Assay of Oral Polymorphonuclear Neutrophils for Assessment of Oral Inflammation in Pregnant Women

by

Sabrina Huda

A thesis submitted in conformity with the requirements for the degree of Master of Science in Pediatric Dentistry

Graduate Department of Dentistry
University of Toronto

© Copyright by Sabrina Huda 2012
Assay of Oral Polymorphonuclear Neutrophils for Assessment of Oral Inflammation in Pregnant Women

Sabrina Huda

Master of Science in Pediatric Dentistry
Graduate Department of Dentistry
University of Toronto
2012

Abstract

Background: A multitude of studies suggest an association between periodontal disease and adverse birth outcomes. Although the evidence is controversial, it is biologically plausible, and the key link maybe inflammation.

Purpose: To correlate levels of periodontal disease as measured by conventional methods with PMN counts from an oral rinse in pregnant women.

Methods: Sixty-three pregnant women were recruited. Periodontal examinations were carried out. Fifteen-second saline rinses were collected. ABTS, a colour changing redox agent was added to each rinse. The intensity of the colour reaction was measured by absorbance to count the oral PMNs.

Results: A statistically significant difference in oral PMN counts was observed between those with periodontal disease and the healthy patients ($P < .05$). The sensitivity and specificity using the bleeding index was 0.94 and 0.76 respectively.

Conclusions: The rinse assay can be used as a screening tool for periodontal disease and oral inflammation in pregnant women.
Acknowledgments

This project pushed me beyond my comfort zone and allowed me to accomplish something remarkable that at times felt impossible. However, this project would not have been achievable without the help and support of numerous people.

Firth and foremost, I want to thank my supervisors Dr. Michael Sigal and Dr. Howard Tenenbaum for their enormous support, encouragement and valuable input. I could always count on Dr. Sigal’s guidance to keep me focused and Dr. Tenenbaum’s enthusiasm to keep me optimistic. Also, Dr. Tenenbaum’s entertaining emails always managed to put a smile on my face.

Next, I want to thank my committee members: Dr. Michael Glogauer, Dr. Wendy Whittle, Dr. Herenia Lawrence and Dr. Michael Goldberg for all their valuable contributions. Special mention to Dr. Whittle for allowing me to access her busy clinic and making it possible to access other obstetric clinics at the Mount Sinai Hospital. I also thank Ashkan Javid for all his help in the laboratory.

My heartfelt thanks to my family who instilled the love of learning in me and always encouraged me to reach for the stars. I also thank my in-laws for all their love and support including the yummy supply of food every week over the busy past three years.

Finally, this thesis is a dedication to my husband Saajid Bhayat. You are my everything.
# Table of Contents

Abstract .................................................................................................................................................. ii  

Acknowledgments ................................................................................................................................. iii  

Table of Contents ................................................................................................................................. iv  

List of Tables ........................................................................................................................................ vii  

List of Figures ........................................................................................................................................ ix  

List of Appendices ............................................................................................................................... x  

Abbreviations ......................................................................................................................................... xi  

Review of the Literature ........................................................................................................................ 1  

1 Preterm Birth ....................................................................................................................................... 1  

2 Low Birth Weight ............................................................................................................................... 5  

3 Sequelae of Adverse Birth Outcomes ............................................................................................... 7  
   3.1 Neurodevelopmental and Behavioural Sequelae ........................................................................ 7  
   3.2 Other Sequelae ............................................................................................................................. 8  

4 Impact of Adverse Pregnancy Outcomes: Preterm and/or Low Birth Weight Infant Delivery ....... 10  

5 Periodontal Diseases ........................................................................................................................ 11  
   5.1 Etiology and Pathogenesis of Gingivitis and Periodontitis .......................................................... 11  
   5.2 Classification of Periodontal Diseases ........................................................................................ 14  

6 Pregnancy and Periodontal Disease ................................................................................................. 17  
   6.1 Impact of Pregnancy on Periodontal Disease ............................................................................ 17  
   6.2 Impact of Periodontitis on Pregnancy Outcomes .................................................................... 18  
      6.2.1 Epidemiological Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes .............................................................. 18  
      6.2.2 Interventional Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes ........................................................................ 20
6.2.3 Mechanistic Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

6.2.4 Possible New Explanation regarding Putative Link between Inflammatory Disease (including periodontitis) and Adverse Pregnancy Outcomes: direct and indirect role of matrix metalloproteinases

7 Polymorphonuclear Neutrophils

7.1 Polymorphonuclear Neutrophil Background and Development

7.2 Polymorphonuclear Neutrophil Function

7.3 Mechanisms of Polymorphonuclear Neutrophil Cell Death

8 Role of Polymorphonuclear Neutrophils in Periodontal Disease

8.1 Polymorphonuclear Neutrophil Disorders and Periodontal Disease

8.2 Polymorphonuclear Neutrophil as Perpetrators of Tissue Damage and Bone Destruction

8.3 Myeloperoxidase and Periodontal Disease

9 Diagnosis of Disease

9.1 Diagnosis of Periodontal Disease

9.1.1 Assessments of Inflammation

9.1.2 Assessments of Damage to Periodontal Tissues

10 Review of Oral Polymorphonuclear Neutrophil Quantification Studies

Rationale

Hypothesis

Objectives

Materials and Methods

1 Study Population

2 Assessment of Oral Polymorphonuclear Neutrophil Levels

3 The Periodontal Examination

4 Maternal and Pregnancy Demographic Data

5 Sample Size Calculation
6 Statistical Analysis ........................................................................................................................................51

Results .........................................................................................................................................................53

1 Descriptive Statistics of the Study Population of Pregnant Women at Recruitment ..................53

2 Relationship between Periodontal Disease and Oral Polymorphonuclear Neutrophil
Counts in Pregnant Women ............................................................................................................................56

3 Usefulness of Oral Polymorphonuclear Neutrophil Counts as a Screening Tool for
Periodontal Disease in Pregnant Women ........................................................................................................64

4 Pregnancy Characteristics of the Study Population that was Followed-up .................................66

5 Birth Outcomes, Periodontal Disease and Polymorphonuclear Neutrophil Counts of the
Study Population ..............................................................................................................................................68

Discussion ...................................................................................................................................................77

Conclusions ....................................................................................................................................................84

References .....................................................................................................................................................85

Appendices ...................................................................................................................................................115
List of Tables

Table 1. Demographic and other details of the study population at recruitment.

Table 2. Periodontal status of the study population at recruitment.

Table 3. Mean values of the oral PMN counts (x 10^6 cells/ml), average probing depths (mm) and bleeding index of the study population at recruitment.

Table 4. Spearman’s rank correlation (r_s) between oral PMN counts and bleeding index, average probing depths, number of teeth, age and gestation age of the study population at recruitment.

Table 5. Mean oral PMN counts (x 10^6 cells/ml) of the study population according to their periodontal status.

Table 6. Pregnancy characteristics of the study population at follow-up.

Table 7. Gestational age at delivery, birth weight, possible indicators and labour, and periodontal status of the six patients experiencing an adverse pregnancy outcome.

Table 8. Incidence overall adverse pregnancy outcomes among the study population at follow-up categorized by certain risk factors, periodontal status and other indicators of the status of periodontal disease.

Table 9. Incidence of preterm birth outcomes among the study population at follow-up categorized by certain risk factors, periodontal status and other indicators of the status of periodontal disease.

Table 10. Incidence of low birth weight outcomes among the study population at follow-up categorized by certain risk factors, periodontal status and other indicators of the status of periodontal disease.

Table 11. Mean values of the oral PMN counts, average probing depths and bleeding index of the study population at follow-up categorized by overall adverse birth outcomes.
Table 12. Mean values of the oral PMN counts, average probing depths and bleeding index of the study population at follow-up categorized by preterm birth outcomes.

Table 13. Mean values of the oral PMN counts, average probing depths and bleeding index of the study population at follow-up categorized by low birth weight outcomes.
List of Figures

Figure 1. Diagrams illustrating clinical measurements for assessment of damage to periodontal tissues.

Figure 2. Relationship between natural log of PMN counts and the bleeding index of the study population.

Figure 3. Relationship between natural log of PMN counts and the average probing depths of the study population.

Figure 4. Comparison of natural log of PMN counts with the modified gingival index of the study population with division of patients based on the visual assessment of inflammation.

Figure 5. Comparison of natural log of PMN counts with the plaque index of the study population with division of patients based on the presence of plaque.

Figure 6. Comparison of natural log of PMN counts with the calculus index of the study population with division of patients based on the presence of calculus.

Figure 7. Comparison of natural log of PMN counts with periodontal status of the study population with division of patients based on their periodontal status.

Figure 8. Sensitivity and specificity values of the oral rinse assay for increasing PMN counts (using bleeding index of 5.5 or more as cut-off point).

Figure 9. Sensitivity and specificity values of the oral rinse assay for increasing PMN counts (using probing depth of $\geq 4$ mm in at least 4 sites on different teeth as cut-off point).
List of Appendices

Appendix A. Information booklet provided to patients.

Appendix B. Patient consent form.

Appendix C. Medical and dental history form.

Appendix D. Definitions of conventional periodontal measures used in this study.

Appendix E. Clinical examination form.
Abbreviations

**ABTS**: 2,2’-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)

**ANOVA**: Analysis of variance

**BOP**: Bleeding on probing

**CAL**: Clinical attachment loss

**CRP**: C-reactive protein

**GCF**: Gingival crevicular fluid

**HBSS**: Hanks’ balanced salt solution

**IL**: Interleukin

**IUGR**: Intrauterine growth restriction

**LBW**: Low birth weight

**LPS**: Lipopolysaccharide

**MPO**: Myeloperoxidase

**MMP**: Matrix metalloproteinase

**NPV**: Negative predictive value

**OD**: Optical density

**OMR**: Orogranulocytic migratory rate

**PGE₂**: Prostaglandin E₂

**PMN**: Polymorphonuclear neutrophil

**PPROM**: Preterm premature rupture of membranes
**PPV:** Positive predictive value

**PTLBW:** Preterm low birth weight

**TNF:** Tumor necrosis factor

**SGA:** Small for gestational age
Review of the Literature

Currently there are a multitude of studies that suggest that periodontitis, an inflammatory disease caused primarily by gram-negative bacteria that destroy tooth supporting connective tissue and bone, is associated with an increased risk of preterm birth, as well as low birth weight among other adverse birth outcomes. Adverse birth outcomes can have major consequences that include neonatal mortality. Therefore, it was thought to be important to explore adverse birth outcomes, mainly preterm birth and low birth weight outcome and inflammatory periodontal disease in this thesis, in the hopes of describing a simple diagnostic test that can identify women at increased risk of adverse birth outcome due to inflammatory periodontal disease.

1 Preterm Birth

Preterm deliveries are those that occur at less than 37 weeks gestational age. The rate of preterm delivery in Canada is approximately 8.1% (Lim et al., 2009). In the United States of America (USA), the reported rate is around 12 – 13% (Goldenberg, Culhane, Iams, & Romero, 2008). The preterm birth rate has risen in most industrial countries, with the Canadian rate increasing more than 25% since the mid 1980s (Lim et al., 2009), and the USA rate increasing from 9.5% in 1981 to 12.7% in 2005 (Hamilton, Martin, & Ventura, 2006). This has occurred despite advancing knowledge of risk factors and mechanisms related to preterm labour, and the introduction of many medical interventions designed to reduce the incidence of preterm birth (Goldenberg et al., 2008).

The obstetric predictors leading to preterm birth are: 1) delivery for maternal or fetal indications, in which labour is either induced or the infant is delivered by prelabour caesarean section (i.e. indicated preterm births), 2) spontaneous preterm labour with intact membranes, and 3) preterm premature rupture of the membranes (PPROM), irrespective of whether the delivery is vaginal or by caesarean section. About 30 – 35% of preterm births are indicated due to medical or obstetrical complications that jeopardize the health of the mother or the fetus, 40 – 45% follow spontaneous preterm labour, and 25 – 30% follow PPROM. Births that follow spontaneous labour and PPROM are designated together as spontaneous preterm births. Preterm births can
also be subdivided according to the gestational age: about 5% of preterm births occur at less than 28 weeks of gestation (extreme prematurity), about 15% at 28 – 31 weeks (severe prematurity), about 20% at 32 – 33 weeks (moderate prematurity), and 60 – 70% at 34 – 36 weeks (near term) (Goldenberg et al., 2008).

Preterm labour is usually defined as regular uterine contractions accompanied by cervical change at less than 37 weeks gestation. The pathogenesis of preterm labour is not well understood, however preterm labour might be caused by early idiopathic activation of the normal labour process or the results of other pathologic insults (Goldenberg et al., 2008). There are multiple mechanisms that have been suggested for the initiation of preterm labour, including local and systemic infection or inflammation, uteroplacental ischemia or hemorrhage, uterine overdistension, stress, and other immunologically mediated processes (Romero et al., 2006). Since a precise mechanism cannot be established in most cases, factors associated with preterm birth have been investigated in order to understand, explain and predict preterm labour. Maternal and fetal characteristics that will be described in more detail below, have been associated with preterm birth including maternal demographic characteristics, nutritional status, pregnancy history, present pregnancy characteristics, psychological characteristics, adverse behaviours, infection, uterine contractions, shortened cervical length, as well as biological and genetic markers (Goldenberg et al., 2008).

Maternal demographic characteristics associated with preterm birth include race, low socioeconomic and educational status, low and high maternal ages, and single marital status. In the USA and in the United Kingdom (UK), women classified as Black, African-American and Afro-Caribbean are consistently reported to be at higher risk of preterm delivery. The reported preterm birth rates are in the range of 16 – 18% for black women compared with 5 – 9% for white women. Black women are also 3 to 4 times more likely to have a very early preterm birth than women from other racial and ethnic groups. These differences have not been explained by medical, psychological or social conditions (Goldenberg et al., 1996). In contrast to Black females, females of East Asian and Hispanic descent typically have low preterm birth rates. Women from South Asia, including the Indian subcontinent have high rates of low birth weight associated with decreased fetal growth. But, preterm delivery does not seem to be increased
substantially for this group of women (Goldenberg et al., 2008). However, the mechanisms by which maternal demographic characteristics are related to preterm birth are unknown.

Maternal nutritional status as indicated by body-mass index (BMI), nutrient intake or assessments of various nutrients in the serum are also associated with increased risk of preterm birth (Hendler, 2005; Scholl, 2005; Tamura et al., 1992). If the BMI is low prior to pregnancy, there could be an increased risk of spontaneous preterm birth, whereas obesity is protective (Hendler, 2005). Women with low serum concentrations of iron, folate or zinc have more preterm births than those with normal levels of these micronutrients (Scholl, 2005; Tamura et al., 1992). Although obesity seems to prevent preterm birth, it has also been observed that obese women who still deliver preterm are more likely to have infants with congenital anomalies such as neural tube defects (Goldenberg et al., 1996). As well, obese women are more likely to develop pre-eclampsia and diabetes; conditions that could also cause indicated preterm birth. Indeed, diabetes and hypertension are two of the most common disorders that complicate pregnancy and are both correlated with increased rates of preterm delivery, many of which are indicated (Goldenberg et al., 2008).

About 15% to more than 50% of women with a previous history of preterm delivery are at increased risk of having other preterm deliveries and this correlates to the gestational age of the newborn. In relation to this, the risk of a woman having an additional preterm birth is related inversely to the gestational age of the previous infant but the mechanism for recurrence is not clear. However, women with a history of early spontaneous preterm births are far more likely to have subsequent spontaneous preterm births and women with indicated preterm births tend to repeat such births (Ananth, Getahun, Peltier, Salihu, & Vintzileos, 2006; Goldenberg, Andrews, Faye-Petersen, Cliver, Goepfert, & Hauth, 2006).

A history of multiple gestations also carries a substantial risk of preterm delivery and is evidently responsible for about 15 – 20% of all preterm births. Nearly 60% of twins are born preterm. About 40% of twins will have spontaneous labour or PPROM before 37 weeks gestation, with others having an indicated preterm delivery because of pre-eclampsia or other maternal or fetal disorders. Uterine overdistension resulting in contractions and PPROM is believed to be the causative mechanism for the rate of increased spontaneous preterm births in relation to multiple gestations (Romero et al., 2006). Multiple gestations associated with assisted reproductive
technologies also carry a significant risk of preterm delivery. Singleton pregnancies after in-vitro fertilization are also at increased risk of preterm birth (Jackson, Gibson, Wu, & Croughan, 2004).

Mothers experiencing high levels of psychological or social stress are also at increased risk of preterm birth (Copper et al., 1996). Women exposed to stressful conditions have increased serum concentrations of biomarkers of inflammation including C-reactive protein (CRP), which suggests that systemic inflammation might be a pathway by which stress could increase the risk of preterm birth (Sheldon, Riches, Gooding, Soni, & Hobbs, 1993). In addition, clinical depression during pregnancy has been reported to be associated with preterm birth (Orr & Miller, 1995). However, individuals who are depressed also tend to smoke, use illicit drugs or alcohol at an increased rate compared to those who are not depressed, and these issues could play an important role with regard to preterm birth in depressed women. Put another way, the relation between depression and preterm birth might be mediated by behavioural factors as opposed to direct effects of depressive illness itself (Zuckerman, Amaro, Bauchner, & Cabral, 1989).

Having said this, it must also be pointed out that in the USA, about 20 – 25% of pregnant women smoke (Cnattingius, 2004). It is known that both nicotine and carbon monoxide are powerful vasoconstrictors, and are associated with damage to the placenta leading to decreased utero-placental blood flow. These problems could both lead to restriction in fetal growth as well as an increase in the number of indicated preterm births. Smoking is also associated with systemic inflammation, another factor that might cause an increase in the prevalence of spontaneous preterm birth (Bermudez, Rifai, Buring, Manson, & Ridker, 2002).

Another important mechanism that can lead to preterm birth is intrauterine infection (Goldenberg, Hauth, & Andrews, 2000). Intrauterine infection might lead to preterm labour as a consequence of activation of the innate immune system (Romero et al., 2006). Furthermore, certain microorganisms are recognized by pattern-recognition receptors (e.g. toll-like receptors), which in turn elicit the release of inflammatory chemokines and cytokines such as interleukin 8 (IL-8), interleukin 1β (IL-1β), and tumor necrosis factor-α (TNF-α) all of which can stimulate preterm birth. Moreover, endotoxins from the invading microorganisms, in addition to the proinflammatory cytokines noted above have been shown to stimulate uterine contractility. Furthermore, degradation of extracellular matrix in the fetal membranes leads to PPROM.
(conceivably induced by upregulated levels of matrix metalloproteinase; MMP, which will be further described below). In relation to this, MMPs are not only produced by bacteria, but they are also produced during the development of the host inflammatory responses to microbial infection. Given this two-pronged effect, it is perhaps not too surprising that intrauterine infection might account for as much as 25 – 40% of preterm births (Goldenberg et al., 2000). However, this might be a minimum estimate because intrauterine infection is difficult to detect with conventional culture techniques (Goldenberg et al., 2008). The organisms are usually vaginal in origin and ascend into the uterus either before or early in the pregnancy. The organisms are often of low virulence and the infections tend to be chronic; persisting for weeks or months before preterm labour or membrane rupture initiates a spontaneous preterm birth (Goldenberg & Culhane, 2007). Ureaplasma urealyticum and mycoplasma hominis are the two most common organisms associated with preterm labour or birth. However, over 30 different bacteria have been identified. Many of these organisms are components of a chronic vaginal infection called bacterial vaginosis, a common condition associated with a 1.5-fold to 3-fold increase in the rate of preterm birth (Hillier et al., 1995; Meis et al., 1995). Several non-genital tract infections, such as pyelonephritis and asymptomatic bacteruria, pneumonia and appendicitis are associated with, and thought to predispose to preterm birth (Goldenberg, Culhane, & Johnson, 2005; Romero, Oyarzun et al., 1989). In line with the putative effects of various infections, it has now been suggested that periodontal disease might play a causal role in the induction of preterm birth as will be discussed in more detail below.

Finally, biological markers such as cytokines, chemokines, estriol, and other analytes from biological fluids (e.g. amniotic fluid, urine, cervical mucous, vaginal secretions, serum, plasma and saliva) have also been assessed and many, especially those related to inflammation, are associated with preterm birth. Genetic association studies have also identified single-nucleotide polymorphisms in several genes such as the genes for TNF-α and interleukin 6 (IL-6) that are associated with preterm labour and PPROM (Goldenberg et al., 2008).

2 Low Birth Weight

Low birth weight (LBW) infants are not a homogenous group. LBW may result from preterm birth, intrauterine growth restriction (IUGR), or a combination of these conditions (Lim et al.,
IUGR is defined as a process of any etiology that can limit the potential for intrauterine growth of the fetus leading to lower birth weight (Valero De Bernabe et al., 2004). The term LBW refers only to infants born weighing 2500 g or less, regardless of gestational age, or the cause of LBW (World Health Organization (WHO), 1961). LBW infants can be classified further as ‘very low birth weight’ (VLBW) (1000 – 1499 g) and ‘extremely low birth weight’ (ELBW) (500 – 999 g) (Valero De Bernabe et al., 2004). Three categories of LBW can be distinguished: 1) premature or preterm LBW infants, that are born before 37 weeks of gestation, 2) term LBW infants, that are born between 37 and 42 complete weeks of gestation, and 3) post-term LBW infants, that are born after 42 weeks of gestation. Therefore, it is important to note that it is possible for both preterm birth and growth restricted infants to weigh greater than 2500 g. Small for gestational age (SGA) is a statistical definition which refers to infants whose weight is less than the lower limit of the confidence interval of the normal curve for weight by weeks of gestation (Valero De Bernabe et al., 2004).

In Canada, about 6% of infants are born with LBW. In the USA and the UK, the reported rates are 8.2% and 7.6% respectively (Lim et al., 2009). Most developed countries have reported an increase in LBW rates in the recent years.

Growth restriction in the womb is thought to be caused principally by placental insufficiency whereby the placenta cannot transfer oxygen, glucose or other vital nutrients and metabolic products to and from the fetus adequately. Other factors that influence fetal growth are essentially similar to the ones for preterm birth, including maternal demographic characteristics (race, low socioeconomic and educational status, low and high maternal ages, and single marital status), poor nutrition during pregnancy, pregnancy history and present characteristics of the pregnancy, substance abuse, and maternal comorbidities such as hypertension and infection (Lim et al., 2009; Valero De Bernabe et al., 2004). Maternal smoking, in particular, has been estimated to account for about one-third of growth-restricted births in economically developed countries (Kramer, 1987). It is estimated that 40% of birth weight is due to heredity, and the remaining 60% is due to environmental factors, many of which are unknown (Valero De Bernabe et al., 2004).
3 Sequele of Adverse Birth Outcomes

The rate of preterm birth and LBW infants in developed countries has risen over time, and unfortunately a significant proportion of infant deaths can be accounted for by this population. However, the mortality rate varies by country, and even different geographic regions within the same country, as well as between various racial and ethnic groups, and this might be related to the level of neonatal care that is available (Saigal & Doyle, 2008). Alternatively, the survival rates of very early preterm births have also increased because of technological advances, and the collaborative efforts of obstetricians and neonatologists in the developed countries. However, biologically preterm infants are more susceptible to complications than their term counterparts. Although, most organs are immature, the brain and lungs are especially susceptible to the consequences of preterm birth. This leads to high rates of long-term neurological and respiratory problems. Some of the conditions associated with LBW infants include perinatal and infant death, physical and cognitive disabilities, and chronic health problems later in life (Goldenberg & Culhane, 2007). The high rates of morbidity arising from these adverse birth outcomes leads to increased health care costs associated with the use of specialized equipment, longer length of hospital stay, and increased use of personal resources in the health care area. In 2005 – 2006, the Canadian average in-hospital cost associated with preterm infants was 9 times higher than for term infants ($9,233 compared to $1,050); while the cost associated with LBW infants was 11 times higher than for those weighing greater than 2500 g ($12,354 compared to $1,084). Moreover, this cost was dramatically higher when an infant was born at ≤ 28 weeks of gestation, with the average hospital cost being approximately $85,103 for a non-SGA infant and $109,286 for a SGA infant (Lim et al., 2009).

3.1 Neurodevelopmental and Behavioural Sequelae

Neurodevelopmental impairments that can present in the early years include cerebral palsy, developmental delay, and sensory impairments such as visual and auditory deficits. Approximately 25% of surviving preterm and/or LBW infants are reported to have substantial neurological morbidity, with the highest rates noted in the most immature survivors based on gestational age. Many infants are also reported to have important lags in development that are
not severe enough to be classified as impairments (Saigal & Doyle, 2008) but exist nonetheless, and are a burden.

Most studies of VLBW infants demonstrate additional sequelae including cognitive deficits, academic underachievement, grade failures, and the need for increased remedial educational assistance during mid-childhood and adolescence (Saigal & Doyle, 2008). These difficulties are apparent even in children without neurosensory impairments and a normal intelligence quotient (IQ), where IQ was measured using the Wechsler Intelligence Scale for Children-Revised and the Wide Range Achievement Test-Revised in a cohort of children from Central West Ontario. Interestingly, these difficulties were more prevalent in boys (Saigal, Hoult, Streiner, Stoskopf, & Rosenbaum, 2000).

Very preterm survivors (gestational age less than 32 weeks) have high rates of dysfunction in other cognitive areas such as attention, visual processing and executive function. The cognitive disadvantage seems to persist into late adolescence and early adulthood (Hack et al., 2002; Lefebvre, Mazurier, & Tessier, 2005; Saigal et al., 2000). However, cognitive dysfunctions are moderated by environmental factors such as parental socioeconomic status and education, two-parent family, neighbourhood effects, schooling, social and racial backgrounds (Aylward, 2005).

The risk of behavioural problems such as attention deficit hyperactivity disorder (ADHD) is increased between 2.6 up to 4.0 times in very preterm infants in early childhood (Aylward, 2005; Delobel-Ayoub et al., 2006; Reijneveld et al., 2006). At school age they continue to be more susceptible to ADHD. They also have emotional troubles that can affect academic function (Breslau & Chilcoat, 2000; Sykes et al., 1997). In addition, preterm infants tend to have traits such as shyness, unassertiveness and social maladaptation, making them appear anxious and withdrawn (Aylward, 2005). In most studies, VLBW adults had slightly lower levels of educational achievement and lower rates of employment and independent living, compared to their normal counterparts (Cooke, 2004; Ericson & Kallen, 1998; Sykes et al., 1997).

### 3.2 Other Sequelae

Compared to infants born at term, preterm infants have higher rates of temperature instability, respiratory distress, apnea, hypoglycemia, seizures, jaundice, kernicterus, feeding difficulties,
periventricular leukomalacia and rehospitalization, especially related to respiratory illnesses (Escobar et al., 2006; Kinney, 2006; Raju, 2006; M. L. Wang, Dorer, Fleming, & Catlin, 2004). Respiratory illnesses also increase the risk of rehospitalization in ELBW children compared to normal birth weight children in the early years (Doyle, Ford, & Davis, 2003). Even at 10-12 years of age, children born before 26 weeks gestation have greater needs for services such as physician visits, occupational or physical therapy, nursing or medical procedures, and compensatory dependency compared to children of normal birth weight (Farooqi, Hagglof, Sedin, Gothefors, & Serenius, 2006). The rates of chronic disorders continue to remain high in ELBW adults compared to young adults of normal birth weight (Hack, Cartar, Schluchter, Klein, & Forrest, 2007; Saigal et al., 2007). Several studies have also reported high systolic blood pressure in VLBW young adults (Doyle, Faber, Callanan, & Morley, 2003; Hack et al., 2007; Keijzer-Veen et al., 2005).

Retinopathy of prematurity is one of the most common morbidities in infants born before 26 weeks gestation. Gestational age affects the rate of blindness or severe visual impairments, or both, with rates of 1 – 2% for infants with a gestational age of 26 – 27 weeks, and 4 – 8% at 25 weeks or below (Farooqi et al., 2006; Marlow, Wolke, Bracewell, Samara, & EPICure Study Group, 2005; Vohr, Wright, Poole, & McDonald, 2005). Myopia and hypermyopia arise in at least 25% of children born before 28 weeks gestation (O'Connor et al., 2002).

VLBW infants have lower growth attainment in weight and length than their normal counterparts during infancy and early childhood (Finnstrom et al., 1998; Hack, Weissman, & Borawski-Clark, 1996; Kitchen, Doyle, Ford, & Callanan, 1992; N. S. Wood et al., 2003). However, by mid-childhood and adolescence substantial catch-up growth is observed. ELBW survivors also achieve normal adult stature, but remain disadvantaged in their height (Doyle, Faber, Callanan, Ford, & Davis, 2004; Hack et al., 2003; Saigal et al., 2006).

Children who are preterm at birth have more behaviour management problems related to their ability to tolerate and cooperate during dental treatment compared to children born full term during the preschool years. However, this difference decreases with increasing age. Preterm birth, low gestational age and LBW are also associated with increased prevalence of molar incisor hypomineralization and developmental defects in enamel (Brogardh-Roth, 2010; Brogardh-Roth, Matsson, & Klingberg, 2011; Lai, Seow, Tudehope, & Rogers, 1997). In
addition, these children exhibit higher plaque levels and a higher degree of gingival inflammation compared to their normal counterparts (Brogardh-Roth, 2010; Brogardh-Roth et al., 2011). However, with respect to dental caries prevalence in the primary and permanent dentition, the findings have been inconsistent. While some studies report a higher prevalence of dental caries in preterm and LBW survivors (Rajshekar & Laxminarayan, 2011; Saraiva, Bettiol, Barbieri, & Silva, 2007), others have not found an increased prevalence of caries compared to normal controls (Brogardh-Roth, 2010; Brogardh-Roth et al., 2011; Cruvinel, Gravina, Azevedo, Bezerra, & Toledo, 2010; Ghasempour, Ahmadpour-Kacho, & Sheikh, 2009; Shulman, 2005).

4 Impact of Adverse Pregnancy Outcomes: Preterm and/or Low Birth Weight Infant Delivery

Based on the above then, it is clear that preterm low birth weight (PTLBW) delivery can lead to many deleterious outcomes and sequelae. This presents a major burden on the healthcare system and even more importantly on the family and children themselves. Therefore, any new information regarding factors that put individuals at risk for PTLBW delivery is extremely important. However, as can also be interpreted from the foregoing, there are so many different presentations and types of PTLBW delivery and infants, that it is almost impossible at this point to correlate risk factors with these outcomes. Given this, it is necessary to define more precisely the types of gestational outcomes under study so that risk factors that might be assessed can be analyzed more accurately and without confounding factors or at least with reduced confounding factors. As alluded to above, regardless of the problems mentioned here, there has been evidence suggesting a linkage between chronic maternal periodontitis and the incidence of PTLBW delivery. However, this putative linkage suffers because of the complexities related to the wide array of PTLBW conditions. Moreover, as will be outlined further, even periodontitis is not one single entity, and is identified using a very wide array of diagnostic markers which do not necessarily correlate with one another. Therefore when attempting to correlate a multifaceted risk factor (periodontitis) with a multifaceted outcome (PTLBW delivery), it might be virtually impossible to demonstrate any linkage at least in with a high degree of reliability.

Given these issues then, it was thought to be important that in order to reduce confounding variables from the oral disease perspective, that periodontitis should be assessed in more detail in
pregnant females. However, prior to outlining the approach to this question it will first be important to describe periodontal diseases below.

5 Periodontal Diseases

Periodontal diseases can be generally divided into two basic categories, gingival diseases and periodontitis. Gingival diseases are those that occur in the periodontium without any loss of attachment to the tooth or any appreciable and irreversible destruction of tissues making up the periodontium. Alternatively, in the case of periodontitis there is destruction of supporting structures such as the periodontal ligament, alveolar bone and cementum; processes that can lead to significant morbidity and tooth loss.

5.1 Etiology and Pathogenesis of Gingivitis and Periodontitis

Gingivitis is caused by an accumulation of substances derived from the microbial plaque, which presents as a biofilm on the tooth surface, at or near the gingival sulcus. All of the other suspected local and systemic etiologic factors either enhance plaque accumulation or retention, or enhance the susceptibility of the gingival tissue to microbial attack (Page, 1986).

In health, the bacteria of dental plaque live in harmony with the host’s immune system, rarely causing systemic infections. The subgingival flora in health contains mainly Actinomyces, Streptococci and Veillonella species. Non-motile species greatly outnumber motile forms by a ratio of about 40 to 1. When oral hygiene practices are stopped, the bacteria in early dental plaque rapidly doubles in size and number. Early colonizers such as Streptococcus gordonii and A. naeslundii provide co-aggregation factors such as adhesins that enable more virulent species to populate, leading to a more complex flora. The biofilm associated with gingivitis releases materials that result in localized tissue inflammation (Darveau, Tanner, & Page, 1997).

The development of gingivitis has been divided into three distinct stages based on the histopathological appearance of the periodontium. The initial lesion appears as an acute inflammatory response i.e. presence of redness, edema, possible bleeding and changes in contour. It presents within four days following initiation of microbial plaque accumulation at the gingival margin. Vasodilation occurs in the tissues adjacent to the sulcus in response to bacterial
products, which provides increased transport of inflammatory cells. Bacteria and their by-products provide chemotactic stimuli for the migration of leukocytes, primarily polymorphonuclear neutrophils (PMNs) to the affected area. The PMNs release MMPs including collagenase and other endopeptidases, resulting in the degradation of collagen in the marginal gingival connective tissues. The early lesion evolves from the initial lesion within one week following the start of plaque accumulation. It is characterized by infiltration of mostly T-lymphocytes along with some macrophages and plasma cells. This stage is characterized by continued destruction of gingival connective tissues and pathologic alteration of resident fibroblasts as well as continued infiltration of PMNs. These altered cells are associated intimately with activated lymphocytes. With time, plasma cells and B-lymphocytes predominate the lesion. The lesion is then referred to as the ‘established lesion’ and appears to have a high degree of organization. Some established lesions remain stable and do not progress for months or years. Others appear to become more active and convert to progressive and in fact destructive lesions that are characteristic of early periodontitis. Importantly, meticulous removal of plaque usually leads to resolution of the chronic gingivitis lesion without residual tissue destruction ("The Pathogenesis of Periodontal Diseases", 1999; Loe, Theilade, & Jensen, 1965; Page, 1986). However, this is not necessarily the case once the lesion has entered a destructive phase.

The histopathological features of periodontitis are in many ways similar to gingivitis. There is a predominance of plasma cells, loss of connective tissue elements in addition to alveolar bone resorption. Despite the histopathological similarities, the pathophysiological aspects of the transition of gingivitis to periodontitis are poorly understood.

The etiology of periodontitis is multifactorial. However, it is widely accepted that the initiation and progression of periodontitis are dependent upon the presence of microorganisms capable of causing disease. Although hundreds of species of microorganisms have been isolated from periodontal pockets, only a few of these species possess the virulence factors thought to be responsible for initiating the periodontal destruction process (such as Porphyromonas gingivalis, Actinobacillus Actinomycetemcomitans, Prevotella intermedia, Treponema denticola and Tannerella forsythia to name a few). At least three characteristics of periodontal microorganisms have been identified that contribute to their ability to act as pathogens: the capacity to colonize,
the ability to evade antibacterial host defense mechanisms, and the ability to produce substances that can directly initiate tissue destruction ("The Pathogenesis of Periodontal Diseases", 1999).

An essential part of the progression of periodontal destruction relates to the inflammatory response of the host, which varies from person to person. Periodontal tissue is destroyed by the modulation of host defenses by bacterial products. This stimulates the host inflammatory process and the local release of pro- and anti-inflammatory cytokines, and enzymes capable of destroying host tissues (with the exception of the anti-inflammatory cytokines). This does not preclude a role for bacterial and virulence factors, but suggests that in most forms of periodontal disease, destruction is a consequence of the host response to these factors (Dennison & Van Dyke, 1997). Genes play an indispensable role in host responses and consequently, in progression of the disease. Certain individuals are disease resistant and will not develop periodontitis despite the presence of long standing and even severe gingivitis. Therefore, periodontitis must be considered to be multifactorial with regard to origin and progression, and the factors involved range from genetic ones to those that are epigenetic (bacteria, smoking, poor oral hygiene) (Yoshie, Kobayashi, Tai, & Galicia, 2007).

In summary, virulent microorganisms capable of initiating or propagating periodontal attachment loss must be present in the local lesion at a critical minimal concentration. In susceptible hosts, or in susceptible periodontal sites within susceptible hosts, protective mechanisms are breached, exposing the underlying tissues and cells to bacterial virulence factors. Consequently, cellular components, including monocytes and fibroblasts, are stimulated by bacterial products such as lipopolysaccharide (LPS) to produce many or all of the cytokines described above. These cytokines are capable of acting alone or in concert to stimulate an inflammatory response and catabolic processes, such as bone resorption and collagen destruction, which are mediated in part by MMPs ("The Pathogenesis of Periodontal Diseases", 1999). Finally, the production of increased levels of the MMPs could play a critical role not only in soft tissue destruction seen in periodontitis, but also in the regulation of other inflammatory diseases including of course periodontitis, but possibly other non-oral conditions as well (Sorsa et al., 2006).
5.2 Classification of Periodontal Diseases

Gingival diseases can be divided into those that are induced by dental plaque, and those which are not. The dental plaque-induced gingival diseases category includes: 1) gingivitis associated with dental plaque only, and 2) gingival diseases modified by systemic factors or medications or malnutrition. The non-plaque induced gingival lesions group includes: 1) gingival diseases induced by bacterial, viral or fungal infection, 2) lesions which are a manifestation of genetic or systemic conditions, and 3) lesions induced by allergy, trauma (self-induced or otherwise) or foreign body reactions (Armitage, 1999). Gingivitis is one of the most common diseases of adults and in fact in the USA, over 50% of adults suffer from this condition (on an average of about 3-4 teeth) (Oliver, Brown, & Le, 1998).

Periodontitis can be divided generally into two primary forms; chronic and aggressive. In addition it can be classified as a manifestation of systemic (i.e. non-oral) disease. Both chronic and aggressive periodontitis can be categorized further on the basis of extent and severity. As a general guide, extent can be characterized as ‘localized’ (less than 30% of sites are involved) or ‘generalized’ (greater than 30% of sites are involved). Severity can be considered on the basis of the amount of clinical attachment loss (CAL) as slight (1 – 2 mm CAL), moderate (3 – 4 mm CAL), or severe (greater than or equal to 5 mm CAL) (Armitage, 1999).

Chronic periodontitis is the most common form of periodontitis and can develop in any age group. Clinically, it is characterized by gingival inflammation, periodontal pocketing, bleeding on probing, alveolar bone loss, gingival recession, tooth mobility and possible tooth loss (Flemmig, 1999). This disease is characterized by varying rates of progression, with varying episodes of quiescence and activity (Socransky, Haffajee, Goodson, & Lindhe, 1984). Indeed, as will be addressed elsewhere, periodontitis can be present in both active and inactive forms but most clinical tests cannot differentiate between the two. Therefore, when trying to correlate the presence of periodontal disease (which might or might not be ‘active’ at the time of presentation) with the incidence PTLBW delivery (another condition that, as shown above, is quite heterogeneous), it can be understood just how difficult it has been and will be to relate these two conditions in a statistically sound manner.
There is significant variation regarding reports in the literature concerning the prevalence of periodontitis. This is due to a lack of use of adequate and consistent methodologies for measuring the disease in epidemiological studies, and this actually stems from an incomplete understanding of the pathophysiology of periodontitis in general, despite extensive research. The apparent prevalence of periodontitis also varies with race and geographic location (Papapanou, 1996). Therefore any prevalence information must be interpreted in light of the population studied and the periodontitis case definition applied. It is estimated that only some 5% to 15% of any population suffers from severe generalized periodontitis, even though moderate disease affects a majority of adults (Burt & Research, Science and Therapy Committee of the American Academy of Periodontology, 2005). The reported prevalence of periodontitis in the USA is around 40% using the case definition of CAL of ≥ 3 mm. However, the reported prevalence drops to around 30% when gauged by presence and number of periodontal pockets ≥ 4 mm on an average of 3 – 4 teeth (Oliver et al., 1998). In contrast to the prevalence reported in the USA, the apparent prevalence of this condition is lower in Canada based on the Canadian Health Measures Survey (Health Canada, 2010). In this case, only about 16% of adults were found to have moderate periodontitis with at least one pocket of 4 or 5 mm in depth in 2007 – 2009, and the prevalence of severe periodontitis is reported to be around 4% based on pocket depths ≥ 6 mm. These data in general indicate that milder forms of periodontitis may be close to universal, however, more severe manifestations of the disease are less prevalent (Burt & Research, Science and Therapy Committee of the American Academy of Periodontology, 2005).

As alluded to above, the lack of universally accepted case definitions for periodontitis has presented challenges for the surveillance of periodontitis and it has been suggested that this is a significant limiting factor in determining and comparing prevalence estimates of periodontitis across studies and surveys (Eke, Page, Wei, Thornton-Evans, & Genco, 2012), and in particular when trying to correlate the prevalence of periodontitis with other diseases (which themselves can be variable in activity and presentation). In order to improve and expand the surveillance of periodontitis in a population in 2007 the Centers for Disease Control and Prevention (CDC) and the American Academy of Periodontology (AAP) developed and proposed a definition of moderate periodontitis as ≥ 2 interproximal sites with CAL ≥ 4 mm or ≥ 2 interproximal sites with probing depth ≥ 5 mm (not on the same tooth); and severe periodontitis as ≥ 2 interproximal
sites with CAL ≥ 6 mm (not on the same tooth) and ≥ 1 interproximal sites with probing depth ≥ 5 mm (not on the same tooth) (Page & Eke, 2007). Recently, the CDC and AAP proposed a definition for mild periodontitis as ≥ 2 interproximal sites with CAL ≥ 3 mm and ≥ 2 interproximal sites with probing depth ≥ 4 mm (not on the same tooth) or 1 site with ≥ 5 mm probing depth (Eke et al., 2012). The total load of periodontitis in a population is suggested to be computed as the sum of mild, moderate and severe periodontitis. These definitions require a full periodontal examination of all the teeth present, which is often considered time consuming when carrying out large population based studies. This might also explain the differences observed between the reported prevalence of the disease in Canada versus the USA in that for the Canadian Health Measures Survey (Health Canada, 2010), only specific teeth were assessed while in the USA, all teeth were assessed.

Aggressive periodontitis has clearly identifiable clinical and laboratory findings, making it possible to reliably differentiate it from chronic periodontitis. The common features of localized and generalized forms of this type of periodontitis are that the patients are generally healthy, while there appears to be a familial aggregation of cases. The disease presents with rapid loss of attachment and destruction of alveolar bone. In addition, the amounts of microbial deposits are usually not proportional to the severity of periodontal tissue destruction unlike what is observed in patients with chronic periodontitis. From a microbiological point of view usually, there are elevated proportions of Actinobacillus actinomycetemcomitans, and in some populations, Porphorymonas gingivalis may be elevated as well. In addition, generally there are phagocyte abnormalities, hyper-responsive macrophage phenotype, including elevated levels of prostaglandin E$_2$ (PGE$_2$) and IL-1β. The localized form of aggressive periodontitis typically has a circumpubertal onset and interproximal attachment loss is usually localized to the first permanent molars and incisors. Patients generally have a robust serum antibody response to the infecting agents. The generalized form usually affects persons under 30 years of age and generalized interproximal attachment loss affects at least three permanent teeth other than first molars and incisors. In contrast to the localized form, patients usually have a poor serum antibody response to the infecting agents (Lang et al., 1999).
6 Pregnancy and Periodontal Disease

6.1 Impact of Pregnancy on Periodontal Disease

Major physiological and hormonal changes occur during pregnancy. The most significant hormonal change is the increased production of estrogens and progesterone. Estrogen levels rise 30-fold and progesterone levels increase 10-fold compared to the concentrations observed during the menstrual cycle. Hormone related changes have been reported to occur in the oral cavity during pregnancy (Laine, 2002; Vogt, Sallum, Cecatti, & Morais, 2012). Studies have shown that pregnant women have more gingivitis compared to non-pregnant women, with a prevalence ranging from 30% to 100% (Laine, 2002; Loe & Silness, 1963; Ziskin & Nesse, 1946). The reported prevalence of periodontitis during pregnancy varies from 20% to 50% (Lieff et al., 2004; Vogt, Sallum, Cecatti, & Morais, 2012). As discussed above, these variations might reflect the differences in definitions of periodontal disease between studies, as well as different populations studied and their characteristics. The prevalence is reported to be higher among certain racial and ethnic minorities, smokers, and women of low socioeconomic status (Lieff et al., 2004).

It has been suggested that pregnancy does not necessarily cause periodontal disease but may exacerbate preexisting unfavourable periodontal conditions (Laine, 2002; Moss, Beck, & Offenbacher, 2005). The depth of periodontal pockets may increase as pregnancy progresses (Moss et al., 2005; Taani, Habashneh, Hammad, & Batieha, 2003), but the level of activity of the disease does not necessarily result in additional or accelerated loss of periodontal clinical attachment (Moss et al., 2005), indicating that these deep pockets may revert following the pregnancy. However, it has also been shown that irreversible clinical attachment loss can occur in susceptible women during pregnancy (Lieff et al., 2004).

The exact mechanism by which hormonal changes in pregnancy increase the susceptibility of gingival tissues to inflammation is not known. Most explanations for pregnancy-related gingival changes have suggested increased vascularity and vascular flow, directly or indirectly as the main factor since both estrogens and progesterone have effects on the vascular system during pregnancy. The increase in progesterone has been shown to result in greater vascular
permeability, gingival edema, crevicular fluid flow levels and prostaglandin production, which may then lead to gingival inflammation (Amar & Chung, 1994). Other proposed mechanisms include changes in the immune system or changes in connective tissue metabolism. Although, the number of peripheral PMNs increases during pregnancy, their function appears to be altered (Barriga, Rodriguez, & Ortega, 1994), which may then render gingival tissues less resistant to challenges caused by bacteria. In addition, progesterone has been shown to decrease IL – 6 production by human gingival fibroblasts, which in turn may lower resistance to infectious challenges (Lapp, Thomas, & Lewis, 1995).

6.2 Impact of Periodontitis on Pregnancy Outcomes

Periodontal disease was first reported to be a potential risk factor for preterm birth in 1996 (Offenbacher et al., 1996). Since then a multitude of studies evaluating the association between a variety of measures of periodontal disease and adverse pregnancy outcomes including preterm birth, LBW, miscarriage or early pregnancy loss, and preeclampsia have been published, some of which will be discussed under the headings of ‘Epidemiological’, ‘Interventional’ and ‘Mechanistic’ studies.

6.2.1 Epidemiological Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

There are several observational (case-control and cohort) studies that suggest that periodontal disease is a risk factor for PTLBW (Khader et al., 2009; Lopez, Smith, & Gutierrez, 2002; Mitchell-Lewis, Engebretson, Chen, Lamster, & Papapanou, 2001; Mokeem, Molla, & Al-Jewair, 2004), LBW (Dasanayake, 1998; Kushtagi, Kaur, Kukkamalla, & Thomas, 2008; Moliterno, Monteiro, Figueredo, & Fischer, 2005; Moreu, Tellez, & Gonzalez-Jaranay, 2005; Offenbacher et al., 2001; Rakoto-Alson, Tenenbaum, & Davideau, 2010; Saddki, Bachok, Hussain, Zainudin, & Sosroneno, 2008), preterm birth (Agueda, Ramon, Manau, Guerrero, & Echeverria, 2008; Bosnjak, Relja, Vucevic-Boras, Plasaj, & Plancak, 2006; Dortbudak, Eberhardt, Ulm, & Persson, 2005; Goepfert et al., 2004; Guimaraes, Silva-Mato, Miranda Cota, Siqueira, & Costa, 2010; Jeffcoat et al., 2001; Lopez et al., 2002; Offenbacher et al., 2001; Pitiphat et al., 2008; Radnai et al., 2004; Radnai et al., 2006; Rakoto-Alson et al., 2010), very preterm birth (Guimaraes et al., 2010; Offenbacher et al., 2006), preeclampsia (Boggess et al.,
2003; Canakci et al., 2004; Canakci et al., 2007; Contreras et al., 2006; Oettinger-Barak et al., 2005; Shetty et al., 2010), and miscarriage or still birth (Moore et al., 2004). Several studies also demonstrated a dose-response relationship, where the risk of adverse pregnancy outcome has been shown to increase as the severity of periodontal disease increases (Jeffcoat et al., 2001; Jeffcoat, Geurs, Reddy, Goldenberg, & Hauth, 2001; Offenbacher et al., 2001). Severe periodontal disease was associated with an even higher risk of very preterm birth (less than 32 weeks), birth weight below 1500 g and early pregnancy loss (Jeffcoat et al., 2001; Offenbacher et al., 2001). Conversely, there are several other studies that did not find periodontal disease to be a risk factor for PTLBW (Buduneli et al., 2005; Davenport et al., 2002; Noack, Klingenberg, Weigelt, & Hoffmann, 2005; Rajapakse, Nagarathne, Chandrasekra, & Dasanayake, 2005; Vettore et al., 2008), LBW (Farrell, Ide, & Wilson, 2006; Hujoel, Lydon-Rochelle, Robertson, & del Aguila, 2006; Lunardelli & Peres, 2005; Moore et al., 2004), preterm birth (Farrell et al., 2006; Holbrook et al., 2004; Lunardelli & Peres, 2005; Moore et al., 2004; Moore, Randhawa, & Ide, 2005; Ryu et al., 2010; Skuldbol, Johansen, Dahlen, Stoltze, & Holmstrup, 2006; S. Wood et al., 2006) or preeclampsia (Lohsoonthorn et al., 2009; Srinivas et al., 2009). It should be noted that, there are several potential biases among the aforementioned studies that could account for the reported differences, one being the inconsistency in periodontal disease definitions. As discussed above, due to a lack of a universally accepted definition for periodontal disease in epidemiological studies, the variety of definitions used has been shown to have a great impact on the apparent prevalence and thus the extent of association between periodontal disease and adverse pregnancy outcomes (Kassab et al., 2011; Manau, Echeverria, Agueda, Guerrero, & Echeverria, 2008). Similarly, there was considerable variation in the definitions of adverse pregnancy outcomes, which would also have a negative impact on the ability to demonstrate a clear relationship between periodontitis and pregnancy outcomes including the concatenation of the terms “low birth weight” and “preterm” as if they were one and the same. In fact the use of such composite outcomes that include “preterm low birth weight” very likely confounds results (Xiong, Buekens, Vastardis, & Yu, 2007), as preterm birth and LBW have distinct etiologies, and should be considered separately. Moreover, since there are multiple social, medical, obstetric, environmental and genetic factors associated with different pregnancy outcomes, there is a strong potential for these confounding items to influence (Michalowicz & Durand, 2007; Wimmer & Pihlstrom, 2008; Xiong et al., 2007) and in some cases suppress (Bassani, Olinto, &
Kreiger, 2007; Lunardelli & Peres, 2005; Vettore et al., 2008) the crude association between periodontal diseases and adverse pregnancy outcomes (Rakoto-Alson et al., 2010). Although, most studies controlled for confounding variables such as smoking, race, socioeconomic status and other important variables using multivariable regression analysis, the variables that were included varies greatly among the studies. It is possible that some residual confounding effects remain (Xiong et al., 2007). Insufficient sample size was also a concern for some of the studies (Xiong et al., 2007).

6.2.2 Interventional Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

Similar to epidemiological studies, clinical interventional studies using different periodontal disease criteria and therapies involving different populations have produced inconsistent results (Han, 2011). It has been suggested that periodontal treatment during pregnancy leads to significant reductions in the rates of preterm birth and LBW (Xiong, Buekens, Goldenberg, Offenbacher, & Qian, 2011) based on randomized controlled trials that were conducted in low socioeconomic status populations or low- and middle-income countries (e.g. Chile and India) (Lopez et al., 2002; Tarannum & Faizuddin, 2007), or that were pilot studies (Jeffcoat et al., 2003; Mitchell-Lewis et al., 2001; Offenbacher et al., 2006), or that had relatively small sample sizes (Radnai et al., 2009; Sadatmansouri, Sedighpoor, & Aghaloo, 2006). However, several large randomized controlled trials conducted in industrialized countries (e.g. USA and Australia) failed to find that periodontal therapy during pregnancy reduced the incidence of preterm birth or LBW (Macones et al., 2010; Michalowicz et al., 2006; Newnham et al., 2009; Offenbacher et al., 2009). Also, trials that involved a single patient enrollment site and a defined sub-population inclined to produce results with a positive effect of periodontal treatment (Jeffcoat et al., 2003; Lopez, Da Silva, Ipinza, & Gutierrez, 2005; Radnai et al., 2009). However, trials involving multiple centers and large general population failed to demonstrate efficacy (Macones et al., 2010; Michalowicz et al., 2006; Offenbacher et al., 2009). Essentially, it has been difficult to establish an association between periodontitis and adverse pregnancy outcomes unequivocally. It has been suggested that periodontal health may affect the birth outcome in a subpopulation rather than in the general population (Han, 2011). Moreover, in some cases in order to ‘standardize’ the treatment being administered, periodontal therapy was limited for all subjects to 1 or 2
appointments for scaling and root planing. Unfortunately, not all patients have the same degree of periodontitis and so a ‘one size fits all’ approach to treatment might not necessarily lead to resolution of the disease in all subjects in a study with a fixed treatment protocol (Offenbacher et al., 2009). In this regard, some patients might require additional initial therapy or perhaps more maintenance therapy throughout gestation (Xiong et al., 2011). Clearly, if periodontitis does contribute to adverse pregnancy outcomes, then it would be expected that upon successful treatment of maternal periodontitis, improved pregnancy outcomes should be achieved (Jeffcoat et al., 2011). In addition, all the published trials to date tested periodontal treatment during pregnancy. However, treating periodontal disease during pregnancy may be too late to reduce the local and systemic inflammation that is related to adverse birth outcomes (Goldenberg & Culhane, 2006; Xiong et al., 2011).

6.2.3 Mechanistic Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

There are two main hypotheses regarding the mechanisms underlying the putative link between periodontal disease and adverse pregnancy outcome. One hypothesis suggests that periodontal disease causes abnormal immunological changes, leading to pregnancy complications (Han, 2011). Chronic periodontal infections can produce oral and non-oral host responses, consequently resulting in up-regulation of the synthesis of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. In addition, the gram-negative anaerobic bacteria thought to be responsible for progressive periodontal disease also provide a reservoir of endotoxins, such as LPS. These cytokines in combination with LPS can stimulate PGE2 synthesis by the human placenta and chorioamnion (Gibbs, Romero, Hillier, Eschenbach, & Sweet, 1992; Romero, Hobbins, & Mitchell, 1988; Romero et al., 1989). Importantly, elevated levels of these factors in amniotic fluids are often observed in women with preterm labour (McGaw, 2002; Romero et al., 1993). These pro-inflammatory cytokines as well as LPS from inflamed periodontal tissues can enter the blood-stream, and reach the maternal-fetal interface. This can trigger or worsen the maternal inflammatory response, and contribute to adverse pregnancy outcomes (McGaw, 2002; Xiong et al., 2007). Serum levels of IL-1β in pregnant women with periodontal disease that gave birth to PTLBW infants have been found to be increased (Sert, Kirzioglu, Fentoglu, Aylak, & Mungan, 2011). Also, moderate to severe periodontal disease has been associated with elevated
systemic inflammation early in pregnancy in African American women, as measured by serum CRP (Horton et al., 2008). It has also been shown that pregnant women with periodontal disease and elevated CRP level are at increased risk for preeclampsia (Ruma et al., 2008).

The second hypothesis suggests that oral bacteria can colonize the placenta directly, causing localized inflammation. This can result in preterm birth and other adverse outcomes (Han, 2011). Translocation of oral bacteria into the placenta has been demonstrated in the mouse animal model for both chronic and acute gingival infections (Han et al., 2004; Lin et al., 2003). In the chronic infection model, *Porphyromonas gingivalis* was inoculated continuously and systemically through a subcutaneous chamber. Using polymerase chain-reaction assays, the organisms were detected in the liver and placenta and were associated with retardation in fetal growth (Lin et al., 2003). In an acute infection model, *Fusobacterium nucleatum* was injected into the mouse tail vein to mimic bacteraemia, which occurs during periodontal infections and following some dental procedures. *F. nucleatum* was shown to cross the endothelium to be capable of colonizing in the placenta (Han et al., 2005; Nithianantham et al., 2009; Xu et al., 2007). Once colonized in the placenta, *F. nucleatum* proliferated quickly and eventually spread to the amniotic fluid (mimicking chorioamnionitis) and the fetus (sepsis). *F. nucleatum* also caused localized inflammation of the fetal-placental unit resulting in direct fetal death, which occurred after 2 to 3 days of hematogenous infection (Liu, Redline, & Han, 2007).

Placental colonization by both *F. nucleatum* and *P. gingivalis* has been associated with intrauterine infections in humans (Han, Shen, Chung, Buhimschi, & Buhimschi, 2009; Katz, Chegini, Shiverick, & Lamont, 2009). *P. gingivalis* has also been detected in chorionic tissues and amniotic fluid of pregnant women diagnosed with threatened preterm labour (Hasegawa-Nakamura et al., 2011; Leon et al., 2007), and in the placenta of women with preterm delivery (Katz et al., 2009), or with preeclampsia (Barak, Oettinger-Barak, Machtei, Sprecher, & Ohel, 2007). The current paradigm in obstetrics indicates that intrauterine infection originates predominantly from the vaginal microflora, with the microorganisms ascending into the otherwise sterile uterus. However, microbial species have been identified from human intrauterine infections that do not belong to the normal vaginal flora (Han et al., 2009). In a case report, *F. nucleatum* was shown to cause stillbirth, where the bacteria originated from the mother’s subgingival plaque and translocated to the placenta and fetus, causing acute
inflammation leading to fetal demise (Han et al., 2010). No fusobacteria were detected in the mother’s vaginal or rectal microflora. Results from these biological studies support a causal relationship between poor oral health and adverse pregnancy outcomes.

However, the clinical influence of specific bacterial species originating from periodontal pockets on preterm birth and/or LBW infants is still questionable (Novak et al., 2008). Although some studies showed higher levels of oral periodontal pathogens such as *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia* in mothers who gave birth to preterm birth and/or LBW infants (Lin, Moss, Beck, Hefti, & Offenbacher, 2007; Mitchell-Lewis et al., 2001), other studies (Noack et al., 2005; Novak et al., 2008) have failed to detect differences in the levels of periodontal pathogens between preterm birth and/or LBW and normal birth mothers (Rakoto-Alson et al., 2010).

### 6.2.4 Possible New Explanation regarding Putative Link between Inflammatory Disease (including periodontitis) and Adverse Pregnancy Outcomes: direct and indirect role of matrix metalloproteinases

Matrix metalloproteinases are a family of structurally related, zinc-dependent endopeptidases whose proteolytic action provides the basis for normal and pathological tissue remodeling. Approximately 20 members of the MMP family have been identified, each capable of degrading multiple substrates, leading to significant overlap in their activities (Visse & Nagase, 2003). MMPs hydrolyze extracellular matrix and are involved in multiple processes that include wound healing, inflammatory states, tumor metastasis, angiogenesis, embryogenesis and implantation, and various other pathological conditions (Cockle, Gopichandan, Walker, Levene, & Orsi, 2007). Within the cervix, MMPs are released from a variety of cells, including fibroblasts, smooth muscle cells, and invading white blood cells such as PMNs (Winkler, 2003). MMP transcription and secretion are thought to increase in preparation for labor, resulting in cervical ripening and dilation and subsequent rupture of the fetal membranes (Cockle et al., 2007). During normal gestation MMP -1, -2, -3, -7 and -9 are found in the amniotic fluid and fetal membranes. MMP-2 and -3 are constitutively present while MMP-9 is barely detectable until labour. In both term and spontaneous preterm labour there is marked increase in placental and fetal membrane MMP-9 (Cockle et al., 2007; Vadillo-Ortega & Estrada-Gutierrez, 2005).
However, the amniotic fluid MMP-9 levels have been reported to be 5-fold higher in the preterm deliveries compared to term deliveries (Athayde et al., 1999) indicating that specific differences in MMP levels likely participate in the pathogenesis of spontaneous preterm labour. Furthermore, alteration in the concentration of other MMPs has been associated with spontaneous preterm labour and PPROM (Cockle et al., 2007). This supports a possible direct role of MMPs in the mechanisms responsible for spontaneous preterm labour and membrane rupture (Cockle et al., 2007). As discussed earlier, chronic periodontal infections can lead to high levels of pro-inflammatory cytokines (such as IL-1β, TNF-α and IL-6). These cytokines can then elicit the differential expression of many participating genes including those responsible for production of MMPs. As noted above, increased production of MMPs could lead to preterm labour. Furthermore, it has been found that MMPs can degrade fetuin (Ochieng & Green, 1996). This is a naturally occurring serum glycoprotein, which is apparently required for the inhibition of pro-inflammatory mediators such as TNF (H. Wang & Sama, 2012). Hypothetically, if there are high levels of MMPs in the serum, it is possible that there may be excessive amounts of these damaging mediators due to the loss of inhibition by active fetuin. Since excessive production of cytokines such as TNF-α during pregnancy can lead to spontaneous abortions (Shaarawy & Nagui, 1997), this may yet be another pathway whereby MMPs play an indirect role in the mechanisms that lead to spontaneous preterm labour.

As elucidated to earlier, PMNs play a major role in the etiology and pathogenesis of periodontal diseases by directly releasing MMPs and other enzymes. Therefore, based on the possible direct and indirect role of MMPs in adverse pregnancy outcomes and in addition, because measurements of oral levels of PMNs will be made (see below; Methods) in order to assay for periodontal disease activity, it was felt important to explore PMNs and their role in the pathogenesis of inflammatory periodontal diseases in this thesis.

7 Polymorphonuclear Neutrophils

7.1 Polymorphonuclear Neutrophil Background and Development

Polymorphonuclear neutrophils are the most common type of white blood cell, comprising about 50 – 70% of all white blood cells (D. R. Miller, Lamster, & Chasens, 1984). These cells are
found in three compartments: the bone marrow, tissues, and vascular compartment. The vascular compartment consists of the circulating or large blood vessel pool and marginating or small blood vessel pool. They are produced in large numbers from pluripotent stem cells residing in the bone marrow. Approximately $10^{11}$ PMNs are produced daily in a healthy adult (Segal & Holland, 2000).

Stem cells either develop into myeloid stem cells or lymphoid stem cells. This occurs through the influence of a combination of stem cell factors and cytokines such as, interleukins and colony stimulating factors. The lymphoid pathway eventually gives rise to T-lymphocytes and B-lymphocytes. The myeloid pathway gives rise to PMNs, monocytes, basophils, and eosinophils. The differentiation and maturation of PMNs from the myeloid stem cells progress through five stages before mature PMNs are released. This occurs over a period of two weeks. The first week is spent in the proliferative phase in which myeloblasts, promyelocytes and myelocytes that are capable of cell division are produced. The second week is spent in maturation and further differentiation phase where metamyelocytes, band PMNs and mature segmented PMNs that are incapable of cell division are produced (Hoffbrand, 2001). Once released from the bone marrow, the half-life of a PMN is 6 – 9 hours in the vascular compartment, and 1 – 4 days in the tissues (Deas, Mackey, & McDonnell, 2003).

### 7.2 Polymorphonuclear Neutrophil Function

Polymorphonuclear neutrophils are the first line of defense when a pathogen is present or tissue damage occurs. The invasion and/or products of pathogens in the tissues initiate a complex series of events that attract PMNs to the site of infection (Deas et al., 2003).

The immediate immune response involves activation of resident mast cells. The mast cells release various signaling molecules such as histamine, which interact with vascular endothelial cells, causing an increase in vascular permeability and dilation. The blood flow in the vessel slows and PMNs move to the periphery of the vessel in a process termed ‘margination’. PMNs then interact with the vessel lining via a cell surface glycoprotein termed L-selectin producing a low affinity cell-to-cell interaction. This results in ‘rolling’ of the PMN along the endothelial surface, increasing its exposure to inflammatory mediators such as histamine, IL-1, TNF-α, complement fragment C5a, leukotriene B₄, IL-8, platelet activating factor and bacterial products.
This in turn stimulates endothelial cells to express P-selectin and E-selectin, which bind PMNs, leading to greater numbers of ‘rolling’ PMNs. The adhesion of PMNs is then strengthened through the induction of β2 (CD18) and α (CD11a,b,c) integrin adhesion molecules. The adhesion to endothelial cells is mediated through the interactions of neutrophil surface integrin complexes that include leukocyte function-associated antigen-1 (LFA-1; CD11a/CD18), macrophage antigen-1 (Mac-1; CD11b/CD18), and leukocyte integrin p-150,95 (CD11c/CD18) with endothelial cell surface receptors that include intracellular adhesion molecules 1, 2 and 3 (ICAM-1, ICAM-2, ICAM-3). Following adhesion, PMNs begin the process of ‘diapedesis’. They accomplish this by flattening out along the endothelium and pushing through intercellular junctions into the extravascular tissues. This process is mediated in part by another glycoprotein called platelet-endothelial cell adhesion molecule-1 (CD31). PMNs secrete gelatinase (MMP-9) from gelatinase granules which degrades type IV collagen in the basement membrane of the vessel thus permitting cellular infiltration into the extravascular tissues (Deas et al., 2003; Dennison & Van Dyke, 1997).

Once into the tissues, PMNs migrate towards the infected area. This is accomplished by chemotaxis, whereby PMNs detect a chemical gradient and migrate in the direction of increasing concentration through mechanisms mediated by intracellular microfilaments consisting of actin and myosin. The chemotactic factors that attract PMNs are produced by both the host and bacterial pathogens. These include TNF, IL-8, PMN chemotactic factor, complement (C5a) and N-formyl-methionyl peptides (fMLP) (Dennison & Van Dyke, 1997).

PMNs then start to ingest and destroy the invading pathogens by phagocytosis. Various immunoglobulins (IgM, IgG₁, IgG₃) and complement fragments (C3b, C3bi), collectively called ‘opsonins’ coat the pathogens, providing a marker for PMN attachment (Deas et al., 2003; D. R. Miller et al., 1984). Fc receptors (CD1, CD32, CD64 and CD89) and complement receptors (CR1 and CR3) on the PMN cell surface recognize the opsonins and form a phagocytic vacuole or ‘phagosome’ that encloses the pathogen (Deas et al., 2003; Dennison & Van Dyke, 1997).

Microbial killing by PMNs can be divided into two systems: oxygen-dependent and oxygen-independent killing. The oxygen-dependent killing relies upon a respiratory burst resulting in the generation of free radicals. Membrane bound nicotinamide adenine dinucleotide phosphate
(NADPH) oxidase catalyzes the reduction of oxygen to superoxide anion in the following reaction:

\[ \text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP}^+ + 2\text{O}_2^- + \text{H}^+ \]

The superoxide anion (\( \text{O}_2^- \)) is rapidly converted into hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) by superoxide dismutase in the following reaction:

\[ 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

Although these free radical metabolites are antimicrobial, the main oxidative attack by PMNs involves the formation of hypochlorous acid (HOCl), hypochlorite (\( \text{OCl}^- \)), and chlorine (Cl) in the presence of the enzyme myeloperoxidase (MPO). These are even stronger antimicrobial agents with a very high oxidizing potential, and are formed through the following reactions (Deas et al., 2003; Dennison & Van Dyke, 1997):

\[ \text{Cl}^- + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \]

\[ \text{HOCl} \rightarrow \text{H}^+ + \text{OCl}^- \]

In the oxygen-independent killing system, the neutrophil relies on the fusion of granules and release of enzymes into the phagosome forming a ‘phagolysome’ (Deas et al., 2003; Nauseef, 2007). This process is called degranulation. Degranulation works in collaboration with the activation of the oxidative pathway to destroy the ingested pathogens.

PMNs contain three types of granules: primary, secondary and tertiary granules. The function of primary granules is to mediate oxygen-independent killing, potentiate oxygen-dependent killing, and modulate tissue inflammation. These granules are identified by their peroxidase content. They contain enzymes MPO and lysozyme, neutral proteinases such as elastase, cathepsin G and proteinase 3, acid hydrolases such as n-acetyl-\( \beta \)-glucosaminidase, cathepsin B, cathepsin D, \( \beta \)-glucorinidase, \( \beta \)-glycerophosphatase and \( \beta \)-mannosidase, and other defensins, cationic proteins, and bactericidal or permeability-increasing factor (Deas et al., 2003; Dennison & Van Dyke, 1997).
Secondary granules are peroxidase negative and are more numerous than primary granules. They help to mediate oxygen-independent killing and regulate inflammatory reactions by releasing their contents into the extracellular space. The contents of these granules include lysozyme, collagenase (MMP-8), lactoferrin, vitamin B₁₂ binding protein, iC₃b receptor (CR3) and cytochrome b₂₄₅. Tertiary granules primarily play a role in promoting adhesion function, regulating respiratory burst and mobility (Deas et al., 2003; Dennison & Van Dyke, 1997; Nauseef, 2007).

It is important to note that the same set of tools available to kill microbes intracellularly can be released by PMNs to the extracellular space in an effort to kill invading microbes. These potent oxidative and enzymatic molecules cause considerable collateral damage to the surrounding connective tissue (Deas et al., 2003; G. Nussbaum & Shapira, 2011).

PMNs have also been found to extrude extracellular fibrillary networks termed ‘neutrophil extracellular traps’ (NETs) (Brinkmann et al., 2004). These networks are composed mainly of DNA, but also contain proteins from the PMN granules. NETs act as a mesh that traps invading pathogens preventing their spread, and in turn facilitates their interaction with PMN derived antimicrobial substances to result in their destruction. These may also serve to limit secondary damage to surrounding tissues by preventing random diffusion of granule contents (Brinkmann et al., 2004; Medina, 2009). Recently, this has been referred to as NETosis, as it results in pathogen induced PMN cell death (Remijsen et al., 2011).

There is a significant level of redundancy in the killing systems of the PMN. The majority of pathogens are killed through a combination of enzymes and cellular products to ensure that most pathogens are destroyed, as some may be resistant to a particular killing system (Dennison & Van Dyke, 1997; Nauseef, 2007).

### 7.3 Mechanisms of Polymorphonuclear Neutrophil Cell Death

Successful resolution of inflammation is a pre-requisite for restoration of healthy tissue. Since acute inflammation is characterized by an abundance of infiltrating PMNs, their elimination, and the restoration of normal numbers of tissue PMNs, is an essential step in the progression towards tissue repair (G. Nussbaum & Shapira, 2011).
Initially, PMN cell death was thought to proceed through either apoptosis or necrosis. However, necrosis is essentially the end result of cell death, and not necessarily a mechanism (Kennedy & DeLeo, 2009). The exact mechanism of PMN elimination or cell death is context-dependent. This can include apoptosis, pyroptosis, oncosis, NETosis, phagocytosis-induced cell death and autophagy (Kennedy & DeLeo, 2009; Witko-Sarsat et al., 2011). These contexts include extracellular factors such as the presence of inflammatory markers, or pH of the extracellular fluid, and intracellular factors such as the abundance of adenosine triphosphate (ATP). For example, under conditions where ATP is depleted or limited, PMN cell death proceeds by oncosis, which entails cell swelling and lysis. In contrast, when ATP is abundant, cell death proceeds by more organized and non-inflammatory mechanisms such as apoptosis or autophagy (Kennedy & DeLeo, 2009).

PMN apoptosis is considered to be the safe process of elimination during the resolution phase of inflammation. It is accompanied by a general decrease in cell function and pro-inflammatory capacity. Apoptosis can be initiated by extrinsic pathway stimuli such as TNF-α or FAS ligand, or intrinsic pathway stimuli such as reactive oxygen species (O$_2^-$, OH-) and/or that mediated by mitochondria. Apoptotic PMNs are subsequently ingested by macrophages. This provides a means to resolve the inflammatory response without releasing cytotoxic molecules that would otherwise damage host tissues (Kennedy & DeLeo, 2009). In contrast, PMN elimination by mechanisms such as oncosis and pyroptosis result in cell lysis, and promote tissue inflammation (G. Nussbaum & Shapira, 2011).

Host-derived factors as well as bacterial and fungal products can delay PMN apoptosis. Cytokines such as IL-1β, TNF-α, interferon-gamma (IFNγ), colony-stimulating factor (CSF), and bacterial products such as LPS and lipoteichoic acid are potent inhibitors of PMN apoptosis. This delay in apoptosis likely promotes persistent inflammatory insult to the surrounding tissues, and progression of inflammation rather than resolution (Kennedy & DeLeo, 2009).

### 8 Role of Polymorphonuclear Neutrophils in Periodontal Disease

Polymorphonuclear neutrophils are the frontline in the acute host response against microbes. Essentially there are two ways PMNs may play a role in the pathogenesis of periodontal disease.
One includes PMN disorders (that affect PMN number or function), which may predispose individuals to periodontal disease. The other includes the normal function of PMNs, which under certain conditions may contribute to the pathogenesis of periodontal disease (Deas et al., 2003; Kantarci, Oyaizu, & Van Dyke, 2003; G. Nussbaum & Shapira, 2011).

8.1 Polymorphonuclear Neutrophil Disorders and Periodontal Disease

Polymorphonuclear neutrophil disorders can be divided into those that affect PMN number and various functions of the PMN. These quantitative and qualitative disorders maybe inherited, acquired or drug-induced, and may result in varying degrees of susceptibility to infection.

A normal healthy adult has an absolute PMN count of 1,800 – 8,000 cells/μl of blood. As the absolute PMN count falls, the risk of infection increases (Deas et al., 2003). Chronic diseases characterized by a reduction in PMN counts can include agranulocytosis, cyclic neutropenia, chronic benign neutropenia, chronic idiopathic neutropenia, familial benign chronic neutropenia, Felty’s syndrome and Kostmann syndrome. These conditions are all associated with severe periodontitis and premature loss of teeth (Deas et al., 2003; G. Nussbaum & Shapira, 2011). Neutropenic patients are often treated with external administration of a haematopoetic colony-stimulating factor such as granulocyte colony-stimulating factor. In these patients, the success in elevating the number of circulating PMNs is usually correlated with improved antibacterial responses to the periodontium and improved clinical outcomes (Hastrik et al., 1998; Ozer et al., 2000). This supports the central role of PMNs in playing a protective role against periodontal destruction in the host (Holmstrup & Glick, 2002; G. Nussbaum & Shapira, 2011).

Disorders of PMN function can involve one or more of the major PMN processes: margination (rolling and adhesion), chemotaxis and migration, phagocytosis, degranulation and killing (Deas et al., 2003). Individuals who manifest functional PMN impairments are also predisposed to increased risk of infections including periodontal disease. One of the examples include leukocyte adhesion syndrome (LAD), which has three variants (LAD I – III). In LAD-I, mutations of the β2 integrin gene (CD18/CD11) result in a profound defect in leukocyte adhesion. This gives rise to serious infections in early life and high infant lethality. In LAD-II, a defective sugar transporter leads to disturbed glycosylation of selectin ligands that primarily affects leukocyte
capture and rolling. Infections in LAD-II are usually milder with patients surviving into adulthood. LAD-III resembles the clinical presentation of LAD-I with β1, β2, β3 integrins normally expressed but inactive (C. Nussbaum, Moser, & Sperandio, 2010). These LAD patients present with rapidly progressing attachment and bone loss in the primary and permanent dentitions, leading to premature tooth loss (Cox & Weathers, 2008; Dababneh, Al Wahadneh, Hamadneh, Khouri, & Bissada, 2008).

Defects in PMN chemotaxis and intracellular killing following phagocytosis have been shown to play a significant role in increased prevalence of periodontal disease in Down syndrome patients. Down syndrome is one of the most common causes of developmental delay in children. It is attributed to trisomy of chromosome 21. Some Down syndrome patients also have aggressive and generalized periodontitis, along with the typical clinical presentations. This leads to subsequent destruction of the supporting tissues, and loss of teeth at an early age (Nualart-Grollmus, Morales-Chvez, & Silvestre-Donat, 2007). Similarly, abnormalities in PMN chemotaxis and migration have been reported in both Papillion-Lefevre syndrome and Chediak-Higashi syndrome. Papillion-Lefevre syndrome is an autosomal recessive disorder, where there is a point mutation of the cathepsin C gene. The syndrome is characterized by palmar-plantar hyperkeratosis, and early onset generalized rapid destruction of periodontal attachment apparatus. This results in premature loss of both primary and permanent teeth (Dhanrajani, 2009). Chediak-Higashi syndrome is a rare autosomal disorder characterized by severe immune deficiency, oculocutaneous albinism, bleeding tendencies, recurrent pyogenic infection, progressive neurologic defect and a lymphoproliferative syndrome. Oral findings include severe gingivitis, ulcerations of the tongue and buccal mucosa, and early onset periodontitis leading to premature loss of both deciduous and permanent dentitions. One of the hallmarks of the Chediak-Higashi syndrome is the presence of large intracellular azurophilic inclusions in the cytoplasm of PMNs. These large inclusions impair PMN migration, possibly by inhibiting cell deformability, and render PMNs unable to metabolize and digest microbes (Deas et al., 2003; Shiflett, Kaplan, & Ward, 2002). Impairment of PMN chemotaxis, adherence, and phagocytosis has also been attributed to the increased prevalence of periodontal disease among patients with diabetes. The mechanism of impaired PMN chemotactic response has been attributed to protein factors in the diabetic serum that competitively bind PMN receptors, thereby, preventing complement mediated phagocytosis (Mealey & Oates, 2006).
Patients who are otherwise healthy, but present with early onset forms of periodontal disease i.e. aggressive periodontitis, have also been reported to have peripheral PMNs with reduced chemotaxis in response to chemotactic signals, as well as impaired PMN phagocytosis and killing (Ryder, 2010). However, there are contradictory findings regarding the nature of the impairments in PMN functions among different populations with aggressive periodontitis (Kinane, Cullen, Johnston, & Evans, 1989a; Kinane, Cullen, Johnston, & Evans, 1989b; Takahashi et al., 2001).

8.2 Polymorphonuclear Neutrophil as Perpetrators of Tissue Damage and Bone Destruction

In the healthy periodontium, a small number of leukocytes migrate towards the gingival sulcus and are found residing in the junctional epithelium. With plaque accumulation at the gingival sulcus, there is increased capillary permeability with very large numbers of PMNs migrating from the dilated gingival plexus into the junctional epithelium and underlying connective tissue (Payne, Page, Ogilvie, & Hall, 1975). In the periodontium, PMNs are found in the epithelium and at the base of the sulcus. PMNs produce NETs to trap bacteria in the gingival sulcus to prevent further ingress, aiding in the defense against periodontal disease (Vitkov, Klappacher, Hannig, & Krautgartner, 2009). Activated PMNs release several mediators as described previously, such as oxygen radicals and proteolytic enzymes, which can directly induce tissue damage. PMN degranulation releases several proteolytic enzymes including elastase, gelatinase (MMP-9) and collagenase (MMP-8), which can hydrolyze several extracellular matrix proteins such as elastin, fibronectin and collagens and fetuin (Kantarci et al., 2003; Oching & Green, 1996). Although the role of PMNs in gingival tissue is primarily a defensive one, their persistence in periodontal connective tissue can result in the release of their ‘arsenal’ of degradative enzymes into the extracellular space. This may lead to an imbalance between repair and breakdown of the connective tissues favouring loss of tissue as seen in periodontal disease. There is evidence that circulating PMNs from patients with periodontitis are already in a ‘primed’ or ‘hyperresponsive’ state as a consequence of bacterial stimulation or sensitization. Therefore, the potential of PMNs to cause tissue damage is enhanced not only as a result of the production and release of degradative enzymes but also by secretion of oxygen radicals, which can also enhance the destruction of tissue (Dias et al., 2011; G. Nussbaum & Shapira, 2011).
In periodontal disease, cytokines and growth factors produced by cells in inflamed periodontal tissue can influence osteoclast differentiation and function. This provides a link between inflammation and the process of bone destruction. The imbalance in bone remodeling that favours resorption is caused by various cytokines in the inflammatory tissue, such as receptor activator for nuclear factor κB ligand (RANKL), TNF-α, IL-1, PGE2, IL-11 and IL-17. Activated PMNs are a major source of many of these osteoclastogenic factors such as prostaglandins, TNF-α, and IL-17, which in turn induces expression of RANKL (Kitami et al., 2010), leading to bone resorption associated with periodontal disease (G. Nussbaum & Shapira, 2011).

8.3 Myeloperoxidase and Periodontal Disease

Myeloperoxidase is a heme-containing peroxidase abundantly expressed in PMNs and to a lesser extent in monocytes. During myelopoiesis in the bone marrow, MPO is actively synthesized in promyelocytes and promonocytes. MPO synthesis normally ceases in fully differentiated myeloid cells. The levels of MPO in PMNs range from 2 – 5% of total cellular protein (van der Veen, de Winther, & Heeringa, 2009). MPO is the product of a single gene, which is 11 kb in size. The gene is composed of 11 introns and 12 exons, and located in the long arm of chromosome 17. The initial translation product is an 80-kD protein, which following modification results in a 90-kD enzymatically inactive apopMPO. ApopMPO is converted to the enzymatically active proMPO with the incorporation of a heme. Mature MPO has a molecular mass of 150 kD, and consists of a pair of heavy-light protomers, with the heavy subunits linked by a disulfide bond (Klebanoff, 2005). This is the form that is found in the primary granules of PMNs, and lysosomes of monocytes.

MPO plays an essential role in the microbicidal activity of PMNs. As described previously, one of the antimicrobial systems formed in the phagosome is: the release of the enzyme MPO (during the degranulation process), hydrogen peroxide (formed by the respiratory burst), and a halide (particularly chloride). The initial product of the MPO-H2O2-chloride system is hypochlorous acid with subsequent formation of chlorine, chloramines, hydroxyl radicals, singlet oxygen and ozone (Klebanoff, 2005). Hypochlorous acid as mentioned previously is a key contributor to the oxygen-dependent microbicidal activity of neutrophils. MPO can also be released extracellularly
along with the oxidant species, which can cause significant tissue damage (van der Veen et al., 2009).

Given the role of MPO, it would be expected that MPO deficiency in individuals would lead to significant morbidity and mortality as a result of infectious diseases. However, in majority of the cases, partial or complete MPO-deficient individuals do not appear to be extremely susceptible to infections (Nauseef, 2007; van der Veen et al., 2009). The prevalence of hereditary MPO deficiencies in the USA and Europe has been reported to be in the range of 1:1000 to 1:4000 (van der Veen et al., 2009). MPO deficient PMNs seem to be able to phagocytose most microbes normally. It has been suggested that MPO-independent microbicidal mechanisms increase their activity to compensate for the lack of MPO (Nauseef, 2007; van der Veen et al., 2009).

MPO has been considered a promising marker of periodontal inflammation. Increased levels of MPO have been found in the gingival crevicular fluid (GCF) of systemically healthy patients with gingivitis and chronic periodontitis (Cao & Smith, 1989; Hernandez et al., 2010). It has been suggested that the increased GCF MPO levels may be related to the overall increase in the quantity of PMNs in the gingival sulcus and degree of gingival inflammation (Cao & Smith, 1989). GCF MPO levels have also been found to decrease in active periodontal disease sites, following both conventional (Hernández et al., 2010; Marcaccini et al., 2010) and surgical treatment (Buchmann, Hasilik, Van Dyke, & Lange, 2002; Jentsch, Sievert, & Gcke, 2004). This supports the role of MPO in the pathogenesis of periodontal diseases.

In biochemistry, 2,2’-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) is a chemical compound that is used to observe the reaction kinetics of specific enzymes and is commonly used as a substrate with hydrogen peroxide. It is a colourless molecule, which oxidizes to a characteristic blue-green colour that can easily be followed with a spectrophotometer (Erel, 2004). The most widely used colorimetric methods for measuring oxidant capacity is based on the use of ABTS. Since MPO is a powerful oxidant, its activity can be detected through the use of ABTS (Parkos, Colgan, Delp, Arnaout, & Madara, 1992).
9 Diagnosis of Disease

As discussed previously, it is difficult to classify the type and/or severity of periodontitis in an unequivocal manner and this not only affects treatment choices for this condition but also has a negative impact on the ability to associate periodontitis with other non-oral conditions (e.g. preterm or LBW infant delivery). The main objective of a diagnostic test is to establish the presence or absence of a certain disease. In addition, a diagnostic test may provide information regarding disease severity, level of current disease activity, and the prognosis of future disease development. Diagnostic tests are usually evaluated using statistical calculations based on Bayes’ theorem. These include sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The sensitivity of a diagnostic test refers to the probability of testing positive given that the disease is truly present. The specificity of a test refers to the probability of testing negative given that the disease is not present. PPV is the probability of having the disease given a positive test, whereas NPV is the probability of not having the disease given a negative test. The values of sensitivity, specificity, PPV and NPV would all equal 1.00 for a perfect diagnostic test. However, in healthcare perfect diagnostics tests do not exist (Pagano, 2000).

9.1 Diagnosis of Periodontal Disease

The diagnosis of periodontal disease is based on the findings from a variety of clinical and radiographic measures. The clinician is usually able to distinguish healthy patients from those with periodontitis based on traditional measures. These measures include assessments of inflammation such as visual detection of signs of inflammation, bleeding on probing and presence of suppuration, and assessments of damage to periodontal tissues such as measurement of attachment loss with a periodontal probe, visual detection of clinical signs of tissue destruction and radiographic detection of bone loss. In general, these traditional diagnostic procedures are cost-effective and easy to use. They provide clinically useful information regarding the location and presence or absence of diseased or damaged tissues. However, these traditional measures do not necessarily provide useful information regarding the severity, morbidity or eventual outcome of periodontal infections, nor whether the disease is in an active or inactive state (Armitage, 1996).
9.1.1 Assessments of Inflammation

Inflamed periodontal tissues may exhibit all five of the cardinal signs of inflammation: redness, swelling, heat, pain and loss of function. However, in most forms of periodontal disease, pain and loss of function are uncommon until an advanced stage of the disease is reached. In addition, inflamed periodontal tissues may exhibit bleeding on probing and suppuration (Armitage, 1996).

There are various measures of periodontal inflammation that have been proposed. The gingival index of Loe (Loe, 1967) describes qualitative changes in the gingival soft tissues based on visual assessment of inflammation. The criteria for the gingival index are: 0 = normal gingiva; 1 = mild inflammation, slight colour change, slight edema, no bleeding on probing; 2 = moderate inflammation-redness, edema and glazing with bleeding on probing; and 3 = severe inflammation-marked redness and edema, ulceration and spontaneous bleeding. These criteria are regularly used by clinicians in the diagnosis of periodontal diseases. However, this index is categorical/ordinal and the numbers do not reflect a specific quantity i.e. whether the inflammation is localized or generalized, nor do they reflect the magnitude of severity i.e. a score of 3 is not three-fold worse than a score of 1, and a score of 2 is not twice as bad as a score of 1.

Bleeding on probing is another widely used relatively objective sign of inflammation as it is either present or it is not (Armitage, 1995; Greenstein, 1984). Bleeding on probing is also one of the earliest clinical sign of gingivitis, preceding discoloration and swelling of gingival soft tissues (Muhlemann & Son, 1971). Inflamed periodontal tissues bleed when probed with a blunt instrument because there are frequently micro-ulcerations in the epithelium that lines the soft tissues of a periodontal pocket (Armitage, 1995).

Suppuration (purulent exudate or pus) is another feature of periodontal inflammation, primarily observed in cases of periodontitis. Suppuration is a result of the accumulation of a large number of PMNs within an infected sulcus (pocket) in response to the presence of a flourishing subgingival flora. The PMN-rich GCF does not readily leave the site via the pocket orifice in some cases but can be detected readily by the gentle application of digital pressure in a coronal direction to the gingival surface. Clinically, suppuration signals the presence of an ongoing infection and its presence has been associated with an increased risk of disease progression (Armitage, 1995). However, suppuration is not a consistent feature of periodontitis. Most studies
report only 3 – 5% of sites with periodontitis demonstrate suppuration (Armitage, 1996) and in some cases, gingival suppuration might be mistaken for the extrusion of subgingival plaque.

Although the above-mentioned measures of inflammation can be used to distinguish between healthy and diseased gingival tissues, they do not distinguish between destructive and non-destructive periodontal diseases reliably, and they do not predict future breakdown. For example, gingival redness and inflammation are features of both gingivitis and periodontitis, and the gingival index scale of Loe cannot distinguish between the two diseases (Armitage, 1996). Similarly, the presence of bleeding on probing can be affected significantly by the pressure with which periodontal probes are inserted by the examiner. It has been reported that bleeding on probing is encountered more frequently when probing forces exceed 0.25 N, as higher probing forces can cause even healthy tissue to bleed (Armitage, 1996). In addition, bleeding on probing has a sensitivity of only 0.29 and PPV of 0.06 making it a poor predictor of periodontal breakdown and recurrence of disease (Lang, Nyman, Senn, & Joss, 1991). Likewise, although the presence of suppuration indicates the presence of an infection, it is not frequently observed. It is also a poor predictor of periodontal disease progression (Badersten, Nilvus, & Egelberg, 1985; Haffajee, Socransky, & Goodson, 1983). None of the traditional measures of inflammation have both high specificity and sensitivity, as desired for an ideal diagnostic test (Haffajee et al., 1983).

9.1.2 Assessments of Damage to Periodontal Tissues

Various methods for assessment of periodontal tissue destruction have been developed. The three general approaches are: 1) measurement of attachment loss with a periodontal probe, 2) visual detection of clinical signs of tissue destruction, and 3) radiographic detection of bone loss (Armitage, 1996).

The most commonly used method of measuring attachment loss is by the use of a calibrated periodontal probe. Probing depth is a measure of the distance from the gingival margin to the base of the periodontal pocket. The primary clinical importance of periodontal pockets is that they are a major habitat for putative periodontal pathogens (Armitage, 1995). In addition, deep pockets are much more difficult for both the patient and the clinician to clean (Armitage, 1995). The complete removal of subgingival plaque by scaling and root planing is highly unlikely in pockets greater than 5 mm in depth (Waerhaug, 1978). Clinical attachment loss is a measure of
the distance from the cementoenamel junction (CEJ) to the base of periodontal pocket. It can also be determined by adding the amount of recession to the probing depth measurement (Armitage, 1995). See Figure 1 below.

**Figure 1.** Diagrams illustrating clinical measurements for assessment of damage to periodontal tissues. “A” represents the pocket depth, “C” represents clinical attachment loss, and “B” is the distance from the crest of the gingiva to the CEJ. The first diagram represents a healthy periodontium with no real pocket and no CAL. The second diagram represents a situation where the attachment has migrated down the root and CAL has to be calculated by subtracting “B” from “A”. The third diagram represents a situation where CAL is calculated by adding the extent of recession “B” to “A”. (Adapted from Health Canada, 2010).

Tooth mobility is a common visual sign of periodontal tissue destruction. Increased tooth mobility is usually graded by the scale developed by Miller (S. C. Miller, 1950), where M1 = first distinguishable sign of movement greater than normal; M2 = movement of crown up to 1 mm in any direction; and M3 = movement of the crown more than 1 mm in any direction and/or vertical depression or rotation of the crown in the socket. Other visible signs of tissue destruction include altered gingival morphology such as interproximal cratering, gingival recession, furcation involvement and tooth migration. However, for some of these signs, their presence does not always mean that damage to the supporting periodontal tissues has occurred (Armitage, 1995).

Dental radiographs can also be used to assess periodontal bone support. They provide a permanent record that can be used for comparison with future examinations as long as the radiographic technique is standardized. This can provide information on the extent of bone loss over a certain period of time (Jeffcoat, Wang, & Reddy, 1995). Generally, 30 – 50% bone loss at
the alveolar bone crest is required for detection with the naked eye using conventional radiography. However, with digital subtraction radiography bone density changes as small as 5% can be detected (Hausmann et al., 1985).

Similar to the diagnostic tests of inflammation, the tests for assessment of tissue destruction are not able to detect active disease or to prognosticate future breakdown. For example, the accuracy and reproducibility of probing depth or CAL measurements can be affected by many factors. These include probing force, probe angulation, probe type, probe position, status of gingival health and amount of pseudopocketing (Caton, Greenstein, & Polson, 1981; Fowler, Garrett, Crigger, & Egelberg, 1982; Persson, 1991; van der Velden, 1978; Van der Zee, Davies, & Newman, 1991). Probing depths of 6 mm or greater has been found to have a sensitivity of 0.26 and a PPV of only 0.11 (Halazonetis, Haffajee, & Socransky, 1989). Also, increased tooth mobility can be caused by a variety of factors other than periodontal disease. These include orthodontic forces, occlusal trauma, periapical lesions and heavy functional loads from prosthetic appliances (Armitage, 1995). Finally, dental radiographs cannot identify active disease or distinguish between treated and untreated cases. Although, subtraction radiography is more sensitive insofar as detection of minute changes in bone levels around teeth is concerned, precise superimposition of serial images is required, making it technique sensitive and time consuming (Reddy, 1992). Therefore, these traditional measures of periodontal disease do not necessarily provide reliable information regarding current disease activity level or risk of progression. These problems with measurement led to research focused on the quantification of oral PMNs as a measure of both the presence of periodontal disease as well as its severity.

10 Review of Oral Polymorphonuclear Neutrophil Quantification Studies

Researchers began studying the number of leukocytes found in saliva over 70 years ago. Early studies showed that the number of leukocytes found in saliva varied vastly between patients, and saliva from patients with periodontitis contained a greater number of leukocytes than saliva from healthy patients (Dreizen, Gilley, & Spies, 1956). Moreover, it was found that edentulous patients had considerably lower oral leukocyte counts than dentate patients (Calouius, 1958).
Therefore, it was concluded that the leukocyte count in saliva might be important in the assessment of pathological processes (Calouius, 1958).

Next, studies found and confirmed that the major source of leukocytes in the oral cavity is the gingival sulcus (Lantzman & Michman, 1970; Schiott & Loe, 1970; Sharry J.J., 1960) and other sites, such as the tonsils, floor of mouth, dorsum of tongue and cheek contribute very few cells (Sharry J.J., 1960). The types of leukocytes found in saliva were also investigated and it was found that the majority of the leukocytes were PMNs (Raeste, 1972; Woolweaver, Koch, Crawford, & Lundblad, 1972).

The ‘Orogranulocytic migratory rate’ (OMR) was first described by Klinkhamer (Klinkhamer, 1963). The OMR was calculated by counting the average number of orogranulocytes present in a series of sequential oral rinses after a plateau level of cells was reached. The technique involved twelve sequential 27-second saline rinses, with a 3-second period for expectoration and replacement with fresh saline. The cells were quantified using a Coulter Counter. The OMR was determined to be a non-subjective, quantitative, and reliable method of determining the rate of gingival leukopedesis based on OMR values from patients with varying degrees of periodontal disease. In addition, the OMR was found to correlate with levels of gingival inflammation (Klinkhamer, 1968; Klinkhamer & Zimmerman, 1969) with the gingival index and with increased pocket depths (Skougaard, Bay, & Klinkhamer, 1969; Woolweaver et al., 1972). However, it was not found to be sensitive to the presence of plaque, calculus or debris (Woolweaver et al., 1972). The original protocol by Klinkhamer was modified by using Hank’s Balanced Salt Solution (HBSS) for the rinse and a hemocytometer for PMN quantification (Schiott & Loe, 1970). The OMR was found to be increased in the presence of gingivitis compared to health, very small numbers of leukocytes being found in the latter state (Schiott & Loe, 1970). However, no direct correlation was found between the gingival index and the OMR. In contrast to previous publications, the study concluded that leukocyte assessment could not be used as an index of gingival and periodontal disease. These results were based on a sample size of 18 patients who did not have periodontal disease.

The idea of using PMN quantification to assess periodontal disease status and the effectiveness of therapy was first proposed by Raeste and Aura in 1978 (Raeste & Aura, 1978). Similar to earlier studies, the OMR was found to be significantly different in patients with periodontitis as
compared to healthy controls. The OMR decreased following periodontal treatment. However, it remained elevated in patients with residual pockets following treatment. There was also a highly significant difference in leukocyte counts between healthy and test subjects in the first rinse of the series. Therefore, it was suggested that the first rinse may have value on its own as a test for disease severity (Raeste & Aura, 1978). A recent study quantified PMNs using a hemocytometer by collecting two concurrent 15 mL rinses of HBSS from each patient (Bender, Thang, & Glogauer, 2006). Oral PMN levels, as determined by the rapid oral rinse method were found to reflect the severity of periodontal disease and the response of this condition to non-surgical treatment. The study concluded that an oral rinse assay is a valid, reproducible and effective means of collecting and quantifying oral PMN levels (Bender et al., 2006) and that this could be used to assess the presence and/or severity of periodontal disease. Another recent unpublished study quantified PMNs using a colourimetric assay, where ABTS, the colour changing redox agent was added to oral rinse samples (Landzberg, 2009). As shown using the hemocytometer, a significant correlation between PMNs and periodontal disease status was found, but in this case it was correlated to colour change in the reagent solution thus providing an even simpler method for measurement of oral PMNs. In addition, the study concluded that a single oral rinse was sufficient for diagnostic purposes (instead of collecting two concurrent rinse samples from each patient), which simplifies this PMN assay even more (Landzberg, 2009).

The quantification of oral PMNs using various techniques other than oral rinses has been described in the literature. Gingival crevicular cells have been harvested using absorbent strips in a given periodontal site (Andersen & Cimasoni, 1993). Elevated PMN counts were found in deeper pockets and bleeding pockets. A decrease in PMN counts following non-surgical periodontal therapy was noted (Andersen & Cimasoni, 1993). A novel intracrevicular lavage technique has also been used to collect and quantify leukocytes before and after non-surgical periodontal treatment (Boretti, Zappa, Graf, & Case, 1995). A statistically significant reduction in the number of vital leukocytes was found following treatment. This was attributed to a reduced influx of leukocytes from the tissues into the pocket due reduction in bacterial load (Boretti et al., 1995). A recent study examining PMNs in plaque samples from deep sites suggested that the use of a periodontal probe for the diagnosis of periodontal disease can be improved upon by quantification of leukocytes in plaque samples. However, the method described, required the use of a microscope (Apsey, Kaciroti, & Loesche, 2006). Although these
methods have significant merit, particularly with regard to assessment of single sites, they are difficult to administer. Moreover, it is suggested here that periodontal inflammation should be considered with regard to the whole periodontium; the *oral inflammatory load*. This is measured best by assaying all of the PMNs in the mouth as opposed to single sites only.
Rationale

As reviewed, adverse pregnancy outcomes such as PTLBW delivery can have major consequences that not only affect the individuals and their families, but also significantly affect the health care system. In addition, despite the multifaceted nature of both periodontal disease and PTLBW delivery, it has still been possible to demonstrate some evidence suggesting a linkage between maternal periodontitis and adverse pregnancy outcomes. In order to investigate this putative linkage, a reliable and valid method of measuring periodontal disease using a non-invasive assay that can be employed by non-specialized staff is required. As described above, the oral PMN quantification assay (Bender et al., 2006) is a non-invasive assay that has been shown to reflect periodontal disease severity and it has the potential to be a simple test that can be employed by non-specialized staff to collect and quantify oral PMNs from a patient. Moreover, it is probable that oral inflammatory load is more impactful insofar as a putative relationship between periodontal disease and PTLBW outcome than assessment of the level of inflammation at single sites. Since PMNs could actually play a role in the pathophysiology of periodontitis, measurement of their levels yields an estimate of both the presence and severity of oral inflammatory load. Moreover, as argued above, there might even be a linkage between PMN-derived proteinases (by way of degradation of fetuin as well as direct effects on placental tissues), which could also explain the apparent but still putative linkage between periodontitis and PTLBW delivery. Therefore, it is proposed here that it would be useful to quantify oral PMNs in a group of pregnant women using the assay system described above so that it can be validated for use as a screening tool for periodontal disease in pregnant women.
Hypothesis

Null hypothesis, $H_0$: An oral rinse assay measuring oral PMN counts does not correlate with levels of periodontal disease as measured by conventional methods in a group of pregnant women.

Alternative hypothesis, $H_1$: An oral rinse assay measuring oral PMN counts correlate with levels of periodontal disease as measured by conventional methods in a group of pregnant women.
Objectives

1. To determine oral PMN counts in a group of pregnant women using a brief oral rinse assay.

2. To correlate levels of periodontal disease as measured by conventional methods with PMN counts from the oral rinse.

Additional Goals

1. To determine the incidence of adverse birth outcomes in this group of pregnant women.

2. To examine any relationship between periodontal disease indicators and adverse birth outcomes in this group of pregnant women.
Materials and Methods

This correlational study was approved by the Scientific and Ethics Review Boards of Mount Sinai Hospital, Toronto and the University of Toronto on February 4th, 2011 prior to commencement of the study. A ‘Data and Biological Transfer Agreement’ was also obtained between Mount Sinai Hospital, Toronto and the University of Toronto to allow transportation of the patient samples to a University of Toronto laboratory (Fitzgerald Building, Room 241, 150 College Street, Toronto, Ontario) for processing.

1 Study Population

Patients in this study were recruited from the ‘low risk’ obstetrical clinics at the Mount Sinai Hospital (700 University Avenue, 3rd Floor, Toronto, Ontario) between February 24th, 2011 and September 2nd, 2011. The clinic appointment schedule was reviewed by the principal investigator (S.H.) on the days of recruitment to identify potential patients by their gestation age. The medical record cover sheets, which listed active medical problems were then reviewed to identify exclusion criteria. The inclusion and exclusion criteria were as follows:

Inclusion criteria

- Pregnant women who were at least 16 years of age and capable of giving informed consent.

- Women who presented with a gestational age greater than 13 weeks and less than or equal to 26 weeks.

- Women who had at least 20 natural teeth.
Exclusion criteria

- Women who presented with multiple gestations, known fetal congenital anomaly or aneupolidy.
- Women with a known documented infection such as urinary tract and/or upper respiratory infection at the time of recruitment.
- Women who had a fetal invasive procedure such as amniocentesis or chorionic villi sampling.
- Women who required antibiotic prophylaxis for periodontal procedures.
- Women who presented with systemic diseases that could affect immune function/neutrophil response such as type I or type II diabetes, immunocompromising diseases, cardiac disease or renal disease.
- Women who had extensive dental tooth decay or were likely to have fewer than 20 teeth.
- Women who presented with lesions of gingiva unrelated to plaque-induced periodontal disease (for example, abscess related to a carious tooth) that might contribute to elevated oral neutrophil levels.

The potential patients were approached by S.H. and the study protocol was explained to each patient using an ‘information booklet’ (Appendix A) that was provided to them. The voluntary nature of the participation was emphasized. It was made explicit that the decision to participate had no bearing on clinical care and could be ended at any point. Written informed consent (Appendix B) was obtained by S.H. prior to commencement of the study.

A medical and dental history was taken from each patient participating in the study using a structured questionnaire (Appendix C). The questionnaire included questions pertaining to maternal general health during pregnancy, recent antibiotic use, medications and supplements, smoking, alcohol consumption, a review of all the systems, oral hygiene habits, and last dental visit and treatment. Maternal demographic information, previous pregnancy history and current gestational age were also recorded from the patient medical records.
2 Assessment of Oral Polymorphonuclear Neutrophil Levels

The protocol used to collect and count oral PMNs was a modification of previous work (Bender et al., 2006; Landzberg, 2009). Pregnant women were asked to rinse their mouth once with 10 mL of 0.9% saline for 15 seconds by gentle swishing, which was demonstrated by S.H.. Study participants were instructed not to eat or drink for a minimum of 30 minutes prior to providing the oral rinse sample to avoid clearance of PMNs prior to donations. Each oral rinse sample was collected in a sterile Falcon tube and stored at 4°C to preserve the cells prior to transportation to the laboratory for processing. The laboratory was located at approximately a five minute walking distance from the obstetrical clinics at Mount Sinai Hospital. The samples were transported in an ice cooler by S.H. by walking over to the laboratory and stored again at 4°C. All samples were then processed within 24 hours of collection by a trained laboratory assistant.

In the lab, the oral rinse sample underwent centrifugation at 2500 RPM for 5 minutes at 21°C (Hettich Rotina 35R, Rare Scientific, Edmonton, Canada). The cell pellet was resuspended in 1 mL of double distilled water. 20 mg of 2,2’-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) (AO; Sigma Chemical, Burlington, Ontario, Canada) was dissolved in 3.6 mL of 1 M phosphocitrate buffer to produce a 1X concentrated solution. 45.6 μL of 30% hydrogen peroxide was added to 3.952 mL of double distilled water to produce a hydrogen peroxide homogenous solution. For each 1 mL concentrated oral rinse sample, 100 μL of the ABTS solution followed by 100 μL of 30% hydrogen peroxide solution was added in order to observe the characteristic blue-green colour change. After the colour reaction was complete, 250 μL of the sample was added to a 96 well plate in triplicate. The absorbance was measured at 420 nm for 10 cycles at 180 seconds per cycle using FLUOstar software (BMG LABTECH GmbH, Offenburg, Germany). The final absorbance value for a sample was given as the average of the absorbance values of the cycles and of the triplicate measurements. The average standard deviation and coefficient of variation (CV) of the optical densities (OD) of the 10 cycles for all the collected samples was 0.09 and 6.34% respectively indicating a very low variation between the cycle absorbance readings.
In order to determine the PMN counts of a given oral rinse sample from the absorbance measure, a standard curve equation was used ($y=3305783.5X-1366$). A series of standard solutions were prepared in the lab as described above, using blood neutrophils of known concentrations. The OD was then measured at 420 nm for 10 cycles at 180 seconds per cycle using the FLUOstar software. The standard curve equation was then obtained by plotting the OD of the standard solutions versus the known concentrations, thus allowing deduction of the PMN counts for a sample using the OD or absorbance value.

3 The Periodontal Examination

Following the oral rinse, a complete periodontal examination was performed on each patient by S.H. using a mirror, explorer, University of North Carolina - 15 periodontal probe, and a single light source. The examination took place at the obstetrical clinics with the patient supine on a hospital bed. The soft tissues of the oral cavity were examined for disease, followed by an examination of hard tissues of the teeth that were present. Bleeding on probing (BOP), probing depths, recession and clinical attachment loss (CAL) at six sites of each tooth, as well as mobility, plaque index, calculus index, and modified gingival index using a standardized method for data collection were recorded (Appendix D). All data were recorded orally onto an iPhone 4 ‘voice memos’ function and then transcribed to a clinical examination sheet (Appendix E). No dental radiographs were taken. The oral examination was usually completed within 30 minutes. Examiner reliability was assessed by calculating intra-examiner Kappa scores between measurements of probing depths and CAL on a volunteer patient lying flat on a hospital bed on two separate occasions (two weeks apart duplicating the study conditions). The intra-examiner correlation or Kappa scores exceeded 0.95 for both measurements, a nearly perfect score demonstrating a high degree of examiner reliability and reproducibility (Landis & Koch, 1977).

Mild periodontitis was defined as $\geq 2$ interproximal sites with CAL $\geq 3$ mm and $\geq 2$ interproximal sites with probing depth $\geq 4$ mm (not on the same tooth) or 1 site with $\geq 5$ mm probing depth (Eke et al., 2012). Moderate periodontitis was defined as $\geq 2$ interproximal sites with CAL $\geq 4$ mm or $\geq 2$ interproximal sites with probing depth $\geq 5$ mm (not on the same tooth), and severe periodontitis as $\geq 2$ interproximal sites with CAL $\geq 6$ mm and $\geq 1$ interproximal sites with probing depth $\geq 5$ mm (not on the same tooth) (R. C. Page & Eke, 2007). These
measurements excluded wisdom teeth. Gingivitis only was defined as the presence of inflammation and presence of bleeding on probing in the patients that did not present with periodontitis as per the definitions above.

All patients received individualized oral hygiene instructions (i.e. brushing and flossing). Patients diagnosed with periodontal disease based on their clinical examination were advised to visit their dentist for periodontal scaling and root planing. Patients who did not have a dentist were assisted in locating a dentist in the community using the Ontario Dental Association website (http://www.oda.on.ca/find-a-dentist.html).

4 Maternal and Pregnancy Demographic Data

Information regarding the course of the pregnancy and the outcome was collected from the antenatal chart and delivery records of the participating patients at the end of the pregnancy by accessing the OB TraceVue records at the Mount Sinai Hospital by S.H. and a co-investigator (W.W.). The data collected included gestational age at delivery, birth weight of the infant, APGAR scores less than 7 at 5 minutes, mode of delivery, and any relevant complications of the pregnancy. Preterm birth was defined as birth at less than 37 weeks gestation. Low birth weight (LBW) was defined as birth weight less than 2,500 g at delivery.

5 Sample Size Calculation

The sample size (n) required to detect a nonzero correlation of at least 0.4 or greater between variation in oral neutrophil counts in pregnant women and variation in measures of periodontal health status at a $\alpha = 0.05$ significance level, using a two sided test ($H_0: \rho = 0$ versus $H_1: \rho \neq 0$) with 90% power ($1 - \beta = 0.90$) was calculated as follows:
Given $Z_{1 - \alpha/2} = Z_{.975} = 1.96$ and $Z_{1 - \beta} = Z_{.90} = 1.28,$

$$n = \frac{(Z_{1 - \alpha/2} + Z_{1 - \beta})^2}{(z')^2 + 3}$$

where $z' = \frac{1}{2} \ln \left(1 + \frac{\rho_{\text{smallest}}}{1 - \rho_{\text{smallest}}}\right)$

$$= \frac{(1.96 + 1.28)^2}{(0.4236)^2 + 3} = \frac{1}{2} \ln \left(1 + \frac{0.4}{1 - 0.4}\right) = 0.4236$$

$$= 61.49 \text{ or } 62 \text{ subjects}$$

Note that $z'$ is the Fisher transformation and ln is the natural logarithm.

The study was not powered to address the additional goals. The patients were followed up as a matter of interest.

6 Statistical Analysis

Descriptive statistics were calculated for all the demographic characteristics, outcomes, and diagnostic factors using means, medians and standard deviations for continuous variables and frequency counts and percentages for categorical factors. Due to the highly positively skewed nature of PMN counts, a natural log transformation was used to normalize the distribution prior to inferential analyses.

Spearman’s rank correlation was used to examine the association between the natural log transformed PMN counts and the bleeding index, average probing depths, age, gestational age at recruitment and the number of teeth. Spearman’s rank correlation, a non-parametric technique was used to account for the non-normal distribution of the outcomes.

The Tukey’s Honestly Significant Difference (HSD) test in conjunction with an analysis of variance (ANOVA) was used to examine the association between the natural log transformed PMN counts and the modified gingival index, calculus index and periodontal status classification (i.e. health, gingivitis, periodontitis). An independent sample t-test was used to examine the association between the natural log transformed PMN counts and the plaque index and history of making a dental visit within the past 6 months. An independent sample t-test was also used to examine the association between the PMN counts, average probing depths and the bleeding index with preterm birth outcomes and low birth weight infant outcomes.
The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the oral rinse assay using a bleeding index of at least 5.5 or more (i.e. \( \geq 10 \) bleeding sites), as well as probing depths of \( \geq 4 \) mm in at least 4 sites on different teeth, were calculated at various PMN counts.

All statistical analyses were conducted in IBM SPSS version 20.0.0 for Microsoft Windows Vista and a Type I error rate of at least 0.05 was used to indicate statistical significance.
Results

1 Descriptive Statistics of the Study Population of Pregnant Women at Recruitment

A total of 83 pregnant patients were approached with an invitation to participate in the study. Seven patients did not fulfill the eligibility criteria and thirteen patients did not wish to participate. Thus, 63 pregnant patients with a mean age of 33.3 ± 3.8 (standard deviation) years and a mean gestational age of 19.3 ± 4.2 weeks were recruited for this study, just fulfilling the predicted sample size required to obtain adequate power for this study. The mean number of teeth present was 28.8 ± 1.8. The patient population was a predominantly Caucasian (69.8%), well-educated group with all the patients either attending or having completed post-secondary education. All the patients reported taking some form of prenatal supplements. None of the patients reported taking antibiotics recently (within two weeks) at the time of recruitment. The two patients that reported smoking claimed to be smoking less than five cigarettes per day during the pregnancy. One patient reported drinking 1 unit of alcohol every 1 - 2 week(s). Nearly two-thirds of the patients had visited a dentist within the past 6 months for a check-up and dental scaling and prophylaxis. See Table 1 for demographic and other details of the study population.
Table 1. Demographic and other details of the study population at recruitment.

<table>
<thead>
<tr>
<th>Factor</th>
<th>N*</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 25 years</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>25 to less than 35 years</td>
<td>39</td>
<td>61.9</td>
</tr>
<tr>
<td>35 years and above</td>
<td>23</td>
<td>36.5</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>44</td>
<td>69.8</td>
</tr>
<tr>
<td>Black</td>
<td>3</td>
<td>4.8</td>
</tr>
<tr>
<td>Asian</td>
<td>6</td>
<td>9.5</td>
</tr>
<tr>
<td>East Indian</td>
<td>5</td>
<td>7.9</td>
</tr>
<tr>
<td>Other**</td>
<td>5</td>
<td>7.9</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attending or completed post-secondary education</td>
<td>63</td>
<td>100.0</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>10</td>
<td>16.0</td>
</tr>
<tr>
<td>Smoking in pregnancy</td>
<td>2</td>
<td>3.2</td>
</tr>
<tr>
<td>Alcohol consumption in pregnancy</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>Oral hygiene habits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brushing at least 2x/day</td>
<td>62</td>
<td>98.4</td>
</tr>
<tr>
<td>Flossing at least 3x/week</td>
<td>32</td>
<td>50.8</td>
</tr>
<tr>
<td>Last dental visit within 6 months</td>
<td>41</td>
<td>65.0</td>
</tr>
</tbody>
</table>

* Sample size, total = 63.

**Other group included 3 women of Hispanic or Latino background and 2 women of Arab background.
Approximately 43% of the patients had gingivitis, while 40% had some form of periodontitis as per the definitions of the study protocol. However, none of the patients presented with severe periodontitis (Table 2). The mean PMN counts from the 15-second rinse were 4.66 x 10^6 cells. The mean levels of average probing depths and the bleeding index are reported in Table 3.

**Table 2.** Periodontal status of the study population at recruitment.

<table>
<thead>
<tr>
<th>Periodontal Status</th>
<th>N*</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>11</td>
<td>17.5</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>27</td>
<td>42.8</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>25</td>
<td>39.7</td>
</tr>
<tr>
<td>Mild periodontitis</td>
<td>20</td>
<td>31.8</td>
</tr>
<tr>
<td>Moderate periodontitis</td>
<td>5</td>
<td>7.9</td>
</tr>
<tr>
<td>Severe periodontitis</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Sample size, total = 63.

**Table 3.** Mean values of the oral PMN counts (x 10^6 cells/ml), average probing depths (mm) and bleeding index of the study population at recruitment.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Mean ± Standard Deviation</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN counts (x 10^6 cells/ml)</td>
<td>4.66 ± 2.06</td>
<td>4.36</td>
<td>2.22</td>
<td>9.53</td>
</tr>
<tr>
<td>Average probing depths (mm)</td>
<td>2.43 ± 0.12</td>
<td>2.42</td>
<td>2.17</td>
<td>2.78</td>
</tr>
<tr>
<td>Bleeding index*</td>
<td>4.44 ± 3.89</td>
<td>3.57</td>
<td>0.00</td>
<td>14.88</td>
</tr>
</tbody>
</table>

*Bleeding index is calculated by number of sites with bleeding divided by total number of sites probed x 100.
2 Relationship between Periodontal Disease and Oral Polymorphonuclear Neutrophil Counts in Pregnant Women

Analyses were conducted to examine the relationship between the level of oral PMNs and severity of periodontal disease. Spearman’s rank correlation showed a significant positive correlation between PMN counts and the bleeding index (Figure 2; $r_s = 0.81$, $p<0.001$). Similarly, analysis with Spearman’s rank correlation also showed a significant positive correlation between the PMN counts and the average probing depths (Figure 3, $r_s = 0.58$, $p<0.001$). There were no significant correlations found between PMN counts and age or number of teeth present, or the gestational age at recruitment, which are reported in Table 4.
Figure 2. Relationship between natural log of PMN counts and the bleeding index of the study population.

Figure 3. Relationship between natural log of PMN counts and the average probing depths of the study population.
Table 4: Spearman’s rank correlation ($r_s$) between oral PMN counts and bleeding index, average probing depths, number of teeth, age and gestational age of the study population at recruitment.

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>$r_s$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding index*</td>
<td>0.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Average probing depths (mm)</td>
<td>0.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of teeth</td>
<td>0.02</td>
<td>0.878</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.09</td>
<td>0.474</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>0.06</td>
<td>0.633</td>
</tr>
</tbody>
</table>

*Bleeding index is calculated by number of sites with bleeding divided by total number of sites probed x 100.

When PMN counts were compared to the modified gingival index, patients with higher visual inflammation exhibited higher PMN counts (ANOVA; p<0.001; Tukey HSD test showed a significant difference between all the groups except between the groups 0 and 1; Figure 4). There were no patients that presented with a modified gingival index of 4 or “severe inflammation”. In addition, comparison of the PMN counts to the plaque index showed that the subjects who presented with plaque had higher PMN counts compared to the subjects who did not (t-test; p<0.05; Figure 5). However, there were no patients that presented with a plaque index score of higher than 1 or mobility of teeth that were pathologic. Similarly, when PMN counts were compared to the calculus index, the patients in group 2 (i.e. moderate amounts of supra and subgingival calculus or subgingival calculus only) exhibited significantly higher PMN counts compared to the other groups (ANOVA; p<0.001; Figure 6). There were no patients that presented with an abundance of supra and subgingival calculus or a calculus index score of 3. There was no significant relationship found between PMN counts and history of making a dental visit within the last 6 months at the time of recruitment (t-test; p=0.484). Finally, the PMN counts from periodontally healthy patients were compared to the PMN counts of the patients
with gingivitis and periodontitis. The patients with gingivitis exhibited a 1.5-fold greater level of PMNs, while the patients with periodontitis exhibited a 2-fold greater level of PMNs compared to the periodontally healthy patients (ANOVA; p<0.05; Tukey HSD test showed a significant difference between all the groups; Figure 7). Furthermore, the PMN counts of the patients with moderate periodontitis were 3-fold greater than those who were periodontally healthy. The mean PMN counts of the patients by their periodontal status are reported in Table 5.
Figure 4. Comparison of natural log of PMN counts with the modified gingival index* of the study population with division of patients based on the visual assessment of inflammation. Tukey HSD test shows significant differences between the groups 0 and 2, 0 and 3; 1 and 2, 1 and 3; 2 and 3 (ANOVA: p < 0.001; n = 3, 32, 18, 10 respectively). **SE = Standard Error

*Modified Gingival Index (Lobene, Weatherford, Ross, Lamm, & Menaker, 1986)

0: Absence of inflammation

1: Mild inflammation: slight change in color, little change in texture of any portion of but not the entire marginal or papillary gingival unit

2: Mild inflammation: criteria as above but involving entire marginal or papillary gingival unit

3: Moderate inflammation: glazing, redness, edema, and/or hypertrophy of the marginal or papillary gingival unit
Figure 5. Comparison of natural log of PMN counts with the plaque index* of the study population with division of patients based on the presence of plaque; n = 12, 51. T-test show statistically significant difference between the groups (t-test: p < 0.05).

*Plaque Index (Ramfjord, 1959)

0: No plaque present

1: Plaque present on some but not all of the interproximal and gingival surfaces of the tooth
Figure 6. Comparison of natural log of PMN counts with the calculus index* of the study population with division of patients based on the presence of calculus. Tukey HSD test shows significant differences between the groups 0 and 2; 1 and 2 (ANOVA: p < 0.05; n = 29, 28, 6 respectively).

*Calculus Index (Ramfjord, 1959)

0: Absence of calculus

1: Supragingival calculus extending only slightly below the free gingival margin (not more than 1mm)

2: Moderate amount of supra and subgingival calculus, or subgingival calculus only
Figure 7. Comparison of natural log PMN counts with periodontal status of the study population with division of patients based on their periodontal status. Tukey HSD test shows significant difference between all the groups (ANOVA: p < 0.05; n = 11, 27, 25 respectively).

Table 5: Mean oral PMN counts (x 10^6 cells/ml) of the study population according to their periodontal status.

<table>
<thead>
<tr>
<th>Periodontal Status</th>
<th>N</th>
<th>Mean ± Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>11</td>
<td>2.86 ± 0.78</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>27</td>
<td>4.31 ± 1.60</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>25</td>
<td>5.83 ± 2.22</td>
</tr>
<tr>
<td>Mild periodontitis</td>
<td>20</td>
<td>5.12 ± 1.86</td>
</tr>
<tr>
<td>Moderate periodontitis</td>
<td>5</td>
<td>8.63 ± 0.91</td>
</tr>
</tbody>
</table>
3 Usefulness of Oral Polymorphonuclear Neutrophil Counts as a Screening Tool for Periodontal Disease in Pregnant Women

In order to determine the usefulness of the oral PMN counts as a tool for detection of periodontal disease in pregnant women, a bleeding index of 5.5 or bleeding upon probing of at least 10 sites was chosen as the cutoff point for the diagnostic test. This is a clinically relevant number of sites, as this can be considered to represent localized inflammation. Similarly, the cutoff point of 4 interproximal sites (excluding wisdom teeth) with a probing depth of at least 4 mm was chosen as a clinically relevant number of probing depths as detection of localized periodontitis would be clinically useful. The sensitivity, specificity, PPV and NPV were determined for the bleeding index cut-off point (Figure 8) and probing depths cut-off point (Figure 9) at various PMN counts. For the bleeding index cutoff, a PMN count of around 4.5 million cells yielded a sensitivity of 0.94 and a specificity of 0.76. The PPV and NPV were calculated to be 0.60 and 0.97 respectively. Similarly, for the probing depth cutoff, a PMN count of approximately 4 million cells yielded a sensitivity of 0.80 and specificity of 0.63. The PPV and NPV were calculated to be 0.59 and 0.83 respectively. The high sensitivity and NPV value of the test using the above mentioned periodontal disease indicators (bleeding index and probing depth cutoff points) indicate that this test is able to ‘rule out’ the presence of disease in patients who do not present with a bleeding index of at least 5.5 or probing depths of at least 4 mm in 4 interproximal sites. Furthermore, the relatively high specificity and PPV indicates that this test is also able to ‘rule in’ the presence of disease in patients who present with a bleeding index of 5.5 or probing depths of at least 4 mm in 4 interproximal sites.
**Figure 8.** Sensitivity and specificity values of the oral rinse assay for increasing PMN counts (using bleeding index of 5.5 or more as cut-off point).

**Figure 9.** Sensitivity and specificity values of the oral rinse assay for increasing PMN counts (using probing depth of $\geq 4$ mm in at least 4 sites on different teeth as cut-off point).
4 Pregnancy Characteristics of the Study Population that was Followed-up

Four of the 63 patients (6.3%) moved away from the city of Toronto and did not deliver at the Mount Sinai Hospital. As a result, the course of the pregnancy and birth outcome data for those four patients could not be collected.

Of the 59 patients that were followed-up, 64% had a history of at least one previous pregnancy. Four (6.8%) of the patients had fertility treatment in the form of in-vitro fertilization to achieve pregnancy. Approximately 24% had a history of at least one spontaneous abortion, with two of the patients having a history of three spontaneous abortions. Two (3.4%) patients had a history of delivering preterm. Over the course of the pregnancy, two (3.4%) patients developed gestational diabetes. Approximately 56% of the patients experienced spontaneous labour, while 22% had labour induced for various reasons that are listed in Table 6 along with the pregnancy characteristics of the patients that were followed up.
Table 6. Pregnancy characteristics of the study population at follow-up.

<table>
<thead>
<tr>
<th>Factor</th>
<th>N*</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Previous pregnancy history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of any pregnancy</td>
<td>38</td>
<td>64.4</td>
</tr>
<tr>
<td>History of spontaneous abortions</td>
<td>14</td>
<td>23.7</td>
</tr>
<tr>
<td>History of induced abortions</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>History of preterm birth</td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Fertility treatment</strong></td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td><strong>Gestational diabetes development</strong></td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Premature rupture of membranes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Term</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>Preterm</td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Delivery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal</td>
<td>40</td>
<td>67.8</td>
</tr>
<tr>
<td>Cesarean</td>
<td>19</td>
<td>32.2</td>
</tr>
<tr>
<td>Elective</td>
<td>13</td>
<td>22.0</td>
</tr>
<tr>
<td>In labour</td>
<td>6</td>
<td>10.2</td>
</tr>
<tr>
<td><strong>Labour</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>33</td>
<td>55.9</td>
</tr>
<tr>
<td>Induced</td>
<td>13</td>
<td>22.0</td>
</tr>
<tr>
<td>No labour and not induced</td>
<td>13</td>
<td>22.0</td>
</tr>
<tr>
<td><strong>Reason for induction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post due dates</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>Term premature rupture of membranes</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>Preterm premature rupture of membranes</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Intrauterine growth restriction</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>4</td>
<td>5.1</td>
</tr>
</tbody>
</table>

*Total patients followed-up = 59.

**Other reasons included gestational diabetes, previous trauma with delivery, oligohydraminos, and fetal anomaly.
5 Birth Outcomes, Periodontal Disease and Polymorphonuclear Neutrophil Counts of the Study Population

Of the 59 patients that were followed-up, five (8.5%) patients delivered preterm and five (8.5%) patients had LBW infants. The average gestation age at delivery was 39.2 ± 1.9 weeks with a minimum gestation period of 31.5 weeks and maximum gestation period of 41.6 weeks. The average infant birth weight was 3241 ± 550 g with a minimum birth weight of 1430 g and a maximum birth weight of 4390 g.

Four patients experienced both adverse pregnancy outcomes: a LBW infant that delivered preterm. One patient delivered a normal weight infant preterm and one patient delivered a LBW infant at term. Therefore, a total of six (10.2%) patients either delivered preterm and/or had a LBW infant. The gestational age at delivery, birth weight, pregnancy characteristics and periodontal status of these six patients have been reported in Table 7. No patients delivered at an ‘extreme preterm gestation age’ (less than 28 weeks gestation). One patient delivered a ‘very low birth weight’ infant.

Regarding the mode of delivery, four patients experienced spontaneous preterm labour and vaginal delivery; three of these patients delivered LBW infants, while the other delivered a normal weight infant (Table 7). Among these patients, one presented with PPROM that precipitated the preterm labour; the other three patients experienced spontaneous preterm labour without any identified cause. Both of the patients that underwent a labour induction and vaginal delivery delivered LBW infants. The indication for induction was intrauterine growth restriction for one patient. The second patient was induced due to PPROM at 34.6 weeks; this patient had also developed gestational diabetes and had been diagnosed with periodontitis at recruitment.
Table 7. Gestational age at delivery, birth weight, possible indicators and labour, and periodontal status of the six patients experiencing an adverse pregnancy outcome.

<table>
<thead>
<tr>
<th>Gestational Age at Delivery (weeks)</th>
<th>Birth Weight (g)</th>
<th>Indicators and Labour</th>
<th>Periodontal Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33.0</td>
<td>None known, Spontaneous preterm labour</td>
<td>Healthy</td>
</tr>
<tr>
<td>2</td>
<td>34.6</td>
<td>PPROM, Induced preterm labour</td>
<td>Periodontitis</td>
</tr>
<tr>
<td>3</td>
<td>31.5</td>
<td>None known, Spontaneous preterm labour</td>
<td>Healthy</td>
</tr>
<tr>
<td>4</td>
<td>37.0</td>
<td>Intrauterine growth restriction, Induced term labour</td>
<td>Healthy</td>
</tr>
<tr>
<td>5</td>
<td>36.0</td>
<td>None known, Spontaneous preterm labour</td>
<td>Healthy</td>
</tr>
<tr>
<td>6</td>
<td>34.5</td>
<td>In-vitro fertilization, Spontaneous preterm labour</td>
<td>Healthy</td>
</tr>
</tbody>
</table>

The incidence of overall adverse birth outcomes, preterm births and LBW outcomes classified by certain risk factors (age, race, pregnancy characteristics, medical history and smoking status), periodontal status (healthy, gingivitis, periodontitis) and indicators of the status of periodontal disease (modified gingival index, plaque index, calculus index) have been presented in Tables 8, 9 and 10 respectively. The majority of patients that experienced an adverse birth outcome were of Caucasian descent. The expected number of preterm deliveries- 5 pregnancies- (based on population statistics) was observed, however, only one of these patients was diagnosed with periodontal disease at recruitment. The best predictor of preterm delivery is the history of a previous preterm birth (odds ratio: 2 fold above baseline risk); in the study population the patients with a history of preterm birth did not experience a repeat preterm birth.
Table 8. Incidence of overall adverse pregnancy outcomes among the study population at follow-up categorized by certain risk factors, periodontal status and other indicators of the status of periodontal disease.

<table>
<thead>
<tr>
<th>Adverse birth outcomes</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm birth and/or low birth weight infant</td>
<td>6</td>
<td>10.2</td>
</tr>
</tbody>
</table>

**Risk factors**

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 35 years and above</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>5</td>
<td>8.5</td>
</tr>
<tr>
<td>Black</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>East Indian</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>History of spontaneous abortions*</td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td>History of induced abortions*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>History of preterm birth*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fertility treatment*</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Gestational diabetes*</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Smoker*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Periodontal status classification**

<table>
<thead>
<tr>
<th>Periodontal status classification</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>5</td>
<td>8.5</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

**Periodontal disease status indicators**

<table>
<thead>
<tr>
<th>Modified gingival index</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plaque index</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>5.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculus index</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*These risk factors are not mutually exclusive.

**Calculated by N divided by the total number of patients followed up i.e. 59 x 100.
Table 9. Incidence of preterm birth outcomes among the study population at follow-up categorized by certain risk factors, periodontal status and other indicators of the status of periodontal disease.

<table>
<thead>
<tr>
<th>Adverse birth outcomes</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm birth</td>
<td>5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 35 years and above</td>
<td>2</td>
<td>3.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Race</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>Black</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>East Indian</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>History of spontaneous abortions*</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of induced abortions*</td>
<td>2</td>
<td>3.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>History of preterm birth*</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertility treatment*</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Gestational diabetes*</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Smoker*</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Periodontal status classification</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Periodontal disease status indicators</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified gingival index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plaque index</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>3.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Calculus index</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*These risk factors are not mutually exclusive.

**Calculated by N divided by the total number of patients followed up i.e. 59 x 100.
Table 10. Incidence of low birth weight outcomes among the study population at follow-up categorized by certain risk factors, periodontal status and other indicators of the status of periodontal disease.

<table>
<thead>
<tr>
<th>Adverse birth outcomes</th>
<th>N</th>
<th>Percentage**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low birth weight</td>
<td>5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk factors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 35 years and above</td>
<td>3</td>
<td>3.4</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>Black</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>East Indian</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>History of spontaneous abortions*</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>History of induced abortions*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>History of preterm birth*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fertility treatment*</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Gestational diabetes*</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Smoker*</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Periodontal status classification</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>4</td>
<td>6.8</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Periodontal disease status indicators</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified gingival index</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>Plaque index</td>
<td>2</td>
<td>3.4</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>5.1</td>
</tr>
<tr>
<td>Calculus index</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*These risk factors are not mutually exclusive.

**Calculated by N divided by the total number of patients followed up i.e. 59 x 100.
The mean oral PMN counts at recruitment for the patients delivering preterm was $4.03 \times 10^6$ cells/ml and for the patients delivering a LBW infant was $3.91 \times 10^6$ cells/ml. The mean values of the oral PMN counts, average probing depths and bleeding index of the patients at recruitment categorized by overall adverse birth outcome, preterm birth and LBW infant outcomes are reported in Tables 11, 12 and 13 respectively. There were no statistically significant differences between the PMN counts, average probing depths and bleeding index of the patients that delivered preterm and the patients that delivered at term (t-test: $p = 0.417$; $p = 0.512$; $p = 0.826$ respectively). Similarly, no statistically significant differences were found between the PMN counts, average probing depths and bleeding index of the patients that delivered a LBW infant and a normal weight infant (t-test: $p = 0.353$; $p = 0.512$; $p = 0.724$ respectively).
### Table 11. Mean values of the oral PMN counts, average probing depths and bleeding index of the study population at follow-up categorized by overall adverse birth outcomes.

<table>
<thead>
<tr>
<th>Periodontal Disease Status</th>
<th>Mean ± Standard Deviation</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTLBW*</td>
<td>No PTLBW</td>
<td>PTLBW</td>
<td>No PTLBW</td>
</tr>
<tr>
<td>PMN counts (x 10⁶ cells/ml)</td>
<td>3.79 ± 2.83</td>
<td>4.73 ± 1.90</td>
<td>2.59</td>
<td>4.47</td>
</tr>
<tr>
<td>Average probing depths (mm)</td>
<td>2.39 ± 0.21</td>
<td>2.44 ± 0.11</td>
<td>2.34</td>
<td>2.43</td>
</tr>
<tr>
<td>Bleeding index</td>
<td>2.18 ± 5.35</td>
<td>4.61 ± 3.64</td>
<td>0.00</td>
<td>3.57</td>
</tr>
</tbody>
</table>

*PTLBW = Preterm and/or low birth weight delivery.
Table 12. Mean values of the oral PMN counts, average probing depths and bleeding index of the study population at follow-up categorized by preterm birth outcomes.

<table>
<thead>
<tr>
<th>Periodontal Disease Status Indicators</th>
<th>Mean ± Standard Deviation</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN counts (x 10^6 cells/ml)</td>
<td>4.03 ± 3.09</td>
<td>2.59</td>
<td>2.40</td>
<td>9.53</td>
</tr>
<tr>
<td></td>
<td>4.69 ± 1.91</td>
<td>4.47</td>
<td>2.22</td>
<td>9.20</td>
</tr>
<tr>
<td>Average probing depths (mm)</td>
<td>2.41 ± 0.22</td>
<td>2.36</td>
<td>2.17</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>2.44 ± 0.11</td>
<td>2.43</td>
<td>2.23</td>
<td>2.66</td>
</tr>
<tr>
<td>Bleeding index</td>
<td>2.62 ± 5.86</td>
<td>0.00</td>
<td>0.00</td>
<td>13.10</td>
</tr>
<tr>
<td></td>
<td>4.52 ± 3.66</td>
<td>3.57</td>
<td>0.00</td>
<td>14.88</td>
</tr>
</tbody>
</table>

*PTB = Preterm birth.
Table 13. Mean values of the oral PMN counts, average probing depths and bleeding index of the study population at follow-up categorized by low birth weight outcomes.

<table>
<thead>
<tr>
<th>Periodontal Disease Status Indicators</th>
<th>Mean ± Standard Deviation</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LBW</td>
<td>No LBW</td>
<td>LBW</td>
<td>No LBW</td>
</tr>
<tr>
<td>PMN counts (x10^6 cells/ml)</td>
<td>3.91 ± 3.14</td>
<td>4.70 ± 1.90</td>
<td>2.58</td>
<td>4.45</td>
</tr>
<tr>
<td>Average probing depths (mm)</td>
<td>2.40 ± 0.23</td>
<td>2.44 ± 0.11</td>
<td>2.33</td>
<td>2.43</td>
</tr>
<tr>
<td>Bleeding index</td>
<td>2.62 ± 5.86</td>
<td>4.52 ± 3.66</td>
<td>0.00</td>
<td>3.57</td>
</tr>
</tbody>
</table>

*LBW = Low birth weight.
Discussion

The altered metabolism during pregnancy may impact on the physiology of the gingiva and the oral mucosa. Conversely, periodontal infection can have effects on other parts and systems of the body. As discussed at length, there are many studies that suggest there is a putative association between maternal periodontal disease and PTLBW delivery. Although it has been extremely difficult to demonstrate conclusively that periodontitis is a significant and unequivocal risk factor for PTLBW delivery due to the multifaceted nature of both periodontitis and PTLBW delivery, it is biologically plausible and the key link is probably inflammation.

Traditionally, the diagnosis of periodontal disease is determined by a periodic professional dental examination of the gingival tissues and measurement of attachment and alveolar bone loss. This involves the insertion of a thin metal probe under the gingival tissues surrounding the teeth for these measurements. Even though this examination gives an accurate portrait of the status of the periodontium at the time of examination, it requires a dental professional to carry out the complete examination. Currently, there is no rapid, inexpensive method of identifying pregnant women who may be at risk of adverse birth outcome due to periodontal diseases (presuming a link). Being able to identify periodontal diseases in pregnant women using a rapid, non-invasive test that can be administered by non-specialized staff has the potential to have a significant impact on this population and the healthcare system. Alternatively, although not tested here, it has been discussed above that treatment of maternal periodontitis prior to pregnancy might be more important insofar as a putative link between pregnancy and adverse pregnancy outcomes are concerned. The assay system described here could be used in virtually any clinical setting to determine whether a female patient who is planning to become pregnant might have periodontitis and should seek treatment. The primary objective of this study was to correlate the levels of periodontal disease as measured by conventional methods with PMN counts from an oral rinse in a group of low risk pregnant women and to determine the oral PMN counts in this group using the rinse assay. The results from this study clearly demonstrated that the levels of periodontal disease as measured by conventional methods (visual assessment of inflammation, presence of plaque and calculus, probing depths and bleeding on probing) correlate significantly with PMN counts from the oral rinse in this study population. Therefore, the null hypothesis can be rejected.
in favour of the alternative hypothesis that “an oral rinse assay measuring oral PMN counts correlate with levels of periodontal disease as measured by conventional methods in a group of pregnant women”. Furthermore, the relatively high PPV, NPV, sensitivity and specificity values for bleeding on probing at 10 or more sites and probing depths of 4 mm or more at PMN counts between 4 million to 4.5 million cells indicate that this oral rinse assay is a very good predictor of the presence of oral inflammation and deep pockets. These findings are consistent with the results from the oral PMN quantification assay study in a group of healthy non-pregnant adults being treated for moderate to severe periodontal diseases (Bender et al., 2006). Importantly though, in the current study, the quantification of oral PMNs was simplified greatly by collecting a single 15 second rinse sample and by measuring the OD of the sample as opposed to the much more labour-intensive cell counting methods described previously. The relative ease and rapidity with which oral PMNs can be collected from a patient and quantified, as well as the low cost of the reagents utilized to quantify the PMNs per patient (approximately CDN$ 5), highlight the simplicity and cost-effectiveness of this assay as a diagnostic test. This study has demonstrated that this oral rinse assay is a valid and reliable method of identifying periodontal disease in pregnant women.

The patient population in the study was healthy and well-educated, with good access to medical and dental care. The patients were all under the care of an obstetrics gynecologist for their pregnancies and 65% had been to a dentist within the past six months at the time of recruitment. The majority of the patients reported having good oral hygiene habits as well. According to the AAP and CDC definitions of periodontitis, the prevalence of periodontitis in this study population was approximately 40% (Eke et al., 2012; R. C. Page & Eke, 2007). Although, the use of heterogeneous definitions of periodontal disease makes comparisons difficult, this figure still falls within the reported range in the published literature (Lieff et al., 2004; Vogt et al., 2012). The severity of periodontitis in this population was of mild to moderate levels with only 8% of the patients presenting with moderate periodontitis. There were no patients that presented with severe periodontitis according to the stringent criteria used in this study and this might also be related to the fact that the population was relatively young, while the incidence of periodontitis increases with age. The AAP and CDC definitions of periodontitis include measurements of both probing depths and CAL at the interproximal sites. Interproximal sites are used since they are considered to be more reliable in detecting true disease while mid-facial or
mid-lingual measurements could be influenced by toothbrush abrasion and non-inflammatory gingival recession resulting in over estimation of disease (Eke et al., 2012). Since oral PMN levels should reflect the current oral inflammatory load and possibly current periodontal disease activity, it can be postulated that measurements of probing depths alone would represent active periodontal disease more accurately as compared to CAL alone, the latter mainly reflecting past disease activity. In order to account for this, oral PMN counts were compared to average probing depths rather than average CAL. However, it was thought to be important to define periodontitis according to the standard definitions to avoid contributing further to the use of diverse definitions of periodontal disease in the literature. The patient population in general presented with shallow pockets. Although average probing depths were utilized instead of frequency distribution of deep pockets (for example pocket depths greater than 5 mm) for comparison to oral PMN counts, it should be noted that the frequency of deep pockets were indirectly determined by defining the periodontal status of the patient population using the standard definitions.

The average oral PMN counts for this group of pregnant women with moderate periodontitis was around 9 million cells, whereas the average figure was around 6 million cells for the group with moderate periodontitis in the study by Bender et al. (2006). Despite finding that the oral PMN counts for pregnant women were comparatively higher, it becomes difficult to make any definitive statements due to the differences in the criteria used to define moderate periodontitis between the two studies (Bender et al. had defined moderate periodontitis as the presence of 10 or fewer pockets > 5 mm). The Bender et al. study did not have a defined mild periodontitis group of patients; therefore, no comparisons could be made for the average oral PMN counts of the group of pregnant women that presented with mild periodontitis in this study. The average oral PMN counts for the patients with healthy periodontal status between the two studies were basically similar.

The incidence of preterm delivery in the patients that were followed-up was 8.5%, while the incidence of LBW outcome was also 8.5%. The overall incidence of PTLBW delivery was 10.2%. In Canada, the national rate of preterm delivery is 8.1% and approximately 6% infants are born at a LBW (Lim et al., 2009). These figures are similar to the findings in this study. However, given that this study population was recruited from a ‘low risk’ clinic, the incidence
may be slightly higher than anticipated. Having said this, it can be stated that the nature of the adverse birth outcomes were not very severe with three patients delivering ‘near term’ and only one patient delivering an infant at a ‘very low birth weight’.

Analysis of the pregnancy characteristics revealed that two patients had their delivery induced due to one experiencing intrauterine growth restriction (IUGR) and the other experiencing PPROM without labour at 34.6 weeks. IUGR by definition refers to any etiology that can limit the growth potential of the fetus resulting in lower birth weight (Valero De Bernabe et al., 2004), which can place the fetus at significant risk. Therefore, IUGR is usually an indication that prompts an intervention at preterm gestational ages (Ananth et al., 2006). IUGR is also a risk factor for spontaneous preterm birth. However, this patient delivered a LBW infant at term. The patient experiencing PPROM had also developed gestational diabetes, and maternal diabetes is a known risk factor of pregnancy complications that include increased rates of indicated preterm births (Goldenberg et al., 2008). The remaining four patients experiencing an adverse birth outcome had spontaneous preterm deliveries. One of these patients experienced PPROM which triggered the preterm labour and delivered a LBW infant. Another patient had received fertility treatment in the form of in-vitro fertilization, also delivered a LBW infant. Compared with spontaneous conception, singleton pregnancies after in-vitro fertilization are associated with significantly higher odds of an adverse birth outcome that include preterm delivery and/or LBW outcome (Jackson, Gibson, Wu, & Croughan, 2004). Of the other two patients that experienced spontaneous preterm labour, one delivered a normal weight infant. Given the above findings, it can be stated that in most of the cases, the patients presented with medical conditions or risk factors that would increase the threat of an adverse birth outcome.

There were no statistically significant differences between the measures of periodontal disease, bleeding index and average probing depths of the patients that delivered at term or had a normal weight infant compared to the patients that delivered preterm or had a LBW infant. Furthermore, no statistically significant differences were found between their oral PMN counts. Only one patient diagnosed with moderate periodontitis at recruitment delivered a LBW infant at preterm in this study. Interestingly, the patient had the highest oral PMN count of this study population. However, this patient had also developed gestational diabetes and as mentioned previously diabetes is a known risk factor for both periodontal disease and adverse pregnancy outcomes and
therefore it would be inappropriate to suggest that the patient’s periodontal condition contributed to the adverse pregnancy outcome in this case. The remaining patients experiencing adverse birth outcomes were periodontally healthy.

Given the associated risk factors that were discovered following the analyses of the patients experiencing adverse birth outcomes and the multifactorial nature of periodontal diseases and PTLBW delivery, these findings are not surprising. However, this study does not have enough ‘power’ to elucidate whether or not a relationship exists. In this regard, the primary objective of this study was not designed to examine the relationship between periodontal disease and adverse birth outcomes. The study population was a relatively homogenous group of pregnant women recruited from a single centre that presented only with severity levels of mild to moderate periodontitis, with only a few patients presenting with moderate periodontitis. Given that the incidence of preterm birth and/or LBW infant outcome is considerably low in the general population, a much larger sample size would be required to detect if there is any significant relationship to periodontal disease. To emphasize issues pertaining to the sample size that would have been needed to determine whether or not there is a relationship between oral inflammatory load and adverse outcomes of pregnancy, it should be noted that there are several studies of this putative phenomenon which have used much larger population sample sizes than used here. For instance, prospective cohort studies that have examined the relationship between maternal periodontal disease and preterm birth and/or LBW infant outcome have used sample populations of well over a thousand subjects (Moore et al., 2004; Offenbacher et al., 2006).

The above notwithstanding, the overarching goal of this research was, first and foremost, to confirm that periodontal disease, or more accurately, oral inflammatory load in pregnant females could be detected and measured reliably using an easily administered test as shown here. Given the findings shown here and in the literature, it can be concluded that by using a rapid non-invasive oral rinse assay it might now be more feasible to study even larger sample sizes (i.e. given the ease of use and analysis of the assay) so that a clearer understanding of the relationship between maternal periodontitis and adverse birth outcomes can be developed in more detail.

It is noteworthy that, as discussed above, PMNs play a major role in the pathogenesis of periodontal disease by releasing MMPs and other enzymes ("The Pathogenesis of Periodontal
Diseases", 1999) and this could have an impact on other systems in the body including the uterus during pregnancy. In relation to this idea, MMPs might even play a direct and/or indirect role in the pathogenesis of adverse pregnancy outcomes (Cockle et al., 2007; Shaarawy & Nagui, 1997). Therefore, the ability to detect periodontal disease reliably and accurately using this assay not only allows for quantitation of oral PMNs and therefore oral inflammatory load, but might also contribute to the understanding of a mechanistic relationship between maternal periodontitis and adverse birth outcomes, if it exists. Furthermore, since the rinse assay reflects the overall oral inflammatory load, it can contribute to understanding the pathogenesis of unfavourable birth outcomes such as preterm birth by studying the relationship between adverse birth outcomes and oral inflammation in general given that there is no single form of periodontal disease (that is, it ranges from gingivitis to periodontitis with several iterations of the latter; but all conditions are inflammatory).

As mentioned earlier, periodontal disease can have other non-oral effects. There is evidence suggesting that periodontal disease is associated with an increase in the risk for cardiovascular disease (Holmlund, Holm, & Lind, 2006; Spahr et al., 2006), diabetes (Jansson, Lindholm, Lindh, Groop, & Bratthall, 2006; Khader, Dauod, El-Qaderi, Alkafajei, & Batayha, 2006), and community and hospital-acquired respiratory infections (Azarpazhooh & Leake, 2006). Furthermore, successful treatment of periodontitis has been shown to lead to reductions in risk markers for cardiovascular disease (Tonetti et al., 2007). Despite the aforementioned findings, periodontal therapy has never been shown to reduce the incidence of acute myocardial infarction. But it has been shown to improve diabetic control by inducing reductions in the levels of haemoglobin A1c in patients with type 2 diabetes (Faria-Almeida, Navarro, & Bascones, 2006) and to also reduce the risk of nosocomial pneumonia (Genuit, Bochicchio, Napolitano, McCarter, & Roghman, 2001; Koeman et al., 2006). Therefore, the treatment of periodontal infections has the potential to improve overall health; an important factor in the delivery of healthcare to pregnant women and others.

Finally, when it is confirmed that increased oral inflammatory load (e.g. periodontitis, gingivitis) is related causally to some non-oral diseases, treatment of oral diseases could have a positive impact on the clinical course of such associated diseases (e.g. diabetes). Alternatively, it is also quite likely that oral inflammatory diseases are co-morbid conditions that, although present,
don’t actually have any impact on the treatment or progression of other non-oral diseases (e.g. cardiovascular disease). Either way, the identification and quantitation of oral inflammatory disease, which should now be simpler with an easy to administer and novel PMN assay as described here, will still provide important healthcare outcomes and place oral healthcare delivery in a pivotal position. Once the issues pertaining to the association/causality findings shown for oral inflammatory diseases and non-oral diseases have been confirmed, the delivery of appropriate oral health care might reduce general disease load. Absent a causal association though, oral healthcare practitioners, on identifying oral inflammatory disease in their patients, will also be in a position to advise these patients of potentially life threatening co-morbid conditions (e.g. cardiovascular disease) and can then direct their patients for appropriate medical consultation, especially if those patients also have other known risk factors for the related diseases (e.g. smoking, obesity, sedentary lifestyle) Either way, the overall health of the population will be affected in a positive manner. Finally it also should go without saying that oral health care for pregnant women; including management of periodontal problems, should be a goal in its own right.
Conclusions

- An oral rinse assay measuring oral PMN counts correlate with levels of periodontal disease as measured by conventional methods in a group of pregnant women.
- The oral rinse assay can be used as a screening tool to detect periodontal disease in pregnant women.
- The mean oral PMN count for this group of pregnant women was 4.66 million cells/ml with a range of 2.22 million to 9.53 million cells/ml.
- The incidence of overall adverse birth outcome was 10.2%, with 8.5% delivering preterm and 8.5% delivering a LBW infant.
- There were no statistically significant differences between the PMN counts, bleeding index and average probing depths of the patients that delivered at term and had a normal weight infant compared to patients that delivered preterm and/or delivered a LBW infant.
References


differentiation via IL-17RA or IL-17RC receptors in RAW264.7 cells. *Biochimie*, 92(4), 398-404.


receptor-1 and -2 levels in periodontal disease, and adverse pregnancy outcomes. *Journal of Periodontology, 82*(12), 1735-1748.


Appendices

Appendix A. Information booklet provided to patients.

For More Information Please Contact:
Dr. Sabrina Neele
T: 416-694-4756 ext. 3044
Dr. Michael Segal
T: 416-698-5174

If you have any concerns relating to your pregnancy, you may contact your doctor, midwife or Mount Sinai Hospital.

If you have any questions about your rights as a research subject, please call Dr. J. Hendegaven, Chair of the Mount Sinai Hospital Research Ethics Board at 416-586-4173. This person is not involved with the research project in any way and calling him will not affect your participation in the study.

INVESTIGATOR
Dr. Michael Segal

CO-INVESTIGATORS
Dr. Sabrina Neele
Dr. Howard Frenkel
Dr. Wendy Whittle
Dr. Michael Geoghegan
Dr. Helena Lawrence
Dr. Michael Goldberg

MOUNT SINAI HOSPITAL
Joseph and Wolf Lebovic Health Centre
200 University Avenue, 2nd Floor
Toronto, Ontario, Canada M5G 1A5

Wedneday, December 15, 2010

Thank you for taking the time to read this leaflet.

We would like to invite you to take part in a research study that compares an oral water rinse test to a standard complete dental examination in pregnant women.

The goal is to find a simple test that can measure gum health status so that we can identify pregnant women who may be at greater risk of having a baby that is too small (low birth weight) or a baby that is born early (pre-term birth) or both, due to gum disease.

Background
Gum disease, also known as periodontal disease, is a very common disease in humans.

There are two main types of gum disease: gingivitis, which only affects the soft tissue around a tooth, and periodontitis, which affects the hard tissues such as bone and connective tissues around the teeth as well. Current studies suggest that mothers with gum disease may give birth prematurely or have a baby with lower birth weight. Further studies show that gum disease may increase the risk of preterm birth. This needs further testing.

What is involved in the study?
A simple test is needed to identify mothers with gum disease. If you agree to participate, you will be asked to rinse your mouth with sterile water for 15 seconds, which will then be collected. You will then have a complete dental examination and a regular examination at your dentist. We will examine your gums (including measurement of the gum pocket depth) and your teeth.

No dental x-rays will be taken.

The findings of your dental examination will be discussed with you and if any oral problems are identified, you will be advised of options regarding follow-up and treatment. In addition, you will receive individualized instructions on oral hygiene (i.e., proper technique of brushing and flossing). The entire procedure should not last longer than 30 minutes and there are no additional hospital visits or investigations that are required as part of this study.

Information will be obtained from your case notes and your baby’s.

This will include details of your pregnancy, labour and birth, as well as details of your baby’s birth weight and progress after birth.

Possible Side Effects
There is no foreseeable harm or injury associated with participating in this study.
Appendix B. Patient consent form.

RESEARCH CONSENT FORM

Novel oral rinse assay for the quantification of oral neutrophils and comparison to the periodontal examination in pregnant women.

INVESTIGATOR: Dr. Michael Sigal, (Mount Sinai Hospital)

CO-INVESTIGATORS Dr. Sabrina Huda, Dr. Howard Tenenbaum, Dr. Wendy Whittle, Dr. Michael Glogauer, Dr. Herenia Lawrence, Dr. Michael Goldberg

TITLE: Novel oral rinse assay for the quantification of oral neutrophils and comparison to the periodontal examination in pregnant women.

Introduction
You are being asked to take part in a research study. Please read this explanation about the study and its risks and benefits before you decide if you would like to take part. You should take as much time as you need to make your decision. You should ask the study doctor to explain anything that you do not understand and make sure that all of your questions have been answered before signing this consent form. Before you make your decision, feel free to talk about this study with anyone you wish. Participation in this study is voluntary.

Background and Purpose
Gum disease also known as periodontal disease is a very common disease in humans. Current studies suggest that mothers with gum disease may give birth preterm or have a baby with low birth weight or both. Another study has shown that if gum disease is treated successfully then it may reduce the risk of preterm birth. This needs further testing.

Guidelines on oral care recommend that every expectant mother should receive a complete oral health evaluation, counseling on proper oral hygiene and necessary dental treatment. This is considered to be an important aspect of overall health in pregnant women.

The purpose of the study is to compare an oral water rinse test that measures intra-oral inflammation by counting neutrophils (a type of white blood cell) to a standard complete oral examination in a group of pregnant women, to evaluate if the oral rinse test reflects their gum health status. In addition, the study will evaluate if poor
gum health status as measured by the oral rinse test is associated with low birth weight baby or preterm birth or both.

**Procedures**

If you agree to participate in the study:

1) You will be asked to complete a medical and dental history questionnaire related to your health with your study doctor, which is designed to take no longer than 5 minutes.

2) You will be asked to rinse your mouth once with 10 mL of sterile water for 15 seconds, which will then be collected. Prior to the rinse, you will be instructed not to eat or drink for a minimum of 30 minutes to avoid clearance of the cells (neutrophils) that we are planning to count.

3) You will then have a complete dental examination similar to a regular examination at your dentist. We will examine your gums (including measurement of the gum pocket depths) and your teeth. No dental x-rays will be taken.

4) The findings of your dental examination will be discussed with you and if any oral problems are identified, you will be advised of options regarding follow-up and treatment. In addition, you will receive individualized instructions in oral hygiene (i.e. proper technique of brushing and flossing).

5) The entire procedure should not take any longer than 30 minutes and there are no additional hospital visits or investigations that are required as part of this study. The care that you receive during your pregnancy will be according to the hospital standards.

6) We will also require your permission to review information about your progress during the antenatal period (before birth), during birth and prior to your discharge home by examining your and your infant's hospital chart.

**Risks**

There is no foreseeable harm or injury as a result of participating in this study. The only inconvenience lies in the time spent participating in the study.

**Benefits**

A comprehensive oral examination has the potential benefit of detecting any oral disease, which may be managed thereafter at a dental clinic. Should any oral problems be identified during the study, you will be notified of your options regarding follow up and treatment. You will also be given individualized oral hygiene instructions to improve your oral health as required.

**Confidentiality**

If you agree to join this study, the study team will look at your personal health information and collect only the information they need for the study. Personal health information is any information that could be used to identify the patient and includes your name, date of birth, medical and dental history. The information that is collected for the study will be kept in a locked and secure area by the study doctor.
for 7 years. Only the study team involved will be allowed to look at your records. Your participation in this study also may be recorded in your medical record at this hospital.

Representatives of the Mount Sinai Hospital Research Ethics Board may look at the study records and at your personal health information to check that the information collected for the study is correct and to make sure the study followed proper laws and guidelines.

All information collected during this study, including the patient's personal health information, will be kept confidential and will not be shared with anyone outside the study unless required by law. You will not be named in any reports, publications, or presentations that may come from this study. Any information about you that is sent out of the hospital will have an assigned number and will not show the patient’s name or address, or any information that directly identifies the patient.

If you decide to leave the study, the information about you that was collected before you left the study will still be used. No new information will be collected without your permission.

Voluntary Participation
Your participation in this study is voluntary. You may decide not to be in this study, or to be in the study now and then change your mind later. You may leave the study at any time without affecting your care. You may refuse to answer any question you do not want to answer, or not to answer an interview question by saying "pass".

In Case You are Harmed in the Study
If you become ill, injured or harmed as a result of taking part in this study, you will receive care. The reasonable cost of such care will be covered for any injury, illness or harm that is directly a result of being in this study. In no way does signing this consent form waive your legal rights nor does it relieve the investigators or involved institutions from their legal and professional responsibilities. You do not give up any of your legal rights by signing this consent form.

Expenses Associated with Participating in the Study
You will not have to pay for any of the procedures involved with this study, including the complete dental examination, which will be done at no cost by the dentist investigator.

Conflict of Interest
The Mount Sinai Hospital Department of Dentistry will pay for the materials used in this study. The study team has an interest in completing this study. Their interest should not influence your decision to participate in this study. You should not feel pressurized to join this study.
Questions About the Study
If you have questions, concerns or would like to speak to the study team for any reason, please call: Dr. Michael Sigal at 416-586-1594 or Dr. Sabrina Huda at 416-979-4750 ext 3096.

If you have any questions about your rights as a research participant or have concerns about this study, call Ronald Heslegrave, Ph. D., Chair of the Mount Sinai Hospital Research Ethics Board (REB) or the Research Ethics office number at 416-586-4875. The REB is a group of people who oversee the ethical conduct of research studies. These people are not part of the study team. Everything that you discuss will be kept confidential.

Consent
This study has been explained to me and any questions I had have been answered. I know that I may leave the study at any time. I agree to take part in this study.

Print Study Participant's Name ___________________________ Signature ___________________________ Date ___________________________

(You will be given a signed copy of this consent form)

My signature means that I have explained the study to the participant named above. I have answered all questions.

Print Name of Person Obtaining Consent ___________________________ Signature ___________________________ Date ___________________________
Appendix C. Medical and dental history form.

---

**Medical and Dental History Form**

<table>
<thead>
<tr>
<th>Question</th>
<th>Y/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of Birth</td>
<td></td>
</tr>
<tr>
<td>Number of Week of Pregnancy</td>
<td></td>
</tr>
<tr>
<td>Are you currently in good health? Y / N</td>
<td></td>
</tr>
<tr>
<td>Have you ever been hospitalized? Y / N</td>
<td></td>
</tr>
<tr>
<td>Do you take any medication(s)? Y / N</td>
<td></td>
</tr>
<tr>
<td>Do you take any supplement(s)? Y/N</td>
<td></td>
</tr>
<tr>
<td>Have you taken any antibiotics in the last 2 weeks? Y/N</td>
<td></td>
</tr>
<tr>
<td>Do you have any allergies? Y/N</td>
<td></td>
</tr>
<tr>
<td>Do you smoke? Y/N</td>
<td></td>
</tr>
<tr>
<td>Do you drink alcohol? Y/N</td>
<td></td>
</tr>
<tr>
<td>Do you use any recreational drugs? Y/N</td>
<td></td>
</tr>
<tr>
<td>Do you take antibiotics before your dental appointment? Y / N</td>
<td></td>
</tr>
<tr>
<td>Do you have any problems related to:</td>
<td></td>
</tr>
<tr>
<td>Heart/Blood Pressure (Cardiovascular system)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Lungs (Respiratory system)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>Y/N</td>
</tr>
<tr>
<td>Immune system</td>
<td>Y/N</td>
</tr>
<tr>
<td>Digestive system (Gastrointestinal system)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Diabetes/Thyroid (Endocrine system)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Bleeding (Hematology)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Kidney/Bladder (Genitourinary system)</td>
<td>Y/N</td>
</tr>
<tr>
<td>Musculoskeletal system</td>
<td>Y/N</td>
</tr>
</tbody>
</table>
Medical and Dental History Form

<table>
<thead>
<tr>
<th>Last visit to the dentist (reason)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brushing frequency ___ 1x / 2x / 3x / &gt;3x per day</td>
</tr>
<tr>
<td>Fluoridated toothpaste ___ Y/N</td>
</tr>
<tr>
<td>Type of tooth brush ___ Electric or Manual</td>
</tr>
<tr>
<td>Flossing ___ Y / N  Frequency ___</td>
</tr>
<tr>
<td>Mouthwash ___ Y / N  Frequency ___</td>
</tr>
</tbody>
</table>
Appendix D. Definitions of conventional periodontal measures used in this study.

Probing depth *(Carranza's clinical periodontology, 2002)* = distance from free gingival margin to base of pocket or crevice

Clinical attachment loss (CAL) *(Armitage, 2004)*
- Defined as probing depth + recession = distance from cementoenamel junction to base of pocket or crevice
- When there is no gingival recession, CAL = pocket or crevice depth minus distance from the cementoenamel junction to the free gingival margin
- Relates to periodontal disease severity: Mild = 1 – 2 mm CAL, Moderate = 3 – 4 mm CAL, Severe ≥ 5 mm CAL

Modified Gingival Index *(Lobene, Weatherford, Ross, Lamm, & Menaker, 1986)*
0: Absence of inflammation
1: Mild inflammation: slight change in color, little change in texture of any portion of but not the entire marginal or papillary gingival unit
2: Mild inflammation: criteria as above but involving entire marginal or papillary gingival unit
3: Moderate inflammation: glazing, redness, edema, and/or hypertrophy of the marginal or papillary gingival unit
4: Severe inflammation: marked redness, edema, and/or hypertrophy of the marginal or papillary gingival unit; spontaneous bleeding, congestion, or ulceration

Plaque Index *(Ramfjord, 1959)*
0: No plaque present
1: Plaque present on some but not all of the interproximal and gingival surfaces of the tooth
2: Plaque present on all interproximal and gingival surfaces, but covering less than one half of entire clinical crown
3: Plaque extending over all interproximal and gingival surfaces covering more than one half of the entire clinical crown

**Calculus Index** (Ramfjord, 1959)

0: Absence of calculus

1: Supragingival calculus extending only slightly below the free gingival margin (not more than 1 mm)

2: Moderate amount of supra and subgingival calculus, or subgingival calculus only

3: An abundance of supra and subgingival calculus

**Bleeding Index** (*Carranza’s clinical periodontology, 2002*)

+ : Bleeding within 10 seconds from probed site

- : No bleeding noted

**Mobility** (S. C. Miller, 1950)

0: Normal mobility (physiologic)

I: First distinguishable sign of movement greater than normal

II: Movement of tooth which allows the crown the move 1 mm from its normal position in any direction

III: Allows the tooth to move more than 1 mm in any direction and/or may be rotated or depressed in their alveoli
Appendix E. Clinical examination form.

### Oral Examination Form

#### BUCCAL

<table>
<thead>
<tr>
<th>Date</th>
<th>18</th>
<th>17</th>
<th>16</th>
<th>15</th>
<th>14</th>
<th>13</th>
<th>12</th>
<th>11</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recession</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pocket Depth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobility</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### PALATAL

<table>
<thead>
<tr>
<th>Date</th>
<th>18</th>
<th>17</th>
<th>16</th>
<th>15</th>
<th>14</th>
<th>13</th>
<th>12</th>
<th>11</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recession</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pocket Depth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### LINGUAL

<table>
<thead>
<tr>
<th>Date</th>
<th>48</th>
<th>47</th>
<th>46</th>
<th>45</th>
<th>44</th>
<th>43</th>
<th>42</th>
<th>41</th>
<th>31</th>
<th>32</th>
<th>33</th>
<th>34</th>
<th>35</th>
<th>36</th>
<th>37</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recession</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pocket Depth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### BUCCAL

<table>
<thead>
<tr>
<th>Date</th>
<th>48</th>
<th>47</th>
<th>46</th>
<th>45</th>
<th>44</th>
<th>43</th>
<th>42</th>
<th>41</th>
<th>31</th>
<th>32</th>
<th>33</th>
<th>34</th>
<th>35</th>
<th>36</th>
<th>37</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recession</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pocket Depth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobility</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Modified Gingival Index

- Date
- Plaque Index
- Calculus Index
- Bleeding Index