Oral Inflammatory Load and Preterm Birth in Women at Risk for Preterm Birth

by

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

Faculty of Dentistry
University of Toronto

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Abstract
The primary aim of this cohort pilot study was to explore the possible association between oral inflammatory load (OIL) and spontaneous preterm birth (SPTB) in pregnant women at risk of preterm delivery. Counts of oral neutrophils were carried out using oral rinse samples. Traditional and non-invasive assessment measures of periodontal health were recorded and a blood sample was collected to assess MMP-9 and the fetuin (Ahsg) serum levels. The 35 participants enrolled in this study had a significant past pregnancy history with an average of 2 (±0.94) previous preterm. Extensive interventions were administered to reduce the risk of SPTB such as antibiotic therapy (77%, n=27). Participants were noted to have a low OIL with a mean neutrophil count of 2.05 (±0.80; 0.86-4.31) x 10^6 cells/ml. Pregnancy outcomes revealed that SPTB occurred in 40% (n=14). Although not statistically significant, increased oral neutrophil counts tended to be associated with SPTB.
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Review of Literature

1 Introduction

Preterm birth (PTB) and low birth weight of the infant (LBW) are adverse birth outcomes which have risen over time in developed countries. Even though technological advancement of the past decades has helped to increase the survival rates of very early preterm birth infants, actual birth prior to 37 weeks of gestation is still the leading cause of perinatal morbidity and mortality (Behrman et al., 2007). Moreover, the costs and morbidities associated with management of PTB are still a significant burden to the healthcare system. Multiple risk factors have been associated with PTB, including maternal infections (Goldenberg et al., 2000). Periodontitis, being, in part, a bacterially mediated infection could allow for periodontopathogenic bacteria to have direct access to the systemic circulation. Therefore, it has been suggested that the increased risk for bacteraemia alone could act as a potential risk factor for PTB. Indeed, multiple observational, interventional and mechanistic studies have been published aiming to describe this potential association, but results are inconclusive. Of course infection could be a key issue and will be discussed in more detail below, but it is also possible that since periodontitis is also a disease of inflammation that increases in oral inflammatory load (OIL) could also play a role as a risk factor either on its own or in concert with the microbes. The concept related to what is OIL as well as its potential impact on PTB will also be discussed in more detail below (see section 4 and 5). In any case, the most critical factor here relates to the putative association between periodontitis and complications of pregnancy. In this regard it is particularly important from the standpoint of the possibility that periodontitis as reflected by high OIL is an actual risk factor for the complications of pregnancy. As will also be discussed below (see section 15), if periodontitis is demonstrated to be a risk factor that, when treated, might improve pregnancy outcomes, the potential impact on infant health, family wellbeing, and the costs of medical management could be enormous.
2 Preterm Birth

2.1 Preterm Birth Definition

According to the World Health Organization, PTB is defined as an infant born before 37 completed weeks of gestation. There are sub-categories of PTB based on gestational age, namely: extreme prematurity (<28 weeks), severe prematurity or very preterm (28-31 weeks), moderate prematurity (32-33 weeks) and near term or late preterm (34-36 weeks).

The preterm birth rate in Canada was 8.1% in 2006-2007 (Behrman et al., 2007) and 12.2% in the USA in 2009 (Martin et al., 2009). Moderate to late preterm birth accounts for the majority of all preterm births, and for 85% of all preterm births in Canada (Moutquin, 2003), whereas very preterm and extremely preterm represent 15% and 5% of the total number of preterm births (Goldenberg et al., 2008).

Despite increasing knowledge about preterm birth risk factors, mechanisms related to preterm birth as well as somewhat improved strategies for prevention of PTB, the rate of preterm births in most industrialized countries has continued to increase, for example from 9.5% in 1981 to 12.7% in 2005 in the USA (Hamilton et al., 2009). This increase has been attributed to the improvement in diagnosis of maternal and fetal indications and as well as the well-documented increase in the prevalence of multiple gestation pregnancies associated with assisted reproductive therapy (Goldenberg & Culhane, 2006), the latter being a known risk factor for preterm birth. In fact, 50% of twins are born preterm (Goldenberg & Culhane, 2006).

PTB can be medically indicated and result from an induced labour or caesarean section for maternal or fetal indications (30-35%). However, in most cases PTB occurs spontaneously (65-75%; Goldenberg et al., 2008). Medical indications for medically indicated (elective) preterm birth include, but are not limited to, maternal hypertension, placental abruption, intrauterine growth restriction (IUGR) and fetal distress (Moutquin, 2003). Spontaneous preterm birth (SPTB) occurs as a consequence of preterm labour (PTL) (40-45%), preterm premature rupture of membranes (PPROM; 25-35%) and/or incompetent cervix (Goldenberg et al., 2008). Spontaneous preterm labour is more frequent in White women, whereas PPROM is more frequent in Black women (Ananth & Vintzileos, 2006).
PPROM is defined as spontaneous rupture of the membranes at less than 37 weeks of gestation at least one hour before the onset of contractions (Goldenberg et al., 2008). The development of intrauterine infection and preterm labour are common complications of PPROM due to the loss of protection from the membranes. After experiencing PPROM, most women will deliver within days, but it may take weeks or months in some others (Goldenberg et al., 2008).

Preterm labour is defined as regular contractions accompanied by cervical change at less than 37 weeks of gestation (Goldenberg et al., 2008).

2.2 Significance of Preterm Birth

As alluded to above, adverse pregnancy outcomes such as PTB delivery can have major consequences that have significant impacts not only on the individuals, but also on their families and the health care system. Although most of the organs of prematurely born infants are immature, the brain and the respiratory system are the systems primarily susceptible to complications arising from PTB (Saigal & Doyle, 2008).

Survival rates of preterm infants have improved greatly in recent years; however, preterm births still account for 75% of perinatal mortality (McCormick, 1985). Understandably then, after delivery, the neonatal intensive care unit plays a crucial role in the survival of the preterm infant and in fact, neonatal PTB mortality rates have been reported to be higher in hospitals without a neonatal intensive care unit (Cifuentes et al., 2002). Immediate morbidities associated with late preterm birth are substantial, including higher rates of temperature instability, respiratory distress, apnea, hypoglycemia, seizures, jaundice, kernicterus, feeding difficulties, periventricular leucomalacia and re-hospitalizations (Escobar et al., 2006). Therefore, for the safety of the mother and the infant, it is generally recommended that high-risk mothers should be transferred to a hospital with a perinatal center prior to delivery (Saigal & Doyle, 2008).

Observational studies on morbidity in infants are often based on data categorized by the infant birthweight described as: low (<2500g), very low (<1500g) and extremely low birthweight (<1000g). LBW may result from preterm birth, intrauterine growth restriction or both (Lim et al., 2009). About 6% of babies are born with LBW in Canada each year (Statistic Canada, 2008).
Preterm infants are at higher risk for the development of medical conditions compared to full term infants, especially during the early years of life. One of the most common morbidities in infants born before 26 weeks gestation is retinopathy of prematurity (Farooqi, 2006). Bronchopulmonary dysplasia is also common, being reported in 40% of very low birth weight (VLBW) infants (Saigal & Doyle, 2008). VLBW infants tend to achieve normal adult stature but they are also inclined to develop problems with growth as well as with weight gain throughout infancy and early childhood (Finnstrom et al., 1998).

Approximately one in four PTB infants are reported to have substantial neurological morbidity such as cerebral palsy, developmental delay and/or sensory impairments (visual or auditory) (Saigal & Doyle, 2008). The prevalence of cerebral palsy is inversely related to the gestational age of the infant (Saigal & Doyle, 2008). In the early years of life, preterm infants also tend to have a higher prevalence of minor neuromotor dysfunction and poorer coordination when compared to term infants (Davis et al., 2007). Cognitive deficits, academic underachievement, grade failures and the need for increased remedial educational assistance are all consequences associated with very low birth weight (VLBW). These cognitive disadvantages seem to persist into late adolescence and early adulthood (Saigal & Doyle, 2008). Adverse birth outcomes can also affect the behavior of premature infants during their childhood and adult life. For example, premature birth has been associated with an increased risk of attention deficit hyperactivity disorder and several traits such as shyness, unassertiveness, social maladaptation, anxiety and withdrawn appearance (Aylward, 2005).

Adverse birth outcomes can also have a deleterious effect on the teeth. Molar Incisor Hypomineralization and enamel developmental defects in molars and incisors are increased in children who were preterm, had low gestational age or were LBW infants (Brogardh-Roth et al., 2011). These children also exhibit higher plaque accumulation and higher associated gingival inflammation; possibly due to the rough surfaces of the teeth and the potential sensitivity associated with these teeth which might interfere with toothbrushing (Brogardh-Roth et al., 2011).

The higher prevalence of mental and emotional delays, visual and hearing deficits, and restriction in activities of daily living and as well as reductions in self-care abilities throughout childhood and adolescence seen in preterm infants affect not only the infant but also the family (Saigal &
Doyle, 2008). This often results in higher stress in the parents of preterm infants (Singer et al., 1999).

The higher rate of mortality and morbidity associated with PTLBW has been correlated with increases in the costs of health care and this is due largely to the need for the use of specialized equipment, longer length of hospital stays, higher rates of hospital re-admission and increased requirements for personal resources for health care, not only during infancy but until adulthood (Saigal & Doyle, 2008). The average hospital cost for a singleton newborn was approximately nine times higher for a preterm newborn than for a term infant. The highest average cost of care, in range of $84,235, has been reported for extremely preterm infants while their average stay in hospital was about 40 times greater than that seen for full term infants (2005-2006 in Canada; CIHI, 2009). These increased costs varied widely from $1,000 to $117,000, being inversely proportional to birth weight (Lim, 2009). Consequently, newborns born both preterm and small for gestational age (SGA; smaller than 90% of babies of the same gestational age) had almost twice the average hospital cost compared to normal weight for gestational age preterm infant ($16,244 compared to $8,558; CIHI, 2009). These costs do not include the increased costs related to maternal care due to management of a high risk pregnancy, nor the cost of frequent re-admission during early childhood of the preterm child. Considering the increasing costs associated with neonatal intensive care, some authors have even questioned whether intensive care is justified for infants of borderline viability (Saigal & Doyle, 2008).

### 2.3 Preterm Birth Pathogenesis

The pathogenesis of PPROM has not been explained completely, but asymptomatic intrauterine infection has been recognized as a common precursor (Romero et al., 1988). The pathogenesis of preterm labour and subsequent preterm birth is not well understood but is suspected to be the result of an idiopathic activation of the normal labour process or of a pathologic insult to normal gestational function, including infection (identified or not), inflammation, as will be emphasized in this investigation, uteroplacental ischemia or hemorrhage, uterine overdistension, abnormal allograft reaction, allergy, cervical insufficiency, hormonal
disorders (progesterone related and corticotrophin-releasing factor related), stress and other immunologically mediated processes (Romero, 2006).

2.4 Risk Factors Associated with Preterm Birth

Multiple variables have been associated with increased risk for adverse outcomes of pregnancy. Previous and present pregnancy history can reveal significant risk factors such as previous preterm delivery, multiple gestations, premature uterine contractions, shortened cervical length and fertility treatment, the latter predisposing to multiple gestation-related risks. Genetic, demographic and environmental factors have also been associated with increased risk in PTB such as maternal nutrition, smoking, stress, depression and infections (Goldenberg et al., 2008). Despite the current knowledge on preterm birth risk factors, more than 50% of preterm births occur in pregnancies with no identified risk factors (Iams et al., 2008).

2.4.1 Demographic and Medical History Risk Factors

Several maternal risk factors have been associated with preterm birth. African-American and Afro-Caribbean women are at higher risk for preterm delivery with a preterm birth rate of 16-18%, whereas East Asian and Hispanic women typically have low preterm birth rates (Goldenberg et al., 2008). Women with a low (<19.8 kg/m²) body-mass index (BMI) are also at greater risk for preterm birth (Mercer et al., 1996), whereas obesity (>35 kg/m²) is thought to reduce the risk (Hendler & Goldenberg, 2005). However, obese pregnant women are at higher risk for congenital anomalies, pre-eclampsia and diabetes (Goldenberg et al., 2008), all of which can lead to significant medical sequelae. Low serum levels of iron, folate and zinc have also been associated with preterm birth (Scholl, 2005; Tamura et al., 1992). Other demographic factors such as low socioeconomic and educational status, low (<16 years old) and higher maternal age (>35 years old) and single marital status are also risk factors associated with preterm birth (Smith et al., 2009; Goldenberg et al., 2008). Tobacco usage is associated with 5-8% of PTB (Cypher, 2012). Maternal medical history consistent with thyroid disease, asthma, diabetes and hypertension has also been associated with an increased risk for PTB (Goldenberg et al., 2008).
2.4.2 Past Pregnancy History Risk Factors

An interpregnancy interval of less than 6 months might increase the risk of preterm birth by two fold (Smith et al., 2003). Women with a previous pregnancy history of preterm birth are more at risk for preterm birth in subsequent pregnancies, with preterm birth rates from 15% to 50% depending on the number of previous preterm birth and the gestational age at which they occurred (Mercer et al., 1999). Women who experienced previous spontaneous preterm birth are more at risk to experience SPTB in the future and likewise, women who previously had an indicated PTB are more likely to have a similar birth outcome in future pregnancies (Ananth et al., 2006). Previous history of cervical cone biopsy or loop electrodautery excision secondary to premalignant disorders has also been recognized as a risk factor for PTB (Goldenberg et al., 2008). Gynecological diseases such as fibromatosis, endometriosis and polycystic ovary syndrome have been correlated with an increased incidence of PTB (Torricelli et al., 2012). Several uterus anomalies, such as the presence of a septum, are associated with an increased risk for PTB (Goldenberg et al., 2008). Genetic factors have been associated with PTB; for example, women with a positive family (sister, grandparents) history of PTB have an increased risk of delivering prematurely themselves (Winkvist et al., 1998). Recent studies have also demonstrated that specific maternal polymorphisms that are associated with inflammation or thrombophilia, might also be correlated to increased risk for PTB (Torricelli et al., 2012).

2.4.3 Pregnancy Risk Factors

Vaginal bleeding, which may be caused by placental abruption or placenta preavia, is also associated with PTB (Goldenberg et al., 2008). Due to the increased serum concentrations of inflammatory markers such as C-reactive protein, women experiencing psychological or social stress during their pregnancy are suspected to be at higher risk for PTB (Wadhwa et al., 2001). Some studies have also suggested that there is a relationship between maternal depression and PTB (Dayan et al., 2006). Intrauterine infection is an important mechanism leading to PTB as it may account for more than 25-40% of preterm births (Goldenberg et al., 2000). Through the activation of the innate immune system by the release of proinflammatory cytokines and prostaglandins, uterine contractions are stimulated and a degradation of extracellular matrix in the membranes can be observed, leading to PPROM (Goldenberg et al., 2008). Microorganisms are suspected to reach the amniotic cavity, most commonly ascending from the vagina but also by systemic dissemination through the placenta, by introduction at the time of invasive procedures
(amniocentesis) or by retrograde spread through the fallopian tubes (Goldenberg et al., 2000). The colonization can happen any time during or before the pregnancy. Intrauterine infection can lead to fetal infection, which has been linked to preterm labour and fetal injury and/or long-term medical consequences such as cerebral palsy and chronic lung disease (Romero et al., 1998). Therefore, genital tract infections such as bacterial vaginosis, but also non-genital tract infections such as pyelonephritis, pneumonia, appendicitis and potentially subclinical asymptomatic bacteriuria, all of which also lead of course to increased inflammatory load, can predispose to PTB (Goldenberg et al., 2008). Approaching labour, the cervix shortens and dilates. A short cervix length of less than 30mm at 16-20 weeks and 25mm (less than 10th percentile) at 20-24 weeks of gestation has been associated with an increased risk for SPTB before 35 weeks. The shorter the cervix, the greater the risk for PTB (Iams et al., 1996). Congenital cervical weakness, surgery or trauma causing cervical insufficiency have been reported to have a causal relationship with PTB (Goldenberg et al., 2008).

2.5 Prevention of Preterm Birth

Intervention aimed at reducing the morbidity and mortality associated with preterm birth can be directed towards all women prior to or during pregnancy (primary interventions), directed at women with known risk factors (secondary interventions) or can be initiated after signs of imminent delivery have been recognized (tertiary interventions) (Iams et al., 2008). The most commonly performed interventions are tertiary and aim primarily at improving the outcomes for preterm infants more than reducing the rate of preterm birth.

2.5.1 Primary Interventions

Public educational interventions can inform the public about avoidable risks associated with PTB and encourage lifestyle changes such as smoking cessation and prenatal supplement usage prior to conception (Iams et al., 2008). Public and professional policies can also be effective as primary intervention. For example, policies aiming to protect pregnant women in their workplace by improving safety and reducing their weekly working hours can immediately reduce the preterm rate (Iams et al., 2008). Primary prevention strategies during pregnancy include the recommendation of treatment such as multivitamin supplementation and the use of screening tests.
such as urine culture to identify risk factors (i.e. urinary tract and/or bladder infection) and provide adequate treatment. A significant reduction in preterm birth rate in women supplemented with calcium was not demonstrated while there was a reduction in pre-eclampsia as reported in a Cochrane review (Hofmeyr et al., 2006). Pre-eclampsia is a disorder of the pregnancy characterized by maternal hypertension, impaired liver and kidney function, proteinuria, thrombocytopenia, pulmonary edema and visual disturbances. This condition can be life-threatening for the mother and therefore, in these cases delivery might need to be induced and this could of course also mean that a preterm delivery will be the result (Hofmeyr et al., 2006). Prenatal and periodontal care have also been suggested as preventive interventions (Iams et al., 2008). Screening of low-risk women for asymptomatic bacteriuria, presence of fetal fibronectin in cervicovaginal fluid, and cervical length through sonography can identify women at risk for PTB and adequate intervention can be established (Iams et al., 2009).

2.5.2 Secondary Interventions

Preventive intervention can be recommended for women at risk for preterm birth, either on the basis of their obstetrical history or following the identification of risk factors in the current pregnancy. Supplementation with omega-3 polyunsaturated fatty acids may lead to a reduction in the incidence of spontaneous preterm birth due to its ability to cause reductions in the production of inflammatory mediators (Olsen et al., 2000). Antibiotic therapy before 20 weeks of gestation has been suggested to reduce the risk of preterm birth, but the results of clinical trials are thus far inconclusive (Iams et al., 2008). Progesterone therapy, through its ability to reduce the formation of gap-junctions, as well as the fact that it antagonizes oxytocin, might be protective against preterm birth. Other mechanisms postulated to explain the protective effects of progesterone also include its ability to maintain cervical integrity as well as the fact that progesterone itself has anti-inflammatory effects (Iams et al., 2008). A meta-analysis including five randomized controlled trials showed that progesterone was associated with a statistically significant reduction in the risk of preterm birth less than 34 weeks (average RR 0.31, 95% CI 0.14-0.69; Dodd et al., 2006; Sanchez-Ramos et al., 2005). As discussed above, cervical length is inversely related to PTB. Consequently, cerclage, a surgically placed suture at the top portion of the cervix, is an appropriate means of reducing PTB risk when this structural defect is identified earlier on in the pregnancy and should strength the cervix thereby lowering the risk for PTB. There are three types of cerclage: elective or prophylactic (cervical insufficiency and history of preterm loss), urgent or indicated
(short cervical length or funneling) and emergency or rescue (advanced dilation or bulging membranes) (Cypher, 2012). In a meta-analysis, the risk of PTB was shown to be reduced by the placement of cerclage in women with a previous PTB history and a cervix of less than 2.5 cm (RR 0.63 95% CI 0.48-0.85; Berghella et al., 2005).

2.5.3 Tertiary Interventions

Following the recognition of imminent preterm labour, established by progressive cervical dilation and effacement or membrane rupture, several treatments can be recommended to reduce neonatal morbidity and mortality, such as antibiotic treatment in case of infection (Iams et al., 2008). Also, a single antenatal dose of corticosteroids to the mother reduces the risk of respiratory distress, intraventricular haemorrhage, necrotizing enterocolitis and patent ductus arteriosus (Wapner et al., 2006). Lastly, the administration of tocolysis drugs such as calcium-channel antagonist drugs, magnesium and oxytocin antagonist, has been suggested to delay the delivery following PTL of 2 days, allowing enough time for corticosteroid and antibiotic administration and the transfer of the pregnant woman to a specialized maternal delivery and neonatal unit (Iams et al., 2008).

3 Periodontal Health

There has been a groundswell of research that has linked the presence of maternal periodontitis to increased risks for adverse outcomes of pregnancy, as alluded to above. If this is indeed the case, and as mentioned previously, successful treatment or prevention of periodontitis (preferably) before pregnancy could hypothetically have an extremely important impact on the reduction of the risk for adverse outcomes of pregnancy. Yet, the nature of periodontitis is such that it is at the same time considered to be both a chronic infection as well as principally a disease of inflammation mediated largely by polymorphonuclear neutrophils (PMNs). Thus in addition to understanding issues pertaining to PTB, it is also critically important to develop an understanding of the underlying pathophysiological mechanisms that regulate the development and progression of periodontitis. Once done, the pathophysiological processes so identified, can be placed more realistically in context with the issues surrounding the complications of pregnancy.
3.1 Periodontal Diseases: Definition and Classification

There are two main categories of periodontal disease: gingivitis and periodontitis (although there are also several subcategories of periodontitis as will be discussed hereunder). Gingivitis is characterized as involving the periodontium with no gingival attachment loss on the tooth, whereas periodontitis involves a certain degree of destruction of supporting structures of the periodontium with attachment loss.

Clinically, gingivitis is characterized by gingival redness, edema, bleeding, changes in contour, loss of tissue adaptation to the teeth and increased gingival crevicular fluid (GCF) flow (American Academy of Periodontology Committee, 1999; Greenstein, 1984). Over 50% of adults in the United States suffer from gingivitis without accompanying periodontitis with an average of 6 or more teeth affected (Oliver, Brown & Loe, 1998). The prevalence of individuals with gingivitis without periodontitis is less reported due to the lack of standardization of disease measurements (Eke et al., 2015). Gingivitis can be either plaque or non-plaque induced. Plaque induced gingivitis can be modified by systemic factors. Several systemic conditions have been noted to cause pathologic changes in the gingival tissues similar to gingivitis such as changes in the endocrine system, affection by conditions mimicking the vascular alterations seen in plaque induced gingivitis or causing cellular infiltration (such as leukocytes), usage of certain medications, and malnutrition (Armitage, 1999; American Academy of Periodontology Committee, 1999).

Periodontitis is generally classified into three main categories: chronic disease, aggressive disease and manifestation of non-oral systemic diseases. Clinical attachment loss, alveolar bone loss, periodontal pocketing and gingival inflammation are the main clinical features of periodontitis (Flemmig, 1999). Chronic and aggressive diseases are subdivided further on the basis of their location (localized or generalized) and severity. The amount of clinical attachment loss (CAL) is the usual evaluation measurement used to determine the severity of the disease (Armitage, 1999). The extent of the disease is described as low in case of 1-10 disease sites, medium for 11-20 sites or high for over 20 affected sites (Flemmig, 1999). As a general rule, periodontitis can be characterized as localized if 30% or less of the sites are involved and generalized if more than 30% of the sites are involved (Armitage 1999). The severity of periodontitis is differentiated as mild (1 to 2 mm), moderate (3-4 mm) or severe (≥5mm) CAL (Flemmig, 1999). A time consuming
complete periodontal examination with probing depths is necessary to establish the severity when this definition is utilized.

Chronic periodontitis is the most common form of periodontitis. 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). Reflecting a lifetime progression of disease accumulation, both the prevalence and the extent of periodontitis diseases increase with age (Oliver et al., 1998). Taking the 1985-86 NIDR national survey, the proportion of adult aged 25-34 year olds with ≥4mm CAL was 13.8% and 53.6% for the 55-64 year olds. The prevalence of this disease is difficult to establish due to the variations in definition and in measurement methodologies of the disease between studies. The prevalence of periodontitis also appears to vary between races and upon geographic location (Papapanou, 1996). Epidemiologic studies have demonstrated that Blacks, adults with less than a high-school degree, adults with an income of less than $20 000, and those who have not seen a dentist in the past 2 years were more affected by periodontitis than their counterparts (Oliver et al., 1991). Several studies have demonstrated that diabetics are more likely to have periodontitis than healthy individuals (Katz et al., 1991). Also, poorly controlled diabetes has been associated with more extensive calculus, pockets and more tooth loss (Tervonen & Oliver, 1993). Smoking has long been a known risk factor for the development and progression of periodontitis (Ismail et al., 1983). Recently, an association between obesity (BMI>29) and periodontitis was found in several studies (Chaffee & Weston, 2010; Morita et al., 2011; Stabholz et al., 2010). Lastly, stress, measured in terms of adverse life events and clinical depression, seems to be associated with progressive periodontitis (Genco et al., 1999).

Overall, the prevalence of the mild form of chronic periodontitis appears to be common, while the more severe forms are less prevalent. According to the last NHANES, in 2009-2012 46% of adults in the United States had periodontitis, with 8.9% having severe periodontitis (Eke et al., 2015). Burt, in 2005, reported that 5% to 15% of any population suffers from severe generalized periodontitis and that the majority of adults have a moderate form of the disease. In 2007-2009 in Canada, the prevalence of severe periodontitis (pocket depth ≥6mm) was reported to be 4%, whereas 16% of adults were found to have moderate periodontitis with at least one pocket with a depth of 4-5 mm (Health Canada, 2010).
There is often a familial pattern associated with aggressive periodontitis. This disease is characterized by rapid loss of attachment and destruction of the alveolar bone (Lang et al., 1999). Also, the amount of microbial deposits is usually inconsistent with the severity of periodontal tissue destruction. This type of periodontitis is often associated with phagocyte abnormalities and a hyper-responsive macrophage phenotype (Lang et al., 1999).

It is noteworthy that the prevalence, extent and severity of periodontitis and gingivitis have decreased since 1981 in the United States (Oliver et al., 1998). This decline has been attributed to improved oral hygiene and increased access to care (Oliver et al., 1998).

3.2 Periodontal Diseases: Etiology and Pathogenesis

Initially, the lesion appears as an inflammatory response characterized by redness and mild swelling of the gingival margin and bleeding on probing of the affected area (American Academy of Periodontology Committee, 1999). Following plaque accumulation, bacteria and their by-products present in the gingival sulcus invade the underlying connective tissue and provide chemotactic stimuli for the migration of inflammatory cells, the PMNs mentioned above, causing pathological changes by direct and indirect means (Page, 1986). The PMNs release collagenase and other endopeptidases, resulting in the degradation of collagen in the marginal gingival connective tissues (Payne et al., 1975). Within a week, T-lymphocytes, macrophages and plasma cells infiltrate the area and along with the PMNs, contribute to the continuing destruction of the gingival connective tissues and the pathologic alteration of the resident fibroblasts (American Academy of Periodontology Committee, 1999; Hellden et al., 1973). The disease is referred to as an 'established lesion' when it appears to have a high degree of organization with plasma cells and B-lymphocytes predominating the lesion (American Academy of Periodontology Committee, 1999). Some established lesions stay stable and do not progress for months or years. In other individuals, lesions stay active and convert into destructive lesions (Jeffcoat et al., 1991). The usual treatment of gingivitis is simple and consists of a meticulous removal of the plaque accumulation. Once the lesions have entered the destructive phase, periodontitis, the treatment needs to be more extensive (American Academy of Periodontology Committee, 1999).
The pathophysiology of the transition from gingivitis to periodontitis is not well understood as periodontitis is not considered an inevitable consequence of gingivitis (American Academy of Periodontology Committee, 1999). It is widely accepted that the initiation and progression of periodontitis are dependent upon the presence of microorganisms capable of causing the disease, however it is also agreed that these microorganisms are necessary but not sufficient to cause periodontitis (Bartold & Van Dyke, 2013). Indeed, it is conceivable that periodontal pathogenic bacteria are opportunistic and become pathologically important only on a canvas of inflammatory disease (Bartold & Van Dyke, 2013). Regardless, both aerobic and anaerobic micro-organisms are present in the mouth, but the progression of chronic periodontitis is associated with increased levels of several gram-negative and anaerobic micro-organisms (Ximenez-Fyvie et al., 2000). *Aggregatibacter actinomycetemcomitans* is often associated with aggressive periodontitis (Schacher et al., 2007) whereas *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* are associated with chronic periodontitis (Socransky & Haffejee, 2005). As alluded to above, periodontitis is a multifactorial disease and destructive organisms are considered necessary but not sufficient to induce the disease (Dennison & Van Dyke; 1997). The progression of the periodontal destruction depends on the host inflammatory response to the virulent organism invasion although as noted previously a bidirectional relationship is now being recognized between host-mediated inflammation and changes in the microbiota associated with progressive periodontal disease. This host response is modulated by both genetic and environmental risks factors (Page et al., 1997). Cellular components such as monocytes and fibroblasts are stimulated by bacterial products such as LPS and will produce cytokines and enzymes capable of destroying the host tissues. This results in an inflammatory response and catabolic processes leading to bone and collagen destruction via the secreted matrix metalloproteinases (MMPs) (Yakob et al., 2013). As a result, the patient presents clinically with periodontal pocketing and apical location below the cemento-enamel junction of the junctional epithelium. Histologically, a loss of collagen fibers subjacent to the pocket epithelium, numerous PMNs in the junctional and pocket epithelium, and a dense inflammatory cell infiltrate with plasma cells, lymphocytes and macrophages can be isolated (Page et al., 1976). Some clinical models suggest that the progression of this chronic disease is marked by alternating episodes of quiescence and activity with varying intensity (Locker et al., 1998; Socransky et al., 1984) as opposed to a slow and continuous pattern of attachment loss over a long period of time.
4 Periodontal Health and Preterm Birth

Epidemiological, interventional and mechanistic studies have been published with sufficient evidence to suggest an association between maternal periodontitis and adverse pregnancy outcomes, but no studies have demonstrated conclusively that periodontitis is a significant and unequivocal risk factor for PTB delivery. This could be due to the multifaceted nature of both periodontitis and PTB delivery or to the relatively unreliable nature of the approaches used for assessment of periodontitis itself as well as an understanding as to whether the condition under study is in fact active or quiescent as noted previously. It is also possible that target populations under study have varied, particularly with regard to socioeconomic status or development, to the point that consistent data regarding the putative linkages between periodontitis and adverse outcomes of pregnancy have been difficult to establish unequivocally.

4.1 Pregnancy and Periodontal Disease

Pregnant women have been shown to have a greater prevalence (30-50%) of gingivitis compared to non-pregnant women (Loe, Silness 1963; Ziskin & Nesse 1946). The reported prevalence for periodontitis in pregnant women is 20-50% (Lieff et al., 2004; Vogt et al., 2012). During the course of their pregnancy, women tend to suffer a decline in their periodontal health and suffer from the exacerbation of preexisting unfavorable periodontal conditions (Laine, 2002; Moss et al., 2005). Hormonal changes through increased production of oestrogens and progesterone are suspected to be the cause of the increased risk for gingival and periodontal diseases during pregnancy (Laine, 2002).

Multiple mechanisms have been suggested to explain how hormonal changes increase the susceptibility to periodontal diseases. Firstly, gingival inflammation could be due to the increased vascular flow caused by changes in hormone levels, resulting in greater vascular permeability, gingival edema and increased prostaglandin production (Amar & Chung, 2000). Also, during pregnancy there is a change in the immune system and change in connective tissue metabolism. For example, the number of neutrophils increases during pregnancy and their function is altered resulting in a gingival tissue which is less resistant to infection (Barriga, Rodriguez & Ortega,
1994). In addition, there is a decrease in IL-6 production, which will also lower the resistance to infection (Lapp, Thomas & Lewis, 1995). The increase in severity and occurrence observed during pregnancy does not seem to cause lasting injuries to the periodontium as the pathologic changes usually subside after parturition (Loe & Silness, 1963).

4.2 Epidemiological Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

Poor periodontal health was first proposed as a potential cause for preterm birth in 1996 (Offenbacher et al., 1996). Numerous studies looking at the possible association between periodontal disease and treatment of periodontal disease and adverse pregnancy outcomes such as preterm birth, LBW, miscarriage and preeclampsia have been published (see Appendix I and II).

Yet, there is considerable variation in the findings published in these mostly observational or retrospective cross-sectional studies concerning the potential association between periodontal disease and different adverse pregnancy outcomes such as PTLBW, LBW, preterm birth, very preterm birth, preeclampsia and miscarriage or still birth (see Table Appendix I). This could be due to the inconsistency in the definitions of both periodontal diseases and adverse pregnancy outcomes (Kassab et al., 2011; Manau et al., 2008). For example, the term PTLBW should not be used as an outcome. Preterm birth and low birth weight have different etiologies and are different albeit related clinical entities; therefore, they should be assessed as separate outcomes (Xiong et al., 2007). Also, spontaneous PTB and indicated PTB have different etiologies and therefore, should be analyzed separately. In addition, difficulty in accounting for the multiple confounding variables in the population recruitment and in the statistical analysis may invalidate some results (Xiong et al., 2007). Some studies also have a small sample size, thus may be lacking power to recognize any correlation (Xiong et al., 2007).

Recently, two meta-analyses revealed that, taken together, the available observational studies suggesting a possible relationship between periodontitis and preterm birth. Vergnes and Sixou (2007) assessed the effect of maternal periodontal disease on preterm delivery and/or birth of low-weight children. Seventeen articles met the inclusion criteria, consisting of 7151 pregnant women, 1056 of whom delivered a preterm and/or low birth weight infant. The meta-analysis suggested a
2.27 times higher chance of preterm birth among mothers with periodontitis (OR 2.27, 95% CI 1.06-4.85, $P<0.05$). The definition for both adverse pregnancy outcome and periodontitis was shown to vary among studies. Periodontal disease measurements were variable across studies and could include, for example, CAL, bleeding on probing (BOP), Gingival Index, Plaque Index, Calculus Index, and periodontal pocket measurements. Also, studies included in this meta-analysis were noted to be of variable quality, especially due to the inconsistency in reporting confounders (Vergnes & Sixou, 2007). Chambrone et al. (2011) aimed to evaluate the association between the incidence of preterm birth and/or low birth weight infants and periodontitis and to assess the methodological quality of the selected prospective cohort studies. Potential articles ($n=1680$) were identified, of which 12 articles were included. A high level of methodological quality was found in 10 of the publications. The meta-analysis demonstrated a risk of preterm delivery in pregnant women with periodontitis (RR 1.70, 95% confidence interval, CI 1.03, 2.81; Chambrone et al., 2011). Both studies highlighted the necessity of further fundamental investigations, well-conducted, large, multicenter observational studies and randomized controlled trials before any causation relationship or strong association can be asserted between preterm birth and periodontitis.

### 4.3 Interventional Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

Similarly, interventional studies have not utilized universal definitions of periodontal diseases and treatments and involve different populations which has likely led to inconsistent results that have made it impossible to generalize to any population (Han, 2011).

After reviewing 11 trials (6558 women), a meta-analysis by Polyzos et al. (2010) concluded that results among studies with high and low methodological quality trials were consistently diverse. Among the high quality studies, treatment had no significant effect on the overall rate of PTB, whereas the lower quality research demonstrated beneficial effects with treatment.

Several randomized controlled trials with large sample sizes conducted in the United States and in Australia failed to find a reduced incidence of preterm birth or LBW following periodontal therapy (Macones et al., 2010; Michalowicz et al., 2006; Newnham et al., 2009; Offenbacher et
al., 2009). Conversely, multiple studies conducted in developing countries, enrolling specific minorities or subgroups (such as women with low socioeconomic status [Mitchell-Lewis et al., 2001] and women at high risk of PTB [Radnai et al., 2009]), pilot studies and clinical trials with small sample size have suggested that periodontal treatment can reduce the incidence of adverse pregnancy outcomes (Lopez et al., 2002; Tarannum et al., 2007) (see Appendix II). Consequently, it has been suggested that periodontal health may affect the birth outcome in a subpopulation rather than in the general population (Han 2011). Large population multicenter trials have failed to demonstrate a positive effect of periodontal treatment (Macones et al., 2010; Offenbacher et al., 2009), while trials involving a defined subpopulation executed in one enrollment center have tended to demonstrate the effectiveness of periodontal treatment (Lopez et al., 2005; Radnai et al., 2009).

It has been proposed that the periodontal treatment provided could also affect the observed result. Indeed, only one or two periodontal therapy and hygiene appointments during the course of the pregnancy may not be sufficient and effective in eliminating the active periodontal disease or in preventing the progression of the disease (Offenbacher et al., 2009). Also, the timing of the periodontal treatment may be inadequate to produce the desired effect. All the published trials intervene during the course of the pregnancy, but treating the patients while they are already pregnant may be too late to reverse the already established local and systemic inflammation (Goldenberg & Culhane, 2006; Xiong, 2011). Conversely, an early treatment may not prevent the periodontal condition of the pregnant woman from worsening during the course of her pregnancy (Han et al., 2011). Less severe periodontitis and gingivitis are more responsive to treatment with faster positive results. Therefore, the successful treatment of such disease in trials may produce a more measurable improvement in birth outcomes as opposed to that observed in someone with a more severe form of periodontal disease (Han, 2011; Polyzos et al., 2010). Lastly, only a few studies verified the success of the periodontal treatment provided during their study. Consequently, if a treatment is ineffective, this could affect the incidence of preterm birth (Polyzos et al., 2010).

Nevertheless, after the completion of a few well-designed, large scale randomized trials (see Appendix II), four comprehensive meta-analyses (Chambrone et al., 2011; Da Rosa et al., 2012; Polyzos et al., 2010; Xiong et al., 2007) have been published examining whether periodontal treatment during pregnancy is associated with a reduction in PTB rate. All recent meta-analyses
consistently concluded that the traditional treatment of periodontal disease with scaling and root planing cannot be considered to be an efficient way of reducing the incidence of preterm birth. Polyzos et al. in 2010 demonstrated no significant effect on the overall rate of preterm birth after periodontal treatment (OR=1.15, 95% CI: 1.15-1.40; P=0.15). Furthermore, they also demonstrated that periodontal treatment did not reduce the rate of low birthweight infants and spontaneous abortions/stillbirths. Da Rosa et al. in 2012 concluded that the treatment of periodontal disease during pregnancy resulted in a non-significant reduction in preterm births (RR= 0.90; 95% CI: 0.68-1.19) and low birth weights (RR= 0.92; 95% CI: 0.71-1.20). Xiong et al. (2007) and Chambrone et al. in 2011 also had similar results and conclusions. Despite the lack of demonstrated efficacy for periodontal treatment in reducing the incidence of PTB, all authors still highlighted the importance of dental and periodontal status assessment in women prior to conception and/or during pregnancy. Moreover, despite the inconsistency, the apparent positive impact of periodontal therapy in patients with a low socioeconomic status, particularly in 3rd world locales, cannot be ignored and points to some biologically relevant interaction between periodontitis and pregnancy outcomes.

### 4.4 Mechanistic Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

The abnormal immunological changes caused by periodontal disease and colonization of the placenta by oral bacteria represent two hypotheses that have been suggested to be the putative link between periodontal disease and adverse pregnancy outcome (Han, 2011).

#### 4.4.1 Oral Inflammation

Chronic periodontal infections produce oral and non-oral host responses, resulting in the synthesis of proinflammatory cytokines such as IL-1, IL-6 and TNF-α. Endotoxins such as LPS are also synthesized by the gram-negative anaerobic bacteria responsible for progressive periodontal disease (Romero et al., 1989). These cytokines and LPS can easily enter the systemic bloodstream and reach the maternal-fetal interface (Gibbs et al., 1992; Romero et al., 1989). They can also stimulate the production of PGE₂ synthesis by the human placenta and chorioamnion. It has been shown that women with preterm labour often have an elevated level of these factors in the
amniotic fluid (McGaw, 2002; Romero et al., 1993). These cytokines can trigger or worsen the maternal inflammatory response and contribute to an increased risk of adverse pregnancy outcomes (McGaw, 2002; Xiong et al., 2007). Pregnant women with moderate or severe periodontitis have been found to have increased systemic inflammation early in pregnancy with increased levels of C-reactive protein (CRP) serum level (Horton et al., 2008). Elevated CRP levels have also been correlated with an increased risk of pregnancy complications such as pre-eclampsia (Ruma et al., 2008). Lastly, PTLBW has been found to be associated with increased serum levels of IL-1β in pregnant women (Sert et al., 2011).

4.4.2 Oral Bacteria

Oral bacteria can colonize the placenta directly and cause localized inflammation which may result in preterm birth and other adverse outcomes (Han, 2011). Porphyromonas gingivalis translocation into the placenta has been demonstrated in a mouse model with chronic gingival infection. Microorganisms in the placenta have been associated with retardation in fetal growth (Lin, 2003). P. gingivalis has also been detected in chorionic tissues and in amniotic fluid of pregnant women who had preterm labour and delivery (Katz et al., 2009; Leon et al., 2007). In an acute gingival infection mouse model, Fusobacterium nucleatum was shown to have the capacity to colonize the placenta (Han et al., 2005; Xu et al., 2007). After colonizing the placenta, F. nucleatum proliferates quickly and spreads to the amniotic fluid. This leads to localized inflammation within the fetal-placental unit and can result in direct fetal death (Liu et al., 2007). In a case report, F. nucleatum originating from the human mother's subgingival plaque was shown to be the cause of stillbirth (Han et al., 2010). Some studies have reported finding 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 et al., 2007; Mitchell-Lewis et al., 2001), whereas other studies failed to detect any association between adverse pregnancy outcomes and the presence of intrauterine oral pathogens (Novak et al., 2008; Rakoto-Also et al., 2010). Nonetheless, F. nucleatum and P. gingivalis have been associated with intrauterine infections in humans (Han et al., 2009; Katz et al., 2009).
5 Periodontal Health Assessment

5.1 Traditional Periodontal Health Assessment

Periodontal disease is commonly assessed using indirect markers such as visual detection of signs of inflammation (visual examination, bleeding on probing, gingival crevicular fluid assessment, presence of purulent exudate) and the assessment of the consequence of the disease, namely, damage to the periodontal tissues (CAL, tooth mobility, radiographic examination; Armitage, 1996). These traditional measures do not necessarily provide useful information regarding the severity, morbidity or eventual outcome of periodontal disease (Armitage, 1996). Nor do these assessments speak to the actual disease activity or other pathophysiological features beyond destruction of periodontal tissues that has occurred as a consequence of the disease. Also, these diagnostic tests can only be executed and assessed effectively by trained dental professionals, often requiring specific dental equipment, thus making assessment of disease impractical at best and impossible at worst. Those barriers associated with traditional measures would make it difficult to implement a systematic approach towards screening for periodontal disease in patients with high risk for spontaneous preterm birth delivery.

5.1.1 Assessment of Inflammation

Periodontal diseases are the result of an inflammatory process of the periodontium. All five cardinal signs of inflammation can be exhibited: redness, swelling, heat, pain and loss of function. Bleeding and presence of suppuration can also be seen in inflamed periodontal tissues. Pain and loss of function are usually uncommon until the disease is at an advanced stage (Armitage, 2000). Multiple measurements of the periodontal inflammation have been suggested and frequently used by clinicians.

The Gingival Index (GI) of Loe and Stillman is one of these (Loe, 1967). Through a visual examination evaluating the color, the form (edema), the texture of the gingival tissues and the occurrence of bleeding on probing, the clinician gives a score from 0 to 3 (0= healthy gingiva) (Loe, 1967). The Modified Gingival Index (MGI), described by Lobene et al., in 1986, is similar to the GI but it has a scoring system from 0 to 4 and evaluates the location of the changes (portion or entire marginal and papillary unity). The development of gingivitis requires the presence of
plaque bacteria, which are thought to induce pathological changes to the gingival soft tissues (Theilade et al., 1966). The plaque accumulation is also an indicator of the oral hygiene and the patient’s compliance to treatment (Loe, 1967). Loe in 1967 proposed an index system for this etiologic factor, the Plaque Index. A score of 0 to 3 (0= no plaque in the gingival area) is attributed depending on the amount and the location (free gingival margin, tooth surface, gingival pocket) of plaque accumulation. Ramfjord in 1959 also developed a Plaque Index (PI) and Calculus Index (CI) to account for those important etiologic factors. Both indices have a 0 to 3 scoring system. The PI of Ramfjord emphasizes on the location and the extent of the plaque accumulation on the crown surfaces whereas the CI scoring system is based on the location of the calculus accumulation (supra and subgingival) and the amount of calculus accumulation (0=absence 3=abundant; Ramfjord, 1959). All these measurements do not give details about the location (localized or generalized), the magnitude of the severity of the disease and cannot distinguish between gingivitis and periodontitis.

Clinicians can also evaluate the gingival inflammation through probing the gingival sulcus. Microulcerations in the epithelium lining of periodontal pockets bleed upon probing (Armitage, 1995). Bleeding on probing is one of the earliest signs of gingivitis and periodontal disease but it has a low sensitivity as a predictor of periodontal disease progression (Newbrun, 1996). Gingival crevicular fluid (exudate) can be collected to quantitatively evaluate gingival inflammation. The increased vascular permeability at affected sites will cause an increase in the production of crevicular fluid, which can be collected with filter paper strips, following which the quantity and the composition of the fluid can be analyzed (Cimasoni, 1983). This method does not differentiate between active gingivitis and periodontitis sites and an increase in gingival crevicular fluid is not necessarily associated with a progressive disease (Armitage, 2000). Gingival inflammation can be assessed by simpler methods, such as the bleeding index; therefore, this diagnostic method is rarely routinely used (Armitage, 2000). Clinicians can also easily assess for purulent exudates from the crevicular sulcus while probing or by digital pressure to the gingival surface. Suppuration occurs as a result of neutrophil accumulation in response to the subgingival bacterial invasion. The presence of suppuration is not a consistent feature of periodontitis, with only 3-5% of the sites with periodontitis having this feature, but when present it usually indicates that there is an increased risk of disease progression (Armitage, 1995).
All of the above measurements do not reliably distinguish between destructive and non-destructive periodontal diseases and do not predict future breakdown. In addition, these traditional measures lack the desirable characteristics required of a diagnostic test: high specificity and sensitivity (Haffajee, 1983). Lastly, all screening indices requiring the use of a dental probe require specific training and are usually sensitive for the patient.

5.1.2 Assessment of Damage to Periodontal Tissues

Damage to the periodontal tissues has traditionally been assessed through measurements of attachment loss with a mechanical probe, visual detection of clinical signs of tissue destruction and through radiographic detection (Armitage, 2000).

The most commonly used method is the measurement of attachment loss with the use of a calibrated periodontal probe (Armitage 1995; Simonton 1925). The attachment loss is deduced after measuring the probing depths and the clinical attachment levels or the relative attachment levels with the periodontal probe (Armitage, 2000). Measurements obtained by the periodontal probes are considered to be a useful approximation of the damage to the periodontal tissues, but this method has been associated with multiple sources of measurement errors. Measurement of pockets can vary with the diameter of the probe used (Keagle et al., 1989), the inflammatory state of the gingival tissues (Caton et al., 1981) and with operator errors (variation in the forces applied, position and angulation of the probe during insertion) (Watts, 1987).

Tooth mobility assessment with the Miller classification is also widely used, as an advanced loss of periodontal support will ultimately result in increasing tooth mobility (Armitage, 1995). A careful evaluation of mobile teeth is indicated, since periodontitis is not the only cause of tooth hypermobility.

The clinician can also notice a change in the gingival morphology, which can take the form of an interproximal cratering, gingival recession, furcation involvement and tooth migration. These are all visual signs of tissue destruction but do not differentiate sites with progressive destruction from well-maintained and treated sites (Armitage, 2000). Although called ‘visual signs of tissues destruction’, the use of a periodontal probe and a curved explorer is warranted to effect a thorough examination.
Dental radiographs can also be used to provide information on the extent of bone loss over a period of time (Jeffcoat et al., 1995). Dental radiographs are late indicators of bone attachment loss, since 30-50% change in bone mineral needs to occur before it can be visible to the clinician on the image (Jeffcoat et al., 1995).

These commonly used tests which are not able to accurately differentiate active disease from past or healing disease require specialized equipment and trained clinicians, and are not able to predict future periodontal breakdown (Haffajee et al., 1983).

5.2 Periodontal Health Assessment: Quantitation of Inflammation and OIL

In light of the above, it is critically important to develop new methods for the assessment of active periodontal disease that are practical and simple to perform and can be used under various clinical situations. Moreover, it is essential that approaches towards the diagnosis of periodontal disease focus more on the disease itself rather than merely measuring its sequelae, an approach that inherently biases against confirming relationships between the disease of periodontitis and other disorders including adverse outcomes of pregnancy.

In searching for more accurate ways to diagnose and measure active periodontal disease, researchers have been studying various underlying pathophysiological aspects of this condition for over 70 years with a major focus on leukocytic infiltrate into the oral cavity as a measure of oral inflammatory load. It was shown that the number of leukocytes varied widely between individuals but more importantly that saliva of patients affected by periodontitis contains a greater quantity of leukocytes than individuals without periodontitis (Dreizen et al., 1956).

Neutrophils/PMNs are the most common type of white blood cell, comprising about 50-70% of all white blood cells (Miller et al., 1984). They are produced from pluripotent stem cells residing in the bone marrow (Miller et al., 1984). Approximately $10^{11}$ neutrophils are produced daily in a healthy adult (Segal & Holland, 2000). Once released from the bone marrow, the half-life of a neutrophil is 6-9 hours in the vascular compartment and 1-4 days in the tissues (Deas et al., 2000).
Neutrophils are the first line of defense against a pathogen or when tissue damage occurs (Miller et al., 1984). The immediate immune response results in an increase in vascular permeability and dilation which enable the PMNs to merge to the periphery of the vessels and roll along the endothelial surface, increasing its exposure to inflammatory mediators such as histamine, IL-1, TNF-α, complement C5a, leukotriene B₄, IL-8, platelet activating factor and bacterial products (Deas et al., 2000). Mediated by some integrin adhesion molecules, the neutrophils then adhere to the endothelial cells (Genco, 1992). Neutrophils secrete gelatinase (MMP-9) to degrade type IV collagen and collagenase (MMP-8) in the basement membrane of the vessel to infiltrate into the tissues. The PMNs will then flatten out along the endothelium into the extravascular tissues which will be mediated by a glycoprotein called platelet-endothelial cell adhesion molecule (Marchesi & Florey, 1960). Through chemotaxis, neutrophils migrate towards the infected area (Murphy, 1976). The chemotactic factors that attract neutrophils are produced by both the host and bacterial pathogens, which include tumour necrosis factor (TNF), interleukin eight (IL-8), neutrophil chemotactic factor, complement (C5a) and N-formyl-methionyl peptides (fMLP) (Dennison & Van Dyke, 2000; Smith et al., 1980). Through phagocytosis, PMNs ingest and destroy the invading pathogens (Dennison & Van Dyke, 2000). This results in the killing of the microorganisms through either an oxygen-dependent or an oxygen-independent system (Miyasaki, 1991). The successful resolution of inflammation is a pre-requisite for the restoration of healthy tissue (Dennison & Van Dyke, 2000). PMNs are the first line of defense against invasive pathogens or when tissue damage occurs, as is seen in periodontal disease (Skapski & Lehner, 1976).

There are two ways that neutrophils may play a role in the pathogenesis of periodontal disease. Firstly, if a host has a neutrophil disorder such that these cells are either non-functional or have been decreased in number, advanced periodontal destruction can occur. Secondly, it is known that as a consequence of the normal function of PMNs, these cells may contribute directly to the destruction of tissue in relation to their responses to increased microbial invasion.

In the healthy periodontium, a small number of neutrophils are found to reside in the junctional epithelium. With plaque accumulation, neutrophils respond the same way to the invading organisms, as described above. Thus, the role of neutrophils is primarily defensive but their persistence in great numbers in the periodontal connective tissue can result in the release of their arsenal into the extracellular space. This may lead to an imbalance between repair and breakdown
of connective tissues and ultimately lead to active periodontitis. Activated neutrophils secrete a number of osteoclastogenic factors such as prostaglandins, TNF-α and IL-17. Those cytokines induce bone destruction by affecting osteoclast differentiation and function (Nussbaum & Shapira, 2011).

Individuals with functional neutrophil impairments, which can affect any of the neutrophil’s processes, are predisposed to an increased risk of infection including periodontitis. Neutrophil disorders can be a quantitative or qualitative alteration of the neutrophils and may be inherited, acquired or drug-induced. These conditions will result in a varying susceptibility to infection. These disorders include: agranulocytosis, cyclic neutropenia, chronic benign neutropenia, chronic idiopathic neutropenia, familial benign chronic neutropenia, Felty’s syndrome, leukocyte adhesion syndrome, Down syndrome, Papillon-Lefebvre syndrome, Chediak-Higashi syndrome, diabetes and Kostmann syndrome. These conditions are all associated with periodontitis which can lead to the early loss of both deciduous and permanent teeth (Deas et al., 2003).

Leukocytes are located on different sites within the oral cavity, such as on the tonsils, the floor of the mouth, the dorsum of the tongue and on the cheek. However, the major source of oral leukocytes are found in the gingival sulcus (Lantzman & Michman, 1970; Schiott & Loe, 1970). Thus, leukocyte saliva counts are suggested to vary with teeth number and gingival inflammation (Miller et al., 1984). Theilade et al. (1966) found that 95-100% of the leukocytes collected in the sulcular crevice of patients with gingivitis were PMNs. The leukocyte count in the saliva and the number of teeth present in the mouth were found to be correlated, the counts being lower in edentulous patients (Calonius, 1958). Observational studies have demonstrated that patients with gingival inflammation tend to have higher numbers of salivary leukocytes (Klinkhamer & Zimmerman, 1969; Skougaard & Klinkhamer, 1969; Woolweaver et al., 1972). Historically, the Oral Migratory Rate (OMR), was used to describe the oral leukocytes counts. The OMR is measured by counting the number of leukocytes in 12 sequential 30 seconds oral rinses with saline. After the 6th rinse, the number usually remains constant and is used as the OMR (Klinkhamer & Zimmerman, 1969). This measurement tool was shown to correlate with the gingival inflammation levels (Klinkhamer & Zimmerman, 1969) and with the increased of pocket depth (Skougaard, Bay & Klinkhamer, 1969; Woolweaver et al., 1972) of the patients with varying degrees of periodontal disease. Studies comparing the OMR (Skougaard & Klinkhamer, 1969) and the salivary leukocytes counts (Schiott & Loe, 1970) to the Gingival Index (GI) have
shown that the number of leukocytes did not, in all patients, reflect the degree of gingival inflammation. OMR has been shown to reflect the oral inflammation rather than the degree of the gingival pockets as the OMR of patients with acute periodontitis has been shown to have an increased OMR compared to patients with chronic periodontitis. Furthermore, this difference was shown to be highly significant \( P<0.001; \) Raeste & Aura, 1978). In the past, other methods of quantifying oral PMNs have been described in the literature, such as the use of absorbent strips in the gingival sulcus (Andersen & Cimasoni, 1993), intracrevicular lavage technique (Boretti et al., 1995) and quantifying the PMNs present on the periodontal probe from deep pocket sites (Apsey, Kacicoti & Loesche, 2006).

After oral hygiene improvement, the number of oral leukocytes has been shown to decrease (Klinkhamer & Zimmerman, 1969; Theilade et al., 1966). One month after periodontal therapy, a lowering of the total number of crevicular leukocytes by 16% to 27% has been observed (Boretti et al., 1995). After the extraction of teeth with periodontal disease, salivary leukocyte counts were observed to be lower than prior to extraction (Lantzman & Michman, 1970).

In a more recent study (Bender et al., 2006), oral salivary PMNs were quantified using a hemocytometer by collecting two concurrent 15 ml rinses of Hanks Balanced Salt Solution (HBSS) from each patient. The researchers concluded that the oral rinse assay was a valid, reproducible and effective means of quantifying oral PMN levels. Most importantly, the PMN counts measured by use of this rinse assay were found to reflect the severity of periodontal disease as well as the response to treatment. Although this assay method was shown to be highly reliable, it was difficult to carry out in the laboratory. In this regard, the use of a hemocytometer is somewhat difficult and time-consuming, and requires training. Therefore, an alternate approach for measurement of PMNs was also developed by creating a reliable colorimetric assay that could be used to measure the levels of PMNs in oral rinse samples, leading to a much simpler and equally as reliable approach for the assessment of the levels of PMNs in saliva rinses. The same study found that a single rinse (as opposed to two; the 1st being a ‘cleansing rinse’) was sufficient for measurement of oral PMN counts (Landzberg, 2009). Given the development of this newer and simpler technology, it was then possible to test populations of patients in various settings that initially were not possible to access. Moosani et al. in 2014 collected oral swab samples to measure neutrophil counts on special needs patients under general anesthesia using a similar laboratory protocol. They demonstrated that the measure of gingival inflammation obtained by the PMN
assay positively correlated with traditional periodontal parameters. This led to the use of an oral rinse assay for the measurement of the oral inflammatory load (i.e., PMN counts) in pregnant women from the 'Low risk' obstetrical clinics at Mount Sinai Hospital (Huda et al., 2015). At that point, it was decided that assessments in high-risk patients was not yet warranted. In any case, oral PMN counts in the saliva of low risk pregnant patients were shown to correlate with the levels of periodontal disease as measured by conventional methods. This rinse assay for inflammation (i.e. OIL) provides a simple, reliable, valid and inexpensive indication of oral inflammatory load in pregnant women and could easily be used by non-specialized personnel to collect and quantify oral neutrophils from pregnant patients as well as any other population group (Huda et al., 2015).

5.3 Other Inflammatory Mediators Associated with Preterm Birth and Periodontitis

5.3.1 Matrix Metalloproteinase (MMP)

Inflamed tissues elaborate various degradative enzymes, including matrix metalloproteinases (MMPs). Matrix metalloproteinases (MMPs) are a family of structurally related, zinc dependent endopeptidases whose proteolytic action provides the basis for normal and pathological tissue remodeling (Cockle et al., 2007). They are released from a variety of cells such as fibroblasts, smooth muscle cells, macrophages, epithelial cells and PMNs (Sorsa et al., 2006; Winkler, 2003). Over twenty MMPs have been identified. They hydrolyze extracellular matrix components and are involved in multiple processes including wound healing, inflammatory states, tumor metastasis, angiogenesis, embryogenesis and implantation (Cockle et al., 2007). MMP activity can be suppressed by one of the four identified types of tissue inhibitors of metalloproteinases (TIMP) in the intercellular space (Kushlinskii et al., 2010; Tency et al., 2012).

During pregnancy, the chorioamniotic membranes are fused with the decidua. To allow for delivery, the membranes need to separate and rupture (Athaye, 1999). MMP is thought to be responsible for the remodeling and dissolution of the intercellular cement and the extracellular matrix of the fetal membranes and cervix, weakening the strength of the membranes and allowing cervical ripening and fetal membrane rupture, leading to placental separation from the maternal uterus and delivery (Athaye et al., 1999; Cockle et al., 2007; Sundrani et al., 2012; Winkler 2003).
Multiple studies have quantified the concentration of MMPs in the amniotic fluid, serum and saliva of pregnant women throughout pregnancy. Pregnant women have a plasma concentration of MMP-9 fifteen times higher than in non-pregnant women (Poon et al., 2009). During normal gestation, MMP-1, -2, -3, -7 and -9 are found in the amniotic fluid. MMP-2 and -3 are constantly present throughout the pregnancy, whereas MMP-9 is barely detectable until labor (Cockle, 2007; Vadillo-Ortega & Estrada-Gutierrez, 2005). The availability of MMP-9 is also increased in the amniotic cavity during microbial invasion (Romero et al., 1997).

An increase in proinflammatory cytokines, such as IL-1 beta, tumor necrosis factor alpha (TNF-α) and IL-8, and the blockage of progesterone action are thought to be the responsible mechanisms for the increase in MMP-9 production and release in women during the common terminal pathway of parturition (Athayde et al., 1999; Christiaens et al., 2008). A higher concentration of MMP-9 may be required to accomplish the separation and rupture of the membranes in preterm gestation than in term gestation (Athayde, 1999). MMP-9 serum concentrations are particularly elevated during preterm labor (Tency et al., 2012). Salivary concentration of MMP-9 is also higher in women with premature rupture of the membranes before preterm delivery compared to nonpregnant women and women in labor with term delivery (Menon et al., 2006). The MMP-9 level in the amniotic fluid is reported to be five folds higher in preterm deliveries compared to term deliveries (Athayde et al., 1999). MMP-9 serum levels measured at 11 to 13 weeks of gestation have shown to be increased in pregnancies during preeclampsia and early spontaneous preterm delivery (Poon et al., 2009). Also, MMP-9 activity is regulated by TIMPs; a variation in the TIMPs concentration and MMP-9: TIMP ratio could affect the MMP-9 availability and the subsequent collagenolysis (Tency et al., 1999). During preterm labor, as TIMP-1 and TIMP-2 concentrations are lower, the serum MMP-9:TIMP-1 ratio and MMP-9:TIMP-2 ratio are in favor of gelatinolysis (Athayde et al., 1999; Tency et al., 2012).

Chronic periodontal infections lead to high levels of pro-inflammatory cytokines which in turn can elicit the differential expression of many participating genes, including those resulting in increased MMP production (Pan et al., 2013). In patients with periodontitis, MMPs are involved in the process of periodontal destruction and remodeling through proteolytic degradation of all extracellular matrix proteins in the periodontium (Kushlinskii et al., 2010; Marcaccini et al., 2010). MMPs are also involved in the modulation of inflammatory responses through their ability to facilitate leucocyte recruitment, and cytokine and chemokine processing, which in turn, can
aggravate periodontal disease (Kushlinskii et al., 2010; Pan et al., 2013). More specifically, MMP-8 and MMP-9 along with granulocyte elastase are involved in tissue destruction associated with chronic periodontitis (Kushlinskii et al., 2010; Sorsa et al., 1988). MMP-8 and MMP-9 have been found to be present in significantly higher levels in oral fluid samples of patients with periodontitis than in patients with a healthy periodontium (Kushlinskii et al., 2010). By inducing a host response, periodontal microorganisms, especially P. gingivalis (Soder et al., 2006) and T. denticola (Yakob et al., 2013), increase the release of MMP-9, also known as gelatinase B, within the gingival crevicular fluid (GCF) (Soder et al., 2006). MMP-9 is believed to either seep into the systemic circulation or through transient periodontal bacteremia and to be upregulated in the blood, contributing to the increased plasma levels of MMP-9 seen in periodontitis patients (Soder et al., 2006). This could indirectly lead to preterm labor.

Three months after periodontal therapy, MMP-8 and MMP-9 levels in the GCF of patients with periodontitis were found to decrease (Marcaccini et al., 2010), whereas periodontal therapy in pregnant women with periodontitis before 21 weeks of gestation was not found to reduce markers of inflammation such as the serum levels of MMP-9 serum level when compared to baseline levels (collected between 13-16 weeks of gestation; Michalowicz et al., 2009).

5.3.2 Fetuin (Ahsg)

Fetuin and its human homologue (a2-HS-glycoprotein, Ahsg) are negative acute-phase proteins that reside in serum and in bones (Albilia et al., 2012). MMPs can also degrade fetuin (Schure et al., 2013). Consequently, a higher level of PMNs will lead to a higher levels of MMPs which can then degrade fetuin and result in an increased levels of the damaging pro-inflammatory mediators (Albilia et al., 2012).

Fetuin is principally secreted by hepatocytes and cells of the monocyte/macrophage family but it can also be found in neurons, fibroblasts and a number of tumor cell lines (Ombrellino et al., 2001). Ahsg circulating levels in healthy adults are 300–600 µg/ml (Wang et al., 1998) but serum level drops significantly (30-50%) during injury and infection (Lebreton et al., 1979; Ombrellino et al., 2001; Wang et al., 1998), and in the presence of inflammatory diseases (Albilia et al., 2012).
Ahsg can suppress the innate (Wang et al., 1998) and cellular immune responses allowing for the resolution of the inflammation process and limiting tissue destruction (Rittenberg et al., 2005). More specifically, this protein, as an anti-inflammatory mediator, is required for macrophage deactivation, restraining the innate immune response (Ombrellino et al., 2001; Wang et al., 1998). Macrophages use fetuin as an opsonin for cationic-deactivating molecules such as spermine, a polyamine released from injured and dying cells at sites of infection, and CNI-1493. Studies suggest that fetuin is required for spermine to suppress TNF production in human monocytes. It has been suggested that the high levels of spermine and fetuin in the fetus and the amnion could counter-regulate TNF production throughout pregnancy and protect the fetus from abortion (Ombrellino et al., 2001; Wang et al., 1997). Furthermore, fetuin is required during lethal systemic inflammation to regulate the systemic accumulation of late inflammatory mediators such as HMGB1, as was suggested in an experimental mice model (Li et al., 2011). Thus it might be possible that reduced serum-levels of this protein could lead to increased risk for adverse outcomes of pregnancy.

It is also noteworthy that intact fetuin regulates osteogenesis (Rittenberg et al., 2005). Ahsg has the ability to antagonize both the osteogenic and anti-proliferative actions of TGF-b cytokines such as bone morphogenetic proteins (BMP) in vitro consequently playing a role as endogenous regulators of calcification (Demetriou et al., 1996). In conditions with repeated inflammatory exacerbation, such as in patients with degenerative joint disease, a low serum level of Ahsg is found (Albilia et al., 2011), which may predispose to an excessive BMP mediated reparative response. In these patients, this contributes to synovial fibrosis, osteophyte and ectopic bone formation with eventual ankylosis (Albilia et al., 2011; Van der Kraan & Van Den Berg, 2007). Hypothetically, through its regulation of calcification, fetuin could play a role in vascular calcification, which could play a role in premature vascular calcification seen in newborns (Rutsch et al., 2003).

Thus, through the inhibition of macrophage-derived cytokines such as TNF, fetuin would be required to limit the development of collateral damage and/or consequences to surrounding tissues (Wang, Sama 2012; Wang et al., 1997). TNF has been found to induce contraction of smooth muscles and may cause fetal expulsion due to uterine contraction or may cause necrosis of implantation sites or of the fetus itself by thrombosing the blood supply (Shaaraway & Nagui, 1997; Silen et al., 1989; Warner & Libby, 1989). Consequently, since an excessive production of
cytokines such as TNF-α during pregnancy as seen in some women with recurrent miscarriages, can lead to spontaneous abortions (Mallmann et al., 1991; Shaaraway & Nagui, 1997), fetuin serum level could also play a role in the mechanisms that lead to preterm birth.

One clinical trial investigated the association between maternal fetuin serum levels and intrauterine growth restriction (IUGR) and showed that there was no significant association between the two variables (Briana et al., 2008). Moreover, there are no previously published studies that have focused on the potential correlation between serum levels of fetuin and preterm birth and/or periodontitis.

6 Periodontal Health Screening in 'High Risk' Pregnant Women

Considering the low incidence of preterm birth and periodontitis in the general population and the multiple confounders affecting the variables, in order to determine a significant relationship between these variables, a well-designed observational study with a large sample size would be necessary. Alternatively, executing the study in a group of pregnant women already at risk of spontaneous preterm delivery may require a smaller sample size to reach a significant correlation between the variables, as adverse birth outcomes are more common in that group. Also, in a systematic review and meta-analysis published in 2012 (Kim et al.), the authors concluded that pregnant women from 'High Risk' groups of preterm birth affected by periodontitis have a statistically significant reduction in risk of adverse birth outcomes following periodontal therapy. This indicates that this population in particular would benefit from early and systematic periodontal health screening. The authors suggest future research should attempt to define those groups in which we see a risk reduction in birth outcomes (Kim et al., 2012). But in general, the assessment of periodontal disease related parameters can be difficult to perform and often requires the utilization of research personnel who have a good level of clinical training in dentistry. This can limit the sample size for any planned investigation in this area. This said, and as outlined further below, we propose that the assessment of OIL by way of using an oral rinse based assay for oral PMNs can be done by investigators who do not have dental training, which means that much larger sample sizes might be attainable.
The primary aim of this study was to explore the association between OIL, defined as the exposure to oral neutrophils and other biological factors that regulate oral inflammation (e.g. MMP-9 and Ahsg), and preterm birth in a group of pregnant women known to be at risk of preterm birth. The second aim of this study was, through a cross-sectional study, to assess the difference in prevalence of OIL between a Low Risk and a High Risk group for preterm birth of pregnant women.

7 Objectives

7.1 Part 1

7.1.1 Primary Objective

- To explore the association between oral OIL and spontaneous preterm birth (SPTB) in a group of pregnant women at risk of preterm birth.

7.1.2 Secondary Objectives

- To correlate the serum MMP-9 levels, the serum levels of Ahsg and traditional, non-invasive and fast periodontal health assessment test in pregnant women at risk of preterm delivery with their oral neutrophil counts.
- To identify a non-invasive, fast and easily widely implemented oral inflammatory load assessment tool that could potentially be used by medical professional with minimal dental training.

7.2 Part 2

7.2.1 Primary Objective

- To compare the OIL, the MGI, PI, and CI between pregnant women at risk of preterm delivery (High Risk group) and pregnant women with no increased risk of preterm delivery (Low Risk group).
Materials and Methods

8 Study Design and Setting
The collection of the exposure variables for this pilot prospective cohort study was carried out at Mount Sinai Hospital at the Frances Bloomberg Centre for Women’s and Infants’ Health (700 University Avenue, 3rd Floor, Toronto, Ontario). More specifically, recruited participants were patients of the Prevention of Preterm Birth Clinic which is one of the Specialty Clinic in the Special Pregnancy Programs (SPP) of Mount Sinai Hospital’s (Toronto) Maternal-Fetal Medicine program. The SPP at Mount Sinai Hospital is dedicated to the investigation and management of the full range of maternal, fetal and placental difficulties that may occur during pregnancy and the specialized group of health professionals included in the care of those patients at risk of preterm birth includes but is not limited to, obstetricians, nurses, sonographers, geneticists, genetic counsellors, nutritionists and psychiatrists.

Ahsg-fetuin and MMP-9 serum levels were analyzed in the research laboratories of Mount Sinai Hospital, while PMN counts for the assessment of OIL, were carried out in the laboratories at the CIHR Matrix Dynamics Group at the University of Toronto (Fitzgerald Building, Room 241, 150 College Street, Toronto, Ontario).

Through a cross-sectional study, two different obstetrical groups were compared, the High Risk group consisted of participants of Part 1 of this study while the data from the Low Risk group were used as historical data from a parallel study being performed at the same hospital but in the General Obstetrics Clinic (Huda et al., 2015).

9 Ethics Approval
This study was approved by the Research Ethics Boards of Mount Sinai Hospital, Toronto. The approved information was provided to the Ethics Boards of University of Toronto prior to the commencement of the study (MSH REB 13-0325-E). A 'Data and Biological Transfer Agreement' was obtained between Mount Sinai Hospital and the University of Toronto to allow transportation of the patient-derived samples to the University of Toronto laboratory (Fitzgerald Building, Room
241, 150 College Street, Toronto, Ontario) for processing. The same ethics proposal and approval as that used by Huda et al. in 2015 was used as the investigations were carried out in a parallel manner in the same laboratories and health institutions overall.

10 Participants

10.1 Selection

Patients in this study were recruited from the Prevention of Preterm Birth Clinic at Mount Sinai Hospital (700 University Avenue, 3rd Floor, Toronto, Ontario). The potential participants were approached in person by Dr Wendy Whittle (W.W.), one of the obstetricians in charge of their care, the nurse coordinator of the clinic or by the receptionist. The study was briefly introduced and, if potential participants agreed to learn more about the study, Dr Marie-Lyne Gosselin (M.G.), a co-investigator, explained in person the study protocol using an 'Information Sheet' (Appendix IV) that was provided to them. The voluntary nature of the participation was emphasized. It was made explicit that the decision to participate would have no bearing on clinical care and that participation could be ended at any time. The medical record was then reviewed to identify any exclusion criteria. Participants were invited to participate in the study if they met all the inclusion criteria and had none of the exclusion criteria (see Appendix III).

Written informed consent (Appendix V) was obtained by M.G. prior to the commencement of the study on the same day or on a separate day than the data collection visit depending on patient's preference and availability.

The study by Huda et al. (2015) had a similar selection process.

11 Variables

11.1 Part 1

11.1.1 Exposure Variables

The exposure to OIL was defined primarily as the exposure to oral neutrophil counts and secondarily as the exposure to inflammatory mediators (serum levels of MMP-9 and fetuin) and exposure to periodontitis by clinical diagnosis as measured by traditional, non-invasive, fast and
easily implemented assessment tools (Modified Gingival Index, Plaque index, Calculus index). Data collection of the exposure variables took place in one assessment carried out between 13 to the 26 weeks of gestation. Patients were notified of their options regarding follow up and treatment if any oral problems were identified during the study.

11.1.1.1 Primary Exposure Variable: Oral neutrophil counts as assessed by an oral rinse

Oral inflammatory load is present in every individual, only the level of inflammation varies.

The protocol used to collect and count oral neutrophils was similar to the protocol used by Huda et al. 2015, which was a modification of previous work carried out by Bender et al. (2006) and Landzberg et al. (2009). Study participants were instructed to not eat or drink for a minimum of 30 minutes prior to providing the oral rinse sample to avoid clearance of neutrophils prior to donations. Participants were instructed to swish with tap water for 10 seconds and expectorate. Two minutes later, pregnant women were asked to rinse their mouth once with 10 ml of 0.9% saline for 30 seconds by gentle swishing, which had been previously demonstrated by M.G.

Each oral rinse sample was collected in a sterile Falcon® tube (Becton Dickinson, Franklin Lakes, NJ, USA) and stored at 4°C to preserve the cells prior to transportation to the laboratory for processing and analysis. The laboratory was located at approximately a five minute walking distance from the Clinic and so viability and intactness of the cells was not considered to be an issue as all samples could be processed within 3 hours of collection by a trained laboratory assistant.

In the laboratory, 9.5 ml of the oral rinse sample underwent centrifugation at 2500 RPM for 5 minutes at 21°C (Hettich Rotina 35 R, Rare Scientific, Edmonton, Canada). The cell pellet was resuspended in 9.5 ml of double distilled water. 30 mg of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (AO; Sigma Chemical, Burlington, Ontario, Canada) was dissolved in 3.6 ml of 1 MM 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 were added in order to observe the characteristic blue-green color change. 250 µl of the colored sample were added to a 96 well
plate in triplicate. The solution light absorbance was measured at 405 nm light after 1 cycle for the 34 samples and for an additional 9 cycles of 1 minute each for 18 of the samples using the FLUOstar software (BMG LABTECH GmbH, Offenburg, Germany). Those 18 samples were used for Part 2 of this study, thus requiring the same methodology used in the historical study used for comparison (Huda et al., 2012). The final absorbance value for a sample was reported as the average of the absorbance values of the cycles. The average standard deviation and coefficient of variance (CV) of the absorbance values of the 10 cycles for all the collected samples was calculated to calculate the variation between the cycle absorbance readings.

In order to determine the neutrophil counts of a given oral rinse sample from the absorbance measure, a standard curve equation was used \( y=3305783.5X-1366 \) based on the use of known numbers of PMN cells. To do this, a series of standard solutions were prepared in the laboratory as described above using saliva neutrophils of known concentrations. The solution light absorbance was then measured at 405 nm for 10 cycles using FLUOstar software. The standard curve equation was then obtained by plotting the absorbance of the standard solutions versus the known concentrations, thus allowing deduction of the neutrophil counts for a sample using the absorbance value.

### 11.1.1.2 Secondary Exposure Variable: Serum Level of Matrix Metalloproteinase 9

Each participant had a venipuncture to collect blood for assessment of serum levels of MMP-9. The blood collection was performed at the same time as the participant presented for routine pregnancy care and venipuncture, thus requiring no additional procedure or visit. All analyses were conducted at the Mount Sinai Hospital Pathology Laboratory, Toronto. The protocol used by Poon (2009) was used and modified to collect and analyze MMP-9 serum levels. Samples were diluted to a concentration of 1 in 3000; this required that a 5 µl of sample had to be diluted with 1495 µl of assay buffer (1:300 dilution), and further diluted on the plate (1:10). The prediluted samples were used to measure MMP-9 concentration by a quantitative sandwich enzyme immunoassay based on monoclonal antibody coated onto a microplate and an enzyme-linked polyclonal antibody added to the well after standards and samples (Quantikine Human MMP-9 Immunoassay, R&D Systems Inc, Abingdon, England). Fresh aliquots of MMP-9 quality control samples of known concentration were measured in duplicate at the beginning and the end of each run to assure an internal reference standard. The mean coefficient of variation was calculated.
This variable was analyzed statistically as a continuous variable because no specific serum levels of MMP-9 has been correlated with different severity of periodontitis in pregnant women prior to labour in the literature.

11.1.1.3 Secondary Exposure Variable: Fetuin (Ahsg) serum level

Blood samples for the serum level assessment of Ahsg were collected on the same occasion and analyzed in the same laboratory that performed the MMP-9 assays. The protocol used by Albilia 2011 was modified and used to collect and analyze the Ahsg serum levels. Blood samples from the study group were collected in serum separator tubes and allowed to coagulate for 30 minutes prior to centrifugation. Tubes were spun at 1,200 g for 10 minutes in a non-refrigerated centrifuge. Serum was then pipetted and stored at -80° C. All samples were split into 1ml aliquots and stored at 253º K until analysis.

ELISA kits for Ahsg were obtained from BioVendor (Candler, NC, U.S.A.). Concentrations in serum of the various analytes were determined using spectrophotometry with samples being contained in a 96 well microtitre plate. A wavelength of 450 nm was used to assess color reactions in the ELISA assays. Once all patients were recruited, serum samples were thawed at room temperature for 20 minutes and assayed using a quantitative sandwich ELISA technique. Assays were performed according to the supplier’s directions except where some modifications were made to improve sensitivity. In order to assure that absorbance values were in the linear range, a final dilution of 1:900 was needed.

This variable was analyzed statistically as a continuous variable because no specific level of fetuin within serum has yet been correlated in the literature, with different degrees of the severity of periodontitis.

11.1.1.4 Secondary Exposure Variables: Traditional, non-invasive periodontal assessment tools

Prior to the collection of the oral rinse assay, a visual examination of the teeth and the supporting soft tissues of the oral cavity was done using a dental mirror and a pocket light, at the Obstetrics Clinics with the patient in a supine position on a hospital bed. The soft tissues were examined visually and by palpation and any pathological lesions were noted. Teeth present were charted and visually detectable caries were noted (see Appendix VI). The principal investigator also
assessed the MGI (Lobene et al., 1986) (see Appendix VII), the PI (Ramfjord, 1959) (see Appendix VIII) and the CI (Ramfjord, 1959) (see Appendix IX) of the patient. The participants were informed of the findings. MGI of 2 and over, CI of 1 and over and PI of 2 and over were considered to have an elevated clinically-measured OIL for statistical analysis.

11.1.2 Outcomes Variables

Information regarding the course of the pregnancy and the outcome were collected from the antenatal chart and delivery records of the participating patients and their infant at the end of the pregnancy by assessing the OB TraceVue records at the Mount Sinai Hospital by M.G. Three participants were contacted by phone because their delivery outcomes could not be obtained through the OB TraceVue records, having delivered in another hospital.

11.1.2.1 Primary outcome variable: SPTB

PTB was defined in this study as spontaneous preterm birth (SPTB) delivery at less than 37 weeks of gestation.

11.1.2.2 Secondary outcome variables

Aiming to describe in further detail the putative link between oral inflammatory load and preterm birth, more specific data on the delivery outcomes were collected. Firstly, the diagnosis or etiology of the delivery was noted as indicated (elective) or spontaneous. Details on the cause for the indicated or spontaneous delivery were noted. After a preterm delivery, the placenta is often sent to Pathology for analysis. The result from this examination was also included in the study as it often helped in confirming the clinical etiology of the preterm delivery. Intrauterine growth restriction (IUGR), resulting in low birth weight, is known to be a major contributor to perinatal morbidity and mortality and may increase the risk for spontaneous or indicated preterm delivery. Thus, the birth weight of the child was recorded and categorized as: adequate for a weight >2,500g or LBW for a weight <2,500g. Participants with a known fetal anomaly at the time of enrollment in the study were excluded. However, a fetal anomaly may have been unknown at the time of recruitment and was noted as a pregnancy outcome. Other pregnancy complications such as stillbirth (intrauterine fetal death at any gestational age; no heart beat present prior or during delivery) and spontaneous abortion (pregnancy loss prior to 14 weeks) were also recorded.
11.1.3 Potential Confounders and Effects Modifiers

Aiming to control the confounders and effects modifiers prior to the commencement of the study, a medical and dental history was taken on each patient participating in the study using a structured questionnaire (Appendix X). This questionnaire included a medical and dental history and specific questions concerning particular habits such as smoking, alcohol and recreational drug usage. Participants who floss at least 3 times a week were considered to be flossing regularly and participants using a mouthwash everyday were considered to use mouthwash on a regular basis. If required, M.G. found missing or incomplete information in medical records of the patients in order to complete all questionnaire-based data as possible.

Some specific information was also recorded from the patient’s medical records such as maternal demographic information (age, race, education level, marital status) and previous pregnancy history (gravity, parity, previously indicated or spontaneous preterm delivery and placental diagnosis). Current medical history including current illness, disease or medical condition and current medications were noted. Several details on the current pregnancy history were collected (gestational age at data collection as diagnosed by ultrasound), cervical length at 20 and at 24 weeks, presence of uterus anomaly, history of cervical cone biopsy, interventions (cerclage (prophylactic, indicated or rescue), progesterone therapy, ASA therapy, history of urinary/genital infections, antibiotic therapy; transient or suppressional throughout pregnancy. Pregnancy complications were also noted such as gestational diabetes, hypertension, antepartum hemorrhage, preeclampsia and intrauterine growth restriction. The nutritional status of the participants was recorded as per their BMI. Women with higher BMI (over 25) were considered to have a protective factor for PTB, compared to women with a BMI lower than 25 for the statistical analysis of this study (SPTB occurring in 8.1% as opposed to 11.3%; Mercer et al., 1996).

11.2 Part 2

11.2.1 Exposure variable: Obstetrical risk group

Pregnant women are separated in two groups: High Risk and Low Risk for preterm delivery. Data for the High Risk group were collected in the present study (Part 1), whereas data for the Low
Risk group were collected in a previous study with a parallel design by Huda et al. (2015) executed in the same institution.

Pregnant women patients are referred to the General Obstetric Clinic (Low Risk group) of the Mount Sinai Hospital (700 University Avenue, 3rd Floor, Toronto, Ontario). According to their obstetrical history (such as history of SPTB), women may be subsequently referred to the Prevention of Preterm Birth Program (High Risk group).

11.2.2 Outcome variable: Oral neutrophil counts as assessed by an oral rinse
The definition and method of data acquisition and measurement were described in Part 1.

11.2.3 Potential Confounders and Effects Modifiers
The potential confounders and effects modifiers were described in Part 1 (page 38).

12 Examiner Standardization
M.G. was the sole examiner for this study. The principal investigator was calibrated with one experienced periodontist to determine inter-rater reliability by independently assessing the PI, CI and MGI of 5 volunteered patients of the University of Toronto dental clinic. The inter-rater reliability was calculated using the kappa statistic and was classified as poor (k<0.20), fair (k=0.21-0.40), moderate (k=0.41-0.60), substantial (k=0.61-0.80) or almost perfect (k>0.81). The Weighted Kappa statistic was used to test the inter-rater reliability. Examiners showed perfect agreement for the CI (k=1) and showed good agreement (k_w=0.68) for both the PI and MGI.

13 Study Sample Size
1. Level of significance set at α= 0.05
2. Power: 80%
3. Incidence of preterm birth in this group of women at risk for preterm delivery as per the hospital records: 25%
4. A meta-analysis of observational studies showed that pregnant women with periodontitis have 2.27 times more chance to deliver prematurely than pregnant women with a healthy periodontal status. The odd ratio used to calculate the sample size was \( OR = 2.27 \) (Vergnes & Sixou, 2007).

The sample size calculation for an observational study of risk was calculated using the program SamplePower and recommended 73 patients per group (73 with periodontitis, 73 without periodontitis) for a total of 146 participants.

14 Statistical Analysis

Participants’ characteristics are categorized in four main domains (demographic and medical, obstetrical, OIL, and pregnancy outcomes). Descriptive statistics was calculated for all the demographic characteristics, exposure and outcome variables, confounders and effects modifiers using means, medians and standard deviations for continuous variables and frequency counts and percentages for categorical factors. The relative risk (RR) for PTB following the exposure to suspected risk factors were calculated. Chi-Square test or Fisher Exact test, for categorical variables, and Student’s t-test for continuous variables were used to describe the association between the exposure and outcome variables, as well as several confounders. Pearson correlation analysis for continuous variables and Student’s t-test for categorical variables were used to explore the association between the oral neutrophil counts, Ahsg and MMP-9 serum levels, traditional, non-invasive periodontal health assessment tests, and confounders. The Chi-Square test and Student's t-test were used to compare the Low Risk group with the High Risk group. All data were analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC).
Results

15 Part 1: OIL and PTB

Between April and August 2014, following specific inclusion and exclusion criteria, 65 pregnant women at risk for PTB who were enrolled in the Prevention of Preterm Birth Clinic of Mount Sinai Hospital were approached, of whom 35 (54%) agreed to participate. After having given her consent to participate in the study and after having provided a blood sample for the MMP-9 and fetuin serum analysis, one participant subsequently refused to proceed with the oral rinse after learning that her pregnancy was threatened by early signs of PTL. Therefore, the oral neutrophil counts and periodontal health assessment indices were available for a total of 34 women for this pilot cohort study. Three blood samples never reached Mount Sinai Pathology Laboratory for analysis and could not be located, therefore 32 women were included for the analysis on MMP-9 and fetuin serum levels. Pregnancy outcome data were available for all participants (n=35) and 15 (43%) who delivered prematurely. The study flow chart is illustrated in Figure 1. Participants’ characteristics are described in Table 1. No statistically significant differences were found between women who delivered prematurely and women who delivered at term in relation to demographic characteristics, medical history, dental history, and obstetrical history.
Figure 1. Flow Chart of this Prospective Cohort Pilot Study

Pregnant women from the High Risk pregnancy group

Identified to participate
n=65

Total recruited
n= 35

Refused to participate
n=30

Lost to follow-up

Refusal to proceed with the traditional periodontal assessment and the oral rinse assay  n= 1

Missing blood sample  n= 3

Data available for analysis

Neutrophils counts (1 cycle analysis)  n= 34
Neutrophils counts (10 cycles analysis)  n= 18
Traditional periodontal assessment  n= 34
MMP-9 and fetuin serum analysis  n= 32

Pregnancy outcomes
n= 35

TB  n= 20

PTB  n=15

Elective PTB  n=1

SPTB  n=14
Table 1. Demographic and Health Characteristics, Obstetric Characteristics, Oral Inflammatory Load Indicators (OIL), and Pregnancy Outcomes Categorized by their Pregnancy Outcome (spontaneous preterm birth (SPTB) and term birth (TB))

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total</th>
<th>TB</th>
<th>SPTB</th>
<th>Unadjusted RR</th>
<th>P value</th>
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<td></td>
<td>n ( % )</td>
<td>n ( % )</td>
<td>n ( % )</td>
<td>RR 95%; CI</td>
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<td><strong>Demographic</strong></td>
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<tr>
<td>Race</td>
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<td>Black</td>
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<td>4 (20)</td>
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<td>Asian</td>
<td>5 (14)</td>
<td>4 (20)</td>
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<tr>
<td>East Indian</td>
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<td>4 (20)</td>
<td>5 (33)</td>
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<td><strong>Education</strong></td>
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<td>College or University completed</td>
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<td>Married/ Common law</td>
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<td>Less than 35</td>
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<td>1.00 (0.46, 2.18)</td>
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<td>Anxiety/Depression</td>
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<td>BMI Group</td>
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<td>More than 25</td>
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<td>Prenatal Supplements</td>
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<td>Brushing at Least Twice a Day</td>
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<td>Regular Mouthwash Usage</td>
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<td>Received a Cleaning in the Past Year</td>
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<td>14 (70)</td>
<td>12 (86)</td>
<td>1.85 (0.52, 6.57)</td>
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<td>Dental Visit Frequency</td>
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<td>Only for emergency care</td>
<td>9 (26)</td>
<td>6 (30)</td>
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<tr>
<td>Regular/occasional check up</td>
<td>25 (74)</td>
<td>14 (70)</td>
<td>11 (79)</td>
<td>1.32 (0.47, 3.68)</td>
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* Significance was assessed by Fisher exact test
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<td>More than 3</td>
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<td>Parity</td>
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<td></td>
<td>0.85</td>
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<tr>
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<td>10 (50)</td>
<td>7 (47)</td>
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<tr>
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<td>8 (53)</td>
<td>1.08 (0.50, 2.33)</td>
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<td>Number of Previous PTB</td>
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<td>1</td>
<td>17 (48)</td>
<td>10 (50)</td>
<td>7 (47)</td>
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<tr>
<td>More than 1</td>
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<td>10 (50)</td>
<td>8 (53)</td>
<td>1.08 (0.50, 2.33)</td>
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<td>History of Spontaneous Abortion</td>
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<td>Uterus Anomaly</td>
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<td>3 (15)</td>
<td>1 (7)</td>
<td>0.55 (0.10, 3.16)</td>
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<td><strong>Pregnancy History</strong></td>
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<tr>
<td>Fertility Treatment</td>
<td>4 (11)</td>
<td>2 (10)</td>
<td>2 (13)</td>
<td>1.19 (0.41, 3.45)</td>
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<td>Urinary/Genital Infection</td>
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<td>11 (55)</td>
<td>7 (47)</td>
<td>0.83 (0.38, 1.78)</td>
<td>0.63</td>
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<tr>
<td>Cervical Length Less than 30mm at 20 Weeks</td>
<td>11 (33)</td>
<td>3 (15)</td>
<td>8 (62)</td>
<td>3.20 (1.37, 7.50)</td>
<td>0.01*</td>
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<td>Cervical Length Less than 25 mm at 24 Weeks</td>
<td>9 (28)</td>
<td>3 (15)</td>
<td>6 (50)</td>
<td>2.56 (1.12, 5.85)</td>
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<td>Cerclage</td>
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<td>11 (73)</td>
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<td>Prophylactic cerclage</td>
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<td>5 (25)</td>
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<td>0.64*</td>
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<td>Indicated cerclage</td>
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<td>3 (20)</td>
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<tr>
<td>Rescue cerclage</td>
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<td>2 (13)</td>
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<td>Progesterone Therapy</td>
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<td>10 (50)</td>
<td>10 (67)</td>
<td>1.50 (0.65, 3.47)</td>
<td>0.32</td>
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<tr>
<td>ASA Therapy</td>
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<td>7 (47)</td>
<td>1.17 (0.54, 2.50)</td>
<td>0.69</td>
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<td>Antibiotic Therapy</td>
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<td>15 (75)</td>
<td>12 (80)</td>
<td>1.19 (0.44, 3.19)</td>
<td>0.99*</td>
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<tr>
<td><strong>Pregnancy Complications</strong></td>
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<tr>
<td>Gestational Diabetes</td>
<td>16 (46)</td>
<td>11 (55)</td>
<td>5 (33)</td>
<td>0.59 (0.26, 1.38)</td>
<td>0.2</td>
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<tr>
<td>Antepartum Haemorrhage</td>
<td>5 (14)</td>
<td>1 (5)</td>
<td>4 (27)</td>
<td>2.18 (1.15, 4.15)</td>
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<tr>
<td>Hypertension</td>
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<td>3 (15)</td>
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<td>0.55 (0.10, 3.16)</td>
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<td>Pre-eclampsia Toxemia</td>
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<td>1 (7)</td>
<td>1.18 (0.28, 4.98)</td>
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<td>IUGR</td>
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* Significance was assessed by Fisher exact test
### Oral Inflammatory Load

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<th>P value</th>
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</thead>
<tbody>
<tr>
<td><strong>Gestational Age (weeks) at Data Collection [Mean; (Standard deviation)]</strong></td>
<td>20.56 (±4.73)</td>
<td>19.95 (±4.98)</td>
<td>21.42 (±4.38)</td>
<td>1.04 (0.95, 1.14)</td>
<td>0.38</td>
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<tr>
<td><strong>Neutrophil Counts x10⁶ cells/ml [Mean; (Standard deviation)]</strong></td>
<td>2.05 (±0.79)</td>
<td>1.94 (±0.74)</td>
<td>2.21 (±0.88)</td>
<td>1.32 (0.79, 2.22)</td>
<td>0.34</td>
</tr>
<tr>
<td>per 1 000 000 more</td>
<td></td>
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<td><strong>MMP-9 x10³ ng/ml [Mean; (Standard deviation)]</strong></td>
<td>1.42 (±0.84)</td>
<td>1.37 (±0.97)</td>
<td>1.49 (±0.64)</td>
<td>1.07 (0.73, 1.57)</td>
<td>0.7</td>
</tr>
<tr>
<td>per 1 000 more</td>
<td></td>
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<tr>
<td><strong>Fetuin x10⁵ ng/ml [Mean; (Standard deviation)]</strong></td>
<td>9.12 (±2.83)</td>
<td>8.71 (±2.46)</td>
<td>9.71 (±3.31)</td>
<td>1.11 (0.93, 1.32)</td>
<td>0.33</td>
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<tr>
<td>per 10 000 000 more</td>
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**Plaque Index:**

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<td>7 (22)</td>
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<td>0.64 (0.19, 2.23)</td>
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**Calculus Index:**

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<td>3 (21)</td>
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<td>26 (76)</td>
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**Modified Gingival Index:**

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<td>28 (82)</td>
<td>17 (85)</td>
<td>11 (79)</td>
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<td>0.67*</td>
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<td>2 or more</td>
<td>6 (18)</td>
<td>3 (15)</td>
<td>3 (21)</td>
<td>1.27 (0.51, 3.20)</td>
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### Pregnancy outcomes

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<td><strong>Neonatal Complications</strong></td>
<td>8 (23)</td>
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<td>8 (53)</td>
<td>3.86 (2.04, 7.30)</td>
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<td><strong>Still Birth or Neonatal Death</strong></td>
<td>2 (6)</td>
<td>0 (0)</td>
<td>2 (13)</td>
<td>2.54 (1.66, 3.88)</td>
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<tr>
<td><strong>Low Birth Weight (2500g or less)</strong></td>
<td>13 (40)</td>
<td>1 (5)</td>
<td>12 (80)</td>
<td>6.77 (2.34, 19.6)</td>
<td>&lt;0.01*</td>
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* Significance was assessed by Fisher exact test
15.1 Demographic and Health Characteristics

15.1.1 Demographic

Participants’ mean age was 33.2 years old (± 4.67). The majority of participants were well educated with 86% (n=30) having completed college and/or university (the education level of two participants was unknown). Similarly, most participants (92%, n=32) were married or in common-law relationship. Participants’ racial backgrounds were diverse, including 23% (n=8) Black and 14% (n=5) Asian.

15.1.2 Lifestyle

One participant reported smoking during her pregnancy. No women reported consuming alcohol or illicit drugs during their pregnancy.

15.1.3 Medical history

Overall, participants were healthy. All medical conditions reported were well-controlled. Three participants had asthma, one woman had Grave’s disease, one had hypertension, and one had Factor V Leiden. Five participants had polycystic ovarian syndrome (PCOS), and one had a history of endometriosis, which are two conditions associated with an increased risk for PTB. Five participants were diagnosed with depression and/or anxiety disorder, but all participants were presumed to have a high anxiety level and were invited to participate in a group or in an individual meeting with the psychology team. All but one (BMI of 16.9) participant had a BMI between 20.5 and 33.5. Only one participant reported not taking prenatal supplementation (3%, n=1). One participant reported having a history of cone biopsy.

15.1.4 Dental history

The majority reported brushing at least twice a day (68%, n= 23), visiting a dentist on a regular to occasional basis (one or more visits per year; 74 %, n=25), and 76% (n=26) received a cleaning in the past year. Eighty percent (n=16) and 50% (n=7) of participants who, respectively, delivered at term and prematurely brushed at least twice a day. This difference was not statistically different (Fisher Exact, P=0.13).
15.2 Obstetrical Characteristics

15.2.1 Obstetrical history

Participants had a significant past pregnancy history with an average of 1.8 (±0.9) previous preterm births. The mean gravidity of the participants was 4.5 (±2.2) and the mean parity was 1.6 (±1.1). All participants had a history of spontaneous preterm birth (SPTB). The majority had a history of spontaneous abortion (52%, n=18). Four participants were diagnosed with uterine anomalies including arcuate uterus, bicornuate uterus and uterine fibroids.

15.2.2 Pregnancy history

Urinary and/or genital tract infections were observed at least once during the pregnancy and treated with antibiotics in 51% (n=18) of the participants. The cervical length at 20 weeks was recorded to be less than 30 mm in 11 (33%) participants. The cervical length at 25 weeks was recorded to be less than 25 mm in nine (28%) participants. A statistically significant relationship was found between SPTB and cervical length at 20 weeks (Fisher Exact test, \( P=0.01 \)) and at 24 weeks (Fisher Exact test, \( P=0.05 \)).

15.2.3 Medical interventions

Extensive interventions were administered to reduce the risk of SPTB, such as antibiotic therapy (77%, n=27), progesterone therapy (57%, n=20), and cerclage (51%, n=18). Two participants were taking antibiotics at the time of data collection to treat a genitourinary tract infection and 25 participants were taking prophylactically antibiotics (erythromycin) throughout their pregnancy. Both women who received a rescue cerclage delivered prematurely. Participants who received a cerclage intervention (54%, n=19) delivered prematurely significantly more often than women who did not receive this intervention (Chi-Square test, \( P=0.05 \)).

15.2.4 Pregnancy complications

Pregnancy complications were common in this cohort, including gestational diabetes (46%, n=16), antepartum hemorrhage (14%, n=5), hypertension (11%, n=4), pre-eclampsia toxemia (6%, n=2), and hypothyroidism (11%, n=4). Participants with diagnosed complications were treated according to their individual condition. Pregnancy complications were not statistically more common in women who delivered prematurely as opposed to women who delivered at term.
15.3 Oral Inflammatory Load

15.3.1 Descriptive data

Antenatal OIL indicators were collected at the mean gestational age of 20.6 weeks (±4.7). All participants were found to have a low OIL (less than 5.12 x10^6 cells/ml) with mean neutrophil counts of 2.05 (±0.80; 0.86- 4.31) x 10^6 cells/ml. Women who delivered prematurely tend to have a higher neutrophil count (2.21 ±0.88 x 10^6 cells/ml) than women who delivered at term (1.94 ±0.97 x 10^6 cells/ml), although this difference was not statistically significant (Student’s t-test, \( P=0.34 \)). The mean MMP-9 serum level of the study population was 1.42 (±0.84) x10^3 ng/ml and the mean fetuin serum level was 9.12 (±2.83) x10^5 ng/ml. Women who delivered prematurely tended to have a higher mean serum level of both MMP-9 and fetuin, but those differences were not statistically different (\( P=0.7; P=0.33 \)). Eighty-three percent (n=28) of the study population had a MGI of 1 or less (corresponding to healthy gingiva or at most, mild gingival inflammation) and 78% (n=27) had a Plaque Index of 1 or less (corresponding to a minimal plaque accumulation). On visual examination, early caries in five participants, geographic tongue in two participants, and traumatic ulcerative lesion on one participant, were observed.

15.3.2 Correlation between neutrophil counts and other indicators of the oral inflammatory load

No significant correlations were found between the neutrophil counts of the participants and traditional and non-invasive assessment measures of periodontal health, and MMP-9 and fetuin serum level as presented in Table 2.

<table>
<thead>
<tr>
<th>OIL Indicators</th>
<th>Correlation coefficient</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP serum level</td>
<td>-0.29</td>
<td>0.12</td>
</tr>
<tr>
<td>Fetuin serum level</td>
<td>0.03</td>
<td>0.87</td>
</tr>
<tr>
<td>MGI</td>
<td>0.07</td>
<td>0.82</td>
</tr>
<tr>
<td>PI (combined 0 &amp; 1 and 2 &amp; 3)</td>
<td>0.26</td>
<td>0.15</td>
</tr>
<tr>
<td>CI</td>
<td>0.17</td>
<td>0.08</td>
</tr>
</tbody>
</table>
15.3.3 Correlation between neutrophil counts and several factors potentially affecting the oral inflammatory load

No significant correlations were found between the neutrophil counts of the participants and several factors potentially affecting the oral inflammatory load as presented in Table 3.

Table 3. Correlation between Neutrophil Counts and Several Factors Affecting the OIL

<table>
<thead>
<tr>
<th>Factors</th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxiety and/or depression disorder</td>
<td>-0.11</td>
<td>0.55</td>
</tr>
<tr>
<td>Brushing at least twice a day</td>
<td>-0.08</td>
<td>0.87</td>
</tr>
<tr>
<td>Number of teeth</td>
<td>-0.11</td>
<td>0.55</td>
</tr>
<tr>
<td>Gestational age at data collection</td>
<td>-0.27</td>
<td>0.13</td>
</tr>
<tr>
<td>Progesterone therapy</td>
<td>-0.35</td>
<td>0.06</td>
</tr>
<tr>
<td>Antibiotic therapy</td>
<td>-0.17</td>
<td>0.34</td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>-0.17</td>
<td>0.35</td>
</tr>
</tbody>
</table>

15.4 Pregnancy Outcomes

Pregnancy outcomes are summarized in Table 4. The average gestational age at delivery was 34.4 (±5.3) weeks for the study population, 37.8 (±1.0) weeks for women who delivered at term, and 29.4 (±5.2) weeks for women who delivered prematurely. SPTB occurred in 40% (n=14) of the pregnancies. For the majority of pregnancies, the onset of delivery was spontaneous (93%, n=14) following preterm premature rupture of membranes (71%, n=10). SPTB also followed preterm labour in 50% (n=7) and incompetent cervix in 57% (n=8) of the participants. One participant with an incompetent cervix and an abdominal cerclage in place electively decided to deliver at 36 weeks with a cesarean section; this pregnancy was considered a success. Among nine histopathological reports of premature placenta available, chorioamnionitis was confirmed in six participants. The mean infant’s birth weight was 2.57 (±0.82) kg. Thirteen (40%) infants had a LBW. Premature infants had significantly more a low birth weight (<2500g) than infants born at term (Fisher Exact test, P<0.01). Eight premature infants required admission to the Neonatal Intensive Care Unit following neonatal complications such as respiratory distress syndrome, sepsis, anemia of prematurity, hypoxic ischemic brain injury, and retinopathy of prematurity. One infant was stillborn after a delivery at 17 weeks of gestation and one neonatal death of a premature infant occurred 11 days post-delivery.
Table 4. Descriptive Table of Pregnancy Outcomes

<table>
<thead>
<tr>
<th>Pregnancy outcomes</th>
<th>Total N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational Age at Delivery</td>
<td>34.43</td>
</tr>
<tr>
<td>Mean (standard deviation)</td>
<td>5.33</td>
</tr>
<tr>
<td>Preterm birth</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>43%</td>
</tr>
<tr>
<td>Very Preterm birth</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>31%</td>
</tr>
<tr>
<td>Onset of delivery:</td>
<td></td>
</tr>
<tr>
<td>Elective</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7%</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>93%</td>
</tr>
<tr>
<td>Preterm premature rupture of membranes</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>71%</td>
</tr>
<tr>
<td>Preterm labour</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>Incompetent cervix</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>57%</td>
</tr>
</tbody>
</table>

15.5 Risk Indicators Associated with Preterm Birth

Figure 2 through 5 present the relative risk for SPTB for identified suspected risk indicators. Only a few factors reached statistical significance in this study. The risk difference of SPTB between participants who brushed at least twice a day versus who did not was 0.3 (or 30%). The relative risk of SPTB in women brushing at least twice a day, although not statistically significant with a confidence interval including the null effect (1), was 48% of the risk when not brushing regularly (RR 0.48, 95% CI 0.22-1.0; P=0.13). Other dental health indicators such as regular dental visit, having received a dental cleaning in the past year and using a mouthwash and flossing regularly were not associated with a reduced risk for SPTB. The risk of PTB in participants who had a cervical length of less than 25mm at 24 weeks was 2.6 times that for those who had a cervical length greater than 25mm (RR 2.6, 95% CI 1.1-5.9; P=0.05). The risk of PTB in participants who had a cervical length of less than 30mm at 20 weeks was 3.2 times that for those who had a cervical length greater than 30mm (RR 3.2, 95% CI 1.4-7.5; P=0.01). The confidence intervals for the cervical lengths were wide, therefore the point estimates and the magnitude of the risks are imprecise. Participants who experienced antepartum haemorrhage were 2.2 times as likely to delivery prematurely (RR 2.2, 95% CI 1.2-4.2; P=0.14). All other characteristics, including all of the OIL indicators, were not identified as significant risk indicators for PTB. Although data were consistent with no effect, participants with a higher oral neutrophil counts tend to be more
likely to deliver prematurely (RR 1.3, 95% CI 0.8–2.2; \(P=0.34\)). Furthermore, the wide confidence interval indicated that a substantial risk is possible. This study did not show any risk or benefit of an elevated MMP-9 serum level, but a weak risk or benefit remain possible (RR 1.1, 95% CI 0.7–1.6; \(P=0.70\)). Fetuin serum level was not found to be associated with any risk or benefit for SPTB (RR 1.1, 95% CI 0.9–1.3; \(P=0.33\)). This study was not very informative in relation to PI, the MGI, and the CI. The confidence intervals for those variables were very wide, so not only was there no clear risk or benefit but also the estimate of risk were so imprecise that determination of substantial risk or benefit was not possible.

Figure 2. Demographic and Lifestyle Characteristics, Medical, and Dental History and Relative Risk of PTB (RR, CI 95%)

*Confidence intervals are represented by horizontal lines and point estimates are represented by rectangles
Figure 3. Obstetrical Characteristics and Relative Risk of PTB (RR, CI 95\%)

*Confidence intervals are represented by horizontal lines and point estimates are represented by rectangles

Figure 4. Oral Inflammatory Load and Relative Risk of PTB (RR, CI 95\%)

*Confidence intervals are represented by horizontal lines and point estimates are represented by rectangles
Part 2: OIL in Different Obstetrical Groups (Low Risk and High Risk)

Eighteen pregnant women enrolled in the Prevention of Preterm Birth Clinic at Mount Sinai Hospital (High Risk group) who were enrolled in the first part of this study had their oral rinse assay analyzed following the same materials and methods used by Huda et al. (2015), thus allowing comparison between the two groups in this cross-sectional study. The historical study by Huda et al. (2015) enrolled 63 pregnant women from the General Obstetrics Clinic (Low Risk group for PTB) from the same hospital. Oral neutrophil counts and pregnancy outcomes were recorded for all participants of both groups.

16.1 Characteristics of Participants

The demographic and lifestyle characteristics, and obstetrical and dental histories of pregnant women from the Low Risk and the High Risk groups are presented in Table 5.
Table 5. Demographic and Lifestyle Characteristics, and Dental and Obstetrical Histories of Pregnant Women Categorized by their Obstetrical Group

<table>
<thead>
<tr>
<th>Participants Characteristics</th>
<th>Low Risk Group</th>
<th>High Risk Group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Demographic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age 35 and above</td>
<td>23 (37%)</td>
<td>6 (33%)</td>
<td>0.80</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Caucasian</td>
<td>44 (70%)</td>
<td>4 (22%)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>3 (5%)</td>
<td>6 (33%)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>6 (10%)</td>
<td>3 (17%)</td>
<td></td>
</tr>
<tr>
<td>East Indian</td>
<td>5 (8%)</td>
<td>5 (28%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>5 (8%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High school completed</td>
<td>63 (100%)</td>
<td>18 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>Lifestyle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking during Pregnancy</td>
<td>2 (3%)</td>
<td>1 (6%)</td>
<td>0.53*</td>
</tr>
<tr>
<td>Drinking during Pregnancy</td>
<td>1 (2%)</td>
<td>0 (0%)</td>
<td>0.99*</td>
</tr>
<tr>
<td><strong>Dental History</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational Age at Data Collection; Mean (standard deviation)</td>
<td>19.2 (4.2)</td>
<td>20.4 (4.6)</td>
<td>0.29</td>
</tr>
<tr>
<td>Brushing at Least Twice a Day</td>
<td>61 (97%)</td>
<td>10 (56%)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Flossing at least 3x/week</td>
<td>39 (62%)</td>
<td>5 (28%)</td>
<td>0.01</td>
</tr>
<tr>
<td>Last dental visit within the past 6 months</td>
<td>41 (65%)</td>
<td>9 (50%)</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Pregnancy History</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertility Treatment</td>
<td>4 (7%)</td>
<td>2 (11%)</td>
<td>0.61*</td>
</tr>
<tr>
<td>History of Previous PTB</td>
<td>2 (3%)</td>
<td>18 (100%)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td><strong>Pregnancy Complications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>2 (3%)</td>
<td>8 (44%)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>1 (2%)</td>
<td>2 (11%)</td>
<td>0.12*</td>
</tr>
<tr>
<td><strong>Pregnancy Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm Birth</td>
<td>5 (9%)</td>
<td>9 (50%)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Onset of Delivery</td>
<td></td>
<td></td>
<td>0.99*</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>4 (80%)</td>
<td>8 (89%)</td>
<td></td>
</tr>
<tr>
<td>Indicated</td>
<td>1 (20%)</td>
<td>1 (11%)</td>
<td></td>
</tr>
</tbody>
</table>

* Significance was assessed by Fisher exact test

16.1.1 Demographics

Women from both groups were well-educated having completed high school, and the majority were under 35 years old (Low Risk: 63%; High Risk: 67%). The majority of the participants from the Low Risk group were Caucasian (70%, n=44), whereas the race of the participants from the High Risk group was more diverse, with 22% (n=4) being Caucasian, 33% Black, 17% Asian and
28% East Indian. This difference in the race distribution between the two groups was statistically significant (ANOVA, \( P=0.0001 \)).

16.1.2 Lifestyle

Two participants from the Low Risk group (3%) and one participant from the High Risk group (6%) reported smoking during their pregnancy. One woman from the Low Risk group (2%) reported drinking alcohol during her pregnancy.

16.1.3 Dental history

The great majority of the participants from the Low Risk group (97%, \( n=61 \)) reported brushing their teeth at least twice a day, compared to 56% (\( n=10 \)) from the High Risk group. This difference was statistically significant (Fisher exact test, \( P=0.00005 \)). Also, women from the High Risk group reported to floss at least three times a week significantly less than women from the Low Risk group (Chi-Square test, \( P=0.01 \)).

16.2 Obstetrical characteristics

16.2.1 Obstetrical history

Significantly fewer participants from the Low Risk group had a previous history of PTB (Fisher Exact test, \( P<0.0001 \)), with 100% from the High Risk group having delivered prematurely in the past, but only 3% of the participants from the Low Risk group had a history of previous PTB.

16.2.2 Pregnancy complications

Forty-four percent (\( n=8 \)) from the High Risk group developed gestational diabetes during their pregnancy, compared to 3% from the Low Risk group. This difference was statistically significant (Fisher Exact test, \( P=0.00005 \)).

16.2.3 Pregnancy Outcomes

Significantly fewer PTB occurred in the Low Risk group compared to the High Risk group: 5 and 9 pregnancies, respectively (Chi-Square test, \( P=0.0001 \)).
16.3 Oral Inflammatory Load

The OIL for both obstetrical groups is summarized in Table 6 and Figure 6. The mean neutrophil count was $4.66 \pm 2.06 \times 10^6$ cells/ml and $2.53 \pm 1.14 \times 10^6$ cells/ml for the Low and High Risk groups, respectively. The MGI was 0-1 (corresponding to an absence of inflammation, mild gingival inflammation) in 56% (n=35) of the Low Risk group and 89% (n=16) in the High Risk group. The OIL of the High Risk group was significantly lower compared to the Low Risk group for the following OIL indicators: neutrophil counts (Student t-test, $P<0.01$), and the Modified Gingival Index (Fisher Exact test, $P=0.01$). However, the Plaque Index was statistically higher in the High Risk group (Fisher Exact test, $P=0.05$).

<table>
<thead>
<tr>
<th>OIL Indicators</th>
<th>High Risk Group</th>
<th>Low Risk Group</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil counts ($\times 10^6$ cells/ml); Mean (standard deviation)</td>
<td>2.53 (±1.14 )</td>
<td>4.66 (±2.06 )</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plaque Index:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 or less</td>
<td>16 (88.9%)</td>
<td>63 (100%)</td>
<td>0.05*</td>
</tr>
<tr>
<td>2 or more</td>
<td>2 (11.1%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Calculus Index:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5 (27.8%)</td>
<td>29 (46.0%)</td>
<td>0.17</td>
</tr>
<tr>
<td>1 or more</td>
<td>13 (5.6%)</td>
<td>34 (54%)</td>
<td></td>
</tr>
<tr>
<td>Modified Gingival Index:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 or less</td>
<td>16 (88.9%)</td>
<td>35 (55.6%)</td>
<td>0.01*</td>
</tr>
<tr>
<td>2 or more</td>
<td>2 (11.1%)</td>
<td>28 (44.4%)</td>
<td></td>
</tr>
</tbody>
</table>

* Significance was assessed by Fisher Exact test

Table 6. OIL of Pregnant Women Categorized by their Obstetrical Group
Figure 6. Box Plot Comparing the Neutrophil Counts Based on the Obstetrical Group (Student’s t-test, P<0.0001)
Discussion

17 Results Summary

A meta-analysis by Kim et al. in 2012 has suggested that women at high risk for PTB (>22.2% of PTB outcome; Kim et al., 2012) showed a significant risk reduction of PTB following periodontal therapy. Periodontitis is, in healthy individuals, a preventable disease and given the significant impacts of PTB not only on premature infants but also on their families and on the social and health systems, a periodontal health screening in high risk population could result in significant cost savings. Therefore, this cohort study aimed to describe further the association between OIL and SPTB in a group of pregnant women at high risk of PTB.

Participants in this study were found to have a low OIL with mean neutrophil counts of 2.05 (±0.80; 0.86-4.31) x 10⁶ cells/ml. A previous and related study done in our laboratory/clinics (Huda et al., 2015) demonstrated that, using the identical assay for OIL, that this correlated with the degree or severity of periodontal disease in a Low Risk group of pregnant women and found that a neutrophil count of 2.86 x 10⁶ cells/ml corresponded to a healthy periodontium. Furthermore, using the periodontal status classification from Huda et al. (2015), only one participant of this study would be classified with gingivitis with a neutrophil count of 4.31 x 10⁶ cells/ml and no participants would be classified with periodontitis (which would require 5.83 x 10⁶ cells/ml).

OIL, as defined as the exposure to oral neutrophil counts (RR 1.3, 95% CI 0.8-2.2, P=0.34) was not identified as a significant risk indicator for SPTB in this study. However, women with increased oral neutrophil counts tended to be more at risk for SPTB. The results found in this study were not statistically significant which could be due to the lack of power or due to chance. To increase the precision of the study and effectively assess the magnitude of the association between OIL and SPTB, another study with a larger sample size would be necessary. Also, this study accounted for multiple suspected risk factors (confounders), but only a few of them were identified as reducing or increasing the risk of SPTB, which suggests the potential existence of other, unidentified risk factors. PTB and periodontal disease may also share a common underlying condition, a genetic or environmental confounder, which would cause an exaggerated inflammatory response that would explain the clinical response to oral pathogens and the
inflammatory process associated with PTB, especially SPTB and PPROM (Offenbacher et al., 2009; Vergnes & Sixou, 2007). Also, this study may have not been able to identify OIL as a risk indicator for PTB because the putative link between both conditions could, as described in detail above, only be through direct oral bacterial placental colonisation (Offenbacher et al., 2009).

MMP-9 expression and degradation of connective tissue activity have been suggested to play a role in the mechanism that leads to PPROM, which is a major cause of spontaneous preterm labour (Vadillo-Ortega et al., 2005). In this study, elevated MMP-9 serum level was not associated with an increased or reduced risk for SPTB (RR 1.1, 95% CI 0.7-1.6; P=0.70). The no effect observed in this study could have been due to the timing of blood collection which occurred in one occasion. Also the timing of the collection varied greatly between participants as samples were being collected between 12 and 26 weeks of gestation. Comparatively, Tency et al. (2012) found that the MMP-9 serum level concentrations were significantly higher in women with PTB in labour compared to women of matched gestational age and who subsequently delivered at term. Poon et al. (2009) also found that the maternal serum concentration of MMP-9 collected early during the pregnancy, between 11 and 13 weeks of gestation, was significantly increased in pregnancies resulting in SPTB. Interestingly, the mean MMP-9 serum level observed in this study population appeared to be high (1.42x10^3 ng/ml) compared to the group of pregnant women in labour who delivered prematurely in Tency et al. study (1.13 x10^3 ng/ml). In this study, all participants were at risk for SPTB following a previous history of SPTB therefore, this group of women may have an inherent predisposition or an unidentified confounder that may be the cause for this increased MMP-9 serum level and the high SPTB incidence.

Similarly, in this study elevated fetuin serum level was not shown to affect the risk for SPTB (RR 1.1, 95% CI 0.9-1.3; P=0.33). The mean level of serum observed in this study was quite high (912μg) in comparison to the mean serum levels of this protein measured in healthy individuals (300-600μg; Wang et al., 1998). Therefore it is conceivable that elevated levels of fetuin could be considered as a risk indicator for pregnant women at risk of SPTB. Whether even greater changes in fetuin might be seen in the presence of periodontitis or not, remains to be seen. In any case, the higher levels of fetuin noted here could help to downregulate TNF production and protect the fetus from abortion (Ombrellino et al., 2001; Wang et al., 1997). Low circulatory fetuin levels can be observed during injury and infection (Ombrellino et al., 2001), and has been associated with preeclampsia (<720μg/ml, adjusted odds ratio 3.69, CI 95% 1.8-7.5; P<0.001; Molvarec et
al., 2009). This study was the first to investigate the association between fetuin serum level and SPTB. But given our findings as well as others’, fetuin might, or not, play either a protective or deleterious role in relation to adverse outcomes of pregnancy depending on the specific adverse outcome being considered.

One of the secondary objectives of this study was to identify a non-invasive, fast and easily implemented assessment tool for OIL that could potentially be used by medical professionals with minimal dental training in order to determine the relative risk their patients might have for adverse outcomes of pregnancy (presuming of course that increased levels of OIL actually do correlate with these adverse outcomes). Three traditional, observational (and therefore non-invasive) periodontal assessment indices were recorded: the PI, the MGI and the CI. These indices were chosen as they require minimal equipment (light and gloves), can easily be taught to medical professionals with minimal dental knowledge, and are well tolerated by patients. These indices do not require the use of a probe, which can be accompanied with discomfort and require specific dental training, but it is also believed that they are likely to underestimate the extent of periodontitis based on the probing depths of periodontal pockets (Oliver, Brown & Loe, 1998). However, gingival inflammation during pregnancy without attachment or bone loss might still be sufficient in some susceptible individuals to affect birth outcomes (Moss et al., 2005). Also, without the use of a probe, the presence of gingival bleeding on probing (BOP) cannot be recorded which is another drawback in regard to the observational assessment of periodontal inflammation used in this investigation. In fact, some authors consider BOP to be the most consistent factor found during the active progression of periodontal disease (Lang et al., 1986; Vogt et al., 2012). In contrast, Haffajee et al. in 1983 showed that neither gingival redness, plaque, suppuration nor bleeding on probing, used as clinical parameters, demonstrated high sensitivity/specificity in regard to the advancement of chronic periodontitis. Therefore these measures might not be adequate for assessment of the activity of periodontitis in a patient population, which underscores why OIL based on PMN assessment is being investigated in our group. This could be one of the reasons why no correlation was found between the oral neutrophil counts and the relatively insensitive periodontal health assessment tests used on this study. Clinical indices assessing plaque and calculus can also be affected by occasional dental brushing. By contrast, gingivitis takes about seven days to develop (Woolweaver et al., 1972) and again, while there could be immediate changes in plaque levels, gingival changes being delayed would not be noticed giving
more reason for the disagreement between the various assessment methods. The oral neutrophil counts relate to the immediate state of gingival or periodontal inflammation and would also not be expected to correlate all that highly with changes in oral debris levels (i.e. plaque and calculus; Woolweaver et al., 1972). Therefore, it might be postulated that assessment of oral PMNs can give real time information regarding inflammation in the mouth and periodontium that is also not as subjective as are most indices that purport to measure inflammation (Klinkhamer & Zimmerman 1969). More importantly, this assessment tool relates to the active disease process, the host inflammatory response, and as discussed above, this is one of the suggested putative links between periodontitis and PTB.

As mentioned above, no significant correlation was found between the PMN counts and traditional periodontal assessment indices. This could potentially be explained by the small number of participants in this study and due to the low OIL in all participants of this cohort study (82%, n=28 with a MGI of 0 or 1). Similarly, Huda et al. in 2015 found that the MGI was significantly correlated to the neutrophil counts, except for a MGI score of 0 (healthy) and 1 (mild gingival inflammation). This may indicate that below a certain level of oral PMNs, the clinical presentation of inflammation can vary between individuals and even if the variance is small, given the low degree of inflammation under study, this could have a negative impact on the ability to demonstrate statistical significance with the variables under study.

This variability with relatively small numerical values might even explain, at least in part, the unexpected increased level for OIL in patients found in the Low Risk group as compared to the High Risk group. As well, despite the fact that the assessments of patients in both the High and Low Risk groups were carried out in parallel experimental designs, they still constitute separate investigations to a certain extent and therefore the data from the Low Risk patients actually represent more of an external control, which might not be as reliable as an internal control. Further along these lines, it should also be considered that studies of PTB in the third world have been more likely to demonstrate clear relationships between periodontitis and adverse outcomes of pregnancy. For example, given the status of individuals in the third world it is known that their burden of periodontal disease is greater than that seen in the 1st world (Tarannum et al., 2007). Clearly, in order to understand more clearly the actual relationships between OIL and adverse outcomes of pregnancy it is essential to focus on patients with more severe periodontitis than those included in the study done here as well as the parallel study done by Huda et al. (2015).
Based on the discussion above then, it should not be all that surprising that another unexpected finding was found; that is, in comparison to those in the High Risk group of patients, significantly more women from the Low Risk group were brushing at least twice a day (Fisher Exact test, \( P=0.01 \)) and flossing regularly (Chi-Square test, \( P=0.01 \)). These unexpected outcomes could be explained by the inherent differences and confounders complicating comparisons of the two groups. These differences could be related to demographic characteristics (race), obstetrical history, pregnancy interventions (including antibiotic therapy in the High Risk group), complications, and outcomes. These results are also based on a small number of participants (Low Risk: 63, High Risk: 18); therefore, findings need to be interpreted with caution and no generalization can be made at this point.

Despite the issues related to above, there were other findings that were more consistent with what might have been expected. In this regard it was demonstrated that brushing at least twice a day tended to be associated with a reduced risk of SPTB (RR 0.48, 95% CI 0.22-1.0; \( P=0.13 \)). Although interesting, this finding might have occurred by chance, especially when considering that a great number of variables were assessed in this pilot study. Conversely, no correlation was shown between the counts of PMN and daily frequencies of brushing by the participants (\( P=0.87 \)). Therefore, further investigations are indicated to explore the association between oral neutrophil counts and reported teeth brushing practices, and their relative risk for SPTB before any generalization and recommendation can be made.

## 18 Study Design Limitations

This study used a convenience sample based on women’s attendance to the Prevention of Prematurity Program and participants were followed over time to assess the occurrence of PTB. Thus, despite its small sample size, this study was considered a cohort study. To investigate causation effects, a comparison group would be needed; instead, levels of exposure to oral neutrophils were used in this study. Ideally, the study population would have been separated in two groups based on their PMNs counts. The definition of the exposed and unexposed groups would have been based on the results of a previous study by Huda et al. (2015), which correlated the neutrophil counts and the periodontal status in low risk pregnant women. Patients with a mean neutrophil count under \( 5.83 \times 10^6 \) cell/ml could have been matched to the unexposed group.
(patients with healthy periodontal status, gingivitis and/or mild periodontal disease). Patients with moderate to severe periodontitis (with mean neutrophil count over $5.83 \times 10^6$ cell/ml) would have been matched to the exposed group. However, all participants of this study had a neutrophil counts below $5.83 \times 10^6$ cell/ml; thus this statistical analysis was impossible. The low oral neutrophil counts observed in the study population could have occurred by chance. A sample size calculation indicated that a sample size of 146 participants would have been required to achieve statistical significance, having only 34 participants enrolled, this was only a pilot cohort study. Due to these study design limitations, results from this pilot cohort need to be interpreted with caution and can not be generalized to all of the high risk population.

19 Selection Bias

The study population was appropriately categorized as a high risk group for PTB, not only because taken together they had a high incidence of PTB (43%), but also individually they were all identified as having obstetrical risk indicators for SPTB. Studies trying to assess the association between periodontal disease and PTB are often of poor quality (Dasanayake, 2013), because they include both high risk and low risk subjects and do not collect all obstetrical history information and confounding variables, rendering the evaluation of the true effect of periodontal disease and/or treatment on PTB risk impossible. Participants in this study were selected from the Prevention of Prematurity Program, a specific clinic dedicated to pregnant women at risk for PTB. Pregnant women received medical and psychological care and treatment, which diminished their overall risk of preterm delivery and also increased the number of confounders. This advanced treatment and prevention protocol could have partially masked the effect of OIL on the risk of PTB. To account for this sampling bias, sociodemographic characteristics and obstetrical characteristics including obstetrical history, obstetrical observations (such as infection), interventions (such as any medication prescribed) and pregnancy complications were recorded and included in the analysis. Results from this study are in agreement with previous obstetrical studies which suggest that short cervical lengths at 20 and 24 weeks are associated with an increased risk for PTB (Iams et al., 1996). Cervical weakness or insufficiency is a known causal etiology for PTB (Goldenberg et al., 2008). Upon diagnosis, a cerclage intervention may be suggested or recommended. In this study, women who had a cerclage intervention were 2.3 times
(RR 2.3, 95% CI 0.9-5.88; \(P=0.05\)) more likely to deliver prematurely than women who did not receive a cerclage. These findings highlight the importance of cervical insufficiency as a risk indicator for PTB and the importance of considering this factor as a confounder. To prevent symptomatic and asymptomatic bacteremia, which have been associated with adverse birth outcomes (Romero et al., 1989), antibiotic treatment with erythromycin was administered to 25 (74%) participants considered at risk for recurrent infections throughout their pregnancy. Although these agents are known to have the ability to reduce periodontal inflammation, the very condition under study as a potential contributing factor leading towards the development of adverse pregnancy outcomes, the administration of antibiotic was not shown to reduce the incidence of SPTB, nor to reduce the oral neutrophil counts of the participants of this study. A previous pilot randomized study also failed to demonstrate any beneficial effect of antibiotic administration and scaling and root planning in pregnant women (Jeffcoat et al. 2003). A meta-analysis was also unable to show any significant effect of antibiotics on the incidence of preterm birth when administered to prevent genitourinary tract infection (Simcox et al., 2007).

Aiming to increase the study quality, pregnancy outcomes were also described in detail in this study. Michalowicz and colleagues in 2006 suggested that early spontaneous preterm birth (before 32 weeks of gestation) and stillbirth, as opposed to all preterm births, may be associated with periodontal disease. In this pilot study, one infant was stillborn and 31% (11 out of 15 infants born prematurely) were born very prematurely (before 32 weeks of gestation). Also, the majority of the preterm deliveries (93%, \(n=14\)) were spontaneous following PPROM in 71% (\(n=10\)) of the pregnancies. The presence of chorioamnionitis can also be a good indication of an active infection of the genitourinary tract, a distant infection, or could occur as a result of the indirect action of translocated bacterial endotoxins and/or maternal inflammatory mediators (McGaw, 2002). Unfortunately, placental pathohistological analysis is not routinely ordered, but among nine histopathological reports of premature placenta available in this study, chorioamnionitis was confirmed in six participants.

Stress, anxiety and depression are known risk indicators for both PTB and periodontitis (Genco et al., 1999; Wadhwa et al., 2001). Five participants in this study were diagnosed with depression and/or anxiety, but in general, pregnant women at risk of PTB are known to have a high anxiety level, especially around the gestational weeks of past pregnancy loss or PTB. This may be one of
the reasons why individuals with diagnosed psychological conditions were not associated with increased risk for SPTB, or increased neutrophil counts in this study.

Women who refused to participate in this study frequently mentioned their “fear of more bad news,” or that their scheduled standard medical evaluation was already “all they could endure.” This may suggest that women who refused to participate may have higher anxiety level and less coping skills, which in turn could affect their oral health and their risk for PTB. One could also hypothesize that the women who refused to participate may have more dental aversion, seeking less dental care and as a result may have more unmet dental and periodontal treatment needs (Armfield et al., 2014). This is yet another selection bias which could be one of the reasons why the OIL of this study population was low.

All participants received a dental examination at the time of data collection. Unmet dental treatment needs such as rampant decay and known/suspected periapical periodontitis (dental abscess) could increase the OIL. Furthermore, these infections could contribute to a systematic spread of infection and inflammatory mediators, increasing the risk for PTB. Patients with unmet dental needs often have unmet periodontal problems. This situation is not uncommon as patients face the same barriers to care such as anxiety and/or finances for both diseases. Therefore, it was decided not to exclude the four participants with caries lesions and the two participants with geographic tongue from the study, and to analyze the oral inflammatory load as opposed to strictly the periodontal inflammatory load of the study population.

20 Measurement Bias

The exposure variable (OIL) indicators were collected only once during each participant’s pregnancy (between 12-26 weeks of gestation). Some authors believe that neutrophil counts vary from person to person and fluctuate for a given person during the day according to sensations such as hunger and heat (Calonius et al., 1958), but vary minimally from one day to the other (Schiotte & Loe, 1970). Bender et al. in 2006 demonstrated that neutrophil counts remain relatively constant in each individual. Precautions were taken to try to minimize this potential measurement bias by asking the participants not to eat 30 minutes prior to the saliva sample collection and to swish with tap water for 10 seconds and expectorate prior to providing the saliva rinse sample. Also,
Despite that in this study the neutrophil counts were not shown to be correlated with the gestational age at data collection, the OIL may also vary throughout pregnancy. Although gingivitis incidence and severity tend to increase throughout pregnancy (Laine, 2002), for most women, the negative influence of the hormonal factors may be counterbalanced by additional plaque control (Amar & Chung, 1994). However, some women with pre-existing gingival conditions or with a particular susceptibility to periodontal disease could be at an increased risk for worsening oral health during their pregnancy following the hormonal changes, despite an improvement in their oral hygiene practices (Moss et al., 2005). Therefore, for each participants, the OIL result could have potentially varied according to the single time at which the sample was collected.

After consent was obtained, oral hygiene instructions were given to the participants of this study. Therefore after data collection but prior to delivery, some pregnant women may have improved their oral hygiene and consequently reduced their OIL prior to delivery. The provision of oral hygiene instructions have been shown to be inconsistently effective in improving the periodontal health of pregnant women. After oral hygiene demonstration during the course of the pregnancy, the plaque scores of pregnant women have been shown to be improved in a study by Lieff et al. in 2004, whereas Yalcin et al. in 2002 found no improvement. In the present study, individualized dental and periodontal recommendations were given following the oral examination. As such, any periodontal treatment and/or dental treatment received by the participants after data collection but before delivery could have biased the results.

21 Future Directions

PMNs counts as an indicator of the OIL could be used as a screening tool by other medical professionals to identify this potential risk indicator for PTB. Although harder to implement away from the clinical milieu, this screening test could be introduced when women are thinking about getting pregnant to reduce their OIL prior to conception. Alternatively, considering the difficulties encountered during recruitment (low participation rate), a similar study using the neutrophil count oral rinse could be executed by nurses as part of the protocol for medical workup for new patients in the High Risk Clinic. In this way, not only could the number of participants could easily be increased, but multiple oral rinse samples could be collected for each patient throughout her pregnancy including a sample prior to the institution of any medical interventions and another
taken at the time of delivery. Women identified with increased oral neutrophil counts could be referred to a dentist and/or a periodontist for a comprehensive periodontal examination and treatment, potentially contributing to reduce the risk of adverse pregnancy outcomes.

Further studies should also aim at describing the OIL differences between different obstetric risk groups to show if a particular group would benefit from oral health screening and education. Ideally, a multicentre case-control study could be implemented. Women from all obstetrical groups could be recruited and their oral neutrophil counts could be assessed at the time of the delivery by trained nurses. Participants delivering prematurely could be matched to women delivering at term allowing for comparison.

Lastly, before being able to implement such an oral health screening program, it would be important to understand obstetricians’ and nurses’ oral health knowledge and how this knowledge impacts on their practice behavior. Only a few studies (da Rocha et al., 2011; Morgan et al., 2009; Wilder et al., 2007) have evaluated the knowledge and attitudes of physicians and nurses toward periodontal and oral health in relation to PTB. Those studies highlighted that, in general, medical professionals have a good understanding of the possible association but that the incorporation of this knowledge into practice is limited.
Conclusions

- The OIL of pregnant women at risk of PTB, was found to be low in this pilot cohort study.
- Although the risk of SPTB could potentially be increased in pregnant women with increased oral neutrophil levels, this possible association was not found to be statistically significant.
- No correlation was found between the OIL and the Modified Gingival Index, Plaque Index, Calculus Index, and MMP-9 and fetuin serum levels in pregnant women at risk for PTB.
- The OIL of pregnant women from a High Risk group was statistically lower than the OIL from a Low Risk group for PTB.
- Due to the limitations of the study design and the small number of participants, results from this study need to be interpreted with caution and no generalization can be made. Moreover, since the participants clearly did not have severe periodontal disease (or even moderate), the measures of inflammation could have gone in any direction but not down, which probably limited the possibility of seeing any effects.
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# Appendix I: Epidemiological Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

<table>
<thead>
<tr>
<th>Condition</th>
<th>Conclusion</th>
<th>References</th>
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<tr>
<td>PTLBW</td>
<td>Positive correlation between periodontal disease and PTLBW</td>
<td>Offenbacher et al., 1996; Offenbacher et al., 2001; Khader et al., 2009; Lopez, Smith and Gutierrez 2002; Mokeem, Molla et al.,-Jewair 2004; Rajapakse et al., 2005; Dortbudak et al., 2005;</td>
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<td>No correlation between periodontal disease and PTLBW</td>
<td>Davenport et al., 2002; Noack et al., 2005; Vettore et al., 2008; Agueda et al., 2008</td>
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<tr>
<td>PTB</td>
<td>Positive correlation between periodontal disease and PTB</td>
<td>Agueda et al., 2008; Bosnjak et al., 2006; Goepfert et al., 2004; Guimaraes et al., 2010; Jeffcoat et al., 2001; Pitiphat et al., 2008; Radnai et al., 2004; Radnai et al., 2006; Rakoto-Alson et al., 2010; Offenbacher et al., 2006; Offenbacher et al., 2001; Sharma et al., 2007</td>
</tr>
<tr>
<td></td>
<td>No correlation between periodontal disease and PTB</td>
<td>Farrell et al., 2006; Holbrook et al., 2004; Lunardelli and Peres 2005; Moore et al., 2004; Moore, Randhawa and Ide 2005; Ryu et al., 2010; Skuldbol et al., 2006; Wood et al., 2006; Srinivas et al., 2009</td>
</tr>
<tr>
<td>Very PTB</td>
<td>Positive correlation between periodontal disease and VPTB</td>
<td>Guimaraes et al., 2010; Offenbacher et al., 2006</td>
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<tr>
<td></td>
<td>No correlation between periodontal disease and VPTB</td>
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### Appendix II: Intervventional Studies Investigating Periodontal Disease and Adverse Pregnancy Outcomes

<table>
<thead>
<tr>
<th>Periodontal treatment during pregnancy (RCT)</th>
<th>Reduction in the incidence of PTB and LBW delivery</th>
<th>Lopez et al., 2002; Tarannum and Faizuddin 2007; Jeffcoat et al., 2003; Mitchell-Lewis et al., 2001; Offenbacher et al., 2006; Radnai et al., 2009; Sadatmansouri, Sedighpoor and Aghaloo 2006</th>
</tr>
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<td>No reduction in the incidence of PTB and LBW delivery</td>
<td>Macones et al., 2010; Michalowicz et al., 2006; Newnham et al., 2009; Offenbacher et al., 2009</td>
<td></td>
</tr>
</tbody>
</table>
Appendix III: Eligibility Criteria

A. Inclusion Criteria

- Women from the Prevention of Prematurity Program with history of previous SPTB or at risk of PTB in the current pregnancy with no previous history of SPTB
- Women who presented with a gestation age equal greater to 12 weeks and less than or equal to 26 weeks
- Pregnant women who are at least 16 years of age and capable of giving informed consent
- Women who have at least 20 natural teeth

B. Exclusion Criteria

- Women who present with systemic diseases that could affect immune function/neutrophil response such as immunocompromising diseases, cardiac disease or renal disease
- Women who present with multiple gestations, known fetal congenital anomaly or aneuploidy
- Women who had a fetal invasive procedure such as amniocentesis or chorionic villi sampling
- Women with a known documented bacterial upper respiratory infection at the time of recruitment
Appendix IV: Information booklet provided to the patients
Appendix V: Patient Consent Form

CONSENT TO PARTICIPATE IN A RESEARCH STUDY

**Title**  
Oral Inflammatory Load as a Risk Factor for Spontaneous Preterm Birth in Women at Risk of Preterm delivery

**Investigator**  
Dr. Michael Sigal, Dr. Howard Tenenbaum

**Co-Investigators**  
Dr. Marie-Lyne Gosselin, Dr. Wendy Whittle,  
Dr. Michael Glogauer, Dr. Michael Goldberg, Dr. Amir Azarpazhooh

**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 or study staff 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 gums disease may have more risk to give birth prematurely. Other studies have 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.
A recent study have demonstrated that an simple oral water rinse test that measures intra-oral inflammation by counting neutrophils (a type of white blood cell) can reflect the gum health status of the patient.

The purpose of the study is to evaluate if poor gum health status as measured by the oral rinse test is associated with preterm birth. In addition, the study will test if some other indicators of inflammation associated with poor gum health present in the mouth and in the blood circulating in the whole body (measured by a blood test) are also associated with preterm birth.

This study will bring about a deeper understanding of the relationship between mother's gum health and preterm birth.

**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 have a dental examination. Your teeth, lips, gums and tongue will be examine with a light and mirror for any signs of significant abnormalities or disease. No dental x-rays will be taken.
3. 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 are planned to count.
4. The findings of your dental examination will be discussed with you and if any 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. This study requires a blood collection, some blood will be drawn to assess for some inflammatory cells present in your body. This will be executed by the scheduled nurse or laboratory technician. This blood collection will be drawn at the same time than your routine medical blood collection. The additional amount of blood that will be drawn for the study will be 1-2 table spoon.
6. This entire procedure should not take any longer than 30 minutes and there are no additional hospital visits that are required as part of this study.
7. The care that you receive during your pregnancy will be according to the hospital standards.
8. 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 medical chart and your infant's hospital chart.
9. Finally, if you agree to participate in this study, we will require your permission to contact you by phone after your child's birth to ask some specific additional information about your teeth and gums health. This phone call will not take more than 2 minutes.

**Risks**

There is no foreseeable harm or injury as a result of participating in this study other than the time spent participating in the study. If you are diagnosed with gums or dental problems, you may feel stress and anxiety knowing you may be more at risk for preterm birth.
**Benefits**

An 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.

**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, postal code, 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 Boards 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 your name or address, or any information that directly identifies you.

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 costs 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, sponsors 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 (or study drug/intervention) involved with this study.

**Conflict of Interest**

The Mount Sinai Hospital Department of Dentistry, the sponsor of this study, will pay the hospital and researcher for the costs of doing this study. The study team has an interest in completing this study. Their
interests should not influence your decision to participate in this study. You should not feel pressured to join this study.

Questions About the Study

If you have any 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 Marie-Lyne Gosselin at 647-338-2600.

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
Was the participant assisted during the consent process? □ YES □ NO

If YES, please check the relevant box and complete the signature space below:

☐ The person signing below acted as a translator for the participant during the consent process and attests that the study as set out in this form was accurately translated and has had any questions answered.

Print Name of Translator  ___________________________  Signature  ___________________________  Date

Relationship to Participant  ___________________________  Language  ___________________________

☐ The consent form was read to the participant. The person signing below attests that the study as set out in this form was accurately explained to, and has had any questions answered.

Print Name of Witness  ___________________________  Signature  ___________________________  Date

Relationship to Participant  ___________________________
Appendix VI: Clinical Examination Form

Case Number:
Date:

**Soft tissues**
Modified gingival index:
Plaque index:
Calculus index:
Other soft tissue pathology and description:

**Hard tissues**
Number of teeth:
Appendix VII: Modified Gingival Index (Lobene et al., 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
Appendix VIII: Plaque Index (S. 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 the entire clinical crown

3: Plaque extending over all interproximal and gingival surfaces covering more than one half of the entire crown
Appendix IX: Calculus Index (S. 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: Abundant amount of supra and subgingival calculus
## Medical History

*Do you have any problems related to:*

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<thead>
<tr>
<th>System</th>
<th>Y/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart/Blood Pressure (Cardiovascular system)</td>
<td></td>
</tr>
<tr>
<td>Lungs (Respiratory system)</td>
<td></td>
</tr>
<tr>
<td>Central nervous system</td>
<td></td>
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<tr>
<td>Immune system</td>
<td></td>
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<tr>
<td>Digestive system (Gastrointestinal system)</td>
<td></td>
</tr>
<tr>
<td>Diabetes/Thyroid (Endocrine system)</td>
<td></td>
</tr>
<tr>
<td>Bleeding (Hematology)</td>
<td></td>
</tr>
<tr>
<td>Kidney/Bladder (Genitourinary system)</td>
<td></td>
</tr>
<tr>
<td>Musculoskeletal system</td>
<td></td>
</tr>
</tbody>
</table>

*Do you take any medication(s)?* Y/N ______________________________

*Have you taken any antibiotics in the last 2 weeks?* Y/N Why________________________

*Do you take any supplement(s)?* Y/N ______________________________

*Do you have any allergies?* Y/N ______________________________

## Dental History

*Do you take antibiotics before your dental appointments?* Y/N ______________________________

*When was your last visit to the dentist?* ___________________________ Reason________________________
When was your last cleaning at the dentist?

Brushing frequency _0x/ 1x / 2x / 3x / >3x per day

Flossing _Y / N _Frequency________ per day/week/month

Mouthwash _Y / N _Type (brand)___________ Frequency________ per day/week/month
Copyright Acknowledgement

Thank you to Dr Sabrina Huda who provided me with the results from her Oral Rinse Assay for Assessment of Inflammation in Pregnant Women study (published in 2015), thus making the Part 2 of this project possible.