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"A MOLECULAR INVESTIGATION OF THE PREVALENCE OF SUSPECTED PERIODONTOPATHOGENS AND THEIR ASSOCIATION WITH PRETERM BIRTH."

By

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Medical Bioscience

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A MOLECULAR INVESTIGATION OF THE PREVALENCE OF SUSPECTED PERIODONTOPATHOGENS AND THEIR ASSOCIATION WITH PRETERM BIRTH

CLAUDE BAYINGANA

KEYWORDS:

- **Preterm birth**
- **Periodontal disease**
- **Cytokines**
- **Pregnancy**
- **Anaerobes**
- **Immunoglobulins**
- **Molecular**
- **Periodontal pathogens**
- **Polymerase Chain Reaction (PCR)**
- **Enzyme Linked Immunosorbent Assay (ELISA)**

ABSTRACT

More than 20 million infants in the world (15.5 % of all births) are born with low birth weight. Ninety-five % of them are in developing countries. Oral colonization of Gram-negative anaerobes has been implicated as a risk factor for preterm delivery of low birth weight (PLBW) infants. The objective of this study was to investigate the association between periodontal pathogens and pre-term delivery of low birth weight (PLBW) infants. The study sample included 200 randomly selected women admitted to the department of obstetrics-gynecology of the teaching hospital of Butare in Rwanda. Mothers were asked to complete a questionnaire in order to identify factors which might pose a health risk to them and their infants. Gingival crevicular fluid (GCF) was collected from each quadrant of the mother's month (using paper points) within 24 hours of delivery. Ten ml of foetal cord serum samples were collected at delivery and 10 ml of maternal serum samples were collected within 48 of delivery. GCF was examined by PCR for the presence of 5 periodontopathogens and ELISA was used for the evaluation of cytokines (IL-6 and IL-10) and immunoglobulins (IgM, IgG) in foetal cord and maternal blood against the periodontopathogens. *P. intermedia* showed significant associations either on its own or in combinations with most indicators of periodontal disease used in this study, while *Aa* and members of the **red complex** were significantly associated with gum bleeding and reduced frequency of tooth brushing. A strong association between PLBW and maternal and foetal cord serum sample levels of IL-10 was observed. Also, a good association was observed between PLBW and FCB sample levels of IL-6. Significant associations were observed between PLBW and maternal IgG against the different peridontopathogens. The findings of this study may suggest that the levels of maternal IgG and foetal IgM against the different periodontopathogens are associated with dissemination of maternal periodontopathogens to the foetus thereby illiciting an inflammatory response which contributes to PLBW.

DECLARATION

I declare that this work is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Claude BAYINGANA **October 2010**

Signed:……..

PEER REVIEWED ARTICLES PUBLISHED FROM THIS STUDY

1. COMPARISON OF PCR AND BANA HYDROLYSIS IN THE DETECTION OF ORAL ANAEROBES IN SUBGINGIVAL PLAQUE.

African Journal of Microbiology Research 2010; 4(9): 771-774 (See abstract, appendix G)

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2. EXAMINATION OF MATERNAL GINGIVAL CREVICULAR FLUID FOR THE PRESENCE OF SELECTED PERIODONTOPATHOGENS IMPLICATED IN THE PRE-TERM DELIVERY OF LOW BIRTHWEIGHT INFANTS.

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3. RISK FACTORS WHICH MAY INFLUENCE PRETERM DELIVERY OF LOW BIRTH WEIGHT (PLBW) IN AN AFRICAN POPULATION.

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DEDICATION

I dedicate this thesis to my wife and children for their love, sacrifice and patience for my success.

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LIST OF ABREVIATIONS USED IN THIS STUDY

+C: Positive control

-C= Negative control

Aa: *Aggregatibacter actinomycetemcomitans*

***Aa*-IgG**: IgG against *Aggregatibacter actinomycetemcomitans*

***Aa*-IgM**: IgM against *Aggregatibacter actinomycetemcomitans*

Ag: Antigen

ANUG: Acute Necrotizing Ulcerative Gingivitis

ATCC: America Type Culture collection

CIPD: Chronic Inflammatory Periodontal Disease

DNA: Deoxyribonucleic Acid

dNTP: Deoxynucleotide-Triphosphate

EDTA: Ethylendiaminetetraacetic acid

EIA: enzyme immunoassay

ELISA: Enzyme-linked Immunosorbent Assay

FCB: Foetal Cord Blood

FIRS: Fetal Inflammatory Response Syndrome

Fn*: Fusobacterium nucleatum*

***Fn*-IgG**: IgG against *Fusobacterium nucleatum*

***Fn*-IgM**: IgM against *Fusobacterium nucleatum*

GCF: Gingival Crevicular Fluid

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IL: Interleukin

JAMA: Judicial Affairs, American Medical Association.

LBW: Low Birth Weight

LPS: Lipopolysaccharide

NIH: National Institutes of Health

NT: Normal Term

MB: Maternal blood

O.D: Optical Density

PA: Protein A

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate buffered Saline

PBS-T: Phosphate buffered Saline- Tween

PCR: Polymerase Chain Reaction

Pg: *Porphyromonas gingivalis*

***Pg*-IgG**: IgG against *Porphyromonas gingivalis*

***Pg*-IgM**: IgM against *Porphyromonas gingivalis*

Pi: *Prevotella intermedia*

***Pi*-IgG:** IgG against *Prevotella intermedia*

***Pi*-IgM**: IgM against *Prevotella intermedia*

PG: prostaglandin

PLBW: Preterm Delivery of Low Birth Weight infants

PT: Preterm

PTB: Preterm birth

PTD: Preterm Delivery

PTL: Preterm Labor

SDS: sodium dodecylsulphate detergent

SIRS: Systemic Inflammatory Response Syndrome

SPSS: Statistical Package for the Social Sciences

STD: sexually transmitted disease

TBE: Tris Borate EDTA

Td*: *Treponema denticola

***Td*-IgG**: IgG against *Treponema denticola*

***Td*-IgM**: IgM against *Treponema denticola*

Th: T-helper

TNF: Tumor Necrosis Factor

TSA: Trypticase Soy Agar

UV: Ultra Violet

WC: Wilkins Chalgren anaerobic broth medium

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. BACKGROUND:

Clinical research has in the past largely excluded women. Diseases that affect them disproportionately were less likely to be studied (Angel, 1993; Schiebinger, 2003) and it is only during the last decade that research has focused on issues relating to women's health. In the US for example, it is only since 1990 when the office of research on women's health within the office of the Director at the National Institutes of Health (NIH), reviewed inequities in biomedical research, treatment and diagnosis of diseases affecting women (Schiebinger, 2003). More research is needed, particularly in pregnant women, of whom three-quarters require drug therapy during pregnancy and use prescription medication for chronic conditions such as diabetes or depression (Schiebinger, 2003).

It has been suggested that oral infection can act as the site of origin for dissemination of periodontopathogens and their toxins as well as induce inflammatory mechanisms to distant body sites, thus linking periodontal diseases to pre-term delivery of low birth weight (PLBW) infants (Offenbacher *et al*., 1996, 1998a,b, 2001; Dasanayake, 1998; Champagne *et al*. 2000; Williams *et al*. 2000; Louro *et al*. 2001; Jeffcoat *et al*., 2001; Mitchell-Lewis *et al*., 2001; Lopez *et al*., 2002; McGaw, 2002; Amar and Han, 2003; Han *et al*., 2004; Russel and Dasanayake, 2006; Lin *et al*., 2007).

Although women take better care of their teeth than men, three-quarters of periodontal office visits are made by women (Zakrzewska, 1996; University of Maryland Medical Center, 2008). Female hormones during puberty, menses, pregnancy, contraceptive use and menopause have been suggested to play an important role in periodontal disease infection (Steinberg, 2000; Sorry, 2000; Blagojevic *et al*, 2002, Krejci and Bissada, 2002, Yalcin *et al*., 2002; Mascarenhas *et al*., 2003; University of Maryland Medical Center, 2008). Periodontal disease is an infection of the tissues surrounding and supporting the teeth (Marsh and Martin, 1992). The increases of estrogen and progesterone concentration in plasma stimulate bacterial growth and are associated with periodontal disease progression (Zachariasen, 1991, 1993; Soory, 2000; Tilakarantne *et al.*, 2000; Bueltmann and Stillman, 2002; University of Maryland Medical Center, 2008).

1.2. PRE-TERM DELIVERY AND LOW BIRTH WEIGHT INFANTS

Preterm birth is defined as birth before 37 weeks of gestation (Offenbacher, 1996; Davenport, 1998; Steer, 2005). Several organ systems in the normal human foetus mature between 34 and 37 weeks, and adequate maturity of the foetus is reached at the end of that period (Steer, 2005). Low birth weight infants are those who weigh less than 2500 gram. Less than 32 weeks of gestation and less than 1500g are defined as very premature and extremely low birth weight respectively (World Health Organization, 1950; Truth and consequences, 2009). One-third of low birth weight infants are due to preterm delivery, however, infants may be underweight for reasons other than preterm delivery (World Health Organization, 2009).

PLBW is increasing extensively and becoming an important problem in both developing and developed countries (McGaw, 2002; Vogel *et al*., 2005). More than 20 million infants in the world (15.5 % o all births) are born with low birth weight. Ninety-five % of them are in developing countries (United Nations Children's Fund and World Health Organization, 2004). The rate of low birth weight (LBW) in developing countries is more than double (16.5 %) that in developed countries (7 %) and in Sub-Saharan Africa, the rate is around 15 %. In the United States of America in 1993, the rate of low birth weight among blacks was 13.3 %, 6.0 % among white and 4.9 % among Chinese (Hack and Merkatz, 1995). In Denmark it has escalated from 5.6 % in 1994 to 6.9 % in 2003, in Canada from 6.4 % in 1981 to 7.1 % in 1996 (Vogel *et al*., 2005). Generally, the rate of preterm birth in Europe and many other developed countries varies from 5 to 9 % (Goldenberg *et al*, 2008).

It is known that PLBW infants are exposed to serious health problems, including, neurodevelopmental disturbances, ear infections, respiratory infections, asthma and death (Shapiro *et al.*, 1980, Cooke, 2006; Hack and Costello, 2007). Ten % of neonatal mortality world-wide is caused by prematurity (Child Health Research Project Special Report, 1999). In the US, 25 % of neonatal mortality is due to prematurity (Mathew and MacDorman, 2006). Preterm delivery is a significant cost factor in healthcare resulting in a considerable cost of long-term care for children with disabilities. A study in US showed a neonatal cost of $ 224, 400 for a newborn at 500-700 g, while only $1,000 for a newborn at over 3,000 g (Gilbert *et al*., 2003).

Different factors have been linked with a higher risk of preterm delivery: Low socio-economic standards, educational level, single motherhood, age at the upper and lower end (> 35 or < 18 years of age), multiple pregnancies (twins, triplets etc…), smoking and alcoholism during pregnancy, maternal medical conditions such as high blood pressure, maternal diabetes, and heart disease (Shiono *et al*., 1995; Gardner *et al*., 1995; Martius *et al*., 1998; Goldenberg *et al*., 1998, Parazzini *et al*., 2003; Rosenberg *et al*., 2005; Goldenberg *et al*., 2008). Infections play a major role in the cause of PLBW (Goldenberg *et al*., 2000). Researchers showed that between 18 and 50 % of all pre-term deliveries are associated with periodontal disease and this appeared to increase more than 7 times after adjusting for smoking, vaginosis treatment history, marital status, previous preterm, race and age (Offenbacher *et al*. 1998a, 2001; Romero *et al*. 1998; McGaw, 2002). In most of the cases of PLBW, the aetiology is unknown and no method to prevent pre-term labour has proven effective (Schellenber, 2003).

1.3. EXPLORING THE ROLE OF PERIODONTAL INFECTIONS

1.3.1. Background

Periodontium or periodontal tissues, are tissues that surround, support and maintain the teeth in the maxillary and mandibular bones. The periodontium is formed by the gingiva, the alveolar bone, the periodontal ligament and the cementum (Manson and Eley, 2000). Like other tissues, the periodontal tissues are subject to a number of diseases. The disease process may be limited to the gingiva or involve the deeper periodontal structures (Manson, 1970).

The 2 forms of periodontal disease are gingivitis and periodontitis. In gingivitis, the gingiva are red, swollen and can bleed easily resulting in false pocket formation. Gingivitis can be treated by improving oral hygiene practice. If it is not treated, toxins from bacteria can penetrate deep tissues of the periodontium and destroy the periodontal membrane and the alveolar bone causing periodontitis. A true periodontal pocket is formed, caused by the migration of the junctional epithelial tissue at the base of the gingiva down the root of the tooth (Levison, 1997). At the late stage of periodontitis, gingiva and alveolar bone can be seriously damaged, resulting in tooth loss (Marsh and Martin, 1992, Bagg *et al*., 1999).

Most periodontal diseases are associated with the presence or overgrowth of anaerobic bacteria either alone or in association. Using statistical analysis, Socransky *et al.,* (1998) clustered frequently occurring bacterial species into complexes which were colour-coded in order to facilitate discussion (Table 1). The **red complex** was found to be most closely associated with pocket depth and bleeding on probing, while the **orange complex** related to pocket depth but less frequently with the other clinical parameters used in the diagnosis of periodontal disease.

Table 1: Bacterial clusters described by Socransky *et al* (1998).

Complex	Bacterial cluster
Red	*Treponema denticola, Porphyromonas gingivalis, Tannerella forsythia*
Orange	*Fusobacterium nucleatum, Prevotella intermedia, Prevotella nigrescens, Micromonas (Peptostreptococcus) micros, Eubacterium nodatum, Campylobacter rectus, Campylobacter showae, Streptococcus constellatus, Campylobacter gracilis*)
Yellow	*Streptococcus sanguis, Streptococcus oralis, Streptococcus mitis, Streptococcus gordonii, Streptococcus intermedius*
Green	*Capnocytophaga, Campylobacter concisus, Eikenella corrodens, Aggregatibacter actinomycetemcomitans* (serotype a)
Purple	*Veillonella parvula, Actinomyces odontolyticus, Aggregatibacter actinomycetemcomitans* (serotype b), *Selenomonas noxia, Actinomyces naesludii*

Among the suspected periodontopathogens associated with PLBW are the **orange** and **red complexes** which were found in 18% of full-term and 100% of preterm delivery cases (Offenbacher *et al.* 1996; Dörtbudak *et al.*, 2005). Microbiology and immunology studies reveal that high maternal immunoglobulin G (IgG) levels against periodontopathogens and high counts of periodontopathic bacteria were found in mothers who delivered PLBW (Hill, 1998; Offenbacher *et al.* 1996; Dasanayake *et al.* 2001**,** 2003; Madianos *et al.*, 2001;

Dörtbudak *et al*., 2005; Lin *et al*., 2007). However, Madianos *et al*., (2001) and Lin *et al*, 2007 reported that lack of maternal IgM antibody to the **red complex** was associated with preterm delivery and that maternal IgG to suspected periodontopathogens was associated with a decrease of PLBW frequency.

No difference was found in the levels of dental plaque periodontopathogens or serum antibody between mothers with PLBW infants and the control group in a study by Jarjoura *et al*., (2005). In a recent meta-analysis of randomized controlled trials (RCTs) of pregnant women with periodontal disease, after pooling the results of 10 published eligible RCTs, the researchers found that treatment for periodontal disease did not reduce the rate of preterm birth (PTB) (Michalowicz *et al*., 2006; Offenbacher *et al*., 2009; Jeffcoat, 2010; Niederman, 2010).

In contrast, greater attachment loss was associated with PLBW. Another study with a small interventional trial found that effective periodontal treatment in the second trimester of pregnancy had 3.8 times reduction in the rate of PLBW compared to the control group with delayed periodontal treatment (Offenbacher *et al*., 2006). Lin *et al* (2007) suggested that microbiological and serologic markers could be used in the regular gynecological checkup during pregnancy to prevent PLBW.

1.3.2. The host immunological response in periodontal disease

The inflammatory defense mechanisms in periodontal disease are defined as non-specific, because the inflammatory stimulus gives rise to similar reactions. The response is quick and designed to stop the spread of infection, but it usually requires an immune response targeted at the specific periodontopathogens present (David *et al.*, 1992). Evidence of the activity of the immune mechanisms in periodontitis include the presence of lymphocytes and plasma cells in the gingiva, circulating antibody to plaque antigens and the correlation of antibody levels with disease severity (David *et al.*, 1992).

Antigens are carried to the local lymph nodes by macrophages and presented to lymphocytes which circulate them through the nodes and tissues (David *et al.*, 1992). The lymphocytes which recognize each individual antigen are activated and secrete antibody under the control of helper and suppressor T lymphocytes. B and T lymphocytes have been implicated as the primary sources of bone resorption lesions in periodontal disease (Kawai *et al.*, 2006). The predominant antibody is IgG (75 %) with a small amount of IgM (7 %). After passing the gingival inflammatory exudates, antibodies pass into the gingival crevice in the crevicular fluid (David *et al.*, 1992).

Due to a continual presence of plaque bacteria in periodontal disease, there is no primary response followed by a larger secondary response like in acute infections. In contrast, there is a prolonged secondary response of constant antibody secretion which will persist at a lower level even after effective treatment (David *et al.*, 1992). Although most of the

antibody in crevicular fluid is derived from the blood, around 15 % of the IgG is produced locally by plasma cells in the tissues.

A high rate of circulating antibodies against different oral bacterial species and many of their products have been detected in adult periodontitis, such as *P. gingivalis* and *A. actinomycetemcomitans* in aggressive periodontitis and *Prevotella intermedia* in Acute Necrotizing Ulcerative Gingivitis (ANUG). Although antibodies have not been shown to be protective in humans, polyclonal activators stimulate B lymphocytes and plasma cells to secrete important cytokines, including interleukin-1 (IL-1) and tumour necrosis factor (TNF) (David *et al*., 1992). Cytokines play different roles in periodontal disease, especially in periodontitis, including coordination of tissue turn-over, immunological and inflammatory processes.

Cell-mediated reactions play a role in fighting against persistent antigens which are resistant to degradation, intracellular pathogens and tumour cells. Few cell-mediated reactions may occur in periodontal disease and may be more important during childhood gingivitis than in adult periodontitis (David *et al*., 1992). Seymour *et al*., (1996) demonstrated the role of cell-mediated mechanisms in the control of periodontal destruction and suggested that cytokine therapy for the treatment of periodontal disease may become a best option.

1.3.3. Role of cytokines in PLBW and their proposed mechanism of action:

IL-1 ß and TNF- α have been reported to regulate trophoblastic cell apoptosis (Yui *et al*., 1994). IL-1β detected in second trimester amniotic fluid has been shown to exhibit an increase in the onset of labour (Romero *et al*. 1989, Opsjøn *et al*., 1993). Konopka *et al*., (2003), found that women with PLBW had a significantly higher level of prostaglandin E_2 ($PGE_{2)}$ and IL-1 ß concentrations in gingival crevicular fluid (GCF). The level of TNF-α was significantly elevated in the serum of mice with foetal growth restriction compared to levels in mice with normal foetuses (Lin *et al*., 2003). Parent (1990), reported that placenta necrosis and foetal resorption could be induced in rats by the injection of recombinant human IL-1 β. Cytokines stimulate PGE_2 by the human placenta and chorioamnion (Romero *et al*. 1988b, Gibbs *et al*. 1992, Kent *et al*., 1993) and the role of prostaglandin in regulating the normal physiology of pregnancy has been well documented (Offenbacher *et al*., 1998).

The biological mechanism by which periodontal disease may trigger PLBW has not been clearly established (Bueltmann, 2002), but an understanding of the response of the mother's immune system to the infection may explain the mechanism postulated by Offenbacher *et al*., (1996, 1998a), Williams *et al*. (2000) and Gibbs *et al*, (2001) which suggest that the infection may activate the liberation of inflammatory mediators, growth factors and cytokines which may provoke PLBW. High levels of inflammatory cytokines (IL - 1, IL - 6, and TNF - α) have been found in the amniotic fluid of patients in preterm labour (Gibbs *et al*., 1992; Offenbacher *et al*., 1996; Dörtbudak *et al*., 2005). Hillier *et al*. (1993) found that infection related to preterm labour before 34 weeks was associated with increased levels of

IL-1, IL-6 and TNF; they proposed that infection is one cause of preterm delivery operating through a mechanism involving the induction of cytokine production. Previous studies reported that mitogen-stimulation of maternal peripheral blood mononuclear cells in women undergoing preterm delivery elicited higher production of Th1 cytokines and decreased production of the Th2 cytokines (Zhang *et al*., 2000; Raghupathy *et al*., 2001; Shokshi *et al*., 2002; Makhseed *et al*., 2003). Gomez *et al*.(1997) reported that while the pathopysiology of preterm delivery is not yet well understood, it is proposed that many of the pathological effects observed in preterm deliveries are mediated by endogenous host molecules such as cytokines.

Different mechanisms have been postulated by Offenbacher *et al*. (1998a), which can explain biological bacterially induced activation of cell-mediated immunity, leading to the production of cytokines including:

1. Translocation of periodontal pathogens such as *F. nucleatum* and *C. rectus* to the fetoplacental unit resulting in the production of IgM (which cannot pass the placenta barrier) obtained from foetal cord serum samples (Offenbacher *et al.* 1999). Transient bacteremia caused by periodontal infection may facilitate bacterial transmission from the oral cavity to the uterus (Han et al., 2004).

2. Translocated bacterial endotoxins such as lipopolysaccharide (LPS) are known to stimulate production of prostaglandins by the placenta and chorioamnion with higher concentrations of LPS measured in the amniotic fluid of PLBW infants (Romero *et al*., 1987, 1988; Offenbacher *et al*. 1996, Gibbs *et al*. 1992). Even when no bacteria are identified in 18% to 49% of histologically inflamed chorioamniotic membranes, the role of

periodontal infection as a possible risk factor for PLWB is still maintained (Mueller-Heubach *et al.* 1990).

3. High concentrations of cytokines produced at sites of chronic periodontitis and found at higher levels in the plasma of patients with periodontitis may cross human foetal membranes and thus contribute to PLBW (Romero *et al.* 1993; Page, 1991, 1998).

1.4. HYPOTHESIS:

It is only recently that research has focused on maternal health and the possible threat to their infants, thus the question about the host response being harmful or beneficial in periodontal disease has not been resolved. More sex-specific research to determine strategies to prevent and treat diseases that have particular impact on women are needed (Krejci and Bissade, 2002; Tauman *et al*., 2005; Keith *et al*., 2007, Ishikawa, 2007). Although isolated studies have established some association of changes in microbiota in different stages of the female life-cycle, there are many unanswered questions relating to the effect oral disease can have on the unborn child. Few studies have been done relating the association between periodontal disease and PLBW focusing on oral microbial infection and their stimulation of antibody responses (Lin *et al*., 2007) and there is a need for further studies with larger sample populations to establish this association (Jarjoura *et al*., 2005; Qureshi *et al*., 2005; Yeo *et al*., 2005; Offenbacher *et al*., 2006; Lin *et al*., 2007). To our knowledge, no studies have been done in Sub-Saharan Africa relating the association of periodontal infections through their inflammatory cytokines with PLBW. This study is designed to use a molecular

approach to test the hypothesis that periodontal pathogens may be associated with preterm birth.

1.5. OBJECTIVES:

The objective of this study was:

- To detect specific suspected periodontopathogens in maternal gingival crevicular fluid using PCR and examine their dissemination following the induction of maternal immune (IgG, IgM) and inflammatory responses (IL-6, IL-10) by examining serum samples from mothers and foetal cords in PLBW and normal birth and relating their associations to PLBW.

CHAPTER 2: MATERIALS AND METHODS

2.1. PATIENT SELECTION

This study requested the participation of 100 cases of PLBW and 100 controls of normal term (NT) obtained from all sequential deliveries which occurred from May to December 2008 in the department of obstetrics and gynecology of the teaching hospital of Butare in Rwanda. Medical records were used and mothers were asked to complete a questionnaire within 48 hours after delivery in order to identify factors such as their medical history, factors predisposing for periodontal disease, previous preterm delivery, and oral hygiene habits (see appendix 2A) which might pose a health risk to them and their infants. The stage of gestation was checked by recording the last date of menstruation of the patient or by ultrasound. Informed consent was obtained from the participants in verbal and written form as defined by the Medical Research Council, 2001 (see appendix 2B). They were informed of the purpose of the study, potential harms, injuries, discomforts or inconveniences which may be experienced as well as potential benefits (see appendix 2C). They were required to sign the form if they agreed to participate in the study and were assured of confidentiality of any disclosures. The study was ethically approved by the Senate Research Committee of the University of the Western Cape.

2.2. CLINICAL EXAMINATION AND SAMPLE COLLECTION

A dentist, a medical doctor, nurses and lab technicians assisted with the clinical examination and sample collection.

2.2.1. Oral examination and gingival crevicular fluid (GCF) sample collection

Samples were collected within 48 hours of delivery**.** Prior to sampling, the patients were examined for signs of gingival inflammation and/or bleeding which might be attributed to lack of oral health care. No attempt was made for an accurate diagnosis of gingivitis or periodontitis using clinical indices or radiographs and instead, gingival inflammation was recorded by the dichotomous score of "presence" or "absence". All clinical examinations and sample collections were done by the same clinician. Gingival crevicular fluid (GCF) was collected from four teeth:

- Maxillary right first molar (16)
- Maxillary left first molar (26)
- Mandibular left first molar (36)
- Mandibular right first molar (46), by inserting sterile filter paper strips (PropFlow, Inc., Amityville, NY) into the base of the pocket for one minute per tooth (Goodson, 2003). Care was taken to prevent saliva or blood contamination during collection. Each paper strip was placed into 50 µl phosphate buffered saline sampling buffer in an Eppendorf tube with added 0.05 % tween-20 (PBS-T) and stored at -80^0 C (Garmonal *et al.*, 2000). Samples were transported on dry ice to South Africa and stored at -80^0 C until analysis.

2.2.2. Blood sample collection

Ten ml of foetal cord blood samples were collected at delivery and 10 ml of maternal blood samples were collected within 48 hours after delivery (Madianos *et al*., 2001), into untreated blood collection tubes (MediPlus, Cat. No: VP4011). The blood samples were stored at + 4^0 C overnight and centrifuged at 3.000 rpm for 10 minutes, then serum collected and stored in aliquots at -80^0C (Papapanou *et al*., 2000).

2.3. EVALUATION OF IL-6 AND IL-10 LEVELS USING ELISA

Maternal serum samples and foetal cord serum samples were used for the evaluation of IL-6 and IL-10 levels using ELISA kits (eBioscience). The preparation of ELISA reagents are explained in appendix F.

2.3.1. ELISA test protocol

Coat corning costar 9018, 96 well ELISA plates were coated with 50 µl/well of capture antibody in coating Buffer. Plates were sealed with a film sealing cycler (Lasec SA Pty Ltd), incubated overnight at 4^0 C, then aspirated and washed 5 times with 250 µl/well wash buffer. Wells were blocked with 100 µl/well of 1x Assay Diluents and incubated at room temperature for 1 hour. Plates were washed as described previously. Fifty µl/well of the standard (Recombinant human IL-6 or IL-10: 1 ug/ml) were added to the top wells and two-

fold serial dilutions of the top standards were performed to make the standard curve. For the IL-6 standards, the following 2-fold serial dilutions were made: 200 pg/ml, 100 pg/ml, 50 pg/ml, 25 pg/ml, 12.5 pg/ml, 6.25 pg/ml, 3.12 pg/ml and 0 pg/ml and for Il-10: 500 pg/ml, 250 pg/ml, 125 pg/ml, 65.5 pg/ml, 31.25 pg/ml, 15.62 pg/ml, 7.81 pg/ml and 0 pg/ml. Fifty µl/well of the samples was added to the appropriate wells (see Table 2). Plates were sealed and incubated at room temperature for 2 hours (or overnight at 4^{o}C for maximal sensitivity).

Table 2. Illustration of ELISA standards and sample disposition.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std1	1	1	10	10	18	18	26	26	34	34
B	Std2	Std2	2	2	11	11	19	19	27	27	35	35
C	Std3	Std3	3	3	12	12	20	20	28	28	36	36
D	Std4	Std4	4	4	13	13	21	21	29	29	37	37
E	Std5	Std5	5	5	14	14	22	22	30	30	38	38
F	Std6	Std6	6	6	15	15	23	23	31	31	39	39
G	Std7	Std7	8	8	16	16	24	24	32	32	40	40
H	Std0	Std0	9	9	17	17	25	25	33	33	41	41

Std: Standards

Plates were aspirated and washed as described previously. Fifty µl/well of detection antibody diluted in 1x assay diluent was added. Plates were sealed and incubated at room temperature for 1 hour. Another step of aspiration and washing was performed as described previously. Fifty µl/well of avidin-HRP diluted in 1x assay diluent was added. Plates were sealed and incubated at room temperature for 30 seconds. A last step of aspiration and washing was performed. In this washing step, wells were soaked in wash buffer for 1 to 2

minutes. This was repeated for a total of 7 washes. Fifty µl/well of substrate solution was added to each well. Plates were incubated at room temperature for 15 minutes. Fifty µl/well of stop solution 2 normal sulfuric acid (2N H_2SO_4) was added to each well. Readings of the optical density (O.D) were made at 450 nm using a microtiter plate reader (Thermo Electron corporation, Multiskan Ex).

2.3.2. Calculation of results

A standard curve was used to determine the amount of IL-6 or IL-10 in the samples. Standard curves were generated by plotting the average O.D (450nm) obtained for the standard concentration on the horizontal axis (X) versus the corresponding IL-6 or IL-10 concentration (pg/ml) on the vertical axis (Y). The main O.D. value for each standard and sample were calculated and all O.D. values were subtracted by the value of the zero-standard before interpretation of results. The standard curve was constructed using Excel.

2.4. ESTIMATION OF MATERNAL SERUM AND FOETAL CORD IMMUNOGLOBULINS

2.4.1. Bacterial strains used and preparation of bacterial antigens

In the development of this assay, the following strains were used as antigens: *Porphyromonas gingivalis* strain America Type Culture collection ATCC 33277, *Treponema denticola* strain ATCC 33521, *Prevotella intermedia* strain ATCC 25611,

Fusobacterium nucleatum strain NTCC 10562, and *Aggregatibacter actinomycetemcomitans* strain ATCC 33396.

Isolation of the above species was done on Trypticase Soy Agar-CY/Dithio (TSA-CT) (See preparation appendix E) in an atmosphere of 80 % N_2, 10 %CO_2 and 10%H_2 at 37^0 C for 5 days. The cultures were transferred in Peptone Yeast Sugars (see appendix E), where they were further grown for 2 to 3 days under the conditions mentioned above and then subcultured onto TSA-CT plates. The purity of the culture was confirmed by colony morphology and Gram stain. The broth was then centrifugated at 5,500 X g at room temperature for 15 min and the bacterial pellet washed 3 times in (PBS). After washing, the bacteria were sonicated for 20 secondes to disperse the cells. The density of the bacterial suspension in the antigen buffer (PBS) was adjusted to give an absorbance of 0.15 at 580 nm and stored in 20 µl aliquots at -80^o C until needed.

2.4.2. ELISA ASSAY

A 1/100 dilution in PBS of the 5 different antigens was made and 50 µl/well used to coat the plates. Two columns were allocated to each antigen for coating and the last two columns were allocated to the protein A from *Staphylococcus aureus* as a conjugate binding substrate (See Table 3). Staphylococcal protein A binds with high and moderate affinity to human IgG and IgM respectively (Sasso *et al*, 1989). This was used as a baseline for calculation of IgG and IgM levels. Protein A was diluted in PBS to a concentration of 0.15 µg/ml and 50 µl/well loaded in the two last columns for coating. The plates were sealed and incubated

overnight at 4^0 C. Plates were aspirated and washed 5 times with 250 µl/well wash buffer. The nonspecific binding was blocked by 200 µl/well of 2 % bovine albumin in PBS at room temperature for 1 hour. Plates were aspirated and washed as described previously. Fifty µl/well of samples diluted 1/500 for IgG and 1/100 for IgM in (2 % bovine albumin in PBS) were loaded perpendicularly to the antigen. The first row was allocated to the positive control, from the second to the sixth were allocated to samples from different patients and the last row was allocated for the negative control in which we put only 50 µl/well of the blocking solution (2 % bovine albumin in PBS). Each sample was loaded in duplicate for the different antigens (See Table 3).

Table 3. Illustration of ELISA for IgG and IgM's

		1	2	3	4	5	6	7	8	9	10	11	12
→+C	A	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag
→S1	B	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag
→S2	C	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag
→S3	D	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag
→S4	E	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag
→S5	F	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag
→S6	G	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag
→-C	H	*Pi*-Ag	*Pi*-Ag	*Pg*-Ag	*Pg*-Ag	*Fn*-Ag	*Fn*-Ag	*Aa*-Ag	*Aa*-Ag	*Td*-Ag	*Td*-Ag	PA-Ag	PA-Ag

Ag = Antigen, *Pi = Prevotella intermedia, Pg = Porphyromonas gingivalis, Fn =Fusobacterium nucleatum, Aa = Aggregatibacter actinomycetemcomitans, Td = Treponema denticola,* PA = Protein A, +C = Positive control, -C= Negative control and Sn = Sample number

The plates were sealed and incubated at room temperature for one hour. Plates were aspirated and washed as described previously. Peroxidase conjugated goat anti-human (IgG or IgM) diluted 1/30,000 in the blocking solution was used as a second antibody. Fifty µl of

the above solution was loaded in each well and the plates were sealed and incubated at room temperature for one hour. Plates were aspirated and washed as described previously but this time for a total of 7 washes. Fifty µl/well of substrate solution was added to each well. Plates were incubated at room temperature for 10 minutes. Fifty µl/well of stop solution (2N H_2SO_4) was added to each well and readings of the optical density (O.D) were made at 450 nm using a microtiter plate reader (Thermo Electron corporation, Multiskan Ex). The main O.D. value for each sample was calculated and all O.D. values were subtracted by the value of the zero-standard (Negative control) before interpretation of results. The IgG or IgM levels were expressed in percentage compared to O.Ds of protein A (100 %).

2.5. 16S rRNA GENE DETECTION BY PCR

Polymerase Chain Reaction (PCR) was used for the detection of the presence of the 5 target bacteria of this study.

2.5.1. Sample preparation

Samples from the freezer were thawed by incubation at 37^o C for 10 min, then centrifuged (10.000 X g) for 15 minutes at 4^o C. The supernatents of the 4 tubes from each patient were pooled to yield a single sample representing all four quadrants of the mouth for each patient (Kim *et al*, 2007). Samples were vortexed for 30 seconds and centrifuged at 2500X g for 2 minutes. The supernatent was removed and the pellet resuspended in 100 µl of distilled water. Another step of vortexing and centrifugation was done and the pellet was

resuspended in 500 µl of distilled water. The suspension was heated at 94^0 C for 10 min and the vials immediately chilled on ice for 5 min.

2.5.2. Preparation of reference DNA

Bacterial cultures were centrifuged at 4000 rpm for 10 minutes at 4^0 C, washed in Tris Hydroxymethyl Methylamine (TES see appendix D**)** buffer, resuspended in 20 ml TES and stored at -80^o C. Cells were lysed by the addition of 1 mg/ml lysozyme and incubated for 10 minutes at 37^o C or until changes in colour or viscosity were seen. Lipid molecules were removed by the addition of sodium dodecylsulphate detergent (SDS see appendix D) to a final concentration of 1 %, followed by another incubation at 37^o C for 10 minutes. Proteinase K (1 mg/ml, dissolved in 0.5 M Tris-HCl pH 7.2) was added and DNA incubated at 37^o C for 30 minutes. Proteinase K was added to facilitate the breakdown of protein molecules and facilitate phenol extraction. Protein was precipitated and extracted with phenol/chloroform as follows: a volume of 500 µl of the aqueous solution of DNA was added to 250 µl phenol solution and 250 µl chloroform, gently mixed by inversion for 1 minute, then spun at 4000 rpm for 10 minutes. Because phenol and water are immiscible, the protein was extracted into the phenol layer (bottom layer), leaving nucleic acids in the aqueous top layer. The aqueous top layer containing nucleic acids was removed. The extraction procedure was repeated twice. DNA was precipitated by the addition of 50 µl of 5M NaCl and 1 ml of 100 % ethanol. After chilling on ice for 3-5 minutes, the tube was gently mixed by inversion and spun for 10 minutes at 4^o C. If the high molecular weight nucleic acids were not precipitated as a white fibrous material, but rather as a colourless, gelatinous material, it indicated that protein was still bound to the nucleic acid. The nucleic

acid was then treated with 24 ribonuclease (RNase) at 65^{o} C for 10 minutes (10 mg in 1 ml TE buffer), followed by another round of proteinase K treatment, phenol/chloroform extraction and ethanol precipitation. The pellet was washed in 70% ethanol without centrifugation (just poured off without mixing) and resuspended in 500 µl sterile distilled water. Fifty µl 5M ammonium acetate (see appendix D) was added, followed by 1 ml cold ethanol. The solution was mixed by inversion and left to precipitate at -20^{o} C for 5 minutes. The DNA was carefully spooled with a glass rod and washed by dipping in 500 µl 70% ethanol, and then in 100 µl TE. The DNA was left overnight at 37^{o} C, if not dissolved immediately. If the DNA preparation appeared very dilute, it was concentrated as follows: 300 µl DNA was mixed with 30 µl 5M NaCl and 750 µl cold ethanol, incubated at -20^{o} C for at least an hour, then spun (10000X g) at room temperature for 10 minutes. The supernatent was removed and the pellet washed with 70% ethanol, dried under vacuum and resuspended in 100 µl TE. This phase was re-extracted with 600 µl TE.

2.5.3. PCR amplification

Chilled samples were centrifuged for 10 seconds at 9000 X g and 5 µl aliquots of the supernatents were used in the PCR assay. Twenty-five µl of the Dreamtaq^{TM} Green PCR Master Mix (2X) (FE K1081, Inqaba biotec), 0.1-1.0 µM of each primer and 18 µl of water nuclease were added to the 5 µl of template DNA. Species-specific primers (Inqaba biotec) were used to detect the presence of the 5 target organisms in this study. The expected product lengths were 404 bp for *P. gingivalis*, 316 bp for *T. denticola*, 307 bp for *P. intermedia*, 500 bp for *A. actinomycetemcomitans*, and 705 bp for *F. nucleatum*.

Confirmation of PCR reaction was achieved by the use of a pair of ubiquitous primers product length (602 bp) which matches most bacterial 16S rRNA genes at the same position. Nucleotide sequences of selected and modified 16S rDNA primer pairs are listed in Table 4.

Table 4: **PCR primer sequences used for detection of our target bacteria**

Target	**PCR primer pairs (5'-3')**	**Source**
Porphyromonas gingivalis: - Forward	AGG CAG CTT GCC ATA CTG CG	Rocas *et al*., (2001)
-Reverse	ACT GTT AGC AAC TAC CGA TGT	
Treponema denticola: - Forward	TAA TAC CGA ATG TGC TCA TTT ACA T	Rocas *et al*., (2001)
- Reverse	TCA AAG AAG CAT TCC CTC TTC TTC TTA	
Prevotella intermedia: - Forward	CAA AGA TTC ATC GGT GGA	Kook *et al* (2005)
- Reverse	GCC GGT CCT TAT TCG AAG	
Fusobacterium nucleatum: - Forward	ATT GTG GCT AAA AAT TAT AGT T	Mayanagi et al., (2004)
- Reverse	ACC CTC ACT TTG AGG ATT ATA G	
Aggregatibacter actinomycetemcomitans:		Avila-campos and Julio (2003)
- Forward	GCT AAT ACC GCG TAG AGT CGG	
-Reverse	ATT TCA CAC CTC ACT TAA AGG T	
Ubiquitous primers: - Forward	GAT TAG ATA CCC TGG TAG TCC AC	Rocas *et al*., (2001)
- Reverse	CCC GGG AAC GTA TTC ACC G	

Preparation of the PCR cocktail

1. A gentle and brief vortex and centrifugation of the Dreamtaq^{TM} Green PCR Master Mix(2X) (Inqaba biotec) was done after thawing. The Dream taq DNA polymerase was supplied in 2X Dream taq green buffer, dNTPs and 4 mM $MgCl_2$ and a Dream Taq green buffer which contained a density reagent and two dyes for monitoring electrophoresis progress.

2. PCR tubes were placed on ice and components for each 50µl PCR reaction were added as follows (See Table 5):

Table 5: Components of the PCR reaction

DreamTaq™ Green PCR Master Mix(2X)	25 µl
Forward primer	0.1-1.0 µM
Reverse primer	0.1-1.0 µM
Template DNA	5 µl
Water, nuclease-free	18 µl
Total volume	50 µl

3. A gentle vortex and spin-down of the sample was done.
4. A volume of 25 µl of mineral oil was added.

The negative control contained 5 µl of distilled water in place of the sample and the positive control consisted of 49 µl from the master mix and 1 µl (100ng) of the reference genomic DNA. A brief vortexing of samples was done. PCR amplifications were perfomed as follows:

P. gingivalis (Rocas *et al.,* 2001): an initial denaturation step at 94^{o}C for 2 minutes, followed by 36 cycles of a denaturation step at 94^{0}C for 30 seconds, a primer annealing step at 60^{0}C for 1 minute, an extention step at 72^{0}C for 2 minutes, and a final step at 72^{0}C for 10 minutes.

T. denticola and ubiquitous primers (Rocas *et al*., 2001): an initial denaturation step at 95^{0}C for 2 minutes, followed by 36 cycles of a denaturation step at 95^{0}C for 30 seconds, a primer

annealing step at 60^{o}C for 1 minute and extention step at 72^{0}C for 1 minute, and a final step at 72^{0}C for 2 minutes.

P. intermedia (Kook *et al* 2005): an initial denaturation step at 95^{0}C for 2 minutes, followed by 36 cycles as one cycle at 94°C for 30 s (denaturation) followed by 55°C for 1 min (annealing) with an elongation of 72°C for 1 minute, and a final step at 72^{0}C for 10 minutes.

A. actinomycetemcomitans and *F. nucleatum* : Same conditions as described previously by Rocas *et al.*, 2001.

The PCR products were analyzed by electrophoresis in 1 % agarose gels using Tris-Borate EDTA buffer at 90 V. A 100 bp size ladder (O'GeneRuler 100 bp DNA ladder, Fermentas) was used as the molecular weight marker. The DNA was stained with ethidium bromide and visualized under UV light.

2.6. DATA ANALYSIS

Data were analyzed using SPSS 14.0. All questionnaires, oral examination and laboratory data were entered into Excel 2003 and then transferred to Statistical Package for the Social Sciences (SPSS) for analysis. Frequencies, means, maximum and minimum were calculated using descriptive statistics. The significance of associations was determined using chi-squared and Fisher's exact test. Statistical differences between groups, cytokine means and antibody levels were determined using Kruskal-wallis test, Mann-Whiteney test and spearman rank correlation. A p value of < 0.05 was considered significant. A multivariate

analysis using a logistic regression of risk factors associated with PLBW was performed to provide an estimate of the odds ratio after adjusting for confounding variables.

CHAPTER 3: RISK FACTORS ASSOCIATED WITH PLBW AS ASSESSED FROM THE QUESTIONNAIRE

3.1. INTRODUCTION

Before sampling and oral examination, mothers were asked to complete a questionnaire in order to identify factors which might pose a health risk to them and their infants and also to obtain some information regarding different factors which have been linked with a higher risk of preterm delivery of low birth weight such as level of education, height of the mother, age, smoking, alcoholism during pregnancy, maternal medical conditions such diabetes, blood pressure and infection. Not all of the participants responded to all of the questions asked, so the results which follow are based on the number of responses to each question, and not the total number of participants in the study. The following sections summarize their responses to the questionnaire.

3.2. PERSONAL DETAILS OF MOTHERS

Maternal Age

Of the mothers who responded (n = 195), the majority were between the ages of 20-30 years (60 %) with a mean age of 28.02 (± 6.089). Generally, age did not appear to influence pregnancy outcomes as there was little difference between normal and PLBW except for a slight increase in mothers aged between 21-25 and ≥ 36 years of age. The association of

PLBW with age was therefore not statistically significant (p =0.557) (Table 6). Ninety-eight of the mothers (50.25 %) delivered normal birth, while 97 (49.74 %) delivered PLBW. Five mothers failed to respond to this question.

Maternal Weight

One hundred and eighty-eight mothers recorded their weights. The mean (SD) weight was 61.92 (± 9.569) kg. Of these, PLBW was significantly increased (p = 0.006) in mothers who weighed less than 65 kg. Of the 53 mothers weighing > 65 kg, only 28.3 % delivered PLBW infants (Table 6). Twelve mothers did not respond to this question.

Table 6: Details of Mothers

	FREQUENCY (%)	Normal birth n (%)	PLBW n (%)	p value
AGE (years) Mean (±SD): 28.02 (± 6.089)				**0.557**
< 20	17 (8.71)	8 (8.2)	9 (9.3)	
20 – 25	60 (30.76)	28 (28.6)	32 (33.0)	
26 – 30	58 (29.74)	31 (31.6)	27 (27.8)	
31 – 35	37 (18.97)	22 (22.4)	15 (15.5)	
≥ 36	23 (11.79)	9 (9.2)	14 (14.4)	
Total	195 (100.0)	98 (100.0)	97 (100.0)	
WEIGHT (kg) Mean (±SD): 61.92 (± 9.569)				**0.006**
≤ 50	16 (8.51)	7 (7.01)	9 (10.0)	
51 – 55	35 (18.61)	13 (13.3)	22 (24.4)	
56 – 60	45 (23.93)	21 (21.4)	24 (26.7)	
61 – 65	39 (20.74)	18 (18.4)	21 (23.3)	
> 65	53 (28.19)	39 (39.8)	15 (16.6)	
Total	188 (100.0)	98 (100.0)	90 (100.0)	
HEIGHT (cm) Mean (±SD): 158.68 (± 7.598)				**0.041**
≤ 150	30 (16.30)	15 (15.6)	15 (17.0)	
151 – 155	41 (22.28)	20 (20.8)	21 (23.9)	
156 – 160	43 (23.36)	16 (16.8)	27 (30.7)	
161 – 165	40 (21.73)	23 (24.0)	17 (19.3)	
> 165	30 (16.30)	22 (22.9)	8 (9.1)	
Total	184 (100.0)	96 (100)	88 (100.0)	
LEVEL OF EDUCATION				**0.355**
No formal education	18 (9.13)	6 (6.1)	12 (12.1)	
Primary school	121(61.42)	59 (60.2)	62 (62.6)	
High School	43 (21.82)	24 (24.5)	19 (19.2)	
university	15 (7.61)	9 (9.2)	6 (6.1)	
Total	197 (100.0)	98 (100.0)	99 (100.0)	
MAIN DIET				**0.197**
Bread	5(2.57)	2 (2.1)	3 (3.1)	
Meat	26(13.40)	15 (15.5)	11 (11.3)	
Fruit and veg	121(62.37)	64 (66.0)	57 (58.8)	
Bread and meat	3(1.54)	3 (3.1)	0 (0.0)	
Bread, fruit and veg	3(1.54)	1 (1.0)	2 (2.1)	
Meat, Fruit and veg	14(7.21)	5 (5.2)	9 (9.3)	
Bread, meat, fruit and veg	22(11.34)	7 (7.2)	15 (15.5)	
Total	194(100.0)	97 (100.0)	97 (100.0)	

Maternal height

Only 184 mothers recorded their height. The mean (SD) maternal height was 158.68 (± 7.569) cm. A significant correlation (p = 0.041) was observed between the height of the mother and PLBW. Mothers with a height ≤ 160 cm were more likely to deliver PLBW while those measuring ≥ 161 cm were more likely to deliver full term and normal weight infants (Table 6).

Level of Education

One hundred and ninety-seven mothers (98.5 %) responded to this question. The majority (61.42 %) of whom had completed their primary school, followed by high school (21.82 %) with 9.13 % having had no formal education and 7.61 % having reached university level (Table 6). Although the number of PLBW increased slightly in mothers with no formal education, no significant association ($p = 0.355$) could be observed between level of education and PLBW.

Diet

Only 6 mothers (3.0 %) did not report on their diet. The majority (62.37 %) of our patients reported fruit and vegetables as their main diet, followed by a predominantly meat diet (13.4 %). There was no significant correlation ($p = 0.197$) between the diet of the mother and PLBW (Table 6).

3.3. MEDICAL HISTORY OF THE MOTHERS

Diabetes

Three (1.53 %) of the 195 (97.5 %) mothers who responded to this question, reported a history of diabetes (Table 7), of which 2 (2.1 %) delivered PLBW and 1 (1.0 %) delivered normal birth. No significant difference was observed for pregnancy outcomes between those with a history of diabetes and those with no history of diabetes ($p = 0.496$).

Table 7: Medical History of the Mothers

CHARACTERISTICS	FREQUENCY (%)	Normal birth n (%)	PLBW n (%)	p value
DIABETIC HISTORY				**0.496**
Yes	3 (1.53)	1 (1.0)	2 (2.1)	
No	192 (99.48)	97 (99.0)	95 (97.9)	
Total	195 (100.0)	98 (100.0)	97 (100.0)	
FAMILY HEART DISEASE HISTORY				**0.528**
Yes	19 (9.74)	9 (9.4)	10 (10.1)	
No	176 (90.25)	87 (90.6)	89 (89.9)	
Total	195 (100.0)	96 (100.0)	99 (100.0)	
URINARY TRACT INFECTION				**0.107**
Yes	45 (23.56)	18 (19.1)	27 (27.8)	
No	146 (76.43)	76 (80.9)	70 (72.2)	
Total	191 (100.0)	94 (100.0)	97 (100.0)	
SEXUALLY TRANSMITTED DISEASE				**0.080**
Yes	28 (14.35)	10 (10.3)	18 (18.4)	
No	167 (85.64)	87 (89.7)	80 (81.6)	
Total	195 (100.0)	97 (100.0)	98 (100.0)	
HISTORY OF ANTIBIOTIC USE				**0.079**
Yes	58 (29.89)	24 (24.7)	34 (35.1)	
No	136 (70.10)	73 (75.3)	63 (64.9)	
Total	194 (100.0)	97 (100.0)	97 (100.0)	
ALCOHOL USE				**0.337**
Never	98 (52.12)	46 (48.4)	52 (55.9)	
Daily	24 (12.76)	10 (10.5)	14 (15.1)	
weekly	29 (15.42)	18 (18.9)	11 (11.8)	
Special occasions	37 (19.68)	21 (22.1)	16 (17.2)	
Total	188 (100.0)	95 (100.0)	93 (100.0)	
SMOKERS				**0.506**
Yes	9 (4.61)	5 (5.1)	4 (4.1)	
No	186 (95.38)	93 (94.9)	93 (95.9)	
Total	195 (100.0)	98 (100.0)	97 (100.0)	
MEDICAL INSURANCE				**0.125**
Yes	183(93.36)	94(95.9)	89(90.8)	
No	13(6.63)	4(9.2)	9(9.2)	
Total	196(100.0)	98(100.0)	98(100.0)	
GYNECOLOGICAL CHECK UP				**0.059**
Never	71(37.96)	37 (37.8)	34 (38.2)	
Once a year	23(12.29)	7 (7.1)	16 (18.0)	
When necessary	93(49.73)	54 (55.1)	39 (43.8)	
Total	187(100.0)	98 (100.0)	89 (100.0)	

Heart disease

Of the 195 (97.5 %) mothers who responded to this question, only19 (9.74 %) reported a history of heart disease (Table 7). No significant association was observed between presence nor absence of heart disease and pregnancy outcomes (p = 0.528).

Urinary tract infections

One hundred and ninety-one (95.5 %) mothers responded to this question with the majority (76.43 %) reporting no history of recurrent or existing urinary tract infections. Those who did, showed a slight increase in PLBW but this was not statistically significant (p=0.107)

Sexually transmitted diseases

Most of the mothers (97.5 %) responded to this question with 28 (14.35 %) reporting a history of sexually transmitted diseases (Table 7). Although those who responded positively to this question showed an increase in PLBW, the association was not statistically significant (p = 0.080). As with the earlier reported histories of diabetes, heart disease and urinary tract infections, the sample number of those responding positively may have been too small for any significant association to be observed.

Antibiotic therapy

Mothers were asked to report on whether they had received antibiotic therapy during their period of gestation. Of the 194 mothers who responded to this question, only 58 (29.89 %) reported that they had received antibiotic therapy during their pregnancy (Table 7). An increase in PLBW was observed in these mothers, but it was not statistically significantly associated (p = 0.079).

Alcohol consumption

One hundred and eighty-eight mothers (94.0 %) responded to this question with 98 (52.12 %) denying any alcohol consumption (table 7). Others reported on daily (12.76 %) and weekly (19.68 %) consumption, while others reported alcohol consumption only on special occasions (18.5 %). Although a slight increase was observed in PLBW when mothers reported daily consumption of alcohol, this association was not statistically significant ($p = 0.337$) since an increase in PLBW was also observed when mothers reported never consuming any alcohol.

Smoking

Of the 200 patients, only 195 (97.5 %) reported on smoking habits, of which 9 (4.61 %) admitted to smoking, while 186 (93.38 %) reported that they had never smoked (Table 7). Smoking did not appear to influence pregnancy outcomes ($p = 0.506$).

Medical insurance and gynecological visits

Although 183/196 (93.36 %) mothers reported having medical insurance which allowed them access to medical care (Table 7), 93/187 (49.73 %) reported visiting a gynecologist only when necessary, 71 (37.96 %) responded never visiting a gynecologist (Table 7) and 23 (12.29 %) claimed that they visited a gynecologist once a year. Again a slight increase in PLBW in mothers never visiting a gynecologist and those visiting once a year, was not

significantly associated (p = 0.059) with pregnancy outcomes, nor was there an association with access to medical insurance (p = 0.125).

3.4. HISTORY OF PREVIOUS DELIVERIES

Health condition of children

Data from 131 (65.5 %) mothers was available regarding the health condition of children in the family. Healthy children were reported by 124 (94.65 %) mothers, while 7 (5.34 %) reported unhealthy children (Table 8). A significant difference (p=0.003) was observed between the health condition of the children in the family and PLBW. Only children born PLBW were reported to be unhealthy (Table 8).

Table 8: History of Previous Deliveries

CHARACTERISTICS	FREQUENCY (%)	Normal birth n (%)	PLBW n (%)	p value
CONDITION OF CHILDREN IN THE FAMILY				**0.003**
Healthy	124 (94.65)	71 (100.0)	53 (88.3)	
Not Healthy	7 (5.34)	0 (0.0)	7 (11.7)	
Total	131 (100.0)	71 (100.0)	60 (100.0)	
HISTORY OF PREVIOUS PREGNANCIES				**0.078**
First pregnancy	75 (38.46)	32 (33.0)	43 (43.9)	
Multiple pregnancies	120 (61.53)	65 (67.0)	55 (56.1)	
Total	195 (100.0)	97 (100.0)	98 (100.0)	
PREVIOUS PRE-TERM BIRTH				**0.000**
Yes	32 (16.0)	8 (11.1)	24 (39.3)	
No	101 (50.5)	64 (88.9)	37 (60.7)	
Total	133 (100.0)	72 (100.0)	61 (100.0)	

History of previous pregnancies

A total of 195 (97.5 %) mothers reported on previous pregnancies (Table 8). Although women in their first pregnancy appeared to be more likely to deliver PLBW than women who reported multiple pregnancies (Table 8), this difference was not found to be statistically significant (p = 0.078).

However, when looking at a history of previous pregnancies, a very good correlation (p = 0.000) was observed between history of previous PLBW and present PLBW (Table 8). Mothers with a history of previous PLBW were more likely to deliver PLBW infants in subsequent pregnancies. Only 133/200 mothers responded to this question so we have no record of the other 67 patients.

3.5. LIVING CONDITIONS

Length of time spent in a particular domain

The stability of length of time spent in a particular domain did not appear to affect pregnancy outcomes (p = 0.995). Most of the patients 130 (67.01 %) lived in their present address for less than 5 years, 38 (19.58 %) between 5 and 10 years and only 26 (13.40 %) lived in their present address for more than 10 years (Table 9). Six (3 %) of them did not respond to this question.

Table 9: Conditions of life in the house

CHARACTERISTICS	FREQUENCY (%)	NORMAL BIRTH n (%)	PLBW n (%)	p value
Duration in the present address				**0.995**
< 5 years	130(67.01)	66 (67.3)	64 (66.7)	
5 – 10 years	38(19.58)	19 (19.4)	19 (19.8)	
> 10 years	26(13.40)	13 (13.3)	13 (13.5)	
Total	194(100.0)	98 (100.0)	96 (100.0)	
Number of family members				**0.029**
0 – 1 people	15(8.02)	4 (4.2)	11 (12.1)	
2 people	36(19.25)	14 (14.6)	22 (24.2)	
3 people	43(22.99)	25 (26.0)	18 (19.8)	
4 people	35(18.71)	15 (15.6)	20 (22.0)	
5 people	22(11.76)	15 (15.6)	7 (7.7)	
6 people and more	36(19.25)	23 (24.0)	13 (14.3)	
Total	187(100.0)	96 (100.0)	91 (100.0)	
Number of children				**0.064**
No child	35(19.12)	13 (14.0)	22 (24.4)	
1 child	71(38.79)	37 (39.8)	34 (37.8)	
2 children	38(20.76)	17 (18.3)	21 (23.3)	
3 children	15(8.19)	12 (12.9)	3 (3.3)	
4 children and more	24(13.11)	14 (15.1)	10 (11.1)	
Total	183(100.0)	93 (100.0)	90 (100.0)	

Number of family members

Table 9 shows a significant correlation (p= 0.029) between number of family members and PLBW. It would generally appear that when there were 2 - 4 people in the house, they were more likely to deliver PLBW while those living ≥ 5 in the house were more likely to deliver a full term baby.

Number of children

No significant correlation (p=0.064) was observed between number of children and PLBW, although PLBW was increased in the 35 mothers who delivered their first child (Table 9). Only 183/200 mothers (91.5 %) reported on the number of other children in their families.

3.6. DENTAL HISTORY OF THE MOTHER

When asked about the frequency of visits to the dentist, 135 (71.42 %) of the participants reported that they never visit a dentist while less than half reported visiting the dentist once or twice a year. There was no significant difference (p = 0.063) between normal birth and PLBW in either of these groups (Table 10).

Table 10: Dental History of the Mother

CHARACTERISTICS	FREQUENCY (%)	NORMAL BIRTH n (%)	PLBW n (%)	p value
FREQUENCY OF DENTAL VISITS:				**0.063**
Never	135 (71.42)	61 (62.9)	74 (80.4)	
Once a year	42 (22.22)	28 (28.9)	14 (15.2)	
Twice a year	8 (4.23)	5 (5.2)	3 (3.3)	
Whenever	4 (2.11)	3 (3.1)	1 (1.1)	
Total	189 (100.0)	97 (100.0)	92 (100.0)	
ACCESS TO MEDICAL OR DENTAL CARE				**0.063**
Yes	33 (17.01)	19 (19.6)	14 (14.4)	
No	161 (85.18)	78 (80.4)	83 (85.6)	
Total	194 (100.0)	97 (100.0)	97 (100.0)	
GINGIVAL INFLAMMATION AND BLEEDING				**0.004**
Yes	104 (55.02)	45 (45.5)	59 (65.6)	
No	85 (44.97)	54 (54.5)	31 (34.4)	
Total	189 (100.0)	99 (100.0)	90 (100.0)	
FREQUENCY OF TOOTH BRUSHING				**0.352**
Once a day	92 (59.35)	44 (58.7)	48 (60.0)	
Twice a day	58 (37.41)	27 (36.0)	31 (38.8)	
After every meal	5 (3.22)	4 (5.3)	1 (1.2)	
Total	155 (100.0)	75 (100.0)	80 (100.0)	
BLEEDING WHILE BRUSHING				**0.522**
Yes	99 (61.11)	48 (61.5)	51 (60.7)	
No	63 (38.88)	30 (38.5)	33 (39.3)	
Total	162 (100.0)	78 (100.0)	84 (100.0)	
PAIN WHILE BRUSHING				**0.104**
Yes	40 (26.14)	23 (31.5)	17 (21.2)	
No	113 (73.85)	50 (68.5)	63 (78.8)	
Total	153 (100.0)	73 (100.0)	80 (100.0)	

One hundred and ninety-four (97 %) provided information on access to medical and dental care. The majority reported having no access to medical and dental care, and although this factor did not appear to directly influence the gestation period and birth weight of the infants ($p = 0.063$), the presence of gingival inflammation and bleeding clinically observed in these mothers, showed a significant correlation ($p = 0.004$) with PLBW (Table 10).

Frequency of tooth brushing

Frequency of brushing did not appear to be associated with normal birth or PLBW outcome ($p= 0.352$). Of the 155 participants who reported on the frequency of tooth brushing, 92 (59.35 %) claimed they brushed once a day, 58 (37.41 %) reported twice a day and 5 (3.22 %) reported that they brushed after every meal (Table 10).

Bleeding and pain while brushing teeth

Forty (26.14 %) reported pain, and 99 (61.11 %) reported bleeding while brushing their teeth, while 113 (73.85 %) and 63 (38.88 %) reported neither pain nor bleeding when brushing their teeth (Table 10). No significant differences could be established in either of these categories for adverse pregnancy outcomes ($p = 0.522$ and $p = 0.104$ respectively).

3.7. DISCUSSION

Not all of the 200 mothers examined responded to all of the questions in the questionnaire with the result that we were not able to determine whether their responses were indeed negative or whether they were reluctant to provide the information requested.

The physical condition of the mothers who responded to the questions on age, height and weight could be summarized as follows: age appeared to influence pregnancy outcomes but not significantly so ($p=0.557$), while a significant correlation was observed between maternal weight and PLBW ($p=0.006$), and maternal height and PLBW ($p= 0.041$). Earlier studies showed that the frequency of preterm delivery was higher in mothers below 18 years and above 35 years of age (Martius *et al*., 1998; Astoffi and Zonta, 1999; Goldenberg 1998, 2008; American College of Obstetricians and Gynecologists, 2001), and it can be expected, as previously reported, that women with a poor nutritional status are at greater risk for preterm delivery (Hendler *et al*., 2005), because the thinner the mother, the weaker she would be and thus less able to carry full term (Mavalankar *et al*., 1994; Siega-Riz et al., 1995; Sekiya *et al*., 2007; Chan and Lao, 2009).

Education plays a major role in providing information on nutrition and health care. It has been suggested in the literature that mothers with no formal education are more likely to deliver PLBW (Goldenberg *et al*., 2008; Astoffi and Zonta, 1999). In this study group, mothers who attended secondary school and university were more likely to deliver full term

and normal weight than PLBW infants, although this association was not significant (p=0.355).

Earlier researchers reported a relationship between diabetes, heart disease and PLBW (Samadi and Mayberry, 1998; Xiong *et al*., 2001; Hedderson *et al*., 2003), but no correlation was found in this group. However, few mothers responded to all the questions asked, thus making it difficult to draw any final conclusions (only 3 cases for diabetes and 19 for heart disease). The impact of smoking and alcohol consumption on maternal health and therefore on pregnancy outcomes, remains uncertain. Contrary to previous studies (Vitoratos *et al*., 1997; Lundsberg *et al*., 1997; Kyrklund-Blomberg *et al*., 2005; American College of Obstetricians and Gynecologists, 2001; Moore and Zaccaro, 2000; Odendaal *et al*., 2009), we found no correlation between alcohol consumption (p =0.337) or smoking (p =0.506) and PLBW in this study. Only 24 reported daily alcohol consumption and of these, 14 (58.33 %) had PLBW and 10 (14.66 %) had normal infants. Although a very low level of alcohol consumption has been associated with pregnancy outcome (Cogswell *et al*., 2003), the weekly and special occasion alcohol consumption of the mothers in this study had no negative outcomes for pregnancy.

No significant correlation (p = 0.079) was observed in this study between antibiotic administration and PLBW although the results show that mothers who took antibiotics while pregnant had more PLBW deliveries than those who didn't. Other studies examining the use of antibiotics have also provided mixed results, with some showing no major benefit while others do (Lamont, 2005; McDonald *et al*., 2007; Iams *et al*., 2008). An important factor to

consider would be whether the antibiotics were administered to treat an infection or whether it was a prophylactic administration to mothers at risk for adverse pregnancy outcomes.

It is a known fact that infection can predispose a mother to adverse pregnancy outcomes (Hillier *et al*., 1995; American College of Obstetricians and Gynecologists, 2001; Wadhwa, *et al*., 2001; Goldenberg, 2002). In this study, mothers who had sexually transmitted disease and urinary tract infections had more PLBW than normal infants, but these correlations were not statistically significant.

No significant correlation was found (p=0.078) between first, multiple pregnancies and PLBW, although the results concur with previous studies which showed that PLBW seemed to be more likely to occur when women were in their first pregnancy (Astoffi and Zonta, 1999) and that mothers with a history of previous PLBW were significantly (p = 0.0000) at higher risk for another preterm birth (Mercer *et al*., 1999; American College of Obstetricians and Gynecologists, 2001).

An association between poor health condition of children in the family and PLBW was observed (p=0.003). Whether this can be attributed to the fact that poor health resulted from PLBW or whether PLBW occurred as a result of poor nutrition is not clear. It is known that poor maternal nutritional status is a very important factor for preterm birth resulting in delivery of PLBW infants who are exposed to serious health problems (Hendler *et al*., 2005).

Oral health care during pregnancy is vital to the prognosis of pregnancy outcomes. Offenbacher *et al*., (2006), found that women who received dental care or treatment before or during their pregnancy had 50 % lower risk of delivering PLBW while some recent studies found that treatment for periodontal disease did not reduce the rate of preterm birth (PTB) (Michalowicz *et al*., 2006; Offenbacher *et al*., 2009; Jeffcoat, 2010, Niederman, 2010). In this study, no significant correlations were found between maternal oral hygiene habits (such as tooth brushing and dental visits), nor pain and bleeding (while brushing teeth) and PLBW. The reasons for lack of dental visitations may include poverty, ignorance and culture. A good association between gingival inflammation and PLBW ($p = 0.004$) was found as in earlier studies (Hill 1998; Dasanayake *et al*., 2001, Madianos *et al*., 2001; Dörtbudak et., 2005; Lin *et al*., 2007), while this was not the case when comparing mothers with PLBW infants and the control group in a study by Jarjoura *et al* (2005).

CHAPTER 4: PCR DETECTION OF SUSPECTED PERIODONTOPATHOGENS IN GINGIVAL CREVICULAR FLUID

4.1. INTRODUCTION

The microbial aetiology of periodontal disease is best explained by the ecological shifts which take place in the dental plaque biofilm. As bacterial plaque accumulates, the inflammatory cells increase in number causing the gingival tissues to bleed, swell, and become edematous. Streptococci are normally the initial colonizers. Nutrients needed by other microbes such as the suspected periodontopathogens become increasingly available due to the bleeding and tissue inflammation initiated by the initial colonizers hence promoting their proliferation (Loesche *et al*., 2001). Various host anti-inflammatory responses against the bacteria are initiated, which can be both destructive and protective to the host's tissues. Among the protective responses are included the removal of bacterial products such as antigens, LPS and enzymes. However, the host's inflammatory response also includes the activation of matrix metalloproteases agents responsible for collagen loss in tissues. Such collagen loss leads to attachment loss, thereby deepening the depression where gingival tissues contact the tooth surface. This further deepening creates the periodontal pocket. The loss of attachment defines the difference between gingivitis and periodontitis.

As one of the objectives of this study was to assess the relationship between proposed risk factors for periodontal disease and PLBW, the detection and identification of suspected anaerobic periodontopathogens from the mother was of great importance. Gingival crevicualar fluid (GCF), which is a mixture of substances derived from serum, leuckocytes, structural cells of the periodontium and oral bacteria (Uitto, 2003), was collected from the mother as explained in Chapter 2. Five periodontopathic bacteria (*Porphyromonas gingivalis, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans)* most frequently associated with periodontal disease and among the suspected periodontopathogens associated with PLBW, were the target of this study. Polymerase Chain Reaction (PCR) was used to achieve this objective.

4.2. PREVALENCE OF THE FIVE SUSPECTED PERIODONTOPATHOGENS IN GCF AS DETECTED BY PCR

Examples of PCR detection of the six periodontopathogens are demonstrated in Figures 1-5. Table 11 reports on their prevalence in GCF. The following abbreviations will be used for all the tables which follow: ***Pg*** = *Porphyromonas gingivalis*, ***Td*** = *Treponema denticola*, ***Pi*** = *Prevotella intermedia*, ***Fn*** = *Fusobacterium nucleatum*, ***Aa*** = *Aggregatibacter actinomycetemcomitans*, **P** = Positive and **N** = Negative. Adjusting for missing data, the valid % positive will be described for each species. The same adjustment will be used for reporting on the frequency of species in all of the Tables.

F. nucleatum was the most prevalent (86.2 %), followed by *P. intermedia* (73.5 %), *A. actinomycetemcomitans* (*Aa*, 45 %), *P. gingivalis* (28.4%) and *T. denticola* (24.3 %) as shown in Table 11.

When examining for their presence in pairs, the valid % of positive combinations in terms of frequency were *Pi / Fn* (64.0 %), followed by the combinations *Aa / Fn* (41.3 %) and *Td / Aa* (12.7 %). Combinations of three showed the most frequent combinations as *Pi / Aa / Fn* (30.7 %), *Td / Pi / Fn* (20.1 %), *Pg / Pi / Fn* (19.0 %) and *Td / Pi / Aa* (12.2 %). The only combination of four was *Td/Pi/Aa/Fn*.

Table 11: Prevalence of the five suspected peridontopathogens in maternal GCF

Bacterial species	Positive n (%)	Negative n (%)	Missing n (%)	Valid Percentage Positive
P. gingivalis (Pg)	54 (27.0)	136 (68.0)	10 (5.0)	28.4
T. denticola (Td)	46 (23.0)	143 (71.5)	11 (5.5)	24.3
P. intermedia (Pi)	139 (69.5)	50 (25.0)	11 (5.5)	73.5
A. actinomycetemcomitans (Aa)	85 (42.5)	104 (52.0)	11 (5.5)	45
F. nucleatum (Fn)	163 (81.5	26 (13.0)	11 (5.5)	86.2
Pg / Td	27 (13.5)	163 (81.5)	12 (6.0)	14.2
Pg / Pi	45 (22.5)	143 (71.5)	12 (6.0)	23.9
Pg / Aa	27 (13.5)	161 (80.5)	12 (6.0)	14.4
Pg / Fn	42 (21.0)	146 (73.0)	12 (6.0)	22.3
Td / Pi	43 (21.5)	146 (73.0)	11 (5.5)	22.8
Td / Aa	24 (12.0)	165 (82.5)	11 (5.5)	12.7
Td / Fn	41 (20.5)	148 (74.0)	11 (5.5)	21.7
Pi / Aa	64 (32.0)	125 (62.5)	11 (5.5)	33.9
Pi / Fn	121 (60.5)	68 (34.0)	11 (5.5)	64.0
Aa / Fn	78 (39.0)	111 (55.5)	11 (5.5)	41.3
Pg / Td / Pi	26 (13.0)	163 (81.)	11 (5.5)	13.8
Pg / Td / Aa	27 (13.5)	162 (81.0)	11 (5.5)	14.3
Pg / Td / Fn	23 (11.5)	166 (83.0)	11 (5.5)	12.2
Pg / Pi / Aa	24 (12.0)	165 (82.5)	11 (5.5)	12.7
Pg / Pi / Fn	36 (18.0)	153 (76.5)	11 (5.5)	19.0
Pg / Aa / Fn	23 (11.5)	166 (83.0)	11 (5.5)	12.2
Td /Pi / Aa	23 (11.5)	166 (83.0)	11 (5.5)	12.2
Td / Pi / Fn	38 (19.0)	151 (75.5)	11 (5.5)	20.1
Pi / Aa / Fn	58 (29.0)	131 (65.5)	11 (5.5)	30.7
Td / Pi / Aa / Fn	22 (11.0)	167 (83.5)	11 (5.5)	11.6

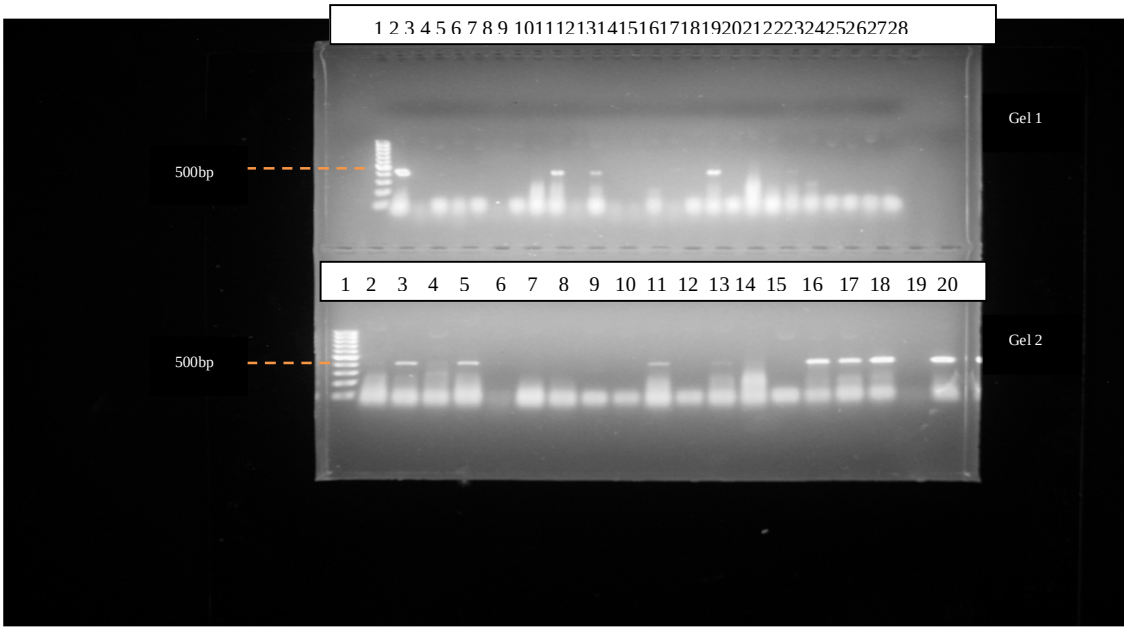

Figure 1: PCR amplification of *P. gingivalis* using Species-specific primers

Expected product size: 404 bp, lane 1: DNA marker 100 bp, lane 2 (Gel 1): positive control, lane 3 (Gel 1): negative control.

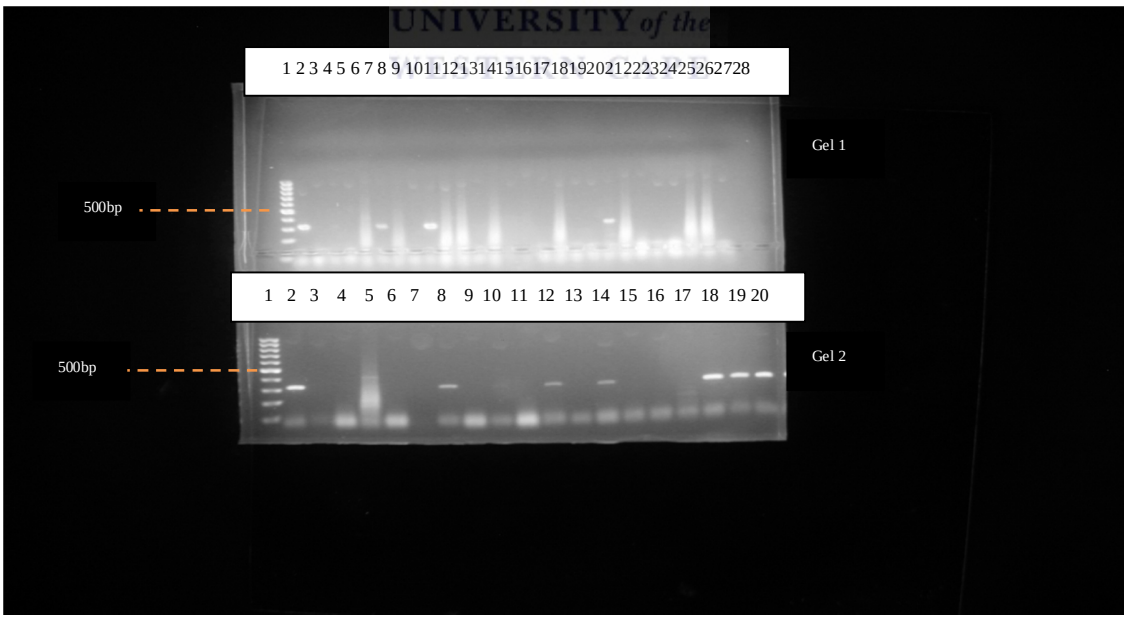

Figure 2: PCR amplification of *T. denticola* using Species-specific primers

Expected product size: 316 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

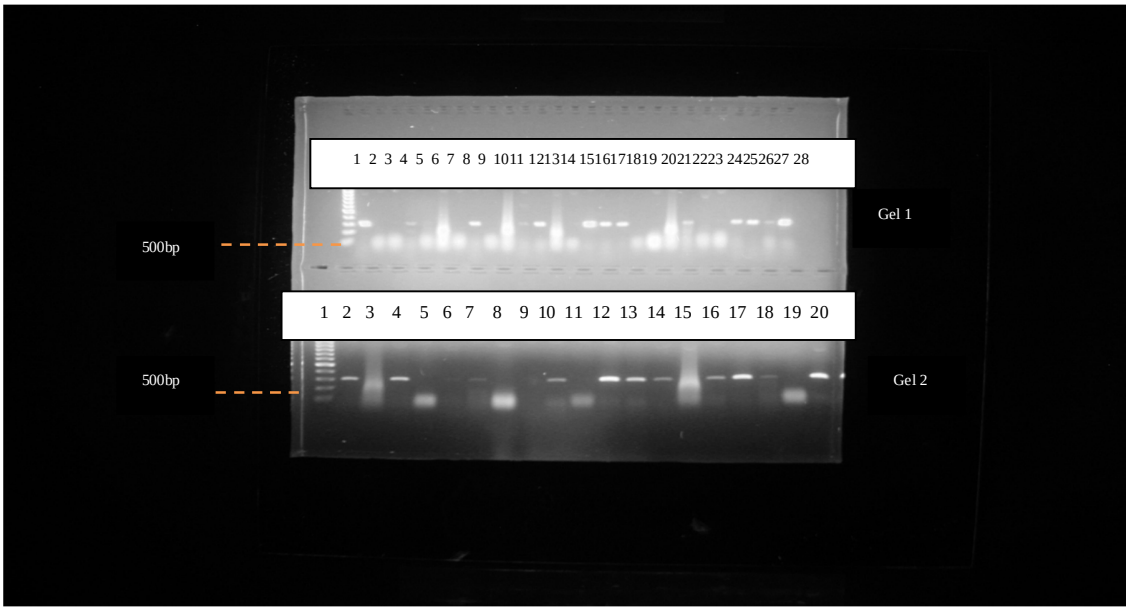

Figure 3: PCR amplification of *P. intermedia* using Species-specific primers

Expected product size: 307 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

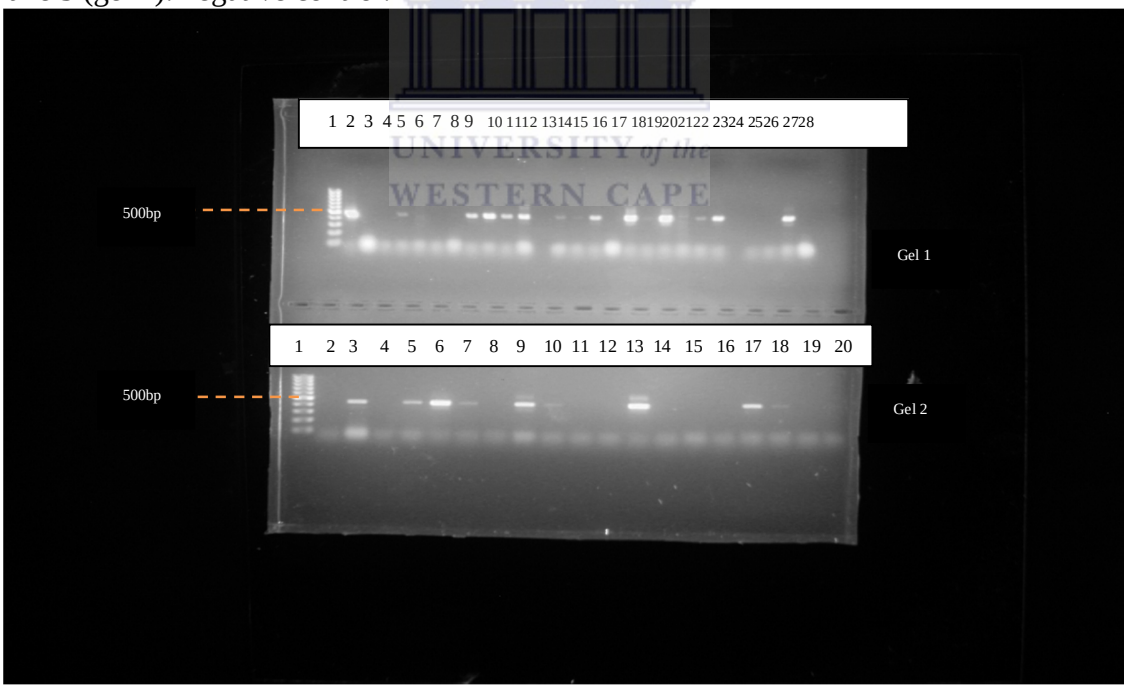

Figure 4: PCR amplification of *A. actinomycetemcomitans* using Species-specific primers. Expected product size: 500 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

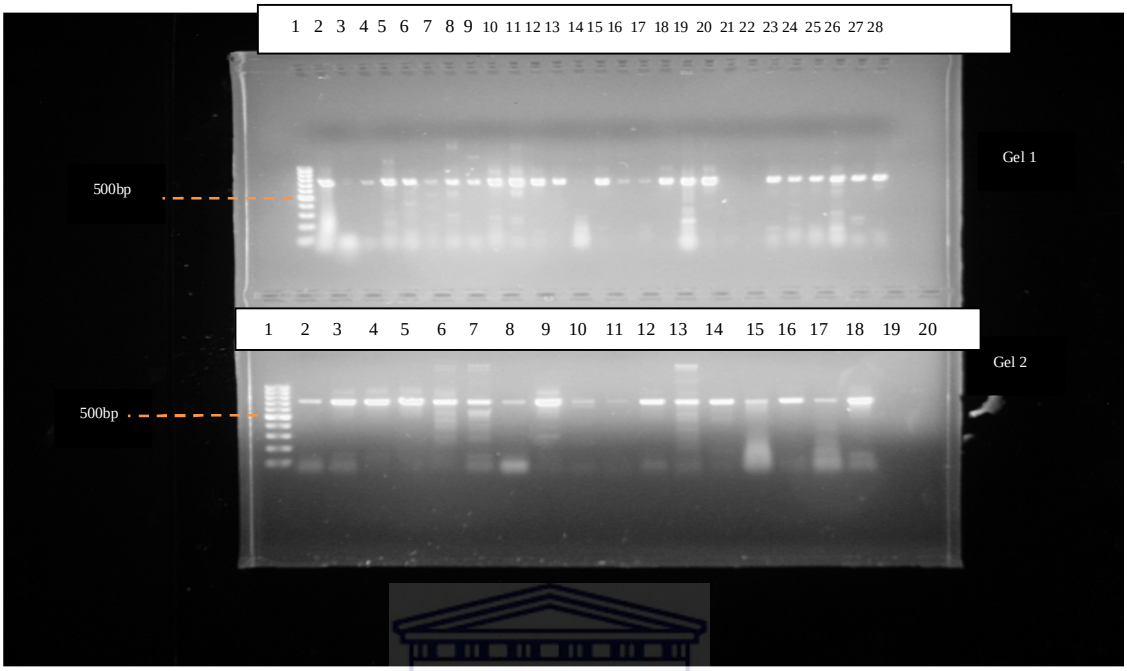

Figure 5: PCR amplification of *F. nucleatum* using Species-specific primers

Expected product size: 705 bp, lane 1: DNA marker 100 bp, lane 2 (gel 1): positive control, lane 3 (gel 1): negative control.

4.3. ASSOCIATION BETWEEN AGE AND THE FIVE ANAEROBIC PERIODONTOPATHOGENS

No significant association could be observed between the age of the mother and the presence of any one of the five anaerobic periodontopathogens when they were detected singly. The p values were 0.258, 0.110, 0.640, 0.229, 0.096 and 0.513 respectively for *P. gingivalis, T. denticola, P. intermedia, A. actinomycetemcomitans,* and *F. nucleatum* (Table 12). However, a significant association was observed between age and the following paired combination Pi/Aa (p value: 0.027).The frequency decreased in the age groups ≤ 20 and 31-35 years, with a slight increase in the age group of ≥ 36 years. No significant association was observed between age and combinations of three or more bacteria (Table 12).

Table 12: Correlation between age and the five anaerobic periodontopathogens

Bacterial species	≤ 20 years n /15 (%)	21 - 25yrs n /57 (%)	26 - 30yrs n /57 (%)	31 - 35yrs n /35 (%)	≥ 36yrs n /22 (%)	p value
P g	3 (20.0)	13 (22.8)	18 (31.6)	15 (42.9)	5 (22.7)	0.258
T.d	3 (20.0)	14 (24.6)	17 (29.8)	6 (17.1)	4 (18.2)	0.640
P i	13 (86.7)	46 (80.7)	37 (64.9)	24 (68.6)	17 (77.3)	0.229
Aa	10 (66.7)	30 (52.6)	19 (33.3)	14 (40.0)	11 (50.0)	0.096
F n	12 (80.0)	49 (86.0)	50 (87.75)	28 (80.0)	21 (95.5)	0.513
Pg / Td	2 (13.33)	6 (10.5)	12 (21.15)	4 (11.4)	3 (13.36)	0.564
Pg / Pi	2 (13.33)	12 (21.1)	18 (31.65)	8 (22.95)	5 (22.7)	0.592
Pg / Aa	3 (20.0)	6 (10.5)	7 (12.3)	6 (17.15)	5 (22.7)	0.579
Pg / Fn	2 (13.3)	8 (14.0)	15 (26.3)	13 (37.1)	4 (18.2)	0.097
Td / Pi	3 (20.0)	14 (24.6)	16 (28.1)	5 (14.3)	4 (18.2)	0.594
Td / Aa	3 (20.0)	8 (14.0)	5 (8.8)	5 (14.3)	3 (13.6)	0.796
Td / Fn	2 (13.3)	13 (22.8)	14 (24.6)	6 (17.1)	4 (18.2)	0.827
Pi / Aa	9 (60.0)	24 (42.1)	12 (21.1)	10 (28.6)	8 (36.4)	**0.027**
Pi / Fn	10 (66.7)	40 (70.2)	33 (57.9)	20 (57.15)	16 (72.7)	0.501
Aa / Fn	8 (53.3)	27 (47.4)	19 (33.3)	13 (37.1)	10 (45.5)	0.454
Pg / Td / Pi	2 (13.3)	6 (10.5)	12 (21.1)	3 (8.6)	3 (13.6)	0.434
Pg / Td / Aa	2 (13.3)	6 (10.5)	12 (21.1)	4 (11.4)	3 (13.6)	0.559
Pg/ Td / Fn	1 (6.7)	5 (8.8)	10 (17.5)	4 (11.4)	3 (13.6)	0.627
Pg /Pi / Aa	2 (13.3)	6 (10.5)	7 (12.3)	4 (11.4)	5 (22.7)	0.687
Pg / Pi / Fn	1 (6.7)	8 (14.0)	15 (26.3)	8 (22.9)	4 (18.2)	0.326
Pg / Aa /Fn	2 (13.3)	4 (7.0)	7 (12.3)	6 (17.1)	4 (18.2)	0.568
Td /Pi / Aa	3 (20.0)	8 (14.0)	5 (8.8)	4 (11.4)	3 (13.6)	0.792
Td / Pi / Fn	2 (13.3)	13 (22.8)	13 (22.8)	5 (14.3)	4 (18.2)	0.783
Pi / Aa / Fn	7 (46.7)	21 (36.8)	12 (21.1)	10 (28.6)	7 (31.8)	0.249
Td / Pi / Aa / Fn	2 (13.3)	8 (14.0)	5 (8.8)	4 (11.4)	3 (13.6)	0.927

4.4. CORRELATION BETWEEN LEVELS OF EDUCATION AND THE FIVE PERIODONTOPATHOGENS

A significant association was observed between educational levels and the presence of *T. denticola* (p = 0.047, Table 13), whether detected singly or in combination with other species. When detected in pairs (Table 13), only *Pg / Td* showed a significant association with the level of education (p = 0.001), while other significantly associated combinations included *Pg / Td / Pi* (p = 0.000), *Pg / Td / Aa* (p = 0.001) and *Pg / Td / Fn* (p = 0.003), (Table 13).

Table 13: Correlation between level of education and the five periodontopathogens

Bacterial species	No education n /18 (%)	Primary school n /111 (%)	High School n /42 (%)	University n /15 (%)	p value
Pg	7(38.9)	32 (28.82)	8 (19.0)	6 (40.0)	0.286
Td	7 (38.9)	24 (21.6)	7 (16.7)	7 (46.7)	**0.047**
Pi	17 (94.4)	84 (75.7)	28 (66.7)	9 (60.0)	0.078
Aa	9 (50.0)	52 (46.8)	17 (40.5)	5 (33.3)	0.683
Fn	16 (88.9)	95 (85.6)	36 (85.7)	13 (86.7)	0.985
Pg / Td	6 (33.3)	11 (9.9)	3 (7.1)	6 (40.0)	**0.001**
Pg / Pi	7 (38.9)	25 (22.52)	6 (14.3)	6 (40.0)	0.086
Pg / Aa	3 (16.7)	15 (13.51)	5 (11.9)	4 (26.7)	0.544
Pg / Fn	6 (33.3)	25 (22.52)	5 (11.9)	5 (33.3)	0.174
Td / Pi	7 (38.9)	22 (19.8)	7 (16.7)	6 (40.0)	0.083
Td / Aa	3 (16.7)	12 (10.8)	5 (11.9)	4 (26.7)	0.358
Td / Fn	6 (33.3)	22 (19.8)	6 (14.3)	6 (40.0)	0.111
Pi / Aa	8 (44.4)	39 (35.1)	13 (31.0)	4 (26.7)	0.694
Pi / Fn	15 (83.3)	71 (64.0)	26 (61.9)	8 (53.3)	0.291
Aa / Fn	7 (38.9)	49 (44.1)	15 (35.7)	5 (33.3)	0.721
Pg / Td / Pi	6 (33.3)	10 (9.0)	3 (7.1)	6 (40.0)	**0.000**
Pg / Td / Aa	6 (33.3)	11 (9.9)	3 (7.1)	6 (40.0)	**0.001**
Pg / Td / Fn	5 (27.9)	10 (9.0)	2 (4.8)	5 (33.3)	**0.003**
Pg / Pi / Aa	3 (16.7)	13 (11.7)	4 (9.5)	4 (26.7)	0.345
Pg / Pi / Fn	6 (33.3)	20 (18.0)	4 (9.5)	5 (33.3)	0.073
Pg / Aa / Fn	2 (11.1)	13 (11.7)	4 (9.5)	4 (26.7)	0.360
Td / Pi / Aa	3 (16.7)	11 (9.9)	5 (11.9)	4 (26.87)	0.288
Td / Pi / Fn	6 (33.3)	20 (18.0)	6 (14.3)	5 (33.3)	0.186
Pi / Aa / Fn	6 (33.3)	36 (32.4)	12 (28.6)	4 (26.7)	0.941
Td / Pi / Aa / Fn	2 (11.1)	11 (9.9)	5 (11.9)	4 (26.7)	0.312

4.5. CORRELATION BETWEEN DENTAL HISTORY AND THE FIVE PERIODONTOPATHOGENS

P.intermedia was the only single species which showed a significant association (p= 0.045) with frequency of tooth brushing (Table 14). No association was observed between frequency of tooth brushing and detection of suspected periodontopathogens in combinations of five and six (Table 14).

No statistically significant association was observed between the single periodontopathogens and gum bleeding while brushing, although the frequency of positive results in patients reporting gum bleeding was higher than those who did not bleed while brushing (Table 15). The following pairs: *Pg / Aa* and *Td / Aa* showed a good association with gum bleeding (p = 0.041 and 0.032 respectively) and again, positive results in patients with gum bleeding were higher than those who reported no bleeding while brushing (Table 15). Combinations of three or four, namely, *Pg / Pi / Aa*, *Td / Pi / Aa*, and *Td / Pi / Aa / Fn* showed a significant association with gum bleeding (p = 0.022, 0.032 and 0.046 respectively. Although the frequency of positive results was slightly higher in patients with gum pain than those who reported no gum pain when brushing (Table 16), no significant association was observed.

Table 14: Correlation between frequency of tooth brushing and the five periodontopathogens.

Bacterial species	Once a day n /87 (%)	Twice a day n /55 (%)	After every meal n /5 (%)	p value
Pg	26 (29.88)	14 (25.5)	2 (40.0)	0.733
Td	21 (24.1)	15 (27.3)	1 (20.0)	0.883
Pi	70 (80.5)	37 (67.3)	2 (40.0)	**0.045**
Aa	34 (39.1)	25 (45.5)	1 (20.0)	0.176
Fn	74 (85.1)	45 (81.8)	5 (100.0)	0.541
Pg / Td	11 (12.64)	7 (12.7)	1 (20.0)	0.887
Pg / Pi	22 (25.28)	10 (18.2)	2 (40.0)	0.399
Pg / Aa	12 (13.79)	6 (10.9)	2 (40.0)	0.193
Pg / Fn	19 (21.83)	10 (18.2)	2 (40.0)	0.497
Td / Pi	20 (23.0)	14 (25.5)	1 (20.0)	0.962
Td / Aa	10 (11.5)	8 (14.5)	1 (20.0)	0.775
Td / Fn	17 (19.5)	14 (25.5)	1 (20.0)	0.704
Pi / Aa	29 (33.3)	18 (32.7)	2 (40.0)	0.947
Pi / Fn	60 (69.0)	32 (58.2)	2 (40.0)	0.225
Aa / Fn	31 (35.6)	23 (41.8)	1 (20.0)	0.129
Pg / Td / Pi	11 (12.6)	7 (12.7)	1 (20.0)	0.891
Pg / Td / Aa	11 (12.6)	7 (12.7)	1 (20.0)	0.891
Pg / Td / Fn	8 (9.2)	6 (10.9)	1 (20.0)	0.723
Pg / Pi / Aa	12 (13.8)	4 (7.3)	2 (40.0)	0.080
Pg / Pi / Fn	17 (19.5)	7 (12.7)	2 (40.0)	0.241
Pg / Aa / Fn	9 (10.3)	6 (10.9)	2 (40.0)	0.129
Td / Pi / Aa	10 (11.5)	8 (14.5)	1 (20.0)	0.775
Td / Pi / Fn	16 (18.4)	13 (23.6)	1 (20.0)	0.752
Pi / Aa / Fn	26 (29.9)	17 (30.9)	2 (40.0)	0.891
Td / Pi / Aa / Fn	9 (10.3)	8 (14.5)	1 (20.0)	0.656

Table 15: Correlation between gum bleeding and the five periodontopathogens

Bacterial species	YES n/97 (%)	NO n/57 (%)	p value
Pg	30 (30.9)	15 (26.31)	0.314
Td	28 (28.9)	10 (17.5)	0.082
Pi	75 (77.3)	38 (66.7)	0.105
Aa	47 (48.5)	20 (35.1)	0.073
Fn	81 (83.5)	49 (86.0)	0.436
Pg / Td	15 (15.5)	5 (8.77)	0.163
Pg / Pi	26 (26.8)	11 (19.29)	0.213
Pg / Aa	18 (18.6)	4 (7.01)	**0.041**
Pg / Fn	19 (19.6)	14 (24.56)	0.279
Td / Pi	27 (27.8)	9 (15.8)	0.064
Td / Aa	16 (16.5)	3 (5.3)	**0.032**
Td / Fn	24 (24.7)	9 (15.8)	0.134
Pi / Aa	37 (38.1)	14 (24.6)	0.059
Pi / Fn	63 (64.9)	34 (59.6)	0.313
Aa / Fn	41 (42.3)	20 (35.1)	0.240
Pg / Td / Pi	15 (15.5)	5 (8.8)	0.173
Pg / Td / Aa	15 (15.5)	5 (8.8)	0.173
Pg / Td / Fn	12 (12.4)	4 (7.0)	0.221
Pg / Pi / Aa	17 (17.5)	3 (5.3)	**0.022**
Pg / Pi / Fn	18 (18.6)	10 (17.5)	0.528
Pg / Aa / Fn	14 (14.4)	4 (7.0)	0.129
Td / Pi / Aa	16 (16.5)	3 (5.3)	**0.032**
Td / Pi / Fn	23 (23.7)	8 (14.0)	0.107
Pi / Aa / Fn	32 (33.0)	14 (24.6)	0.179
Td / Pi / Aa / Fn	15 (15.5)	3 (5.3)	**0.046**

Table 16: Correlation between pain while brushing and the five periodontopathogens

Bacterial species	YES n/39 (%)	NO n/108 (%)	p value
Pg	12 (30.8)	30 (27.8)	0.436
Td	11 (28.2)	25 (23.1)	0.335
Pi	31 (79.5)	78 (72.2)	0.253
Aa	19 (48.7)	45 (41.7)	0.283
Fn	34 (87.2)	90 (83.3)	0.389
Pg / Td	6 (15.4)	12 (11.1)	0.330
Pg / Pi	10 (25.6)	24 (22.22)	0.420
Pg / Aa	8 (20.5)	13 (12.03)	0.157
Pg / Fn	9 (23.1)	22 (20.37)	0.452
Td / Pi	11 (28.2)	23 (21.3)	0.253
Td / Aa	7 (17.9)	12 (11.1)	0.205
Td / Fn	10 (25.6)	22 (20.4)	0.318
Pi / Aa	15 (38.5)	35 (32.4)	0.311
Pi / Fn	27 (69.2)	67 (62.0)	0.274
Aa / Fn	18 (46.2)	40 (37.0)	0.209
Pg / Td / Pi	6 (15.4)	12 (11.1)	0.330
Pg / Td / Aa	6 (15.4)	12 (11.1)	0.330
Pg / Td / Fn	6 (15.4)	9 (8.3)	0.173
Pg / Pi / Aa	7 (17.9)	12 (11.1)	0.330
Pg / Pi / Fn	8 (20.5)	18 (16.7)	0.376
Pg / Aa / Fn	7 (17.9)	10 (9.3)	0.124
Td / Pi / Aa	7 (17.9)	12 (11.1)	0.205
Td / Pi / Fn	10 (25.6)	20 (18.5)	0.234
Pi / Aa / Fn	14 (35.9)	31 (28.7)	0.261
Td / Pi / Aa / Fn	7 (17.9)	11 (10.2)	0.359

4.6. CORRELATION BETWEEN SMOKING, ALCOHOL CONSUMPTION AND THE FIVE PERIODONTOPATHOGENS

Neither alcohol nor smoking could be associated with the presence of the five periodontopathogens (Table 17 and 18). However, the number of patients who reported smoking may be too small for a definite conclusion to be drawn.

Table 17: Correlation between alcohol consumption and the presence of suspected periodontopathogens

Bacterial species	Never n/91 (%)	Daily n/24 (%)	Weekly n/28 (%)	Special occasion n/35 (%)	p value
Pg	27 (29.67)	5 (20.8)	10 (35.7)	11 (31.42)	0.676
Td	24 (26.4)	6 (25.0)	4 (14.3)	10 (28.6)	0.562
Pi	69 (75.8)	20 (83.3)	22 (78.6)	23 (65.7)	0.434
Aa	36 (39.6)	14 (58.3)	12 (42.9)	18 (51.4)	0.327
Fn	75 (82.4)	23 (95.8)	25 (89.3)	29 (82.9)	0.353
Pg / Td	13 (14.28)	2 (8.3)	3 (10.7)	8 (22.85)	0.346
Pg / Pi	23 (25.3)	5 (20.8)	8 (28.6)	8 (22.85)	0.929
Pg / Aa	11 (12.1)	3 (12.5)	4 (14.3)	8 (22.85)	0.439
Pg / Fn	20 (22.0)	4 (16.7)	8 (28.6)	9 (26.5)	0.727
Td / Pi	24 (26.4)	5 (20.8)	4 (14.3)	8 (22.85)	0.605
Td / Aa	12 (13.2)	2 (8.3)	2 (7.1)	6 (17.1)	0.602
Td / Fn	20 (22.0)	6 (25.0)	4 (14.3)	9 (25.71)	0.710
Pi / Aa	30 (33.0)	11 (45.8)	8 (28.6)	12 (34.3)	0.594
Pi / Fn	58 (63.7)	19 (79.2)	20 (71.4)	19 (54.3)	0.216
Aa / Fn	33 (36.3)	13 (54.2)	11 (39.3)	16 (45.7)	0.404
Pg / Td / Pi	13 (14.28)	2 (8.3)	3 (10.7)	7 (20.0)	0.585
Pg / Td / Aa	13 (14.28)	2 (8.3)	3 (10.7)	8 (22.9)	0.389
Pg / Td / Fn	10 (11.0)	2 (8.3)	3 (10.7)	7 (20.0)	0.480
Pg / Pi / Aa	11 (12.1)	3 (12.5)	3 (10.7)	6 (17.1)	0.865
Pg / Pi / Fn	17 (18.7)	4 (16.7)	7 (25.0)	7 (20.0)	0.873
Pg / Aa / Fn	9 (9.9)	2 (8.3)	3 (10.7)	8 (22.9)	0.212
Td / Pi / Aa	12 (13.2)	2 (8.3)	2 (7.1)	5 (14.3)	0.744
Td / Pi / Fn	20 (22.0)	5 (20.8)	4 (14.3)	7 (20.0)	0.851
Pi / Aa / Fn	28 (30.8)	10 (41.7)	7 (25.0)	10 (28.6)	0.604
Td / Pi / Aa / Fn	11 (12.1)	2 (8.3)	2 (7.1)	5 (14.3)	0.785

Table 18: Correlation between smoking and the presence of suspected periodontopathogens

Bacterial species	YES n/9 (%)	NO n/175 (%)	p value
Pg	3 (33.3)	51 (29.14)	0.518
Td	1 (11.1)	44 (25.1)	0.307
Pi	7 (77.8)	129(73.7)	0.570
Aa	4(44.4)	79(45.1)	0.621
Fn	9 (100.0)	149 (85.1)	0.246
Pg / Td	1 (11.1)	26 (14.85)	0.612
Pg / Pi	3 (33.3)	42 (24.0)	0.388
Pg / Aa	1 (11.1)	26 (14.85)	0.606
Pg / Fn	3 (33.3)	39 (22.28)	0.341
Td / Pi	1 (11.1)	41 (23.4)	0.349
Td / Aa	1 (11.1)	23 (13.1)	0.668
Td / Fn	1 (11.1)	39 (22.3)	0.380
Pi / Aa	2 (22.2)	61 (34.9)	0.350
Pi / Fn	7 (77.8)	111 (63.4)	0.312
Aa / Fn	4(44.4)	72 (41.1)	0.552
Pg / Td / Pi	1 (11.1)	25 (14.3)	0.629
Pg / Td / Aa	1 (11.1)	26 (14.9)	0.609
Pg / Td / Fn	1 (11.1)	22 (12.6)	0.687
Pg / Pi / Aa	1 (11.1)	23 (13.1)	0.668
Pg / Pi / Fn	3 (33.3)	33 (18.9)	0.247
Pg / Aa / Fn	1 (11.1)	22 (12.6)	0.687
Td / Pi / Aa	1 (11.1)	22 (12.6)	0.687
Td / Pi / Fn	1 (11.1)	36 (20.6)	0.428
Pi / Aa / Fn	1 (11.1)	55 (31.4)	0.433
Td / Pi / Aa / Fn	1 (11.1)	21 (12.0)	0.707

4.7. CORRELATION BETWEEN CLINICAL SIGNS OF GINGIVAL INFLAMMATION AND THE FIVE PERIODONTOPATHOGENS

The only periodontopathogen which appeared to be significantly associated with patients showing clinical signs of gingival inflammation was *P. intermedia*, whether alone (p = 0.013) or with another species viz Pg / Pi and Pi / Fn (p = 0.040 and 0.043 respectively).

P. gingivalis was closely but not significantly associated (p = 0.050). Combinations of three, four and five showed no association with clinical signs of gingival inflammation (Table 19).

Table 19: Correlation between clinical signs of gingival inflammation and the presence of the five periodontopathogens

Bacterial species	No sign of gingival inflammation n/83 (%)	Signs of gingival inflammation n/103 (%)	p value
Pg	18 (21.7)	35 (33.98)	0.050
Td	17 (20.5)	28 (27.2)	0.187
Pi	54 (65.1)	83 (80.6)	**0.013**
Aa	42 (50.6)	40 (38.8)	0.072
Fn	71 (85.5)	89 (86.4)	0.515
Pg / Td	12 (14.5)	14 (13.59)	0.504
Pg / Pi	14 (16.86)	30 (29.1)	**0.040**
Pg / Aa	10 (12.04)	16 (15.5)	0.333
Pg / Fn	15 (18.3)	26 (25.2)	0.171
Td / Pi	16 (19.3)	26 (25.2)	0.215
Td / Aa	12 (14.5)	11 (10.7)	0.289
Td / Fn	15 (18.1)	25 (24.3)	0.200
Pi / Aa	30 (36.1)	32 (31.1)	0.283
Pi / Fn	47 (56.6)	72 (69.9)	**0.043**
Aa / Fn	38 (45.8)	37 (35.9)	0.113
Pg / Td / Pi	12 (14.5)	13 (12.6)	0.439
Pg / Td / Aa	12 (14.5)	14 (13.6)	0.515
Pg / Td / Fn	10 (12.0)	12 (11.7)	0.555
Pg / Pi / Aa	9 (10.8)	14 (13.6)	0.369
Pg / Pi / Fn	12 (14.5)	23 (22.3)	0.119
Pg / Aa / Fn	9 (10.8)	13 (12.6)	0.445
Td / Pi / Aa	12 (14.5)	10 (9.7)	0.221
Td / Pi / Fn	14 (16.9)	23 (22.3)	0.229
Pi / Aa / Fn	27 (32.5)	29 (28.2)	0.313
Td / Pi / Aa / Fn	11 (13.3)	10 (9.7)	0.298

4.8. CORRELATION BETWEEN THE FIVE PERIODONTOPATHOGENS AND PREGNANCY OUTCOMES

No significant association was observed between the presence of the five periodontopathogens and preterm delivery of low birth weight infants (Table 20).

Table 20: Correlation between the presence of the five periodontopathogens and pregnancy outcomes

Bacterial species	Normal Birth n/98 (%)	PLBW n/91 (%)	p value
Pg	28 (28.57)	26 (28.6)	0.546
Td	22 (22.4)	24 (26.4)	0.323
Pi	69 (70.4)	70 (76.9)	0.198
Aa	48 (49.0)	37 (40.7)	0.158
Fn	82 (83.7)	81 (89.0)	0.197
Pg / Td	14 (14.28)	13 (14.3)	0.570
Pg / Pi	23 (23.46)	22 (24.2)	0.538
Pg / Aa	15 (15.30)	12 (13.2)	0.407
Pg / Fn	22 (22.4)	20 (22.0)	0.524
Td / Pi	21 (21.4)	22 (24.2)	0.391
Td / Aa	11 (11.2)	13 (14.3)	0.340
Td / Fn	19 (19.4)	22 (24.2)	0.267
Pi / Aa	36 (36.7)	28 (30.8)	0.238
Pi / Fn	59 (60.2)	62 (68.1)	0.163
Aa / Fn	45 (45.9)	33 (36.3)	0.115
Pg / Td / Pi	14 (14.3)	12 (13.2)	0.498
Pg / Td / Aa	14 (14.3)	13 (14.3)	0.582
Pg / Td / Fn	12 (12.2)	11 (12.1)	0.576
Pg / Pi / Aa	14 (14.3)	10 (11.0)	0.323
Pg / Pi / Fn	20 (20.4)	16 (17.6)	0.379
Pg / Aa / Fn	14 (14.3)	9 (9.9)	0.242
Td / Pi / Aa	11 (11.2)	12 (13.2)	0.424
Td / Pi / Fn	18 (18.4)	20 (22.0)	0.331
Pi / Aa / Fn	33 (33.7)	25 (27.5)	0.222
Td / Pi / Aa / Fn	11 (11.2)	11 (12.1)	0.516

4.9. DISCUSSION

The objective of this chapter was to examine an African population for the presence of anaerobic bacteria frequently associated with periodontal disease and implicated in adverse pregnancy outcomes such as PLBW. We elected to use gingival crevicular fluid instead of dental plaque because it enabled us to use the same sample to examine for bacterial species (using PCR) as well as for inflammatory cytokines (next chapter) which may serve as biomarkers for preterm delivery.

One sample was collected from each quadrant of the mouth and the four samples pooled to yield a single sample for each patient (Kim *et al*, 2007). PCR was used to detect five suspected periodontopathogens and the PCR results matched with selected risk factors (age, level of education, smoking, alcohol consumption, oral health care) and indicators (bleeding when brushing, gingival inflammation) for periodontal disease. In the absence of adequate diagnosis of periodontal disease, we concede that the associations may be inconclusive and for that reason, we present these results as an association between proposed risk factors for periodontal disease and PLBW rather than the presence of periodontal disease and PLBW. Although the five anaerobic bacteria examined for in this study have been implicated in PLBW, no overt association exists.

Significant associations were observed between the level of education and the presence of *T. denticola* and its combinations with other bacteria. The patients with no formal education and those who attended University were more likely to harbour the two bacteria and its

combinations with other bacteria. With the exception of *P intermedia* which showed a significant association with clinical signs of gingival inflammation ($p = 0.013$) and also with the frequency of tooth brushing ($p = 0.045$), none of the other species could be significantly associated with age, smoking, alcohol consumption, frequency of brushing, pain and bleeding when brushing, nor gingival inflammation when detected on their own. Bacterial combinations however, yielded significant correlations especially when *Aa* and *P. intermedia* were present. Gingival bleeding when brushing was the only variable where *T. denticola* showed any association, particularly when detected along with *P. gingivalis*. Because anaerobic spirochaetes are known indicators of periodontal disease (Loesche *et al*., 1988), one may speculate that these patients reporting bleeding when brushing their teeth had, or were at risk for, periodontal disease.

The association with gingival inflammation supports the positive correlation reported by other researchers between poor periodontal health and PLBW (Madianos *et al*., 2001; Dörtbudak *et al*., 2005; Jarjoura *et al*., 2005; Bosnajak *et al*., 2006; Lin *et al*., 2007). However, our dichotomous recording of "presence" or "absence" of gingival inflammation as opposed to the use of periodontal indices as a measurement of periodontal health or disease provides a rather crude assessment of the periodontal health of the mother and thus caution should be exercised in interpretation of this finding.

In those studies where differences in the presence of the five bacteria were reported when comparing NT and PLBW, the results yielded no statistical significance between the groups when bacterial load was taken into account (Papapanou *et al*., 2000; Noack *et al*., 2005;

Skuldbol *et al*., 2006) and therefore, as in our study, failed to establish a direct association between these species and PLBW. It could be interpreted that the detection of all five species in both cases and controls, may suggest a "carrier" state for all of these species. However, the lack of quantification of bacterial load, in all of the samples, presents an obstacle in drawing a finite conclusion.

In general, *P. intermedia* showed significant associations either singly or in combinations with most indicators of periodontal disease used in this study (gum bleeding, frequency of tooth brushing and clinical signs of gingival inflammation). *Aa* and members of the "Red Complex" were significantly associated with gum bleeding and frequency of tooth brushing only.

The consistent finding of *Aa* in all the bacterial combinations which showed a significant correlation with the variables described deserves some discussion. *Aa* has been significantly associated with aggressive periodontitis (Imbronito *et al*., 2008). It has also frequently been found in healthy individuals (Buduneli *et al*., 2005). Studies of different population groups have revealed that a unique *Aa* clone (JP2) is responsible for the aggressive form of periodontitis observed in adolescents from north and west Africa (Haubek *et al*., 1997, 2008) and from individuals of African origin living in Europe and USA. Being an Africa population, the prevalence of *Aa* in these subjects is not surprising.

It could be argued that the presence of *Aa* is nothing more than an indicator of a "Carrier" state, but the increased significance of the associations of the bacteria in combination with

the variables described, would suggest otherwise. All of the combinations significantly associated with gingival bleeding when brushing include *Aa*. It has been suggested that *Aa* may occur in 2 forms, either as an opportunistic pathogen occurring world-wide with a diversity of *Aa* clones or as an exogenous pathogen with a particular clone (JP2) racially restricted to persons of African origin (Haubek *et al*., 1997). Further studies on this population group are underway and will inform us of the actual role played by *Aa* in this population and therefore in PLBW.

CHAPTER 5: EVALUATION OF IL-6 AND IL-10 LEVELS IN MATERNAL AND FOETAL CORD SAMPLES

5.1. INTRODUCTION

Cytokines are soluble proteins and peptides that act as humoral regulators at nano to picomolar concentration and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. Cytokines also mediate interaction between cells directly and regulate processes in the extracellular environment. Some cytokines behave like classical hormones in that they are involved in biological phenomena such as inflammation, systemic inflammatory response syndrome, and acute phase reaction, wound healing, and the neuroimmune network. Unlike hormones, cytokines are not produced by specialized cells organized in specialized glands and act on a wider range of target cells than hormones (Saini and Arora, 2011). The different types of cytokines include interleukins that were first seen to be produced by white cells (leucocytes), lymphokines, initially thought to be produced by lymphocytes; monokines, initially thought to be produced by monocytes; interferons, initially thought to participate in antiviral responses; colony stimulating factors CSF), initially thought to help the growth of cells and chemokines and thought to participate in chemotaxis (Saini and Arora, 2011).

T helper cells, also known as Th cells are a sub-group of lymphocytes that play an important role in establishing and maximizing the capacity of the immune system. Type 1 cytokines are those produced by Th1 T-helper cells and include IL-2, IL-6, IL-12, IFN-gamma and

TNF-β, while type 2 cytokines are produced by Th2 T-helper cells and include IL-4, IL-5, IL-10 and IL-13. Th1-type cytokines produce the proinflammatory response which helps the killing of intracellular parasites and perpetuate the autoimmune responses. Th2 cytokines tend to produce an anti-inflammatory response and counteract the excessive proinflammatory responses which can lead to uncontrolled tissue damage.

The human immune system should produce a well balanced Th1 and Th2 response (Jarocki *et al*., 2007). Maternal Th1 cells that mediate allospecific cytotoxic responses are inhibited during pregnancy to prevent rejection of the fetal allograft, while Th2 cells which mediate antibody responses are increased to prevent complete immunosuppression (Warning *et al*., 2011). Earlier studies found that the host immune response could be a causative factor in the induction of preterm labor. Accumulation of pro-inflammatory cytokines by the host response can induce the release of substances like prostaglandin from gestational tissues that induce the uterus to contract and cause preterm labour. Elevated levels of inflammatory cytokines were found in women with preterm labour (Casey and MacDonald, 1988; Romero *et al*., 1988; Jarocki *et al*., 2007). Previous research showed that IL-6 and IL-10 have opposed biological effects in preterm labour. IL-6 is an inflammatory cytokine which can cause the release of prostaglandins and cause uterine contraction while IL-10 (anti inflammatory cytokine) is a body regulator of the inflammatory cytokine pathway (Jarocki *et al*., 2007; Perinatal Research Center, 2009).

Among the different mechanisms postulated by which periodontal disease may trigger PLBW, are the increased levels of cytokines in plasma of patients with periodontal disease

which may cross foetal membranes (Offenbacher *et al*. (1998a). The presence of suspected periodontopathogens in GCF of these mothers, would elicit a host response to the antigens presented. One of the objectives of this study was to evaluate IL-6, and IL-10 levels in blood serum samples from the mother and foetal cords in PLBW and normal birth.

The evaluation of IL-10 and IL-6 levels in blood serum from the mother and foetal cords was done by ELISA as explained in the material and methods chapter of this study. In this chapter, the following abbreviations will be used: **MB**: Maternal blood and **FCB**: Foetal Cord Blood

5.2. IL-10 AND IL-6 RESULTS

The means (standard error of the mean) were: 47.32 pg/ml (± 6.526), 21.26 (± 4.413), 46.19 (± 5.517), 67.49 (± 9.482) respectively for IL-10 MB, IL-10 FCB, IL-6 MB and IL-6 FCB. Minimum and maximum were -2.521 and 813.8, -22.05 and 443.4, 0.327 and 665.2, -3.168 and 826.2 also respectively for IL-10 MB, IL-10 FCB, IL-6 MB and IL-6 FCB (Table 21).

Table 21: Descriptive statistics of IL-10 and IL-6 results

	Minimum	Maximum	Mean	Standard deviation	Standard Error
IL-10 MB (pg/ml)	-2.521	813.8	47.32	91.83	6.526
IL-10 FCB (pg/ml)	-22.05	443.4	21.26	62.09	4.413
IL-6 MB (pg/ml)	0.327	665.2	46.19	77.64	5.517
IL-6 FCB (pg/ml)	-3.168	826.2	67.49	133.1	9.482

When examining the association between maternal medical records and IL-10 and IL-6 (Table 22), a significant association was observed between IL-6 MB and age ($p = 0.018$). The level of IL-6 MB for patients > 36 years was very low (72.93 pg/ml) compared with other age groups. IL-10 levels in FCB showed a significant association with maternal weight ($p = 0.024$) with mothers weighing ≤ 60 kg more likely to have a higher level of IL-10 than those weighing > 60 kg. The p values were 0.021 and 0.008 respectively for IL-10 MB and IL-10 FCB in these two groups. Also, the levels of IL-6 were higher in patients weighing ≤ 60 kg than in patients weighing > 60 kg but the differences were not significant (Table 22). Significant associations were also observed between the height of the mother and IL-10 and IL-6 results. Elevated levels of IL-10 MB, IL-10 FCB and IL-6 MB were associated with short rather than tall mothers ($p = 0.002$, 0.020 and 0.048 respectively) while levels of education could not be associated with IL-10 and IL-6 in this study (Table 22).

Higher levels of IL-10 FCB were observed when mothers reported being in their first pregnancy than those who reported multiple pregnancies ($p = 0.040$). Also the level of IL-6 was generally higher for mothers in their first pregnancy than those with multiple pregnancies but the difference was not significant (Table 22). However, no significant association was observed between mothers with induced abortion and IL-10 and IL-6 results

(Table 22). Previous preterm deliveries resulted in the expression of a higher level of IL-10 FCB than when mothers had no history of previous preterm deliveries (Table 22).

No significant associations were observed between IL-10 and IL-6 and maternal history of urinary tract infection, STD and heart disease (Table 22). Neither were any significant associations observed between IL-10/IL-6 and gum bleeding, pain while brushing, frequency of tooth brushing, access to medical aid and alcohol consumption of the mothers (Table 23), although a significant association ($p = 0.032$) was observed between smokers and IL-6 MB, ($p = 0.032$) (Table 23). There was also a significant association ($p = 0.036$) between antibiotic administration and IL-6 MB. Patients who took antibiotics before delivery had a lower level of IL-6 MB (82.82 pg/ml) than those who didn't (Table 23). No significant differences were observed between clinical signs of gingival inflammation as observed by the clinician, and the levels of IL-10 and IL-6 (Table 23).

Table 22: Association between maternal medical history and IL-10 and IL-6

	n	IL-10 MB mean pg/ml	IL-10 FCB mean pg/ml	IL-6 MB mean pg/ml	IL-6 FCB mean pg/ml
AGE (years)					
< 20	17	94.00	120.50	109.35	96.47
20 – 25	57	108.67	99.05	111.42	97.57
26 – 30	58	108.67	88.47	84.74	86.47
31 – 35	37	83.53	93.81	100.69	107.14
≥ 36	23	105.09	97.00	72.93	97.95
p value		0.192	0.332	**0.018**	0.509
WEIGHT (kg)					
≤ 50	16	110.06	90.34	69.22	88.69
51 – 55	34	103.63	112.69	106.37	105.37
56 – 60	44	97.65	100.69	99.08	96.56
61 – 65	38	90.74	91.96	88.88	87.74
> 65	53	78.79	75.53	89.51	85.31
p value		0.131	**0.024**	0.178	0.467
WEIGHT (kg)					
≤60	94	101.93	103.27	96.63	98.40
> 60	91	83.78	82.39	89.25	86.33
p value		**0.021**	**0.008**	0.348	0.124
HEIGHT (cm)					
≤ 50	30	115.15	110.13	107.60	105.92
151 – 155	40	99.08	95.29	105.04	86.05
156 – 160	43	89.07	90.37	82.20	88.76
160 – 165	38	88.51	92.55	81.57	84.24
> 165	30	62.00	65.08	80.25	91.48
p value		**0.002**	**0.020**	**0.048**	0.474
LEVEL OF EDUCATION					
No formal education	17	127.32	108.26	116.76	109.18
Primary school	120	97.54	99.44	98.90	102.06
High School	42	85.14	93.96	90.13	86.58
university	15	97.97	79.70	85.13	72.20
p value		0.077	0.486	0.317	0.102
NUMBER OF PREGNANCIES					
First	74	95.57	106.89	106.38	97.91
Multiple	118	97.08	89.99	90.31	94.79
p value		0.855	**0.040**	0.051	0.704
INDUCED ABORTION					
Yes	33	77.65	71.79	61.36	74.92
No	103	65.57	67.45	70.79	65.76
p value		0.125	0.582	0.232	0.242
PREVIOUS PRETERM DELIVERIES					
Yes	31	71.69	81.84	66.66	74.71
No	100	64.24	61.09	65.80	62.62
p value		0.339	**0.008**	0.912	0.119
URINARY TRACT INFECTION					
Yes	44	89.02	91.77	93.85	102.18
No	114	96.17	95.33	94.70	91.48
p value		0.446	0.704	0.928	0.252
STD					
Yes	26	81.67	89.81	97.15	111.87
No	166	98.82	97.55	96.40	93.50
p value		0.143	0.509	0.949	0.115
HISTORY OF HEART DISEASE					
Yes	19	107.97	102.08	101.92	108.84
No	173	95.24	95.89	95.90	94.58
p value		0.343	0.645	0.654	0.286

Table 23: Association between maternal smoking, alcohol consumption and oral health care and IL-10 and IL-6

CHARACTERISTICS	n	IL-10 MB mean pg/ml	IL-10 FCB mean pg/ml	IL-6 MB mean pg/ml	IL-6 FCB mean pg/ml
FREQUENCY OF TOOTH BRUSHING					
Once a day	91	74.71	73.22	78.11	73.66
Twice a day	57	80.53	84.39	75.96	81.04
After every meal	5	78.50	61.60	68.60	75.80
p value		0.737	0.241	0.875	0.612
BLEEDING WHILE BRUSHING					
Yes	99	80.12	81.09	99(80.89	81.51
No	61	81.12	79.55	79.87	77.57
p value		0.894	0.839	0.892	0.600
PAIN WHILE BRUSHING					
Yes	40	70.79	73.18	81.53	76.65
No	111	77.88	77.02	74.01	75.08
p value		0.379	0.634	0.351	0.845
ACCESS TO MEDICAL AID					
Yes	33	105.56	95.68	95.27	103.76
No	159	94.62	96.67	96.75	94.38
p value		0.303	0.926	0.889	0.375
ALCOHOL CONSUMPTION					
Never	96	90.62	92.15	95.32	87.12
Daily	23	105.15	104.30	90.07	107.07
Weekly	29	85.59	82.00	100.45	99.28
Special occasions	37	97.43	96.80	82.97	91.95
p value		0.535	0.483	0.550	0.367
SMOKING					
Yes	8	115.19	64.75	137.63	98.00
No	184	95.69	97.88	94.71	95.91
p value		0.331	0.099	**0.032**	0.917
ANTIBIOTIC ADMINISTRATION					
Yes	55	91.76	90.25	82.82	87.26
No	136	97.71	98.33	101.33	98.86
p value		0.501	0.360	**0.036**	0.188
GINGIVAL INFLAMMATION AND BLEEDING					
Yes	83	90.15	88.00	89.41	89.18
No	103	96.20	97.93	96.80	96.11
p value		0.446	0.211	0.352	0.382

Table 24: Association between periodontopathogens and IL-10 and IL-6

Bacterial species		IL-10 MB mean pg/ml	IL-10 FCB mean pg/ml	IL-6 MB mean pg/ml	IL-6 FCB mean pg/ml
	n/187				
P. gingivalis	54	87.45	86.29	98.44	91.23
p value		0.292	0.214	0.474	0.713
	n/186				
T. denticola	46	97.57	98.35	97.41	91.76
p value		0.555	0.481	0.570	0.856
	n/186				
P. intermedia		95.68	95.28	99.19	93.87
p value		0.363	0.458	**0.017**	0.718
	n/186				
A. actinomycemcomitans	85	96.62	93.65	94.20	92.59
p value		0.469	0.973	0.871	0.923
	n/186				
F. nucleatum	160	94.62	94.78	93.30	96.32
p value		0.482	0.420	0.900	**0.037**

Table 24 shows the association between the five periodontopathogens and the levels of IL-10 and IL-6. Only *P. intermedia* showed a significant association with IL-6 MB (p = 0.017) and *F. nucleatum* with IL-6 FCB (p = 0.037).

Table 25: Association between paired combinations of periodontopathogens and IL-10 and IL-6

Bacterial species		IL-10 MB mean pg/ml	IL-10 FCB mean pg/ml	IL-6 MB mean pg/ml	IL-6 FCB mean pg/ml
	n/187				
Pg / Td	27	99.35	96.70	109.50	91.56
p value		0.579	0.779	0.108	0.839
	n/185				
Pg / Pi	45	84.57	82.88	97.46	83.49
p value		0.225	0.145	0.521	0.192
	n/185				
Pg / Aa	27	83.33	91.31	97.20	94.74
p value		0.310	0.860	0.659	0.813
	n/185				
Pg / Fn	42	84.27	82.82	97.56	93.67
p value		0.230	0.161	0.530	0.872
	n/186				
Td / Pi	43	99.92	98.08	97.67	92.47
p value		0.373	0.525	0.562	0.940
	n/186				
Td / Aa	24	98.81	99.52	99.90	94.58
p value		0.604	0.557	0.533	0.877
	n/186				
Td / Fn	41	96.65	94.12	97.32	95.59
p value		0.672	0.933	0.607	0.726
	n/186				
Pi / Aa	64	92.23	91.20	99.25	88.30
p value		0.816	0.672	0.291	0.385
	n/186				
Pi / Fn	118	96.62	96.10	99.44	97.09
p value		0.297	0.385	**0.047**	0.173
	n/186				
Aa / Fn	78	96.42	93.47	94.82	93.09
p value		0.529	0.996	0.776	0.984

When the five periodontopathogens were detected in pairs, only *Pi / Fn* showed a significant association with the level of IL-6 MB (Table 25) (p = 0.047).

Table 26: Association between combinations of three or more periodontopathogens and IL-10 and IL-6 results

Bacterial species	n/186	IL-10 MB mean pg/ml	IL-10 FCB mean pg/ml	IL-6 MB mean pg/ml	IL-6 FCB mean pg/ml
Pg / Td / Pi	26	98.71	94.00	107.79	89.19
p value		0.595	0.959	0.145	0.696
Pg / Td / Aa	27	99.17	96.41	108.69	91.33
p value		0.554	0.762	0.113	0.861
Pg / Td / Fn	23	98.46	91.26	111.50	96.30
p value		0.637	0.831	0.087	0.752
Pg / Pi / Aa	24	80.42	88.13	100.10	90.79
p value		0.202	0.600	0.520	0.829
Pg / Pi / Fn	36	83.49	79.89	99.15	87.03
p value		0.214	0.091	0.483	0.456
Pg / Aa / Fn	23	83.41	88.11	98.80	96.87
p value		0.337	0.608	0.614	0.711
Td / Pi / Aa	23	98.28	96.93	98.50	92.30
p value		0.649	0.744	0.634	0.947
Td / Pi / Fn	38	99.24	93.49	97.61	96.68
p value		0.461	0.999	0.598	0.634
Pi / Aa / Fn	58	91.82	91.22	99.24	88.62
p value		0.774	0.697	0.328	0.452
Td / Pi / Aa / Fn	22	98.86	95.20	100.98	90.77
p value		0.619	0.874	0.488	0.835

No significant association was observed with levels of IL-10 and IL-6 and the detection of five periodontopathogens in combinations of 3 and more (Tables 26). A strong association between PLBW and the levels of IL-10 MB, IL-10 FCB and IL-6 FCB (p = 0.002, 0.000 and 0.000 respectively) was observed (Table 26).

Table 27a: Association between pregnancy outcomes and IL-10 and IL-6 results

	PLBW		NORMAL		
	Number	Mean pg/ml	Number	Mean pg/ml	p value
IL-10 MB (pg/ml)	97	111.78	100	86.60	0.002
IL-10 FCB (pg/ml)	97	122.83	100	75.89	0.000
IL-6 MB (pg/ml)	97	93.89	100	103.96	0.215
IL-6 FCB (pg/ml)	97	116.30	99	81.06	0.000

Table 27b: Correlation between cytokines ratios (IL-6M/ IL-10M and IL-6M/ IL-10M) with the 5 periodontopathogens, gingival inflammation and PLBW

		IL-6M/IL-10M	IL-6F/IL-10F
T. denticola	**r**	0.054	0.117
	p	0.464	0.113
	n	186	185
P. gingivalis	**r**	0.091	0.006
	p	0.216	0.935
	n	187	186
P. intermedia	**r**	0.143	0.104
	p	0.051	0.159
	n	186	185
A. actinomycetemcomutans	**r**	0.033	-0.091
	p	0.656	0.217
	n	186	186
F. nucleatum	**r**	-0.036	-0.000
	p	0.628	0.997
	n	186	185
Gingival inflammation	**r**	0.061	-0.018
	p	0.406	0.804
	n	186	186
PLBW	**r**	0.088	-0.016
	p	0.220	0.824
	n	197	196

r = coefficient of correlation, p = p value, n = number of patients

IL-10 MB, IL-10 FCB and IL-6 FCB were significantly associated with PLBW. No significant association was found between PLBW and IL-6 MB (Table 27a). No correlation was found between the ratio of the two cytokines (IL-6 and IL-10) in maternal blood or in foetal cord blood with the 5 periodontopathogens, gingival inflammation nor PLBW (Table 27b).

5.3. DISCUSSION

The objective of this chapter was to evaluate the relationship between PLBW, Interleukin- 6, Interleukin - 10 and the presence of periodontopathogens. For this purpose, maternal and foetal cord blood samples were collected for the evaluation of IL-6 and IL-10 levels using the ELISA test as these two cytokines were found to play an important role in PLBW.

Age, level of education, urinary tract infections, STD, history of heart disease, gum bleeding, pain while brushing, frequency of tooth brushing, signs of gingival inflammation, access to medical aid, and alcohol consumption may influence the levels of IL-6 and IL-10 directly through infection or indirectly by contributing to the occurrence of an infection. All the above factors didn't show any association with IL-6 and IL-10 in this study. The lack of association between signs of gingival inflammation and the levels of IL-6 and IL-10 in this study is unclear, and may be attributed to the coarse assessment of gingival inflammation in the absence of accurate measurement of clinical indices or radiographs.

Mello *et al*., (2008), in their study on the effect of weight-loss on cytokines, found that weight reduction resulted in an increased expression of IL-6. In this study maternal weight appeared to be significantly associated with IL-10 and IL-6. This could be associated with malnutrition of the mothers which may increase the risk for of infection and therefore increase the production of inflammatory cytokines. Little is known about the history of previous preterm deliveries and number of previous pregnancies and cytokine levels. In our study, a significant association was demonstrated between number of pregnancies and IL-10 FCB, while mothers with previous preterm deliveries had a higher level of IL-10 FCB.

Smoking is strongly associated with periodontal disease and poor response to periodontal therapy. Also the upregulation of LPS-mediated monocyte secretion of PGE2 by nicotine may play an important role in the pathogenesis of periodontal disease (Haber and Kent; 1992; Crossi *et al*., 1994; Payne *et al*., 1996; Kamma *et al*., 1999). In this study, smokers had a higher level of IL-6 MB than non-smokers ($p = 0.032$), and this significant finding is

in agreement with a study by Giannopoulou *et al*. (2003) who found that IL-6 levels significantly correlated with smoking.

Antimicrobial agents have been reported to be involved both *in vivo* and *in vitro* in the modification of the inflammatory and the immune response (Morikawa *et al*., 1996). There was a significant association (p = 0.036) between antibiotic administration and IL-6 MB in this study. Patients who took antibiotics before delivery had a lower level of IL-6 MB (82.82 pg/ml) than those who didn't (101.33 pg/ml). This may be due to eradication of bacteria by antibiotics as described in a study by Morikawa *et al*., (1996) who found that dexamethasone deeply suppressed the synthesis of IL-6 and IL-10, fosfomycin enhanced the synthesis of both cytokines while clarithromycin enhanced only IL-10.

Earlier studies on IL-10 levels differ. Some found a higher level of IL-10 in mothers with PLBW (Apuzzio *et al*., 2004), while others found low levels of IL-10 in mothers with PLBW (Greig *et al*., 1995; Lin *et al*., 2003; Jarocki *et al*., 2007). In the present study a strong association between PLBW and maternal and foetal cord serum sample levels of IL-10 was observed (p = 0.002 and 0.000 for IL-10 MB and IL-10 FCB respectively). High maternal and foetal cord blood levels of IL-10 were found in PLBW. Many contradictions are reported in the literature regarding the role of IL-10 in PLBW and its role at the maternal – foetal interface remains controversial (Blidane and Stamatini 2002, Thaxton *et al* 2010). Some researchers reported a reduced level of IL-10 in PLBW (Dudley 1997, Bayouni *et al* 2003) and CIPD (Yamakazi *et al* 2001, Gautoudi *et al* 2004), while others reported elevated levels of IL-10 with PLBW (Matoba *et al* 2009). The elevated IL-10 levels in MB and FCB

may be due to the fact that the inflammatory responses are of foetal origin and may play a role in pregnancy loss. A recent study demonstrated that in women with pregnancy loss, some polymorphisms of IL-10 gene promoter could contribute as risk factors for PLBW (Cochery-Nouvellon *et al.*, 2009). Menon *et al.*, (2010) found that genetic regulation of cytokine concentrations in PTB differ with ethnicity and that cytokine concentrations were associated with interactions between genotype and PTB in African Americans, but not in Caucasians.

Previous studies conducted to determine IL-6 levels in amniotic fluid of patients who presented with PLBW found that IL-6 could be a marker for PLBW (Apuzzio *et al.*, 2004; Figueroa *et al.*, 2005; Jarocki *et al.*, 2007). A good association was observed between PLBW and FCB sample levels of IL-6 in this study. However no significant association was found in this study between PLBW and maternal blood levels of IL-6. The higher level of IL-10 in maternal blood found in this study could have an inhibitory effect on IL-6 thus decreasing its bioavailabilty. Previous studies also found that IL-6, TNF and IL-8 were regulated at the transcriptional level by IL-10 thus decreasing their bioavailability for action (Fortunate et al., 1996; 1997, 1998).

It was suggested that cytokines may be used as markers in the diagnosis of the pathogenesis of periodontal disease (Giannopoulou *et al.*, 2003). In this study only *P. intermedia* and *F. nucleactum* showed a significant association with IL-6. In both cases, patients with *P. intermedia* and *F. nucleatum* had higher maternal serum levels of IL-6 and foetal cord serum levels of IL-6 respectively. An association between *P. gingivalis* and IL-6 has been reported

previously (Hirose *et al*., 1997; Lin *et al*., 2003) and to our knowledge, this is the first human study showing the relationship between different periodontopathogens, maternal serum and feotal cord serum levels of IL-6 and IL-10. The relationship between pro- and anti –inflammatory cytokines and pregnancy outcomes could not be clearly established in this study, because *F. nucleatum* showed a significant correlation with IL-6 levels in FCB and when present with *P.intermedia*, a correlation with IL-6 was also observed in MB. *P. intermedia* and *F. nucleatum* are both associated with pregnancy gingivitis which may add to the oral inflammatory burden, thus increasing the risk for PTL. It was unexpected to find no correlation between IL-6 and IL-10 and gingival inflammation in this study. Studies by Skuldbol *et al* (2006) and Mitchell-Lewis *et al* (2001) also found no association between PTL and CIPD.

CHAPTER 6: EVALUATION OF IgG AND IgM LEVEL AGAINST FIVE PERIODONTOPATHOGENS

6.1. INTRODUCTION

Antibodies (also called immunoglobulins or Ig) are proteins which can bind specifically to a wide range of antigens and give a response that is important in antimicrobial defense. Antibodies are important for the prevention and/or cure of infections (bacterial or viral). There are different types of immunoglobulins that react specifically with the antigen that induced their formation. Five immunoglobulin classes are defined on the basis of their heavy chain composition, namely, IgG, IgM, IgA, IgE and IgD. Their functions in the host immunity are different and their synthesis occurs at different stages during an immunological response to an infection (Kenneth, 2011).

Immunoglobulin G (IgG) is a protein with a molecular weight of 150,000 daltons. It consists of two identical heavy (H) chains and two identical light (L) chains. The two L chains are connected to the H chain and the two H-chains are connected to one another by disulfide bridges giving the molecule a Y- shaped appearance. IgG is the most predominant immunoglobulin in the serum (75% of the total serum immunoglobulin). It can diffuse out of the blood stream into the extravascular spaces and its concentration in tissue fluid is high during inflammation. Predominantly, IgG antibodies are involved in the secondary immune

response. IgG is very effective in neutralizing bacterial exotoxins and viruses. It crosses the placenta and provides passive immunity to the foetus and infant for the first six months. Its small size and persistence in the serum of the mother facilitates its sharing with the foetus in utero, thus explaining why an infant is born with the full complement of the mother's IgG (Kenneth, 2011).

Immunoglobulin M (IgM) is the first Ig to be synthesized by infants and the first to appear in the blood stream when an infection occurs. IgM makes up around 10% of the total serum immunoglobulin. The most important role of IgM is its ability to function early in the immune response against blood-borne pathogens. It is very efficient in agglutinating particulate antigens. IgM binds to a microbe rendering the microbe more susceptible to phagocytosis. On the surface of mature B cells, IgM functions as an antigen receptor capable of activating B cells when bound to the antigen (Kenneth, 2011).

Previous studies found that patients who presented with PLBW had an increased level of antibodies against certain periodontopathogens (Hill, 1998; Dasanayake *et al.* 2001**,** 2003; Dörtbudak *et al*., 2005) while other studies found the opposite (Madianos *et al.*, 2001; Jarjoura *et al*., 2005; Lin *et al*., 2007). In the present study, the ELISA test was used to investigate the IgG and IgM levels in maternal and foetal cord blood samples respectively, directed against 5 suspected periodontopathic bacteria (*Porphyromonas gingivalis, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans)* in order to establish the host immunological response of the mother

to these periodontal pathogens and their association with PLBW. The details of the methodology are outlined in chapter two.

6.2. IgG AND IgM RESULTS

The IgG or IgM levels were expressed in percentage compared to O.Ds of protein A (100 %) from *Staphylococcus aureus* which is an immunoglobulin binding substrate. In this chapter the following abbreviations were used: *Pi*-IgG = IgG against *Prevotella intermedia*, *Pg*-IgG = IgG against *Porphyromonas gingivalis*, *Fn*-IgG = IgG against *Fusobacterium nucleatum*, *Aa*-IgG = IgG against *Aggregatibacter actinomycetemcomitans*, *Td*-IgG = IgG against *Treponema denticola*, *Pi*-IgM = IgM against *Prevotella intermedia*, *Pg*-IgM = IgM against *Porphyromonas gingivalis*, *Fn*-IgM = IgM against *Fusobacterium nucleatum*, *Aa*-IgM = IgM against *Aggregatibacter actinomycetemcomitans*, *Td*-IgM = IgM against *Treponema denticola*.

Only *Aa*-IgM showed a significant difference with maternal age ($p = 0.008$) and level of education (0.007) (Tables 28 and 29). The level of IgM against *Aa* in mothers aged < 20 years and also in mothers with no formal education was very low compared with other groups. No significant differences were associated with IgG and IgM against the other periodontopathogens and age or the level of education (Tables 28 and 29), nor were any significant associations observed between oral health care and IgG and IgM levels (Tables 28 and 29). Only *Pg*-IgG and *Pi*-IgM showed a significant association with maternal gum bleeding with p values of 0.013 and 0.029 respectively (Tables 28 and 29). *Pg*-IgG and *Td*-

IgM showed a good correlation with maternal gingival pain during tooth brushing (p= 0.003 and 0.002 respectively, Tables 28 and 29). No significant association was observed between smoking, alcohol consumption and IgG nor IgM except for *Pg*-IgM which showed an association with mothers who smoke (p = 0.045, Tables 28 and 29). PCR detection of *A actinomycetemcomitans* and *P. gingivalis* showed significant associations with their specific IgG (p = 0.012) and IgM (p = 0.011) levels respectively. No significant associations were observed between the other bacteria and their respective IgG or IgM levels (Tables 28 and 29).

Higher levels of specific IgG and IgM against different periodontopathogens were observed in mothers with gingival inflammation, with only *Fn*-IgG and *Pg*-IgM showing a significant association (p = 0.026 and p = 0.021 respectively). Mothers with no sign of gingival inflammation had higher levels of *Fn*-IgG with low levels of *Pg*-IgM in FCB. (Tables 28 and 29). Positive correlations between IgG levels and infection with *P. gingivalis* and *Aa* have been reported previously (Nakagawa et al., 1994).

There was a significant association between PLBW and IgG against the different peridontopathogens with the following p values: 0.002, 0.002, 0.016, 0.000 and 0.023 respectively for *Pi*-IgG, *Pg*-IgG, *Fn*-IgG, *Aa*-IgG and *Td*-IgG. Mothers with PLBW babies had a low level of IgG against the different periodontopathogens compared to those with normal babies. No significant associations were observed between PLBW and IgM against the 5 periodontopathogens although higher levels of IgM were observed in FCB with PLBW (Tables 28 and 29).

Table 30 shows the correlation between the level of cytokines (IL-6 and IL-10) in maternal blood and foetal cord blood, and the levels of IgG and IgM against the 5 periodontopathogens. The following association showed a positive correlation: *Pi*-IgG with IL-6 FCB (p = 0.045), *Aa*-IgG with IL-10 FCB (p = 0.046) and *Td*-IgG with IL-6 MB (p = 0.003) while the following showed negative correlations with IL-10 MB: *Pi*-IgM (p = 0.044), *Pg*-IgM (p = 0.001) and *Aa*-IgM (p = 0.019). In general, most positive correlations were seen with IgG and negative correlations with IgM (Table 30).

Table 28: Association between IgG against periodontopathogens and maternal history

Parameters	n	*Pi*-IgG (Median %)	n	*Pg*-IgG (Median %)	n	*Fn*-IgG (Median %)	n	*Aa*-IgG (Median %)	n	*Td*-IgG (Median %)
Age in years:										
< 20 years	17	34.99	17	42.41	17	27.65	17	30.84	17	25.68
21 - 25	58	37.03	58	52.43	58	36.30	58	39.79	58	25.89
26 - 30	58	36.75	58	51.14	58	33.94	58	42.36	58	23.48
31 - 35	37	37.13	37	50.97	37	24.30	37	37.09	37	14.59
≥ 36	23	42.59	23	46.62	23	38.22	23	36.73	23	20.43
p value		0.772		0.865		0.552		0.756		0.212
Education:										
No education	18	40.04	18	36.60	18	33.21	18	33.86	18	23.73
Primary School	119	37.11	119	50.97	119	36.21	119	43.42	119	25.07
High School	42	28.79	42	45.82	42	25.62	42	25.95	42	19.26
University	15	46.33	15	55.85	15	29.19	15	46.62	15	29.04
p value		0.401		0.587		0.387		0.331		0.439
Dentist Visit frequency:										
Never	132	37.26	132	47.22	132	28.21	132	33.52	132	21.59
Once a year	42	31.55	42	50.09	42	38.15	42	42.83	42	27.17
Twice a year	8	33.17	8	45.65	8	27.95	8	29.77	8	18.11
When ever	4	28.39	4	59.99	4	37.38	4	84.28	4	16.90
p value		0.891		0.934		0.589		0.359		0.597
Frequency of tooth brushing:										
Once a day	91	36.94	91	45.83	91	33.54	91	36.67	91	22.13
Twice a day	57	39.50	57	48.61	57	27.05	57	23.91	57	19.89
After every meal	5	35.90	5	75.40	5	30.48	5	49.49	5	48.49
p value		0.880		0.643		0.955		0.488		0.259
Gum bleeding:										
Yes	99	36.95	99	51.23	99	33.75	99	37.24	99	23.20
No	61	30.45	61	36.99	61	27.50	61	25.76	61	17.33
p value		0.412		**0.013**		0.340		0.063		0.231
Pain while brushing:										
Yes	40	42.60	40	67.64	40	37.54	40	40.73	40	25.75
No	112	35.41	112	38.92	112	28.46	112	30.79	112	22.17
p value		0.292		**0.003**		0.186		0.080		0.236
Smoking:										
Yes	9	45.60	9	38.70	9	33.06	9	37.09	9	14.74
No	183	36.94	183	49.57	183	32.51	183	36.67	183	22.54
p value		0.914		0.409		0.638		0.895		0.437
Alcohol consumption:										
Never	95	35.90	95	46.62	95	28.72	95	34.02	95	22.54
Daily	24	42.96	24	53.24	24	38.88	24	37.59	24	21.09
Weekly	29	38.17	29	47.81	29	30.48	29	37.09	29	19.52
Special occasion	37	32.61	37	44.54	37	34.80	37	33.79	37	23.20
p value		0.735		0.707		0.369		0.828		0.733
PCR:										
Positive	137	36.94	54	60.99	161	33.06	85	49.48	82	27.95
Negative	50	41.24	133	44.38	26	34.70	102	32.99	104	21.78
p value		0.496		0.158		0.861		**0.012**		0.190
Gingival inflammation:										
Yes	82	39.56	82	51.10	82	36.67	82	44.23	82	25.58
No	104	31.82	104	46.23	104	27.70	104	33.07	104	21.35
p value		0.054		0.389		**0.026**		0.109		0.054
Term:										
PLBW	98	27.48	98	38.77	98	27.25	98	26.43	98	20.90
Normal	99	44.55	99	59.01	99	36.86	99	56.84	99	26.77
p value		**0.002**		**0.002**		**0.016**		**0.000**		**0.023**

Table 29: Association between IgM against periodontopathogens and maternal history

Parameters	n	*Pi*-IgM (Median %)	n	*Pg*-IgM (Median %)	n	*Fn*-IgM (Median %)	n	*Aa*-IgM (Median %)	n	*Td*-IgM (Median %)
Age in years:										
< 20 years	15	17.30	15	11.41	15	10.62	15	-1.53	15	4.80
21 - 25	55	19.00	55	13.31	52	13.45	54	7.90	54	5.88
26 - 30	57	21.93	57	15.04	57	16.01	57	6.48	57	5.94
31 - 35	37	20.31	37	20.90	37	13.80	37	5.29	37	6.04
≥ 36	22	13.62	22	15.40	22	17.11	22	8.83	22	8.82
p value		0.777		0.592		0.575		**0.008**		0.706
Education										
No education	18	17.45	18	13.30	18	12.38	18	0.46	18	4.41
Primary School	116	18.33	116	13.81	112	13.91	115	6.29	114	5.86
High School	40	19.85	40	15.11	40	15.10	40	9.55	40	4.42
University	14	34.16	14	27.29	14	31.63	14	9.69	14	10.78
p value		0.309		0.165		0.273		**0.007**		0.190
Dentist Visit frequency:										
Never	127	18.42	127	13.63	123	15.47	126	6.62	125	5.418
Once a year	42	20.85	42	20.90	42	13.42	42	6.31	42	8.69
Twice a year	7	17.39	7	12.28	7	16.84	7	5.20	7	5.61
When ever	4	31.02	4	30.97	4	26.53	4	8.65	4	0.92
p value		0.778		0.118		0.720		0.819		0.196
Frequency of tooth brushing:										
Once a day	88	18.46	88	16.63	84	14.10	87	6.29	86	5.83
Twice a day	55	17.30	55	13.84	55	12.41	55	5.29	55	7.38
After every meal	5	30.80	5	21.08	5	37.86	5	8.43	5	3.98
p value		0.305		0.337		0.128		0.779		0.821
Gum bleeding:										
Yes	94	24.28	94	21.48	90	15.77	93	5.40	92	5.66
No	60	14.76	60	12.88	60	14.73	60	8.40	60	7.922
p value		**0.029**		0.096		0.427		0.119		0.124
Pain while brushing:										
Yes	38	16.13	38	13.48	38	13.38	38	5.10	38	3.99
No	107	20.67	107	16.53	103	16.52	106	7.95	105	8.15
p value		0.146		0.576		0.219		0.058		**0.002**
Smoking:										
Yes	9	12.95	9	7.04	9	12.44	9	6.22	9	3.401361
No	177	19.125	177	15.04	177	15.18	177	6.51	177	5.982906
p value		0.294		**0.045**		0.379		0.873		0.672
Alcohol consumption:										
Never	92	15.71	92	15.18	91	15.18	92	5.85	92	6.34
Daily	24	21.45	24	26.00	21	15.47	23	7.69	22	6.65
Weekly	29	21.03	29	10.07	29	15.54	29	7.96	29	5.511
Special occasion	35	24.64	35	16.00	35	12.62	35	6.40	35	5.98
p value		0.266		0.110		0.953		0.982		0.643
PCR:										
Positive	131	18.68	52	23.92	153	13.62	80	5.71	43	7.10
Negative	50	18.58	130	12.67	24	15.36	100	7.29	136	5.77
p value		0.624		**0.011**		0.920		0.096		0.734
Gingival inflammation:										
No	83	17.23	83	12.56	81	12.25	82	5.44	82	5.86
Yes	99	20.45	99	20.00	97	16.66	99	7.53	98	6.34
p value		0.118		**0.021**		0.057		0.236		0.667
Term:										
PLBW	92	16.02	92	14.24	88	16.90	91	6.85	90	7.23
Normal	99	19.37	99	14.12	99	12.97	99	6.40	99	5.61
p value		0.105		0.907		0.153		0.555		0.058

Table 30: Spearman rank correlation between cytokines (IL-6 and IL-10) and IgG and IgM against periodontopathogens

		Maternal blood (MB)		Fetal cord blood (FCB)	
		IL-6	**IL-10**	**IL-6**	**IL-10**
***Pi*-IgG**	**r**	0.110	0.072	0.144	-0.048
	p	0.124	0.316	**0.045**	0.502
	n	196	196	195	196
***Pg*-IgG**	**r**	0.092	-0.027	0.003	-0.080
	p	0.202	0.704	0.971	0.264
	n	196	196	195	196
***Fn*-IgG**	**r**	0.030	0.110	0.128	-0.051
	p	0.681	0.124	0.076	0.478
	n	196	196	195	196
***Aa*-IgG**	**r**	0.098	0.095	0.068	0.143
	p	0.170	0.186	0.346	**0.046**
	n	196	196	195	196
***Td*-IgG**	**r**	0.210	0.051	0.018	-0.124
	p	**0.003**	0.474	0.800	0.084
	n	196	196	195	196
***Pi*-IgM**	**r**	0.114	-0.146	-0.024	-0.066
	p	0.116	**0.044**	0.748	0.363
	n	190	190	189	190
***Pg*-IgM**	**r**	-0.025	-0.250	-0.099	-0.088
	p	0.731	**0.001**	0.174	0.229
	n	190	190	189	190
***Fn*-IgM**	**r**	-0.099	-0.058	-0.043	0.025
	p	0.180	0.430	0.564	0.732
	n	186	186	185	186
***Aa*-IgM**	**r**	0.072	-0.171	-0.025	0.074
	p	0.323	**0.019**	0.730	0.310
	n	189	189	188	189
***Td*-IgM**	**r**	-0.010	-0.030	0.050	0.079
	p	0.889	0.687	0.496	0.279
	n	188	188	187	188

r = coefficient of correlation, p = p value, n = number of patients

6.3. DISCUSSION

The objective of this chapter was to evaluate the host immunological response of the mother to 5 periodontopathogens and its association with PLBW. In this regard IgG and IgM levels were determined in maternal and foetal cord blood samples respectively. ELISA test was used for this purpose as described in point 2.4.

A population of low level of education and also a very young population may be exposed to periodontal disease due to a lack of proper dental care. In this study only the foetal IgM against *Aa* showed a significant association with age and with level of education.

Gum bleeding and pain when brushing are common indicators of periodontal disease. Chronic periodontal infections can produce local and systemic host responses (Gibbs *et al*., 2001). Offenbacher *et al*., (2006) reported that 40 % of all pregnancies are associated with foetal IgM antibody response to periodontopathogens. The presence of specific IgG corresponds to maturation of the antibody response while IgM is involved in primary response. Reduced IgG in MB offers no protection against infection by that particular species while higher levels of IgM indicate that a new infection is present. In this study, the lack of maternal (IgG) against *P.* gingivalis was strongly associated with gum bleeding and pain while brushing teeth, while the foetal IgM against *P. intermedia* and *T. denticola* were associated with gum bleeding and maternal pain during tooth brushing, respectively.

Ebersol et al., (1982), found that serum antibody levels to suspected periodontopathogens are useful markers for periodontal infection. A study by Herminajeng *et al*., (2001) found increased levels of specific IgG antibodies in immunized mice after being challenged with live *A. actinomycetemcomitans*. In the present study, only *A. actinomycetemcomitans* showed a significant association with its specific IgG ($p = 0.012$). Madianos *et al*., (2001) demonstrated an association between FCB-IgM and *P. gingivalis*. In our study, *P. gingivalis* also showed a significant association ($p = 0.011$) with its specific FCB-IgM. Once again we may interpret that the detection of periodontopathogens in these mothers as "carrier" state for these species and that the lack of quantification of bacterial load in all of the samples, presents an obstacle in drawing a finite conclusion. PCR is a very sensitive detection method for periodontopathogens, therefore the risk of detecting a very small number of bacteria in sites which are considered to be healthy is also higher (Lee *et al*., 2003; Klein and Goncalve 2003).

The following associations showed positive correlations: *Pi*-IgG with IL-6 FCB, *Aa*-IgG with IL-10 FCB and *Td*-IgG with IL-6 MB while *Pi*-IgM, *Pg*-IgM and *Aa*-IgM showed negative correlations with IL-10 MB. In agreement with our findings in chapter 4 regarding the association of *P. intermedia*, *Aa* and the members of the "Red complex" with most indicators of periodontal disease used in this study, the specific IgG and IgM against the same periodontopathogens are associated with IL-6 and IL-10.

Madianos *et al*., (2001), found that maternal periodontal infection without protective maternal antibody response is associated with the dissemination of periodontopathogens

and therefore with PLBW. In this study, significant associations were observed between PLBW and IgG against the different peridontopathogens. No significant associations were observed between PLBW and IgM against the 5 periodontopathogens but the higher levels of IgM were observed in FCB with PLBW. The findings of this study may suggest that the low level of maternal IgG and the high level of foetal IgM against the different periodontopathogens are associated with dissemination of periodontopathogens to the foetal cord thereby suggesting that foetal infection may contribute to PLBW.

CHAPTER 7: SUMMARY AND CONCLUSION

The aim of this study was to investigate the association between selected periodonto-pathogens and pre-term delivery of low birth weight infants. This aim was reached, firstly by recording maternal risk factors which may contribute to preterm delivery of low birth weight infants; secondly, by identifying periodontopathogens (*Porphyromonas gingivalis, Treponema denticola, Prevotella intermedia, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans)* from maternal gingival crevicular fluid using PCR; and thirdly, by using ELISA to determine the host response (levels of IL-10, IL-6, IgG and IgM) to the above suspected periodontopathic bacteria in MB and FCB respectively. We ommitted to examine GCF for IgG and IgM because there was not enough sample available.

Two hundred women who were admitted to the obstetrics and gynecology unit of the teaching hospital in Rwanda constituted the sample group for this study. One hundred delivered full-term normal weight infants, while the other hundred delivered PLBW infants.

Table 31 shows the principle findings of this study, while Table 32 shows a multivariate analysis using a logistic regression of risk factors associated with PLBW after adjusting for confounding variables. All factors which were significantly associated with PLBW were included in this analysis, except for the health status of the children in the family which presented with many missing data.

Table 31. Principle findings of the study

ASSOCIATIONS	p value
Pregnancy outcome vs Weight	0.006
Pregnancy outcome vs Height	0.041
Pregnancy outcome vs health condition of children in the family	0.003
Pregnancy outcome vs Previous pre-term birth	0.000
Pregnancy outcome vs Gingival inflammation and bleeding	0.004
Pregnancy outcome vs IL-10 MB	0.002
Pregnancy outcome vs IL-10 FCB	0.000
Pregnancy outcome vs IL-6 FCB	0.000
Pregnancy outcome vs *Pi*-IgG	0.002
Pregnancy outcome vs *Pg*-IgG	0.002
Pregnancy outcome vs *Fn*-IgG	0.016
Pregnancy outcome vs *Aa*-IgG	0.000
Pregnancy outcome vs *Td*-IgG	0.023
Frequency of tooth brushing vs *Pi*	0.045
Gum bleeding vs *Pg / Aa*	0.041
Gum bleeding vs *Td / Aa*	0.032
Gum bleeding vs *Pg / Pi / Aa*	0.022
Gum bleeding vs *Td / Pi / Aa*	0.032
Gum bleeding vs *Td / Pi / Aa / Fn*	0.046
Signs of gingival inflammation vs *Pi*	0.013
Signs of gingival inflammation vs *Pg / Pi*	0.040
Signs of gingival inflammation vs *Pi / Fn*	0.043
IL-6 MB vs *Pi*	0.017
IL-6 MB vs *Pi / Fn*	0.047
IL-6 FCB vs *Fn*	0.037
Fn-IgG vs Gingival inflammation	0.026
Pg-IgG vs Gum bleeding	0.013
Pg-IgG vs Pain while brushing	0.003
Aa-IgG vs PCR	0.012
Pi-IgM vs Gum bleeding	0.029
Pg-IgM vs PCR	0.011
Pg-IgM vs Gingival inflammation	0.021
Td-IgM vs Pain while brushing	0.002
IL-6 MB vs *Td*-IgG	0.003
IL-10 MB vs *Pi*-IgM	0.044
IL-10 MB vs *Pg*-IgM	0.001
IL-10 MB vs *Aa*-IgM	0.019
IL-6 FCB vs *Pi*-IgG	0.045
IL-10 FCB vs *Aa*-IgG	0.046

Factors associated with preterm birth include, amongst others, low maternal body weight, maternal smoking and maternal infections (Kramer 2003, Bayingana 2010b). Socioeconomic and ethnic factors may account for 50% of the risk for PLBW (Moore *et*

al., 2000; Goldenberg et al., 2000; Hendler *et al*., 2005; Menon *et al* 2008, 2010; Velez *et al* 2008) and the remaining risks are deemed to be due to immunologic factors (Choudhury and Knapp, 2000). Using bivariate analysis in this study, maternal weight, height, history of previous preterm, health status and number of family members, along with maternal gingival inflammation, showed a significant relationship with PLBW. Smoking and reduced frequency of tooth brushing were significantly associated with Pg-IgM and Pi-PCR respectively, while maternal level of education, number of previous pregnancies, urinary tract infection, sexually transmitted disease, antibiotic administration, diabetes, history of heart disease, alcohol consumption and smoking showed a positive but not significant relationship with PLBW (Table 31).

Using multivariate analysis, it was demonstrated that mothers weighing ≤ 50 kg, 51-55 Kg, 56-60 Kg and 61-65 were more likely to deliver PLBW infants than mothers weighing > 65kg (Table 32). Maternal height didn't show significant differences between PLBW and FT birth (Table 32). Mothers with a history of previous PLBW were 5.165 times more likely to deliver PLBW infants in subsequent pregnancies than mothers with no history of PLBW infants. The analysis also shows that mothers with no gingival inflammation were less likely to deliver PLBW infants than mothers with gingival inflammation (Table 32).

Table 32: Multivariate analysis of risk factors associated with PLBW

	PLBW vs FT	
	OR	**95% CI**
WEIGHT (kg)		
> 65 (Reference)		
≤ 50	6.150	0.698-54.154
51 – 55	7.151	1.086-47.093
56 – 60	3.353	0.735-15.302
61 – 65	3.132	0.664-14.768
HEIGHT (CM)		
> 165 (Reference)		
≤ 150	1.438	0.038-2.393
151 – 155	1.153	0.073-3.608
156 – 160	0.525	0.310-8.013
160 – 165	1.033	0.082-3.068
PREVIOUS PRE-TERM BIRTH		
No (Reference)		
Yes	5.165	1.524-17.510
GINGIVAL INFLAMMATION		
Yes (Reference)		
No	0.385	0.138-1.076
IL-10 MB	1.003	0.997-1.009
IL-10 FCB	1.019	0.993-1.046
IL-6 FCB	1.000	0.996-1.004
***Pi* -IgG**	0.997	0.986-1.007
***Pg* -IgG**	0.995	0.986-1.004
***Fn*-IgG**	0.999	0.978-1.020
***Aa*-IgG**	0.998	0.991-1.004
***Td*-IgG**	1.016	0.991-1.041

OR: Odds ratio; 95% CI: 95 % confidence interval.
PLBW =preterm low birth weight; FT = Full term

No significant associations were observed between the PCR detection of the five periodontopathogens (and their combinations) and preterm delivery of low birth weight infants. Gingival inflammation was significantly associated with combinations of *Pi* and *Pg* with *Fn* and *Td* (Table 31). *Aa* was found to be an essential element for the significant association with gingival bleeding, thus exacerbating the gingival inflammation caused by the combinations of periodontopathogens. One can speculate that the aggressive *Aa* clone referred to in the previous chapter may be associated with this exacerbation.

Maternal and foetal immune responses are composed of cytokine expression with a shift in the pregnant mother's immunity from a balanced Th1 and Th2 response to a predominant production of Th2 cells which result in the down-regulation of the synthesis of cellular immunity and the release of pro-inflammatory cytokines which promote antibody production (Fried *et al,* 1998; Marzi *et al.*, 1996, Mossman and Sad 1996, Ugwumado *et al*, 2002). IL-6 has been suggested to be an indicator of intrauterine infection and a predictor of PLBW (Dudley 1997; Greig *et al* 1997; Perenyi *et al* 1999; Romero *et al* 1998; Blidane and Stamatin 2002; Apuzzio *et al* 2004; Figuera *et al* 2005; Jarocki *et al* 2007). IL-6 was also found to play a role of diagnostic marker in chronic inflammatory periodontal disease (CIPD) (Giannopoulou *et al* 2003, Atilla *et al* 1998, McGee *et al* 1998, Wu *et al* 2001, Quenoz *et al* 2008, Dashash *et al* 2008). Elevated levels of maternal IL-6 have been associated with SIRS (Systemic Inflammatory Response Syndrome), a diagnosis which has been associated with PLBW in the absence of early aetiologic organisms (Dudley, 1997). Genotypes of IL-6 were found to be both a risk factor for PLBW due to CIPD (Offenbacher et al, 1998) as well as protective against intrauterine growth restriction (Dashash *et al* 2008).

It is not known whether inflammatory cytokines that may trigger PLBW are of foetal or maternal origin, nor is it clear whether they are expressed as a cause or consequence of PLBW (Elovitz, 2006). Offenbacher *et al*., (1999) reported that information regarding translocation of periodontopathogens to the fetoplacental interface can be obtained from foetal cord serum samples. If inflammation rather than bacterial invasion *per se* is suspected to be the cause of PTB, then it is meaningful to examine for specific

inflammatory markers in the mother and neonate. In this study, we examined maternal (MB) and fetal cord blood (FCB) for IL-6 and IL-10 levels in order to assess their role as indicators of PLBW and their association with selected periodontopathogens implicated in preterm delivery. IgG and IgM were also detected in MB and FCB respectively.

Elevated IL-6 expression in FCB was found to correlate with amniotic fluid levels (Romero *et al* 1998), but not with maternal serum levels of mothers in labour (Jokie *et al* 2000, Romero *et al* 1998; Skogstrand *et al* , 2008), indicating that IL-6 does not easily cross the placenta and that IL-6 in FCB may be of foetal origin (Aaltonen *et al* 2005, Reisenberger *et al* 1996, Bhartiya *et al* 2000), initiating labour along-side inflammation of chorioamniotic membranes (Romero *et al.*, 1998). In this study, we examined FCB for IL-6 in order to determine whether foetal inflammation was present before birth, thus initiating PLBW. Using the bi-variate analysis, we found that over expression of IL-6 in FCB was significantly associated with PLBW ($p = 0.000$), but no association was found between IL-6 MB and PLBW. In the multivariate analysis, no association between IL-6 in FCB and PLBW was found. Nemes *et al*. (2009) reported that multivariate analysis tends to systematically overestimate odds ratios (OR) when the sample size is less than 500. If that is the case, then one has to bear in mind that these results are based on a sample size of only 200 mothers. Elevated levels of IL-6 in foetal blood are known to be associated with FIRS (Foetal Inflammatory Response Syndrome), the response of the foetus to the hostile intrauterine environment (Romero et al., 2007).

The relationship between pro- and anti-inflammatory cytokines and maternal gingival inflammation could not be clearly established in this study, because *F. nucleatum* showed a significant correlation with IL-6 levels in FCB and when present with *P.intermedia*, a correlation with IL-6 was also observed in MB. Since *P. intermedia* and *F. nucleatum* are both associated with pregnancy gingivitis (Africa *et al* 2010) which may add to the oral inflammatory burden, thus increasing the risk for PLBW, it was unexpected to find no correlation between IL-6 and IL-10 and gingival inflammation in this study.

Contradictions in the literature create confusion regarding the role of IL-10 in PLBW and its apparent multifarious role at the maternal – foetal interface remains controversial (Blidane and Stamatini 2002, Lin *et al* 1993, Thaxton *et al* 2010). Infants are known to have a dominant anti-inflammatory cytokine profile (Adkins et al, 2004) that is reported to be reduced in PLBW (Dudley 1997, Bayoumi *et al* 2008) and CIPD (Yamakazi *et al* 2001, Gautoudi *et al* 2004). Others have reported elevated levels of IL-10 with PLBW (Matoba *et al* 2009). In this study (using bivariate analysis), significant associations of IL-10 MB and IL-10 FCB were observed with PLBW. Bayoumi *et al* (2008) recorded IL-10 FCB to be significantly lower than IL-10 MB or placental sera ($p<0.001$). No significant differences were observed in this study between IL-10 MB and IL-10 FCB with PLBW when multivariate analysis was used, although levels were slightly increased (Table 32).

In summary, this study revealed that:

a) Positive correlations were found between:
    - Pi-IgG and IL-6 FCB
    - Aa-IgG and IL-10 FCB
    - Td-IgG and IL-6 MB
b) Negative correlations were found between:
    - IL-10 MB and PI-IgM
    - IL-10 MB and Pg-IgM
    - IL-10 MB and Aa-IgM
c) IgG to each of the 5 periodontopathogens showed a significant association with PLBW
d) IL-6 FCB, IL-10 MB, IL-10 FCB were all significantly associated with PLBW.
e) Significant associations were found between Pg-IgG and reduced frequency of tooth brushing, as well as between pain and Pg-IgG and Td-IgM (Table 32).
f) IgM and the 5 periodontopathogens showed no statistical association even though higher levels of IgM were observed in FCB of PLBW infants.
g) A significant association between gingival inflammation and PLBW was demonstrated.

As with IL-6, the presence of IgM in FCB signifies foetal synthesis and may be considered to be a non-specific indicator of intra-uterine infection. Since IgM does not cross the placenta, its presence would demonstrate foetal response to a transplacental transfer of oral microbial antigens (Madianos et al, 2008). It can therefore be concluded

that foetal exposure to the oral pathogens *Pi, Pg* and *Aa*, evidenced by an IgM response as well as an inflammatory response (IL-6 FCB, IL-10 FCB), coupled with maternal IgG response to the 5 periodontopathogens and the release of inflammatory cytokines (IL-6 MB and IL-10 MB), all of which,(except IL-6 MB) could be significantly associated with PLBW, may provide a mechanism by which maternal periodontopathogens may infect the foetus and thus increase the risk for PLBW. The increased expression of inflammatory mediators may trigger the release of PGE_2 resulting in premature contractions in an effort to deliver the foetus from a threatening environment.

Limitations of this study include the crude evaluation of gingival inflammation and failure to establish whether the association between levels of inflammatory markers and PLBW correlated with the degree of prematurity. Although PCR has often been proposed as the new gold standard for detecting pathogens in clinical samples (Edwards *et al*., 2003; Klein and Gonçalves, 2003), detection of endogenous microbes (as in the oral cavity) can only yield meaningful results if bacterial load is quantified (Ashimoto *et al*., 1996; Papapanou *et al*., 2000). Also, PCR is a sensitive detection method for periodontopathogens, and thus there is a risk of detecting even a very small number of bacteria in an otherwise healthy periodontium. (Klein and Gonçalves, 2003; Africa *et al*., 2009; Bayingana *et al*., 2010a).

With so many contradicting reports regarding the presence and role of the oral anaerobes and PLBW, research in this area continues in the quest for conclusive evidence of their role in adverse pregnancy outcomes. Some studies show a significant correlation, while

others do not (Madianos *et al*., 2001; Mitchell-Lewis et al., 2001; Dörtbudak *et al*., 2005; Jarjoura *et al*., 2005; Noack *et al*., 2005; Skuldbol *et al*., 2006; Lin *et al*., 2007). Factors such as race, geographical location and socio-economical standards should be adequately adjusted for and thus studies done in USA and Europe differ from those in Latin America or Africa largely because of socio-economic factors such as affordability and easy access to adequate health care.

Whether or not to treat pregnant mothers for periodontal disease remains controversial. Most interventions to prevent preterm birth have in the past been unsuccessful (Michalowicz *et al*., 2006; Offenbacher *et al*., 2009; Jeffcoat, 2010; Niederman, 2010), and in the few cases that reported success, these are not known to be universally effective and are only applicable to a small number of women at risk for preterm birth (Goldenberg *et al*., 1998). The quest for a better understanding of the mechanism leading to preterm delivery continues. In the meanwhile, pregnant women are required to fulfill the basic rules of proper hygiene and nutrition, weight control and regular visits to dentist and gynecologist to ensure that they maintain good health throughout their pregnancies and deliver healthy infants. It should be emphasized that this was not an assessment of periodontal disease *per se* although an association was found between the host responses to selected periodontopathogens implicated in PLBW. It is viewed that this study has paved the way for more detailed examination and assessment of the oral health status of mothers with particular emphasis on periodontal disease and its association with adverse pregnancy outcomes.

Until a fully standardized protocol is available to establish the exact role of periodonto-pathogens in PLBW, comparison with other studies will always be hampered by differences in diagnosis, sampling and size of sample, detection methods and data analysis. This, compounded by differences in race, socio-economic status and geographical position may lend itself to speculation and often affect interpretation of results in such a way as to favour a proposed hypothesis.

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APPENDIX TO CHAPTER 2(A)

Health Risk Assessment Questionnaire

Dear patient,

As part of this study we need to collect information pertaining to your lifestyle so that an assessment of your health risks may be made. Any information contained on this sheet will be held in the strictest confidence and I would urge you to respond to the questions with accuracy. No details of personal identification will be included for your protection.

Age…….....

Weight of the mother…… Height of the mother……

Weight of the new born…… Term of the new born…….

1. What is your level of formal education? High school / primary school/ university/ no formal education	**2**. Do you live in a House / flat / shack/ homeless
3. How many people share your home?	**4**. (i) How long have you been living at your present address? < 5 Years / 5-10 years / >10 years
5. (i) How many children do you have ? (ii) Are they healthy? Yes / No (iii) If No, what is the problem? (iv) How long has it been?	**6**. (i) Is this your first pregnancy ? Yes / No (ii) If No, how many have you had and did you carry full term? Yes / No (iii) Have you ever had an induced abortion? Yes / No (iv) What stage of your pregnancy was it terminated? First / second / third trimester
7. (i) Have any of your children been born preterm or with low birthweight ? Preterm / low birthweight	**8.** How often do you visit your doctor for gynecological check ups? Never / once a year / only when necessary

9. (i) Do you have frequent urinary tract infections ? Yes / No	**10**. (i) Have you ever had a sexually transmitted disease ? Yes / No (ii) If yes, do you know what it was and was it treated? iii) How many sexual partners have you had? 1/ <5 /5-10/ ›10
11. (i) Are you diabetic? Yes/No (ii) If yes, what is the duration?.....years (iii) Are you being treated for diabetes? Yes/No	**12.** (i)Do you or any of your family have heart disease? Yes/No (ii) If yes who? (iii) Duration and treatment?.....years
13. (i) How frequently do you visit the dentist ? Never / Once a year / Twice a year / whenever (ii) When was the last time you visited a dentist? (iii) What was the reason? (iv) Do your gums bleed when you brush your teeth? Yes / No (V) Do you feel pain when brushing your teeth? Yes / No (v) How frequently do you brush your teeth? Once a day / twice a day / after every meal (Vi) Do you have bad breath? Yes/ No	**14.** Do you have easy access to medical or dental care? Yes / No
15. Do you have medical insurance? Yes / No	**16.** (i) How often do you have a drink containng alcohol? Never / daily / weekly/ special occasions (ii) How many drinks would you consume when you do drink? 1-2 / 3-5 / >6
17. (i)Do you smoke? Yes / No/ Sometimes (ii)If yes, How many a day? <5 / 5-10 / >10 (iii)How long have you been smoking? <5 / 5-10 />10yrs	**18.** (i) What does your diet mainly consist of? Bread / meat / fruit and veg /
19. (i) Did you take antibiotics in past days? Yes/No (ii) If yes when?	

Thank you for your participation

APPENDIX TO CHAPTER 2(B)

CONSENT FORM FOR PARTICIPATION IN RESEARCH PROJECT

Title of Project: A molecular investigation of the prevalence of suspected periodontopathogens and their association with preterm birth.

Names of Researchers: Prof Charlene WJ Africa, Dr Janet Kayitenkore, Mr Claude Bayingana (Ph.D student)

If you would like to participate in this study please tick the relevant boxes:

1. Have you read the attached information sheet and has the purpose of the research project been explained to you? Yes NO
2. Do you understand the method of sample collection and any risks involved?

Yes NO

3. Do you grant permission for information from your medical records to be disclosed to the research team as and when necessary? Yes NO

4. Do you agree that samples collected for research or diagnostic testing can be stored for possible use in future research projects conducted by the above named researchers and /or other research collaborators? Yes NO

I declare that my participation in this research project is voluntary and that I am free to withdraw my approval for use of the sample(s) at any time without giving a reason and without my medical treatment or legal rights being affected. I understand that any information contained in my file will remain confidential and that I (or my doctor) will be informed if any of the results of the medical tests done (as part of the research) have implications for my health. I know how to contact members of the research team should I change my mind about participating in this study.

……	…….	……
Name of patient	**Date**	**Signature**
(BLOCK CAPITALS)		
……	…….	…….
Name of person taking consent	**Date**	**Signature**
……	…….	……
Name of researcher	**Date**	**Signature**

THANK YOU FOR AGREEING TO PARTICIPATE IN THIS RESEARCH

APPENDIX TO CHAPTER 2(C)

INFORMATION SHEET

Prospective participants are requested to read this information sheet carefully and to ask questions where necessary, before signing the attached consent form. This sheet must be detached and retained by the participant and the consent form filed for record.

Periodontal infection (gum disease) of the mother may be a potential risk factor for preterm low birth-weight (PLBW)). Research data suggests that 18.2 %-50 % of the (PLBW) deliveries occurring each year might be attributed to periodontal disease. The objective of this study is therefore to asses the relationship between periodontal diseases of the mother and PLBW.

The clinical procedure will entail the detection of 5 bacteria most often associated with periodontal disease and the measurement of cytokine levels in gingival crevice fluid, maternal serum samples and foetal cord serum samples. Specific antibodies (IgG) against the above bacteria will be estimated in gingival crevice fluid and in maternal serum. The sample collections are safe and will be carried out with the utmost care to ensure the comfort of the patient.

Patients will be required to sign the attached form granting consent for these procedures to be carried out and for the subsequent use of the samples donated and clinical parameters recorded. The patient will also be required to grant permission for her medical history to be disclosed. Participants will not be recorded by name, but samples and information will be coded to protect the identity of the individual. However, the coding will be used by the clinic to trace the individual if relevant information (as a result of the study) should be passed to her or her doctor. Where necessary, participants with high level of inflammatory cytokines or participants testing positive for bacteria most often associated with periodontal disease, will be referred for treatment. Permission will also be sought for the use of additional biological material collected in the clinic, which is usually discarded but which the researchers may find useful for future research.

Participation in this study is voluntary and refusal to participate will not prejudice the treatment of the patient in any way. Consent to participate will be recorded by completing the attached form. Should individuals agree to participate and later change their minds, they may withdraw by calling the following persons:

Prof C. Africa, University of the Western Cape, Department of Medical Biosciences, Tel: 021 959234 or Mr. Claude BAYINGANA, Tel: 0782896940.

APPENDIX TO CHAPTER 2(D)

TES buffer

50 mM Tris hydroxymethyl methylamine

pH 8.0

15 mM Ethylenediaminetetra-acetic acid disodium salt.

91

20 % SDS

20g sodium dodecyl sulfate (SDS) dissolved in 100 ml water. Sterilize by filtration through a 0.2 μm filter.

5 M Ammonium acetate

Dissolve 38.54g ammonium acetate in 100ml distilled water.

10X TE :

100 mM Tris-Cl pH 7.5

10 mM EDT

For 1 liter: Dissolve 12.11 g Tris and 3.72 g EDTA in around 700 ml distilled water.

Adjust pH to 7.5 with concentrated HCl. Make volume up to 1 liter. Autoclave.

10XTBE

108g Tris, 55g boric acid and 9.3g EDTA. Dissolve and make up to 1 liter.

Ethidium Bromide (EtBr)

Dissolve 0.1 g in 10 ml of water. Shake well to dissolve. Wear gloves and never breath the dust.

Loading buffer

0.25% bromophenol blue and 0.25% xylene cyanol in 30% of glycerol in distilled water.

APPENDIX TO CHAPTER 2(E)

1. PEPTONE YEAST GLUCOSE (Label with sugar name): 400 ml

- Peptone (Difco Protease peptone No 3)…….2.0 gr
- Trypticase Peptone (BBL)…….2.0 gr
- Yeast Extract (Difco)…….4.0 gr
- Resazurin……1.6 ml
- Salts Solution (VPI Salts)……..16.0 ml
- Glucose……4.0 ml
- Distilled water…….400.0 ml
- Boil on hot plate with chimney till goes yellow
- Put into ice + CO_2 gas with thermometer till 25^0 C
- Add vitamin K /Hemin (for sugars)……4.0 ml

  L-cyteine HCL (BDH)……...0.20 gr
- pH 6.9 with 8N NaOH
- Change gas to N_2
- 5 ml per tube

2. <u>TRYPTICASE SOY AGAR-CY/DITHIO (TSA-CT): 500 ml</u>

- Trypticase soy Agar (BBL) ……..20.0 gr
- Yeast extract (DIFCO)…….2.5 gr
- Salts Solution (VPI salts)……20.0 ml
- Resazurin solution…….2.0 ml
- Distilled water…….450 ml
- Steam and add cysteine/dithio solution (make up fresh)……....5.0 ml
- Autoclave and add (Vit K/hemin 5.0 m + horse blood 25.0 ml): fridge

3. <u>CYSTEINE/DITHIOTHREITOL SOLUTION (made up fresh)</u>

- BDH Cystein HCL……0.5 gr
- Dithiothreitol (fridge)……0.1 gr
- Add 10 ml of distilled water
- Use 5 ml for 500 ml medium

4. <u>Hemin</u> (For plate)

- hemin (sigma stored at 4^{o} C)……0.5gr
- N NaOH……1.0 ml
- Add 100 ml of distilled water
- Autoclave
- For vit K/Hemin for plates, add 1 ml stock K3.

5. Salts solution

- $CaCl_2$ (anhydrous)……..0.2 gr
- $MgSO_4$* (anhydrous)……...0.2 gr
- K_2HPO_4…….1.0 gr
- KH_2PO_4…….1.0 gr
- $NaHCO_3$……...10.0 gr
- NaCl……..2.0 gr

Mix $CaCl_2$ and $MgSO_4$ in 300 ml distilled water until dissolved. Add 500 ml water and, while swirling, slowly add remaining salts. Continue swirling until all these salts are dissolved. Add 200 ml distilled water, mix, and store at 4^o C.

or use 0.48 gr $MgSO_47H_2O$.

6. RESAZURIN SOLUTION

Dissolve 25 mg resazurin in 100 ml distilled water.

APPENDIX TO CHAPTER 2(F)

PREPARATION FOR ELISA REAGENTS:

a. ELISA/ELISPOT Coating Buffer Powder

One packet of ELISA eBioscience Coating Buffer Powder was added and mixed to 1 liter distilled water until dissolved, and was filted using a 0.22um filter.

b. Capture Ab: Purified anti-human IL-6 or IL-10

For 1 plate, 20 µl of Ab solution was to 4980 µl coating buffer (Dilution: 1/250)

c. Wash Buffer: 10 X PBS, 0.5 % Tween-20

The following reagents were dissolved in 1 liter dH_2O:

80g NaCl

2g KCl

17.7g $Na_2 HPO_4.2H_2O$

2.4g KH_2PO_4

Add 5ml Tween-20

One part of the above solution was added to 9 parts of dH_2O to make 1XPBS, 0.05% Tween-20.

d. 5x Assay diluent

To make 1X Assay Diluent, 5 ml of 5X Assay Diluent was added to 20 ml of distilled water. This was the working solution for 1 plate.

e. Standard: Recombinant human IL-6 or IL-10 (1 ug/ml)

For 1 plate, 5 µl of standard solution was added to 2500 µl of assay diluent (Dilution: 1/500) to prepare the top standard solution.

f. Detection Ab: Biotin-conjugate anti-human IL-6 or IL-10

For 1 plate, 20.4 µl of Ab solution was added to 5079.6 µl of assay diluent (Dilution:1/25).

g. Enzyme: Adividin-HRP

For 1 plate, 20.4 µl of enzyme solution was added to 5079.6 µl of assay diluent (Dilution: 1/250).

h. Substrate: 1xTMB solution

Working solution (5000 µl for 1 plate)

i. <u>Preparation of the stop solution</u>: 2N Sulfuric acid (H_2SO_4)

Slowly add 6mL concentrated H_2SO_4 to 90mL water. Make to I00mL with water. The solution is stable indefinitely.

Note: Always add acid to water, not water to acid, to avoid excess heat formation and spitting of acid. Stir solution when adding acid.

APPENDIX G

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Full Length Research Paper

Comparison of PCR and BANA hydrolysis in detecting oral anaerobes in subgingival plaque

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Abstract

Culture has always been recognized as the gold standard for detecting oral anaerobes in dental plaque samples. However, many of the bacterial morphotypes observed by microscopy are difficult to culture, thus necessitating the need for alternative methods of detection. The objective of this study was to evaluate the sensitivity and specificity of BANA (N-benzoyl-DL-arginine-B-naphthylamide) and PCR (Polymerase Chain Reaction) as reliable detection methods for detecting oral anaerobes such as *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* (often referred to as the "red complex") in subgingival dental plaque. Of the 372 samples analysed, 7.25% tested positive for the BANA test and 36.29% yielded a positive PCR test. This study showed that PCR was more sensitive than BANA in detecting members of the "red complex" in plaque samples.

Key words: Polymerase chain reaction (PCR), N-benzoyl-DL-arginine-B-naphthylamide (BANA), red complex, subgingival plaque, periodontal disease.

APPENDIX H

Research Paper

Examination of maternal gingival crevicular fluid for the presence of selected periodontopathogens implicated in the pre-term delivery of low birthweight infants

Charlene Africa, Janet Kayitenkore and Claude Bayingana

Virulence: Volume 1, Issue 4
July/August 2010; Landes Bioscience

Background: Reports show that more than 20 million infants world-wide are born prematurely with 95 % of all pre-term births occurring in developing countries. Oral colonization of Gram-negative anaerobes has been implicated as a risk factor for preterm delivery of low birth weight infants.

Materials and Methods: This study comprised 200 women admitted to the department of obstetrics and gynecology of the teaching hospital of Butare in Rwanda. Gingival crevicular fluid was collected from each quadrant of the mother's mouth (using paper points) within 24 hours of delivery. A dichotomous score of presence or absence of gingival inflammation was recorded for each patient along with demographic data such as age, marital status etc. Samples were examined by PCR for the presence of *Aggregatibacter actinomycetemcomitans* and selected members of the red and orange complexes described by Socransky *et al*., (1998), and their presence associated with age, gingival inflammation and pregnancy outcomes.

Results: Association of bacterial species with the risk of periodontal disease and thus the risk of preterm delivery was only observed when they occurred in pairs or groups of three or more. *Aa* appeared to be a necessary co-factor for significant associations of bacterial groups with the variables recorded.

APPENDIX I

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Full Length Research Paper

Risk factors of preterm delivery of low birth weight (PLBW) in an African population

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Abstract

More than 20 million infants in the world (15.5% of all births) are born with low birth weight. Ninety-five percent of them are in developing countries. The objective of this study was to examine different factors which may contribute to preterm delivery of low birth weight (PLBW) in a recent sample of Rwandan birth. The study sample included 200 randomly selected women admitted to the department of obstetrics-gynecology of the teaching hospital of Butare in Rwanda. Mothers were asked to complete a questionnaire and obstetrics records were used in order to identify factors which might pose a health risk to them and their infants. Maternal weight, height, history of previous preterm and healthy conditions of the children in the family showed a significant relationship with PLBW. Maternal level of education, number of pregnancies of the mother, urinary tract infection, sexually transmitted disease, antibiotic administration, diabetes, history of heart disease, alcohol consumption and smoking showed a relationship with PLBW but the relationship was not significant. More studies are required for a better understanding of the mechanism leading to preterm delivery of low birth infants.

Key words: Africa, pregnancy, preterm delivery, low birth weight infants.