tooth	9			10			11			12			13			14			15		
BUCCAL																					
SITE	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D

Tooth	16			14			13			12			11			10			9		
LINGUAL																					
SITE	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M

Tooth	8			7			6			5			4			3			2		
LINGUAL																					
SITE	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D

MANDIBULAR

Tooth	31			30			29			28			27			26			25		
BUCCAL																					
SITE	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M

Tooth	24			23			22			21			20			19			18		
BUCCAL																					
SITE	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D

Tooth	18			19			20			21			22			23			24		
LINGUAL																					
SITE	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M

Tooth	25			26			27			28			29			30			31		
LINGUAL																					
SITE	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D

Site Decoding: B=Buccal D=Distal L=Lingual M=Mesial

Examiner's Signature_____ Date_____

Appendix 8. Final Institutional Review Board approval document for the study entitled "Bacteremia and the Systemic Host Response Following Toothbrushing in Healthy Adults"

INTERDEPARTMENTAL COMMUNICATION
Research Compliance Administration (RCA)
Indiana University - Purdue University Indianapolis

DATE: April 10, 2007

TO: Michael J. Kowolik
Periodontology/Oral Biology
DS 260
IUPUI

FROM: Michele Garvin
Research Compliance Administration

SUBJECT: Final Approval

Study Number: 0703-68
Study Title: Bacteremia and the Systemic Host Response Following Toothbrushing in Healthy Adults (07-I-027) - Sponsor: NIH

The study listed above has received final approval from the Institutional Review Board (IRB-04) under Expedited Categories 2 and 4. Please note that subjects must be provided with and sign a current informed consent document containing the IRB approval stamp.

Special requirements for the inclusion of prisoners: Please note that unless your study has received approval for the inclusion of prisoners, you may not enroll and/or otherwise involve a prisoner in your study. Special requirements apply if an individual enrolled on the study either is a prisoner or has become a prisoner during the course of his/her study participation (and the study has not been previously granted approval for the enrollment of prisoners as a subject population). If the investigator becomes aware that a subject is a prisoner, all research interactions and interventions with the prisoner-participant must cease. If the investigator wishes to have the prisoner-participant continue to participate in the research, Research Compliance Administration (RCA) must be notified immediately (317-274-8289). In most cases, the IRB will be required to re-review the protocol at a convened meeting before any further research interaction or intervention may continue with the prisoner-participant. Refer to the IUPUI/Clarian Standard Operating Procedure (SOP) on *Involving Prisoners in Research* for further information. The SOP can be found at http://www.iupui.edu/~respoly/human-sop/human-sop-index.htm.

As the principal investigator of this study, you assume the responsibilities as outlined in the SOP on *Responsibilities of Principal Investigators*, some of which include (but are not limited to):

1. **CONTINUING REVIEW** - No less than annually, a status report must be filed with the IRB. The RCA staff will generate these reports for your completion. This study is approved from **April 10, 2007** to **April 10, 2008**. **If your study is not re-approved by this date, the study will automatically expire, which means that all research activities, including enrollment of new subjects, interaction and intervention with current participants, and analysis of identified data, must cease.**

2. **STUDY AMENDMENTS** - You are required to receive prospective approval from the IRB for ANY changes to the research study, including changes to protocol design, dosages, timing or type of test performed, population of the study, and informed consent statement, prior to implementation. This request is made via an amendment form, which can be obtained at: http://www.iupui.edu/%7Eresgrad/spon/download2.htm.

3. **UNANTICIPATED PROBLEMS INVOLVING RISKS TO SUBJECTS OR OTHERS AND NONCOMPLIANCE** - You must promptly report to the IRB any event that appears on the **List of Events that Require Prompt Reporting to the IRB.** Refer to the SOP on *Unanticipated Problems Involving Risks to Subjects or Others and Noncompliance* for more information and other reporting requirements. The SOP can be found at: http://www.iupui.edu/~respoly/human-sop/human-sop-index.htm. NOTE: If the study involves gene therapy and an event occurs which requires prompt reporting to the IRB, it must also be reported to the Institutional Biosafety Committee (IBC).

4. **UPDATED INVESTIGATIONAL BROCHURES, PROGRESS REPORTS and FINAL REPORTS** - If this is an investigational drug or device study, updated clinical investigational brochures must be submitted as they occur. These are submitted with an amendment form. Progress or final reports must be provided to the IRB with your written assessment of the report, briefly summarizing any changes and their significance to the study.

5. **ADVERTISEMENTS** – You can only use IRB-approved advertisements to recruit participants for your study. If you will be advertising to recruit study participants and the advertisement was not submitted to the IRB at the time your study was reviewed and approved, a copy of the information contained in the advertisement and the mode of its communication must be submitted to the IRB as an amendment to the study. These advertisements must be reviewed and approved by the IRB PRIOR to their use.

6. **STUDY COMPLETION** – You are responsible for promptly notifying the IRB when the study has been completed (i.e. there is no further subject enrollment, not further interaction or intervention with current participants, including follow-up, and no .further analysis of identified data). To notify the IRB of study completion, please obtain a close-out form at http://www.iupui.edu/%7Eresgrad/spon/irb_submit.htm and submit it to the RCA office.

7. **LEAVING THE INSTITUTION** - If the principal investigator leaves the Institution, the IRB must be notified as to the disposition of EACH study.

PLEASE REFER TO THE ASSIGNED STUDY NUMBER AND THE EXACT TITLE IN ANY FUTURE CORRESPONDENCE WITH OUR OFFICE. In addition, SOPs exist which cover a variety of topics that may be relevant to the conduct of your research. See link: http://www.iupui.edu/~respoly/human-sop/human-sop-index.htm. All documentation related to this study must be neatly typed and must also be maintained in your files for audit purposes for at least three years after closure of the research; however, please note that research studies subject to HIPAA may have different requirements regarding file storage after closure. If you have any questions, please call Research Compliance Administration at 317/274-8289.

Enclosures:
- ☒ Documentation of Review and Approval
- ☒ Authorization Form(s)
- ☒ Informed Consent Statement(s)
- ☐ Advertisement(s)
- ☐ Assent(s)
- ☐ Other:

Appendix 9. Informed consent statement form for the study entitled "Dental Plaque Accumulation as a Risk Factor for CHD"

IUPUI and Clarian Informed Consent Statement for "Bacteremia and the Systemic Host Response Following Toothbrushing in Healthy Adults (07-G-027)"

You are being invited to participate in a research study because you have signed a consent form allowing us to contact you for future studies or you called in expressing an interest in participating in research studies. The researcher for this study is Dr. Michael Kowolik of the Indiana University School of Dentistry. You should read this consent form carefully and ask him or his staff any questions you have before agreeing to be in this study.

Purpose
The purpose of this study is to determine if Bacteremia (bacteria in the blood stream) occurs in persons when they brush their teeth for the first time after not performing any oral hygiene techniques (brushing, flossing, mouthrinse etc.) for one week. This will be done by studying samples of your blood before and after you stop brushing your teeth for a one week period.

Number of People Taking Part in the Study
If you agree to take part in this study, you will be one of up to 50 persons screened and up to 25 accepted into this study.

Products
There are no experimental products in this study.

Procedures
This study will require you to make five visits to the Oral Health Research Institute in four weeks. These visits are described as follows:

Visit 1 (30-45 minutes): You will be asked to read this consent form and an authorization for the release of health information for research form. The information in these forms will be explained to you, and you will have the chance to ask questions and to decide if you want to be in this study. Our study dentist will examine your mouth (oral soft tissue exam) to see if you have any oral conditions (like mouth sores) that could keep you from being in this study and ask you questions to determine if you can participate. You will be asked to blow into a machine (carbon monoxide machine or CO reading) to show that you are not a cigarette smoker. You will receive an exam of your gums to show that your gum tissue is healthy enough to be in the study. If you qualify to participate you will be scheduled for a dental cleaning.

Visit 2 (60- 75 minutes): A study dentist will review your medical history, and a dental hygienist will clean your teeth. You will take another CO reading to show that you are not smoking. You will receive instructions on good brushing and flossing techniques and will be expected to follow these good oral hygiene instructions for the following week.

Visit 3 (30 to 45 minutes): This visit will need to occur in the morning. Your medical history will be reviewed, an oral soft tissue exam of your mouth will be performed and a CO reading will be taken. You will have been instructed to not brush your teeth this morning and to not eat any solid food (fast) from midnight on the night before but you will be encouraged to drink as much water as you want before your visit (this can sometimes help make the blood draw easier to perform). The arm from which your blood will be taken will be cleaned with alcohol and with another antiseptic (germ fighting) cleaner like Betadine or Chlorhexidine. A fasting blood sample will be taken in which about 6 teaspoons of blood will be drawn from a vein in your arm (generally at the crook of the elbow). A device called a Butterfly will be attached to a syringe or blood collection tube and used to collect your blood. Dental exams for plaque levels (sticky build up on teeth) and gum disease will also be performed by a study dentist. A snack will be provided following your blood draw and dental exams. You will be instructed to stop all oral hygiene techniques including brushing, flossing, mouthrinse and chewing gum until your next visit in seven days.

Visit 4 (5 to 5 ½ hours): This visit will need to occur in the morning. Your medical history will be reviewed, an oral soft tissue exam of your mouth will be performed and a CO reading will be taken. You will have been instructed to continue your no brushing period and not brush the morning of this visit and to not eat any solid food from midnight on the night before but you will be encouraged to drink as much water as you want before your visit. Both of your arms will be used for blood draws during this visit. Both arms will be cleaned with alcohol and Betadine or Chlorhexidine. The blood collection site from which the first sample will not be taken will be covered

with a large bandaid to keep it clean. A baseline fasting blood sample will be taken in which about 6 teaspoons of blood will be drawn from the vein in your arm without the bandaid using a butterfly device. You will also have a dental exam for plaque level and gum disease. Following the baseline blood draw and dental exams, you will brush your teeth for two minutes using toothpaste and a toothbrush. The bandaid will be removed and about 8 teaspoons of blood will then be drawn immediately following brushing. Each arm will again be cleaned right before drawing blood at five minutes after brushing (6 teaspoons) and 30 minutes (six teaspoons) after brushing, once from one side and once from the other. After the 30 minute blood draw you will be allowed to eat something and to leave the building, if you wish. You will need to return at 4 hours after the brushing for a final blood draw. At this blood draw, your arm will only be cleaned with alcohol and about 2 teaspoons of blood will be drawn. After the 4 hour blood draw, you will receive a dental cleaning, fluoride treatment and oral hygiene instructions.

Visit 5 (30-45 minutes): This visit will need to occur in the morning. Your medical history will be reviewed, an oral soft tissue exam of your mouth will be performed and a CO reading will be taken. You will have been instructed to not brush your teeth this morning and to not eat any solid food from midnight on the night before but you will be encouraged to drink as much water as you want before your visit. The arm from which your blood will be taken will be cleaned with alcohol and Betadine or Chlorhexidine. Using the Butterfly device, a fasting blood sample will be taken in which about 2 teaspoons of blood will be drawn from a vein in your arm. Dental exams for plaque levels and gum disease will also be performed by a study dentist. A snack will be provided. Your participation in this study will then end.

Risks

Because we are asking that you not eat solid foods from midnight on at Visits 3, 4 and 5 you might begin to feel uncomfortable from not eating. If you have trouble going without eating for extended periods of time, you should not participate in this study. If you experience dizziness or other symptoms associated with not eating during the study, you should notify study personnel. You will be allowed to drink SlimFast, if needed, without hurting your participation in the study.

Not brushing your teeth for seven days between visits 3 and 4 will produce some gum irritation, possibly including some bleeding of the gums. You may have bad breath. The bad breath and irritation of the gum tissue should go away quickly after you receive the Visit 4 dental cleaning and resume your normal oral hygiene routine. If by the end of Visit 5 your gum tissue has not returned to its pre-study healthy state, you will receive another dental cleaning and follow up dental exams until the dental team is comfortable that your gum tissue has returned to normal.

The exam for gum disease involves rubbing a dental instrument over the gum tissue and this may cause mild bleeding. The amount of bleeding is scored to determine how healthy the gum tissue is. This bleeding is light like the bleeding that may occur while brushing your teeth, if your gums are not as healthy as they could be.

You could experience bruising, swelling, pain or infection in the area of the blood draw. You could also experience irritation or sensitivity in the area of the blood draw resulting from the scrubbing of the site with alcohol and an antiseptic. You could feel nauseous or lightheaded in association with not eating and getting your blood drawn. To help prevent this, an experienced medical assistant trained in drawing blood will take your blood sample following strict infection control procedures. You will be encouraged to drink water before each visit and during visit 4 to help keep your body hydrated (enriched with water) which may help the medical assistant draw the blood easier. During Visit 4 multiple blood draws will occur in a short period of time (30 minutes) which may increase your risk for bruising, swelling, pain, irritation, sensitivity or infection in the area of the blood draw (both arms). A light snack and juice will be provided at the end of the 30 minute blood draw. You may eat as much as you want between the 30 minute and four hour blood draws.

As with any dental procedure, irritation or accidental injury to the oral soft tissues (gums, cheek linings, etc) is a possibility. Cross contamination (germ spreading) is also a risk but will be reduced through the use of experienced personnel using strict infection control procedures (germ fighting steps like wearing gloves, plastic coverings, sterilizing instruments, etc) as outlined by the Infection Control Committee of the Indiana University School of

Dentistry. Additionally, accidental loss of confidentiality of dental records is also possible. To reduce this risk, all records and data created by this study will be stored in locked cabinets that only study personnel can get into. With the exception of the forms with your name on them (like this consent), a study number and your initials will be the only thing that identifies your records.

Benefits
You will receive 2 dental cleanings as a part of this study. No other benefits should be expected from participating in this study. This is a research study and should not replace your regular dental check-up.

Costs/Compensation for Research Related Injury
There is no cost to you for being in this study.

In the event of physical injury resulting from your participation in this research study, necessary medical treatment will be provided to you and billed as part of your medical expenses. Costs not covered by your health care insurer will be your responsibility. Also, it is your responsibility to determine the extent of your health care coverage. There is no program in place for other monetary compensation (payment) for such injuries. However, you are not giving up any legal rights or benefits to which you are otherwise entitled. To report such an incident, please call Dr. Michael Kowolik or his staff at (317) 274-8822, Mon-Fri from 8:00-5:00.

Payment for Participation
You will receive $20 for completing the screening visit, whether you qualify or not. This will be in the form of a check sent to you in the mail. If you qualify to participate in the study, you will receive $50 each for completing Visits 2, 3 and 5 and $250 for completing Visit 4. If you complete all 5 visits, you will receive an additional $100 for a study total, excluding the screening visit, of $500. You will receive this payment in the form of a check either in the mail or at the visit as follows: $100 at the completion of Visit 3, $250 at the completion of Visit 4 and $150 ($50 if all visits not completed) at the completion of Visit 5. A partial payment of $20 may be given if upon reporting to the study visit, it is determined you cannot participate in the study visit (i.e., you have a mouth sore, you can't stay for the entire visit, you smoked cigarettes etc.). Otherwise, you will only be paid for visits completed.

Alternatives to Participating in this study
You are not being treated for a dental condition; so, there is no alternative therapy. An alternative to participating in this study is to not take part.

Confidentiality
Efforts will be made to keep your personal information confidential. We cannot guarantee absolute confidentiality. Your personal information may be given out if required by law. While the results of this research project may be published in a scientific journal at a future date, your name and records will be kept in strict confidence. Copies of records may be submitted to the Sponsor, the IUPUI/Clarian Institutional Review Board and its designees (people working with them), and some federal and state regulatory institutions, such as the United States Food and Drug Administration (FDA) and the Office for Human Research Protection (OHRP) for the purpose of quality assurance (safety) and data analysis. Your contact information (name, telephone number and address) will be kept on record in the event we need to contact you with additional information.

Contacts for Questions or Problems
The researcher conducting this study is Dr. Michael Kowolik and you may contact him or his staff at (317) 274-8822 to discuss any study related questions you have. If you cannot reach him during regular business hours (8:00 am–5:00 pm weekdays), call the IUPUI/Clarian Research Compliance Administration office at (317) 278-3458 or toll free at (800) 696-2949. After hours call (317) 562-8447 and page the dentist on call by entering your telephone number after the prompt.

For questions about your rights as a research participant or to discuss problems, complaints or concerns about a research study, or to obtain information, or offer input, call the IUPUI/ Clarian Research Compliance Administration office at (317)278-3458 or (800)696-2949.

Voluntary Nature of Study

Participation in this study is strictly voluntary, and you may choose not to take part or may withdraw for any reason without loss of benefits you are otherwise entitled to. You will be informed in a timely manner of any significant new findings developed during the course of the research that may affect your willingness to continue. Your participation may end before the study is completed if the investigator feels it is in your best interest for safety concerns or if he feels you are not properly following the study instructions.

If you are willing to be in this study, initial the first three pages of this consent and sign and date the Subject's Consent section.

Sincerely,

Dr. Michael Kowolik, Principal Investigator

Subject's Consent

I have read the above details and give my consent to take part in this research study. I may withdraw from the study at any time without having to give a reason. I acknowledge receipt of a copy of this informed consent statement.

_____

Subject's Name (Please Print)

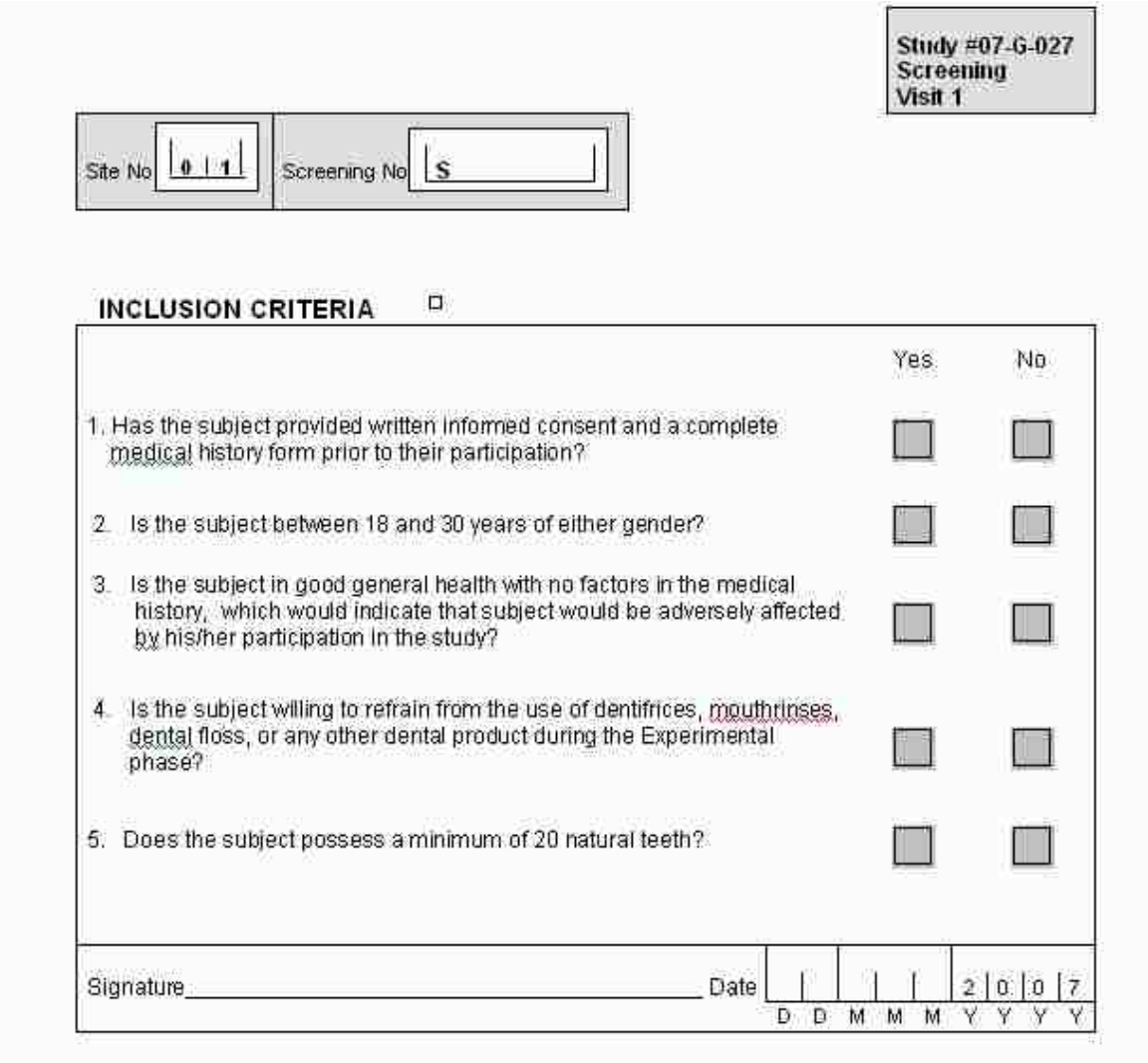
_____

Subject's Signature Date

_____

Name of Study Personnel Obtaining Informed Consent Date

Appendix 10. Inclusion criteria form for subject enrollment in the study entitled "Bacteremia and the Systemic Host Response Following Toothbrushing in Healthy Adults"



Study #07-G-027
Screening
Visit 1

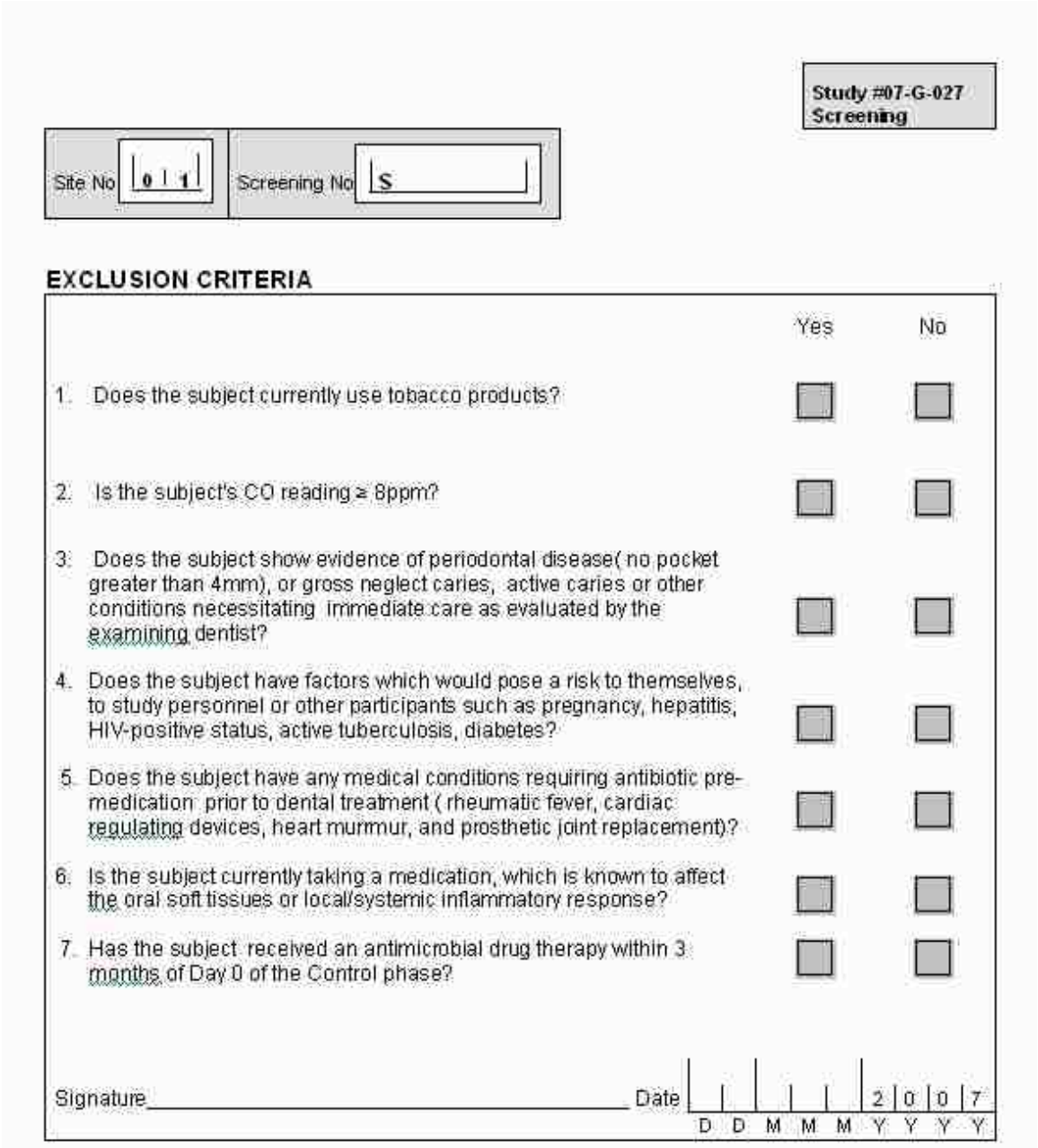
Site No 0 1 | Screening No S

INCLUSION CRITERIA ☐

	Yes	No
1. Has the subject provided written informed consent and a complete medical history form prior to their participation?	☐	☐
2. Is the subject between 18 and 30 years of either gender?	☐	☐
3. Is the subject in good general health with no factors in the medical history, which would indicate that subject would be adversely affected by his/her participation in the study?	☐	☐
4. Is the subject willing to refrain from the use of dentifrices, mouthrinses, dental floss, or any other dental product during the Experimental phase?	☐	☐
5. Does the subject possess a minimum of 20 natural teeth?	☐	☐

Signature_____ Date | | | | | | | | | | 2 0 0 7
D D M M M Y Y Y Y

Appendix 11. Exclusion criteria form for subject enrollment in the study entitled "Bacteremia and the Systemic Host Response Following Toothbrushing in Healthy Adults"



Study #07-G-027
Screening

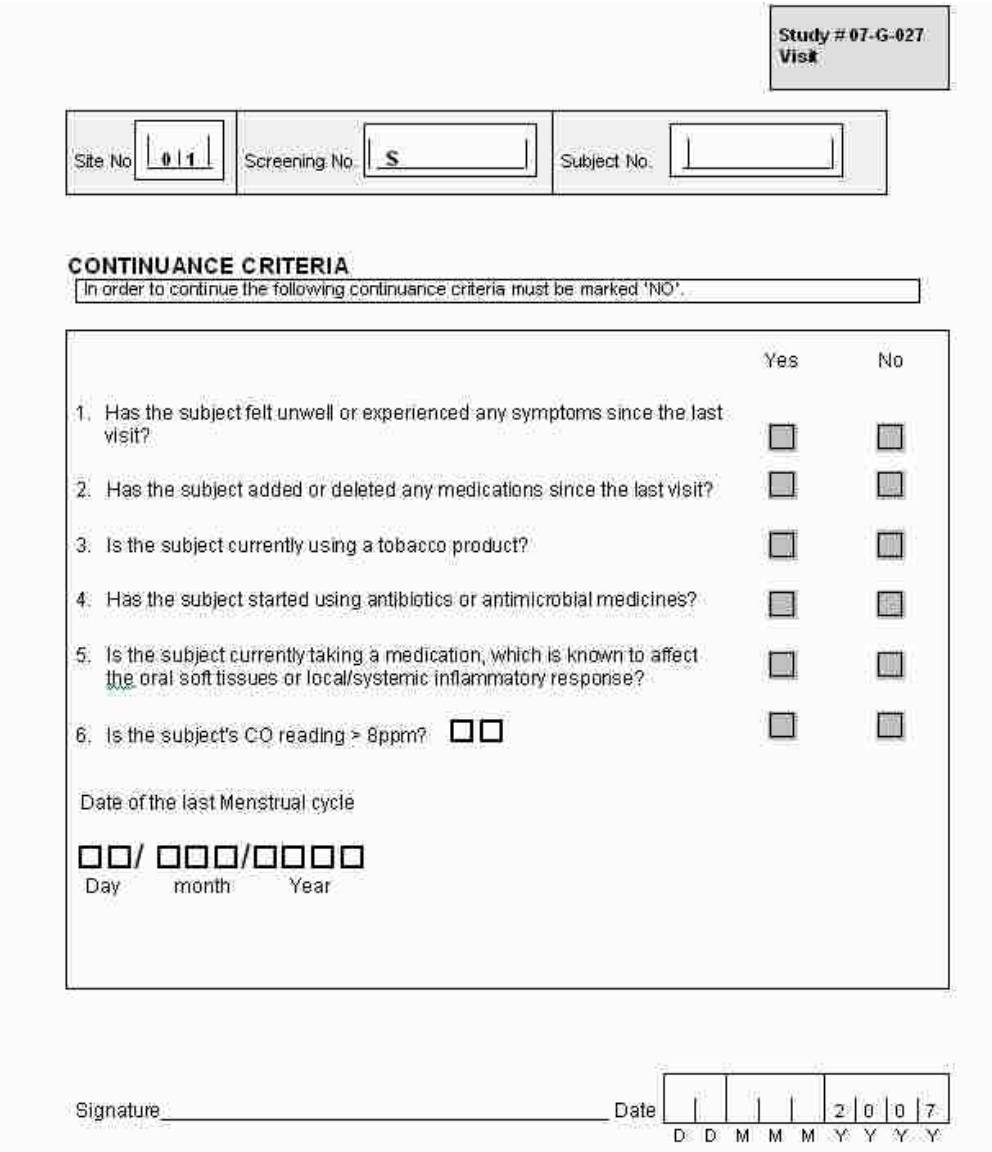
Site No 0 1 1 Screening No S

EXCLUSION CRITERIA

	Yes	No
1. Does the subject currently use tobacco products?	☐	☐
2. Is the subject's CO reading ≥ 8ppm?	☐	☐
3. Does the subject show evidence of periodontal disease(no pocket greater than 4mm), or gross neglect caries, active caries or other conditions necessitating immediate care as evaluated by the examining dentist?	☐	☐
4. Does the subject have factors which would pose a risk to themselves, to study personnel or other participants such as pregnancy, hepatitis, HIV-positive status, active tuberculosis, diabetes?	☐	☐
5. Does the subject have any medical conditions requiring antibiotic pre-medication prior to dental treatment (rheumatic fever, cardiac regulating devices, heart murmur, and prosthetic joint replacement)?	☐	☐
6. Is the subject currently taking a medication, which is known to affect the oral soft tissues or local/systemic inflammatory response?	☐	☐
7. Has the subject received an antimicrobial drug therapy within 3 months of Day 0 of the Control phase?	☐	☐

Signature_____ Date | | | | | 2 0 0 7
D D M M M Y Y Y Y

Appendix 12. Continuance criteria form for the study entitled "Bacteremia and the Systemic Host Response Following Toothbrushing in Healthy Adults"



Study # 07-G-027
Visit

Site No 0 1 | Screening No S | Subject No.

CONTINUANCE CRITERIA

In order to continue the following continuance criteria must be marked 'NO'.

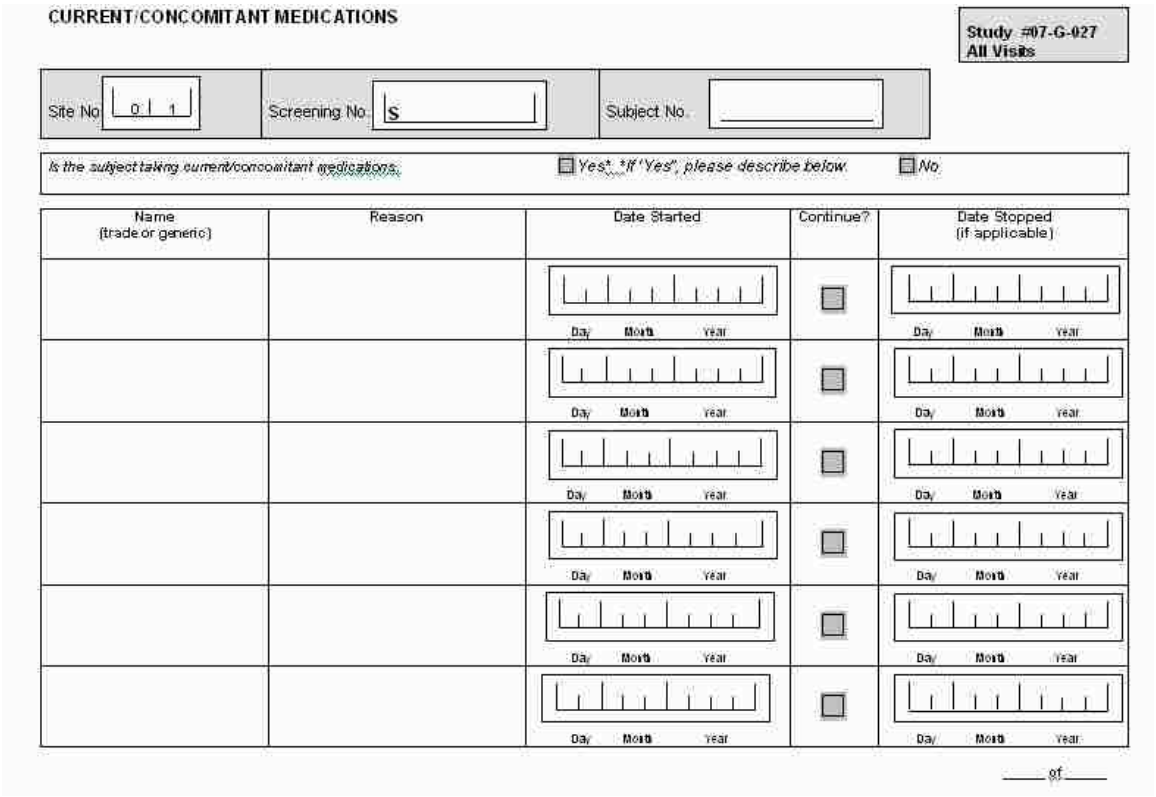
	Yes	No
1. Has the subject felt unwell or experienced any symptoms since the last visit?	☐	☐
2. Has the subject added or deleted any medications since the last visit?	☐	☐
3. Is the subject currently using a tobacco product?	☐	☐
4. Has the subject started using antibiotics or antimicrobial medicines?	☐	☐
5. Is the subject currently taking a medication, which is known to affect the oral soft tissues or local/systemic inflammatory response?	☐	☐
6. Is the subject's CO reading > 8ppm? ☐☐	☐	☐

Date of the last Menstrual cycle

☐☐/ ☐☐☐/☐☐☐☐
Day month Year

Signature_____ Date | | | | | 2 0 0 7
D D M M M Y Y Y Y

Appendix 13. Current/concomitant medications form.



CURRENT/CONCOMITANT MEDICATIONS

Study #07-G-027
All Visits

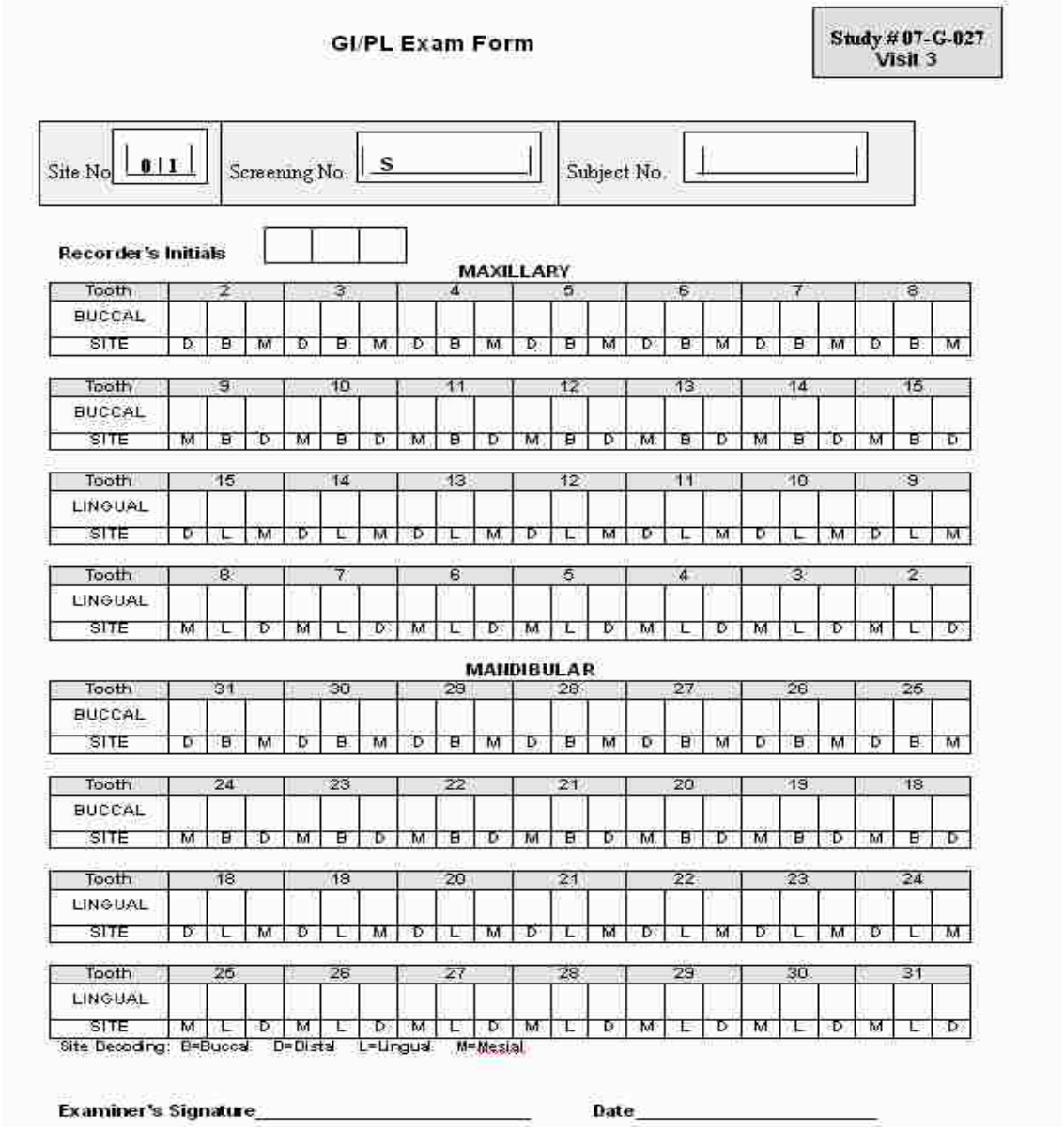
Site No. 0 1 | Screening No. S | Subject No. _____

Is the subject taking current/concomitant medications. ☐ *Yes*, *If "Yes", please describe below* ☐ *No*

Name (trade or generic)	Reason	Date Started	Continue?	Date Stopped (if applicable)
		Day Month Year	☐	Day Month Year
		Day Month Year	☐	Day Month Year
		Day Month Year	☐	Day Month Year
		Day Month Year	☐	Day Month Year
		Day Month Year	☐	Day Month Year
		Day Month Year	☐	Day Month Year

____ of ____

Appendix 14. Form for recording the periodontal and gingival indices.



GI/PL Exam Form

Study # 07-G-027
Visit 3

Site No. 0 1 | Screening No. S | Subject No.

Recorder's Initials

MAXILLARY

Tooth	2			3			4			5			6			7			8		
BUCCAL																					
SITE	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M

Tooth	9			10			11			12			13			14			15		
BUCCAL																					
SITE	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D

Tooth	15			14			13			12			11			10			9		
LINGUAL																					
SITE	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M

Tooth	8			7			6			5			4			3			2		
LINGUAL																					
SITE	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D

MANDIBULAR

Tooth	31			30			29			28			27			26			25		
BUCCAL																					
SITE	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M	D	B	M

Tooth	24			23			22			21			20			19			18		
BUCCAL																					
SITE	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D	M	B	D

Tooth	18			19			20			21			22			23			24		
LINGUAL																					
SITE	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M	D	L	M

Tooth	25			26			27			28			29			30			31		
LINGUAL																					
SITE	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D	M	L	D

Site Decoding: B=Buccal D=Distal L=Lingual M=Mesial

Examiner's Signature_____ **Date**_____

Appendix 15. Final Institutional Review Board approval document for the study entitled "The In vitro Response of Human Peripheral Blood Neutrophils to *Fusobacterium Nucleatum*"

INTERDEPARTMENTAL COMMUNICATION
Research Compliance Administration (RCA)
Indiana University - Purdue University Indianapolis

DATE: February 11, 2008

TO: Michael J. Kowolik
Periodontology/Oral Biology
DS 260
IUPUI

FROM: Michele Garvin
Research Compliance Administration

SUBJECT: Final Approval

Study Number: 0712-67
Study Title: The In vitro Response of Human Peripheral Blood Neutrophils to Fusobacterium Nucleatum - Sponsor: NIH

The study listed above has received final approval from the Institutional Review Board (IRB-04) under Expedited Category 5. The IRB has granted a Waiver of Informed Consent under 45CFR46.116(d).

Special requirements for the inclusion of prisoners: Please note that unless your study has received approval for the inclusion of prisoners, you may not enroll and/or otherwise involve a prisoner in your study. Special requirements apply if an individual enrolled on the study either is a prisoner or has become a prisoner during the course of his/her study participation (and the study has not been previously granted approval for the enrollment of prisoners as a subject population). If the investigator becomes aware that a subject is a prisoner, all research interactions and interventions with the prisoner-participant must cease. If the investigator wishes to have the prisoner-participant continue to participate in the research, Research Compliance Administration (RCA) must be notified immediately (317-274-8289). In most cases, the IRB will be required to re-review the protocol at a convened meeting before any further research interaction or intervention may continue with the prisoner-participant. Refer to the IUPUI/Clarian Standard Operating Procedure (SOP) on *Involving Prisoners in Research* for further information. The SOP can be found at http://www.iupui.edu/~respoly/human-sop/human-sop-index.htm.

As the principal investigator of this study, you assume the responsibilities as outlined in the SOP on *Responsibilities of Principal Investigators*, some of which include (but are not limited to):

1. **CONTINUING REVIEW** - No less than annually, a status report must be filed with the IRB. The RCA staff will generate these reports for your completion. This study is approved from **January 29, 2008** to **January 29, 2009**. **If your study is not re-approved by this date, the study will automatically expire, which means that all research activities, including enrollment of new subjects, interaction and intervention with current participants, and analysis of identified data, must cease.**

2. **STUDY AMENDMENTS** - You are required to receive prospective approval from the IRB for ANY changes to the research study, including changes to protocol design, dosages, timing or type of test performed, population of the study, and informed consent statement, prior to implementation. This request is made via an amendment form, which can be obtained at: http://www.iupui.edu/%7Eresgrad/spon/download2.htm.

3. **UNANTICIPATED PROBLEMS INVOLVING RISKS TO SUBJECTS OR OTHERS AND NONCOMPLIANCE** - You must promptly report to the IRB any event that appears on the **List of Events that Require Prompt Reporting to the IRB.** Refer to the SOP on *Unanticipated Problems Involving Risks to Subjects or Others and Noncompliance* for more information and other reporting requirements. The SOP can be found at: http://www.iupui.edu/~respoly/human-sop/human-sop-index.htm. NOTE: If the study involves gene therapy and an event occurs which requires prompt reporting to the IRB, it must also be reported to the Institutional Biosafety Committee (IBC).

4. **UPDATED INVESTIGATIONAL BROCHURES, PROGRESS REPORTS and FINAL REPORTS** - If this is an investigational drug or device study, updated clinical investigational brochures must be submitted as they occur. These are submitted with an amendment form. Progress or final reports must be provided to the IRB with your written assessment of the report, briefly summarizing any changes and their significance to the study.

5. **ADVERTISEMENTS** – You can only use IRB-approved advertisements to recruit participants for your study. If you will be advertising to recruit study participants and the advertisement was not submitted to the IRB at the time your study was reviewed and approved, a copy of the information contained in the advertisement and the mode of its communication must be submitted to the IRB as an amendment to the study. These advertisements must be reviewed and approved by the IRB PRIOR to their use.

6. **STUDY COMPLETION** – You are responsible for promptly notifying the IRB when the study has been completed (i.e. there is no further subject enrollment, not further interaction or intervention with current participants, including follow-up, and no further analysis of identified data). To notify the IRB of study completion, please obtain a close-out form at http://www.iupui.edu/%7Eresgrad/spon/irb_submit.htm and submit it to the RCA office.

7. **LEAVING THE INSTITUTION** - If the principal investigator leaves the Institution, the IRB must be notified as to the disposition of EACH study.

PLEASE REFER TO THE ASSIGNED STUDY NUMBER AND THE EXACT TITLE IN ANY FUTURE CORRESPONDENCE WITH OUR OFFICE. In addition, SOPs exist which cover a variety of topics that may be relevant to the conduct of your research. See link http://www.iupui.edu/~respoly/human-sop/human-sop-index.htm. All documentation related to this study must be neatly typed and must also be maintained in your files for audit purposes for at least three years after closure of the research; however, please note that research studies subject to HIPAA may have different requirements regarding file storage after closure. If you have any questions, please call Research Compliance Administration at 317/274-8289.

Enclosures:
- ☒ Documentation of Review and Approval
- ☐ Authorization Form(s)
- ☐ Informed Consent Statement(s)
- ☐ Advertisement(s)
- ☐ Assent(s)
- ☐ Other:

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CURRICULUM VITAE

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Education

PhD in Dental Sciences (2010)

Indiana University (IU), Indianapolis, IN

MSD (Master of Science in Dentistry) in Preventive Dentistry (2004)

IU School of Dentistry, Indianapolis IN

BDS (Bachelor of Dental Surgery) (1997)

University of Jordan, Amman, Jordan

Honors and Awards

- Volpe Prize Finalist in the 2009 Volpe Prize International Periodontal Competition. Columbus, OH.
- 2009 IU School of Dentistry Maynard K. Hine Award for Excellence in Dental Research (for Best Research Manuscript) – 1st Place
- 2008 IU School of Dentistry Maynard K. Hine Award for Excellence in Dental Research (for Best Research Manuscript) – 1st Place

Research Experience

Doctoral Candidate (2004-2010)

Department of Oral Biology, IU School of Dentistry

- Mentor: Dr. MJ Kowolik, Professor, Department of Periodontics, IU School of Dentistry
- Dissertation: "The Systemic Inflammatory Response to Dental Plaque Accumulation"
- Range of areas addressed:
    - A clinical investigation that employed the classical experimental gingivitis model to examine the role of dental plaque accumulation as a risk factor for Coronary Heart Disease and moreover, to determine whether gender/racial disparity in the systemic inflammatory response to dental plaque accumulation exists
    - A clinical investigation that employed a modified experimental gingivitis model to assess the role of toothbrushing, following dental plaque accumulation, in inducing bacteremic episodes and systemic inflammatory responses in young adults
    - An in vitro investigation of the role of *Fusobacterium nucleatum* in activation of Systemic human neutrophils
- This investigation was supported by NIH # R01 DEO15145-01

MSD (2001-2004)

IU School of Dentistry

Major: Preventive Dentistry

Minors: Operative Dentistry and Dental Materials

- Mentor: Dr. RL Gregory, Professor, Department of Oral Biology, IU School of Dentistry

- Thesis: Role of Oral Biofilm Proteins in S*treptococcus mutans* Adherence and Caries Formation
- Issues addressed:
    - Enzyme-linked immunosorbent assays were conducted to assess binding between *S. mutans* and colostral IgA, whole saliva, amylase, lysozyme, and lactoferrin as potential receptors for *S. mutans*

    - An in vitro caries model and confocal laser scanning microscopy were used to determine the role of oral biofilm proteins in dental caries lesion formation

Teaching Experience

Training and mentoring PhD and Masters dental students during the conduction of their research projects.

Student Tutor (2001-2005)

IU School of Dentistry

Tutoring dental students in problem-based learning**.**

Clinical Experience (1997-1999)

General dental practice, Amman, Jordan

Published Abstracts

- **Wahaidi VY** *, Catt DM, and Gregory RL (2003). Role of Oral Biofilm Proteins in Streptococcus mutans Adherence. J Dent Res 82 (Spec Iss A): 0710. Presented at the 32nd annual meeting and exhibition of the American Association of Dental Research (AADR), San Antonio TX, USA.

- Wahaidi VY *, Eckert GJ, Dowsett SA, Krushinski CA, Lukantsove L, Mau M, and Kowolik MJ (2007). Experimental Gingivitis Model: Experience and Clinical Outcomes by Race/Gender. J Dent Res 86 (Spec Iss A): 2338. Presented at the IADR/AADR/CADR 85th General Session and Exhibition, New Orleans LA, USA.

- Wahaidi VY *, Eckert GJ, Dowsett SA, Allen SD, and Kowolik MJ (2008). Bacteremia and the Systemic Host Response following Toothbrushing in Adults. J Dent Res 87 (Spec Iss B): 2732. Presented at the 86th annual meeting and exhibition of the International Association of Dental Research (IADR), Toronto Ontario, Canada.

- Wahaidi VY, Allen BL, Dowsett SA, Eckert GJ, and Kowolik MJ * (2009). Dental Plaque as a Risk Factor for Coronary Heart Disease. J Dent Res 88 (Spec Iss A): 99. Presented at the 87th General Session and Exhibition of the IADR/AADR/CADR, Miami Beach FL, USA.

- Wahaidi VY ***,** Eckert GJ, Galli DM, R.L. Gregory RL, and Kowolik MJ (2009). The In-vitro Response of Human Neutrophils to Fusobacterium nucleatum. J Dent Res 88 (Spec Iss A): 2731. Presented at the 87th General Session and Exhibition of the IADR/AADR/CADR, Miami Beach FL, USA.

* Primary Presenter

Invited Presentations

- The Systemic Inflammatory Response to Dental Plaque Accumulation. Presented at the Indiana Section of American Association of Dental Research, Indianapolis, IN (2009)

- The Systemic Inflammatory Response to Dental Plaque Accumulation. Presented at the Research Seminar Series on Infectious Diseases, IU School of Medicine, Indianapolis, IN (2009)

Publications

- **Wahaidi VY**, Dowsett SA, Eckert GJ, and Kowolik MJ. Neutrophil Response to Dental Plaque by Gender and Race. J Den Res. 2009;88:709-14.

- **Wahaidi VY**, Dowsett SA, Eckert GJ, Allen SD, and Kowolik MJ. Bacteremia and the Systemic Host Response Following Toothbrushing (In Preparation).

Professional Affiliations

- International Association of Dental Research/American Association of Dental Research

- Indiana Section of the American Association for Dental Research