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The Role of C-Reactive Protein as a marker for Preterm Delivery

by

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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.

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ABSTRACT

Background: Pregnancy associated maternal morbidity and mortality along with adverse pregnancy outcomes have gained momentum over the past few years, particularly in Sub-Saharan Africa and Southern Asia despite the advances in medical science. Adverse pregnancy outcomes are associated with low birth weight, growth restriction, developmental and cognitive abilities in infants and children. Medical care for preterm babies is costly, requires advanced equipment and qualified trained staff. Recently, levels/concentrations of cytokines have been used to predict and determine potential risk in various medical conditions. Biomarkers have shown to be helpful in many medical conditions and could be used to reduce the number of preterm deliveries in developing countries. The **aim** of this study was to determine whether a highly elevated CRP serum concentration was associated with preterm delivery in a population of Rwandan mothers.

Material and Method: Maternal (N=116) and foetal cord serum (N=171) samples were collected from mothers in the obstetrics unit at the Medical Hospital of Butare, Rwanda. The study sample comprised of 46 mothers who delivered full-term (control group) and 90 mothers who delivered preterm. Samples were analysed using an in-house DAS ELISA to determine the concentration of serum CRP.

Results: The full-term maternal group had a slightly higher mean CRP concentration (75.94ng/ml) compared to the preterm maternal group (56.96ng/ml), but no statistical difference was observed (P= 0.300) between the sample groups. Further comparison between full term and preterm groups demonstrated no association (P= 0.944), between the levels of CRP in foetal cord serum (3.99ng/ml and 4.16ng/ml respectively), while significant differences were observed between the two groups for maternal weight and level of education. Other risk factors such as maternal age, BMI, infant birth weight, parity, recurrent UTIs and STIs, showed no association with pregnancy outcomes.

Conclusion: Although highly elevated CRP levels were observed in all subgroups examined, no significant correlation was found between the elevated CRP levels and pregnancy outcomes. A conclusion can thus be drawn that CRP concentration was not a useful biomarker for predicting preterm delivery in these mothers and should be used in combination with a more specific biomarker for the prediction of adverse pregnancy outcomes.

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ACRONYMS AND ABBREVIATIONS

AIDS	Autoimmune Deficiency Syndrome
ANOVA	One Way Analysis of Variance
AV	Aerobic Vaginitis
BMI	Body Mass Index
BV	Bacterial Vaginosis
CA	Coating Antibody
CD	Cluster of Differentiation
CDC	Centre for Disease Control
CHEAP	Chicken pox and shingles, Hepatitis C, Enteroviruses, AIDS (HIV infection), Parvovirus B19
CS	Caesarean Section
DA	Detecting Antibody
ELBW	Extremely Low Birth Weight
FAS	Foetal Alcohol Syndrome
FH	Factor H
FIRS	Foetal Inflammatory Response Syndrome
HIV	Human Immunodeficiency Virus
IL-	Interleukin
INF	Interferon
IUGR	Intrauterine Growth Restriction
ITAM	Immune-Receptor Tyrosine-Based Activation Motif
ITIM	Immune-Receptor Tyrosine-Based Inhibition Motif
LBW	Low Birth Weight
MAC	Membrane Attack Complex
MDG	Millennium Development Goals
MMR	Maternal Mortality Rate

MMP	Matrix Metalloproteinase
NAS	Neonatal Abstinence Syndrome
NGAL	Neutrophil Gelatinase Associated Lipocalin
NK	Natural Killer
OD	Optical Density
P_4	Progesterone
PTD	Preterm Delivery
PBS	Phosphate Buffered Saline
pH	Potential of Hydrogen
PLROM	Pre-Labour Rupture of the Membranes
PPROM	Preterm Premature Rupture of Membranes
PTSD	Post-Traumatic Stress Disorder
T-Cells	T-Lymphocytes
TMB	Tetramethylbenzidine /Hydrogen Peroxidise Substrate
TNF	Tumour Necrosis Factor
TORCH	*Toxoplasma Gondii*, Rubella Virus, Cytomegalovirus and Herpes Simplex
VLBW	Very Low Birth Weight

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1 Introduction

It has been estimated that a woman dies every minute during pregnancy or child birth as a result of complications during pregnancy (Nour 2008). These complications may include post-partum haemorrhage, infection, unsafe abortion, eclampsia, and obstructed labour, all of which may lead to death. Other indirect causes include anaemia, malaria and heart disease during pregnancy (Nour 2008; Mctavish *et al*., 2010).

In 1987, attention was drawn to the severity of the maternal mortality rate (MMR) in developing countries at the International Safe Motherhood Conference in Kenya. The Safe Motherhood initiative aimed to reduce maternal mortality by 50% by 2000. Unfortunately, the goal showed signs of unattainability and new Millennium Development Goals were established (Nour 2008).

Millennium Development Goal (MDG) 5 set in 1990, aimed to improve maternal health by reaching a target of 75% maternal mortality reduction by 2015. Additionally, the Sustainable Development Goal 3.2 is also aimed at ending preventable maternal mortality (Golding *et al.,* 2017). These goals have proven to be challenging, as many women are still dying from complications during pregnancy and child birth each year (WHO 2015), many of which are unaccounted for, due to compromised registration of births and deaths in rural communities .

In 2015, Sub-Saharan Africa and Southern Asia were reported to have the highest maternal deaths globally, with 302 000 and 201 000 deaths, respectively reported annually. It was estimated that 99% of maternal deaths occurred in developing regions. Developing countries such as Nigeria and India were shown to contribute to a third of the world's maternal deaths, estimated at 58 000 and 45 000 deaths, respectively. Eighteen countries with the highest MMR are located in Sub-Saharan Africa, with a MMR ranging from 500 to 999 deaths per 100 000 live births (WHO 2015).

Countries that have shown no progression have usually been impacted by natural disasters or outbreaks such as malaria and HIV/AIDS. For example, South Africa was reported to have the

highest maternal AIDS related deaths in the world in 2015, with 2% of all maternal deaths being AIDS related.

Maternal reproductive history plays an important role in having a normal and healthy pregnancy or determining adverse pregnancy outcomes. Usually when health professions are aware of the mother's reproductive history, preventative measures are put in place to avert adverse pregnancy outcomes. One of the major complications that can occur during pregnancy is preterm delivery (PTD), accounting for 13% of neonatal deaths in South Africa (South Africa Every Death Counts Writing Group 2008).

PTD is defined as the birth of an infant before the 37th completed week of gestation (Kim *et al*., 2013; Basu *et al*., 2017) and has been implicated as one of the leading causes of death in children under the age of 5 years (WHO 2018). Babies that are born extremely premature (≤ 28 weeks) and very premature (≤ 32 weeks) are frequently affected by mortality and morbidity (Haram, Mortensen and Wollen 2003; Tucker and Mcguire 2004; Temu *et al.*, 2016).

Several studies have used maternal biomarkers to predict adverse pregnancy outcomes and elevated C-reactive protein (CRP) concentrations during the early stages of pregnancy have been reported to increase the risk of adverse outcomes (Tjoa et al., 2003; Pitiphat *et al.*, 2005; Sorokin *et al*., 2010; Maguire *et al.*, 2015; Vecchié *et al.*, 2018). However, a review of the literature revealed a paucity of studies reporting on CRP levels during or immediately after delivery, so this review will focus on the perceived risk factors for adverse pregnancy outcomes such as PTD and the potential biomarkers which may be used for the early detection of maternal and neonatal morbidity and mortality.

2 Review of the literature

2.1 Epidemiology of PTD

An annual estimation of 15 million babies are born preterm with an approximation of a million babies dying each year due to complications during birth (WHO 2018). A study conducted in 2014 showed that 13.4% and 8.7% of PTDs were reported from Northern Africa and Europe, respectively, whereas 78% and 81.1% of PTDs were reported from Asia and sub-Saharan Africa, respectively, thus demonstrating an increase in the amount of PTDs over the past few years (Chawanpaiboon *et al.*, 2019).

PTD, being a risk factor that results in 50% of all neonatal infections (Blencowe *et al*., 2013), has been shown to be the leading cause of neonatal mortality and morbidity (da Fonseca *et al*., 2003; Kim *et al*., 2015). Survivors of PTD often have low birth weight (LBW) and experience an array of complications such as respiratory and feeding problems during infancy. Other medical conditions such as cerebral palsy, visual and hearing impairment, developmental delays, as well as life-time learning disabilities might also exist in preterm survivors (Tucker and Mcguire 2004; WHO 2018; CDC 2019). Other lifelong effects of PTD may include chronic lung disease, cardiovascular disease and other non-communicable diseases (WHO 2018; CDC 2019).

Although the advancement in perinatal care assisted with a reduction in babies born prematurely and their outcomes, PTD still remains a major problem, especially in third world countries. Underdeveloped countries or low-income countries were shown to have the highest maternal mortality rates with nearly half of all babies being born before the 32nd week of gestation (Tucker and Mcguire 2004; Blencowe *et al*., 2013).

2.2 Predisposing factors for PTD

The exact causes of many PTD cases are unknown (Basu *et al.*, 2017). Approximately, 50 % of PTDs are idiopathic (Haram, Mortensen and Wollen 2003). Other possible causes of spontaneous PTD are spontaneous preterm labour, multiple pregnancy, cervical incompetence/ uterine malformation, antepartum haemorrhage, intrauterine growth restriction (IUGR), pregnancy associated hypertension and preterm premature rupture of membranes (PPROM).

In essence, most causes are linked to maternal physiology being unable to sustain the pregnancy (Tucker and Mcguire 2004).

2.2.1 Maternal reproductive history

Maternal risk factors for PTD include: nulliparity, multiple pregnancy, assisted reproduction, mothers having a history of PTD (Laughon *et al*., 2014), genetic predisposition, epigenetics, environmental factors, maternal age, short intervals between pregnancies, low maternal body mass index and infection (Tucker and Mcguire 2004; Blencowe *et al.*, 2013).

2.2.1.1 Maternal age

The age of the mother plays a large role in her reproductive health, as many young females live a more perilous life style and older females are more likely to suffer from co-morbidities, increasing the risk of an adverse pregnancy in both age groups (Frederiksen *et al*., 2018). As a female ages, her reproductive system starts to deteriorate, causing impaired functionality (due to lack of elasticity). There is also an increase in chromosomal disorders amongst the offspring of matured mothers due to meiotic dysfunction. Adolescent females that fall pregnant too soon after menarche (their first menstrual cycle/period) also pose a health risk, as both infant and mother are growing and compete for nutrition and other resources (Frederiksen *et al*., 2018; Menon 2008).

2.2.1.2 Mode of delivery of previous pregnancy

Many women have opted for delivering via Caesarean section (CS). A large number of women fear labour pain and vaginal birth and therefore many obstetricians convince women to choose surgery since they find it convenient to plan and schedule CS instead of spending hours in and out of delivery rooms for unplanned natural birth (Hopkins 2000).

The induction of labour, via elective CS has also been recommended in some incidences where the length of labour was risky or the mother reported a previous traumatic delivery experience. Mothers that have delivered via CS are often encouraged to wait a year or two before their next pregnancy, as the CS wound needs to heal completely in order to avoid complications (Tucker and Mcguire 2004; Basu *et al.*, 2017; Chevallier *et al.*, 2017).

2.2.1.3 Gravidity

Gravidity refers to the number of times a mother has been pregnant. The outcomes of previous pregnancies influence the current pregnancy and may give an indication of the potential outcome. Women who have had a miscarriage, still birth, CS, short or lengthy interval between pregnancies or an abortion have indicated an association with PTD (Asgharnia, Varasteh and Pourmarzi 2020). Studies have found that women with a history of multiple abortions have a significantly higher risk of LBW and PTD (Brown, Adera and Masho 2008). Short intervals ($\leq$ 6 months) between abortions and pregnancies have also been associated with increased risk of maternal anaemia, PROM, LBW, PTD and very low birth weight (VLBW) (DaVanzo *et al*., 2007). Short intervals between pregnancies are correlated with insufficient maternal recovery time, hormonal imbalances and post-partum nutritional stress, whereas longer intervals (>18months depending on socio economic factors and demographics) showed diminished physiological privileges which the previous pregnancy allowed for (Asgharnia, Varasteh and Pourmarzi 2020).

2.2.1.4 Parity

Parity refers to the number of times a mother has given birth. The incidence of PTD is higher in developing countries than developed countries (da Fonseca *et al.,* 2003) due to the high fertility rate of these regions (WHO 2018). Eswaran and Kotwal (2004) provided a number of reasons why developing countries have high fertility rates including high mortality rates and an above average number of children.

Looking at three consecutive pregnancies, it was found that early gestational age of the first pregnancy significantly increased the risk of PTD in subsequent pregnancies (Laughon *et al*., 2014). The risk of delivering a third baby at preterm increased 5.5-fold if the mother had two prior PTDs compared to mothers who had delivered two babies at term (Laughon *et al*., 2014). Additionally, the authors concluded that if the second child was born preterm, the risk of the third child being born preterm is 3.5-fold, despite the first child being delivered at term.

2.2.2 Maternal Life style

Cigarette smoking, excessive alcohol consumption, drug abuse, laborious work, long periods spent standing as well as bad oral hygiene are life style factors that may contribute to PTD (Pitiphat *et al*., 2005). Most of these life style factors can be adjusted and reduce the risk of PTD, creating a positive observable effect on the mother and baby's health.

2.2.2.1 Cigarette smoking

Cigarette smoking is considered the most important adjustable risk factor for PTD. Positive effects on birth weight are instantly observed upon smoking cessation. Additionally, the number of cigarettes smoked is highly associated with the decrease in birth weight. Roughly 20% to 30% of LBW births have been linked to mothers who have smoked during pregnancy (Chomitz, Cheung and Lieberman 1995). Smoking during pregnancy was also shown to retard foetal growth by 150-320 grams compared to non-smoking mothers. The reduction in weight is mainly due to IUGR and very preterm birth (Windham *et al*., 2000). The physical and mental effects are not only observed in infancy but have shown to impair cognitive abilities in the long run (Chomitz, Cheung and Lieberman 1995).

The nicotine and carbon monoxide in cigarettes are recognized potent vasoconstrictors that can hinder blood circulation to the utero-placental region and damage the placenta. Blood flow restriction has been associated with foetal growth restriction, LBW and PTD (Goldenberg *et al*., 2008). Some effects of maternal smoking during pregnancy such as thickening of the basement membranes, decrease in vascularisation and nutrients to the foetus, may result in growth restriction. These adverse effects on foetal lung development and structure due to toxins such as nicotine have also been documented. Structural changes include enlargement of the alveoli and bronchiole leading to decreased functionality and inhibition in lung development and maturation (Zacharasiewicz 2016).

2.2.2.2 Recreational drug use

Maternal drug abuse has been linked to serious medical conditions in both mother and infant. Drugs such as methamphetamine, cocaine , heroin and other synthetic derivatives have clearly been associated with various adverse birth outcomes including, PPROM, foetal retardation, abruptio placentae, foetal distress, perinatal death, LBW, PTD, high blood pressure (Whiteman et al. 2014) preeclampsia, pregnancy and delivery complications (Chomitz, Cheung and Lieberman 1995; Berkowitz *et al.*, 1998). Drug addicts commonly present with multiple factors such as malnutrition, sexually transmitted diseases and a combination of other drug abuse which may also contribute to adverse pregnancy outcomes (Chomitz, Cheung and Lieberman 1995; Whiteman *et al.*, 2014).

Apart from the effects during pregnancy, the aftermath is just as severe. Babies born from drug addicted mothers often suffer from neonatal abstinence syndrome (NAS) (Whiteman *et al.*, 2014). During pregnancy, the drug is transferred through the placenta and the foetus becomes dependent on the drug. After delivery, the baby experiences withdrawal symptoms due to the discontinuation of the drug. These symptoms include incessant crying, irritability, seizures, feeding problems and vomiting (Stanford children's Hospital n.d)

2.2.2.3 Alcohol consumption

A strong association was seen between mothers who consumed alcohol during pregnancy and the reduction in birth weight and gestational age (Nykjaer *et a*l., 2014). Excessive alcohol consumption, often referred to as "heavy drinking "or 'binge drinking" is defined as a single session of drinking that leads to intoxication (Brittain *et al.*, 2017). Excessive alcohol consumption could be referred to as 1.5 drinks a day or seven or more drinks per week. When alcohol is consumed it crosses the placenta resulting in equal concentrations of alcohol in mother and foetus (Nykjaer *et al*., 2014). Alcohol intoxication during pregnancy is related to a set of severe birth defects, defined as foetal alcohol syndrome (FAS) (Dale, Bakketeig and Magnus 2016). Defects commonly seen in FAS include prenatal and postnatal growth retardation, central nervous system disorders and distinct abnormal cranio-facial features (Guerri, Bazinet and Riley 2009). Alcohol consumption causes structural changes to the shape, volume, surface area of the overall brain and certain regions of the brain. It also reduces white matter and increased grey matter densities in corresponding areas, resulting in learning disabilities and decreased academic achievement, including difficulties in verbal and nonverbal learning and memory (Guerri, Bazinet and Riley 2009).

Excessive alcohol consumption is the leading preventable cause of mental retardation in babies (Nykjaer *et a*l., 2014). The effect of alcohol consumption in moderation is not completely documented, although reduced risk or no risk of adverse pregnancy outcomes is associated with lowered consumption of alcohol (Dale, Bakketeig and Magnus 2016). A cohort study by Dale, Bakketeig and Magnus (2016), deduced that mothers who had been drinking throughout their pregnancy were less likely to deliver preterm provided that these mothers were drinkers before their pregnancy. This protective dose-response effect was noticed in mothers who consumed 4-5 drinks a week.

2.2.3 Maternal Infection

2.2.3.1 Endogenous Oral infection

It is estimated that 50-70% of women develop periodontal disease and gingivitis between the second and 8th month of pregnancy, with a connection established between high levels of pregnancy hormones and a decline in oral health care (Wu, Chen and Jiang 2015). A strong probability of an adverse pregnancy outcome was seen in mothers with periodontal disease compared to mothers with no periodontal disease and normal delivery (Turton and Africa 2017).

Before an actual connection between periodontal disease and PTD was made, studies performed in the late 1990's suggested that gingival crevice organisms caused intrauterine infection via maternal bacteraemia and the trans-placental route (Goldenberg *et al*., 2008).

Periodontal infections during pregnancy cause gingival inflammation due to oral bacterial species interacting with host tissue, resulting in the elevation of inflammatory cytokines and chemokines which not only exacerbate the damage to periodontal structures (Kumari *et al*,. 2014), but may also contribute to PTD (Kesrouani *et al*., 2016).

2.2.3.2 Endogenous Vaginal infections

Maintaining a healthy vaginal biofilm during pregnancy is important to prevent opportunistic infections that may result in adverse pregnancy outcomes (Kaambo and Africa 2017). The hormonal change in pregnancy can lead to many imbalances in the microbial biofilm community as well as compromised immunity (Ramos-e-Silva, Martins and Kroumpouzos 2016). The vagina is colonised by a diversity of microbes which play a protective function. When the microbial homeostasis is disrupted, it can impact reproductive health negatively. The

hormonal changes (particularly elevated sex hormones) during pregnancy could result in an imbalance within the microbial community and a reduction of lactobacilli (Sun, Qiu and Jin 2017). The normal pH of the vagina ranges from 3.8 to 4.8 with an average of 4.5 (Garg *et al.*, 2010). However, an imbalance can promote aerobic bacterial growth and the resultant alkalinity may result in aerobic vaginitis (AV). The inflammation of the vaginal mucosa and submucosa connective tissue can spread to a deep uterus infection and eventually trigger PTD (Kaambo and Africa 2017; Sun, Qiu and Jin 2017). Thus, the increased alkalinity and suppressed lactic acid bacteria allow pre-existing microbiota to thrive and reduce protection against the risk of PTD.

The conversion from a dominated *Lactobacillus* environment to a multi-species anaerobic environment causes bacterial vaginosis (BV) which presents without inflammation (Kirjavainen *et al.*, 2009; Ramos-e-Silva, Martins and Kroumpouzos 2016*;* Kaambo and Africa 2017).

Vulvovaginal infections are common amongst sexually active women and include infectious vaginitis, trichomoniasis and BV. Abnormal vaginal microflora or vaginal dysbiosis during early pregnancy has also been associated with adverse pregnancy outcomes (Tellapragada *et al*., 2016).

Oestrogen may alter susceptibility to infection at different stages of the menstrual cycle, pregnancy, reproductive senescence, contraceptives and supplement usage (Sugarman and Mummaw 1990; Steeg and Klein 2016;). Microbes with an affinity for sex hormones express enzymes that allow them to respond to host sex steroids and their metabolites to regulate their growth and metabolic requirements. The presence of high affinity oestrogen binding-sites in many microorganisms may change their virulence. The mechanism by which it does this is through oestrogen-mediated alteration of the immune response of the host, alterations in the host defence factors, as well as alteration in mammalian cell structure (Sugarman and Mummaw 1990). For example, *Candida albicans* contains an oestrogen binding protein that has a high affinity for estradiol, which can stimulate transition of the yeast into a hyphal form that may increase fungal virulence (Steeg and Klein 2016). Symptomatic infection caused by *Candida* species during pregnancy, unlike the usual asymptomatic presentation in non-pregnant females, has been correlated to PPROM, preterm labour, chorio-amnionitis and congenital candidosis (Ramos-e-Silva, Martins and Kroumpouzos 2016).

2.2.3.3 Intra-uterine infections

Microorganisms frequently responsible for intra-uterine infections include; *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, Ureaplasma urealyticum,* group B streptococci and *Bacteroides* species and have mainly been associated with PPROM and other adverse pregnancy outcomes (Krohn *et al*., 1991; Donders *et al*., 1993). Microbes such as the CHEAP TORCHES (chicken pox and shingles, hepatitis C, enteroviruses, AIDS (HIV infection), parvovirus B19, *Toxoplasma gondii,* rubella virus, cytomegalovirus and herpes simplex) group have been shown to migrate across the placenta during gestation (Ford-Jones and Kellner, 1995; Kumari *et al.,* 2011).

Intrauterine infection is commonly asymptomatic until labour is initiated or membrane rupture occurs. There is no guarantee that signs and symptoms will be present during labour, although histological and blood tests confirm the infection (Thomakos *et al*., 2010).

While most neonatal infections may occur early or during late postpartum there are infections that can manifest during the pregnancy and increase the risk of PTD (Placzek and Whitelaw 1983). Microbes may gain access through the cervix, migrate across to the amniotic cavity or reach the placenta haematogenously (Goldenberg *et al*., 2008; Vinturache *et al*., 2016). There is also evidence that non-pregnant women with BV have intrauterine colonisation associated with chronic plasma cell endometritis and is linked to prelabour (Goldenberg *et al.,* 2000).

Studies have revealed that there is an association between a women's socio-economic status, educational status, urban/rural residence, proximity to a health clinic, and use of maternal health care (Mctavish *et al*., 2010). A high income has shown to have no effect on the gestational period. However, a mother with a socio- economic disadvantage status could indirectly be influenced by unhealthy behaviour and increased stress levels, which could shorten gestation (Kramer *et al*., 2001).

It is also evident that premature babies born in high income countries survive very preterm conditions in comparison to premature babies born in low income countries, where they are less likely to receive proper health care (Eswaran and Kotwal 2004). In many underdeveloped countries, there is a lack of resources and skilled medical attendants (Nour 2008). For example, in South Africa, due to a lack of incubator availability, nearly 45% of PTD cases result in neonatal death and have to resort to kangaroo mother care, (South Africa Every Death Counts Writing Group, 2008).

2.2.4 Socio-economic factors

2.2.4.1 Ethnicity

In the USA and UK, black women are reported to be at higher risk of PTD than any other ethnicity (Goldenberg *et al*., 2008). Reasons for this include the fact that they are more socio-economically disadvantaged, access medical care less frequently or at a later stage and tend to have more severe disease and comorbidity as well as increased risk for hypertension (Harper *et al*., 2007). Thus, black women are more likely than white women to be exposed to stress early in life and during pregnancy (referred to as one's allostatic load) with higher rates of unintended pregnancy, partner-associated stress, separation or divorce within the first 12 months of delivery. Although Wallace and Harvillle (2013) reported no direct association between racial ethnicity and preterm birth, traumatic stress was found to correlate with PTD (Wallace and Harvillle 2013). Stress was shown to change immune function, leading to susceptibility to intra-uterine infection and inflammation with high risk behaviour reported as a means of coping (Lu and Chen 2004).

In South Africa, a "coloured" population of mothers (either of mixed ancestry or direct descendants of the indigenous people of South Africa) were found to have a high rate of mid-trimester abortions, PTD and infants that were small for gestational age, as well as high stillbirth/neonatal death ratios. These high rates were as a result of excessive smoking and drinking amongst the mothers (Brink et al., 2019).

Another study conducted in the Western Cape, South Africa, examined a population of mothers that were predominately black and mixed ancestry and found that 80% of the mothers delivered infants vaginally at FT and that only 17% of the mothers delivered at PT. Despite the pregnancy acquired medical condition, HIV, food insecurity and low socio-economic status of these mothers, only 7% of the infants required hospitalisation after delivery (Zar et al., 2019).

2.2.4.2 Maternal educational levels

In high income countries, the reason for elevated risk of PTD is a combination of life style habits in the minority of women with a low/no level of education (Jansen *et al.,* 2009). In low-income countries, the majority of people face challenges such as poverty, inadequate or no health care nor education, corruption and lack of employment. Studies have shown that mothers from poor income countries with lower levels of education were less likely to seek medical

health care compared to their counterparts from high income countries. Women with a higher level of education have a lower risk of delivering prematurely (Wike, 2017).

2.2.4.3 Access to health care

In Africa, many countries are still rural with a minor portion been urbanised, thus pregnant women are faced with the calamity of having to travel far distances for regular check-ups, particularly during the last trimester of pregnancy (McTavish *et al*., 2010). Other constraints include high population density in health care facilities, long waiting periods for consultation or treatments, expensive medical treatment and untrained staff (van Schalkwyk *et al.*, 2018).

2.3 Immunology of pregnancy

2.3.1 Protection of the foetus

There are three stages of immune response during pregnancy, depending on the stage of pregnancy. The first stage of immunological response occurs during implantation, placentation and the first trimester, which is a pro-inflammatory immunological response. Most women experience morning sickness as a result of the pro-inflammatory response resulting from various changes to her body. By the second immune response phase, the mother's body has successfully adapted to her pregnancy and no longer suffers harsh side effects towards the foetus. With the exception of some mothers experiencing morning sickness sporadically the second immune phase mimics a relatively calm anti-inflammatory immune response. The final phase consists of a pro-inflammatory phase, where parturition is signalled by the increase in immune cells and cytokines into the myometrium. This influx creates an inflammatory response that will initiate uterus contractions for the expulsion of the baby and placenta (Mor and Cardenas 2010). Under normal circumstances the foetus is protected from immune defence through tightly controlled cytokine levels at the maternal-foetal interface. Some aspects of immunity are suppressed due to the increased levels of hormones, for example, the functionality of neutrophils (Wu, Chen and Jiang 2015). The semi-allogenic foetus is not targeted by the maternal innate immune system due to some immunological privileges being lost during pregnancy. The maternal immune system produces anti-foetal, anti-placental and anti-paternal antibodies, which indicates detection of the foetus by the maternal immune system but does not compromise the pregnancy (Coulam 2000).

In addition, the human decidua contains high levels of T-cells, macrophages, Natural Killer (NK) cells, T-lymphocytes and leukocytes (Mor and Cardenas 2010). Some cytokines have shown to increase gradually throughout pregnancy until term or when parturition mechanisms are activated. Parturition mechanisms include blocking of progesterone (P_4) receptors on the myometrium with elevation of Interleukin (IL)-1β, IL-6, IL-8 and Tumour Necrosis Factor (TNF)-α in the local region of the cervix, membranes and myometrium (Peltier 2003). The abovementioned cytokines, increase in production during cervical ripening, due to the increase in neutrophils and macrophages at the cervix. IL-1β may act upon prostaglandin E_2 assisting with cervical dilation. These pro-inflammatory cytokines all aid with migration of neutrophils, macrophages and other cells to the region, assisting cervical ripening. A similar process occurs at the membranes and myometrium, resulting in the rupture of membranes and rhythmic contractions, respectively.

Normally, the full effects of these pro-inflammatory cytokines are suppressed by IL-10 and other immune-modulators such as P_4. Immuno-modulators inhibit or limit inflammation at the Maternal –foetal interface, which contribute to the survival of the foetal allograft (Peltier 2003; Perunovic *et al.*, 2016).

2.3.2 Response to infection and Markers for predicting PTD

Premature activation of cytokines such as IL-1β, TNF-α and Interferon (INF) ϒ activate pro-inflammatory cascade mechanisms, with inflammation at the maternal-foetal interface resulting in adverse pregnancy outcomes (Peltier 2003; Perunovic *et al.*, 2016).

Early detection of a high-risk pregnancy could predict and prevent PTD. Mothers at possible risk should be encouraged to get enough bed rest and might be administered prophylactic progesterone during spontaneous PTD, antibiotic treatment or a cervical cerclage depending on the circumstance (da Fonseca *et al*., 2003; Vinturache *et al*., 2016).

Traditional methods of predicting women at risk for PTD were generally based on the obstetric history, tocometry, biochemical markers and ultrasonography of the cervix. However, these methods were not entirely effective at lowering the PTD rate (Menon 2008). In recent years, molecular techniques employing biomarkers have been widely used to detect and diagnose non-communicable diseases, such as cancers (Hiss 2012) and adverse pregnancies (Garshasbi *et al*., 2011).

With the advancement in technology, maternal serum biomarkers can be used to detect high risk pregnancies such as intrauterine infection linked to PPROM within a shorter period than scenarios where isolation of the pathogen, or other techniques might have taken several days to perform in order to diagnose a patient, (Loukovaara *et al*., 2003; Cháfer-Pericás *et al.*, 2015). Biomarkers used include foetal fibronectin, salivary oestriol, corticotrophin releasing hormones, IL-6, CRP, CD163 thrombin-antithrombin complex, IL-8, matrix metalloproteinase -8 (MMP-8), ferritin, placental alkaline phosphatases and relaxin (Menon 2008; Kim *et al.*, 2015). Although able to predict high risk pregnancies, some are ineffective at determining the time period appropriate for the gestational age and delivery (Menon 2008).

2.3.2.1 Matrix metalloproteinase and salivary oestriol

Both matrix metalloproteinase-9 (MMP-9) and salivary oestriol were observed to be useful in the prediction of late PTD, with MMP-9 found to rise rapidly 24 hours before the initiation of labour (Kesrouani *et al*., 2016). Prediction of late preterm is not as important as predicting early PTD because most deaths or morbidity occur during the early preterm stages (Menon 2008).

2.3.2.2 Foetal fibronectin

Foetal fibronectin is a marker that indicates chorio-decidual disruption. This glycoprotein (present in cervical fluid) was found to be one of the most powerful biochemical markers used in PTD prediction (Menon 2008). The marker is usually absent from the 20th week of gestation and therefore, the presence of foetal fibronectin in maternal serum and vaginal secretions after the 20th week could indicate possible leakage of amniotic fluid and risk for PTD (Vogel *et al*., 2005; Menon 2008). Foetal fibronectin was considered to accurately predict spontaneous PTD within 7 to 10 days among mothers with symptoms of PTD before advanced cervical dilatation (Coleman *et al.*, 2001; Honest *et al*., 2002). However, another study of 58 women with positive foetal fibronectin reported no positive cases of foetal fibronectin in amniotic fluid, thereby negating its efficiency as an ideal predictor of PTD. Other clinical limitations in this study included the recruitment of patients who had sexual intercourse 24 hours prior to sampling, cervical cerclage, PPROM, preeclampsia and bleeding, all of which are factors believed to provide false positive results from the vaginal discharge (Vogel *et al*,. 2005).

2.3.2. 3 Neutrophil Gelatinase Associated Lipocalin (NGAL)

NGAL was shown to be a promising biomarker for pre-eclampsia. High levels of NGAL were seen in the serum samples of pre-eclamptic patients during the first and second trimester, which explains damaged endothelia in pre-eclamptic patients (Petla *et al.,* 2013).

2.3. 2.4 Interleukins (IL-)

Several studies have shown success using biomarkers such as interleukins and TNF-α to determine high risk intrauterine infection during a genetic amniocentesis taken in the mid-trimester. These cytokines have proven to be useful in the detection of PTD and as a 'surrogate' test to determine inter-amniotic infection (Cobo *et al*., 2009; Thomakos *et al*., 2010; Kunze *et al.,* 2016; Nadeau-Vallée *et al,*. 2016).

The cytokine IL-6 is secreted by the T cells and macrophages in order to stimulate an immune response. IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine, mediating fever and antibacterial response (Konstantinov *et al.,* 2013) and is probably the most researched biomarker for intrauterine infection. Elevated levels in amniotic fluid are associated with sterile inflammation and intrauterine infection (Sadowsky *et al.,* 2006; Thomakos *et al.,* 2010; Kim *et al*., 2013; Dulay *et al.,* 2015; Mustafa *et al.*, 2015), especially for chorio-amnion colonisation that may lead to PTD and active labour (Coleman *et al*., 2001;Cobo *et al*., 2009; Kim *et al*., 2013).

However, many studies have conflicting results about IL-6 as an independent marker of PTD as well as the accuracy and cut-off value of prediction. A study conducted by Sadowsky *et al,* (2006) independently infused IL-1B, TNFα, IL-6 and IL-8 in to the amniotic fluid of pregnant rhesus monkeys. The results indicated that IL-6 and IL-8 alone did not primarily induce preterm labour. The authors then concluded that preterm labour may be induced by a number of inflammatory or immune substances (Sadowsky *et al.,* 2006). The Cobo *et al* (2009) study, combined IL-6 and proteomic biomarkers and was unsuccessful in improving the predictive value for PTD and neonatal morbidity. Kesrouani *et al* (2016) also found that there was no statistical signifant difference in amniotic fluid levels of IL-6 and MMP of full term and PTD cases, a limitation of this study being the small sample size (N=39) which needs to be taken into consideration when interpreting this finding. Both IL-6 and IL-8 were reported to predict imminent PTD occurring within 2 or 7 days (Cobo *et al*., 2009).

IL-8 is a chemokine frequently associated with inflammation, activation and migration of cells (Konstantinov *et al.*, 2013). IL-8 is synthesized by macrophages and other cell types, such as epithelial cells in the cervix. Production of IL-8 may be enhanced by IL-1β. Some studies have shown that IL-1β and IL-8 play a role in cervical ripening (Olah *et al.*, 1996) and dilatation (Konstantinov e*t al.*, 2013), with an increase in IL-8 observed within the cervical stroma and squamous epithelium after spontaneous labour and vaginal delivery as well as in the amniotic fluid of women who delivered prematurely (Konstantinov *et al*., 2013).

Tanaka *et al*, (1998) proposed that IL-8 stimulates the release of elastase and collagenase, which degrade collagen fibres in the uterine cervix. The degradation of the fibres results in cervical ripening during preterm or labour, while Sakamoto *et al*, (2004) concluded that the increased levels of IL-8 play an important part in cervical dilatation, but not pre-labour cervical ripening. However, results were based on positive foetal fibronectin results in mothers experiencing cervical ripening. Limitations of the study include the small sample size and the inclusion of favourable cervices. In addition, leucocytes were found to express the IL-8RA and IL-8RB receptors, linking IL-8 to the recruitment of leucocytes during cervical dilatation (Sakamoto *et al.*, 2004).

2.3.2.5 C-Reactive Protein (CRP)

CRP is a sensitive marker of systemic inflammation and responds to infection and tissue injury. Being an acute phase reactant of inflammation, it may be present in some cases of chronic inflammatory disorders. (Pitiphat *et al.*, 2005). CRP has also shown to exert an anti-inflammatory effect as a protective measure within the host (Kim *et al.*, 2015). This sensitive marker is mainly produced in the hepatocytes (Pitiphat *et al*., 2005). However, there have been rare incidences where CRP has been synthesised in locations other than the hepatocytes, such as neurons, atherosclerotic plaque, monocytes and lymphocytes. Not much is known about the production of CRP outside of the liver, with the exception of plasma CRP levels not being influenced by the external concentration (Black, Kushner and Samols 2004; Jialal, Devaraj and Singh 2006).

CRP is a member of the pentraxin family and is made up of five identical non-covalently bound subunits. This structure also has a calcium (Ca^{+2}) dependent binding site specificity for phosphocholine, amongst ligand binding sites and other binding sites (Agrawal *et al.*, 2001; Black, Kushner and Samols 2004; Thirumalai *et al.*, 2017).

CRP induction and activation has been described as follows. In the presences of an apoptotic cell, pro-inflammatory cytokines, namely, IL-6, IL-1 and TNF-α are released into circulation. These cytokines activate transcriptional factor C/EBP family that will promote the synthesis of CRP from the hepatocytes. Once released into circulation, CRP has various mechanisms by which it may function.

Firstly, CRP can bind to a particular receptor such as CLq, thereby activating the classic complement cascade by activating C3 Convertase which in turn activates the C3 and C4 cascades, eventually leading to phagocytosis. This particular interaction is said to be the most efficient execution of CRP (Marnell, Mold and Du Clos 2006). The early stage of this cascade is CRP mediated. However, CRP reacts with factor H (FH), which is a C5 inhibitor or regulatory protein in the late stage that promotes the disintegration of C3 and C5 convertases. As a result, the late stage, which is a highly inflammatory host defence that generates chemotactic peptides such as the membrane attack complex (MAC), is limited. Therefore, CRP is able to promote an anti-inflammatory response and delay the number of neutrophils to the region. This phenomenon allows CRP to function pleiotropically (Black, Kushner and Samols 2004). In the absence of C1q receptors, CRP activates the mannose binding lectin pathway or alternative pathways providing downstream activation of the complement pathway (Gershov *et al*., 2000).

Secondly, CRP can bind to several ligands such as chromatin, small nuclear ribonucleoproteins, histones, laminins and many more, which may trigger one of the complement pathways (Gershov *et al.*, 2000; Black, Kushner and Samols 2004).

Additionally, CRP can bind to immunoglobulin receptors FcϒRI and FcϒRII that are stimulated by an effecter molecule, immune-receptor tyrosine-based activation motif (ITAM) or immune-receptor tyrosine-based inhibition motif (ITIM) which may or may not trigger opsonisation (Black, Kushner and Samols 2004; Marnell *et al.*, 2006).

3 Summary and Objectives

Developing countries have extremely high PTD cases. Many mothers and infants are dying due to inferior medical care during antenatal visits and delivery. PTD is the biggest killer of children under the age of five years and causes up to 50% of morbidity and mortality in neonates. Predicting the chances of PTD during pregnancy could reduce the risk of spontaneous PTD and the effects of PTD during infancy and childhood.

CRP has yielded promising results when predicting adverse pregnancy effects. This proinflammatory marker has been widely used as a sign of infection in several westernised clinical settings. There is a paucity of studies relating maternal and foetal cord blood CRP levels to different pregnancy outcomes on the African continent. The **aim** of this study was therefore to evaluate the use of CRP as a predictive marker for PTD in a cohort of Rwandan women.

The objectives of the study were:

- To detect and compare the concentrations of CRP in maternal and foetal cord serum samples at delivery
- To compare the mean concentration of CRP in maternal and foetal cord blood in full term and preterm cases
- Investigate a possible association between the various risks factors for PTD and mean CRP within the sub-groups

3.1 Hypothesis

$H_{0:}$ Elevated CRP concentrations cannot be used as a predictive biomarker of PTD in Rwandan mothers.

$H_{1:}$ Elevated CRP concentrations can be used as a predictive biomarker of PTD in Rwandan mothers.

CHAPTER 2

MATERIAL AND METHODS

2.1 Patient selection

Convenience sampling was employed for the collection of blood samples from 200 mothers who had given birth in the obstetric and gynaecological units of the teaching hospital in Butare, Rwanda. A research-collaboration between the teaching hospital and the University of the Western Cape governed the sample collection from the Rwandan population. Permission to conduct the study was granted by the ethical committees of the University of the Western Cape and the teaching hospital of Butare in Rwanda (Registration number 08/1/31).

The study complied with the declaration of Helsinki (2013). Mothers were informed of the nature and purpose of the study as well as possible injuries, inconveniences, discomforts and potential benefits as well as the right to withdraw from or refuse to participate in the study. Thereafter, mothers were asked to sign a consent form (see appendix 2A) to obtain maternal and foetal cord blood samples for investigation and storage for future investigations. Confidentiality of their information and anonymity were ensured by allocating a number to each sample collected instead of a name.

Two hundred mothers gave written consent to participate in the study. Information about the maternal demography, medical history, previous pregnancies, life style habits, diet and dental visits were collected by means of a questionnaire in order to identify factors that might pose a health risk to herself and unborn child (see appendix 2B).

2.1.1 Inclusion criteria

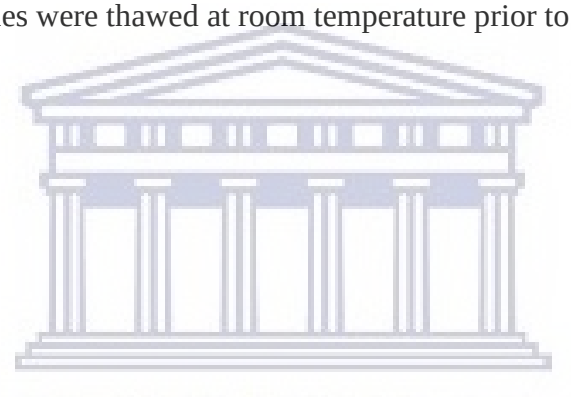
Mothers were included in the study if they were admitted for delivery to the obstetric and gynaecological units of the teaching hospital in Butare. Mothers between the ages of 16 and 50 years who were of Rwandan ethnicity were included. Gestational age was determined from the mothers last period or ultrasound.

2.1.2 Exclusion criteria

Excluded from the study were all mothers with active infections, mothers who had recently been on antibiotic treatments, or who reported co-morbidities presumed to pose a risk to themselves or their infants.

2.2 Blood Sample Collection

Medical doctors, nurses and laboratory technicians assisted with the sample collection and clinical examination. Ten ml of maternal blood was collected 48 hours after delivery and 10ml of foetal cord blood (FCB) was collected at delivery into untreated blood collection tubes (Medi Plus, Cat. No: VP4011). Blood samples were stored with patient consent at 4°C overnight, centrifuged at 3000rpm for 10 minutes and serum stored in 1.5ml aliquots at -80°C (Papapanou *et al.,* 2000). Serum samples were thawed at room temperature prior to testing.



2.3 Preparation of ELISA reagents

2.3.1 Coating antibody

A 1/1000 dilution of the 2mg/ml capture antibody was constituted in 1 x phosphate buffered saline (PBS) (Lonza , Cat. No: 17-517Q and Sigma, Cat. No: P4417-100TAB). Thereafter, Maxisorp NUNC 96 well plates (Thermofisher, Cat. No: 442404) were coated with 50µl of anti-CRP capture antibody (abcam, Cat. No: ab8279). These pre-coated plates were stored at -20°C for future use.

2.3.2 Washing Buffer

Wash buffer was constituted using 5ml of 0.1% Tween 20 (Merck, Cat. No: 9005-64-5) in 1 X PBS (100ml of 10XPBS and 895ml of distilled H_2O).

2.3.3 Blocking Agent

Blocking agent was constituted using 2% blotting grade blocker non-fat dry milk blocking agent (Bio-rad, Cat. No: 170-6404) dissolved completely in 1 X PBS and centrifuged for 2 minutes at 4000rmp before being added to the plate or being used to constitute assay diluent.

2.3.4 Assay Diluent

Assay diluent was constituted from a 1-part blocking agent and 9 parts wash buffer solution.

2.3.5 Detecting Antibody

A 1/1000 dilution of 0.7mg/ml detecting antibody (abcam, Cat. No: ab24462) was constituted in the assay diluent (preparation mentioned above).

2.3.6 CRP Standard preparation

The CRP standard was reconstituted as per instructions on the Bio Aim Human CRP ELISA kit (BioAim, Cat. No: 3010026) by diluting 5ng/ml of CRP standard in 200µl of 1/5 assay diluent to obtain a concentration of 1000pg/ml.

CRP standard was later replaced with Human CRP (Sino biological Cat. No: 11250HNAH) reconstituted as instructed in the data sheet. CRP (0.25mg/ml) was dissolved in sterile distilled water. The CRP standard was aliquoted and stored at -80°C. Upon usage, aliquots were thawed

at room temperature, centrifuged and further diluted to 5000pg/ml. The CRP standards were run together to compare the standard validity.

2.4 Optimization of coating and detecting antibody concentration for the ELISA

The Checkerboard ELISA method was used to determine the optimum concentrations of capture and detecting antibody for developing the in-house CRP ELISA.

A volume of 50µl of 1XPBS was added to all rows with the exception of row H, where 100 µl of capture antibody solution was added to row H and serially diluted vertically, ranging from 1/500 to 1/32000 (Figure 1). Row A would just have 1XPBS in. The microtiter plate was then incubated overnight at 4°C.

Capture antibody was decanted and the plate was rinsed five times with Wash Buffer. Thereafter, 200µl of 2% blotting grade blocker non-fat dry milk blocking agent was added and left to incubate for an hour on a shaker at room temperature.

Columns 8-12 contained the antigen and served as the positive control. A volume of 50µl of CRP standard (1000pg/ml) was added to columns 8-12 on the plate while columns 1-7 contained assay diluent only (negative control). The plate was then incubated overnight at 4°C (Figure 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	1/8000	1/4000	1/2000	1/1000	1/500	Dil	1/8000	1/4000	1/2000	1/1000	1/500
B	1/32000					DA						DA
C	1/16000					DA						DA
D	1/8000					DA						DA
E	1/4000					DA						DA
F	1/2000					DA						DA
G	1/1000					DA						DA
H	1/500	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA

Figure 1: Checkerboard ELISA plate layout. Yellow region (column 7-12) indicates the positive CRP control and the blue region (column 1-6) indicates the negative CRP controls. Detecting antibody (DA) was added to columns 6 and 12 and Coating antibody (CA) was added to row H

Post incubation, the antigen was decanted and the plate was washed several times with Wash Buffer. A volume of 100µl of the detecting antibody was added to columns 6 and 12 then horizontally serially diluted (doubling dilution) to columns 2 and 8, respectively. The detecting antibody was incubated at room temperature for an hour, followed by the wash step.

Fifty micro litres of tetramethylbenzidine/hydrogen peroxidise substrate (TMB) (Cat. No: 5120-0074) was heated to 37°C and added to the plate. Thereafter, the plate was placed in a (dark) drawer and allowed to incubate for 25-40 minutes at room temperature, whilst observing colour change. Stop solution (Merck, sulphuric acid, H_2SO_4 (0.5mM) was added and the plate was read at 450nm (Multiskan EX, Thermo Electron Corporation).

2.4.1 Optimal coating and detecting antibody dilution range

Once the optimum concentrations for the antibodies were determined, the microtiter plate was coated at 1/500 for columns 1- 6 and 1/1000 for columns 7-12, which were incubated overnight at 4°C. Capture antibody was decanted and washed several times, as explained above, after which blocking agent was added and incubated at room temperature on a plate shaker for an hour. The plate was washed and dabbed dry, followed by the addition of five different preterm maternal samples diluted 1/1000 in assay diluent. A volume of 100 µl of CRP standard was added to designated wells A1 and A7, which were vertically serially diluted to construct the standard curve and incubated overnight at 4°C. After overnight incubation, the antigen was decanted and the plate was washed. Detecting antibody was added at dilutions of 1/500 and 1/1000 respectively, then incubated for an hour on a microtitre plate shaker. TMB substrate was added for approximately 15-20 minutes before the reaction was stopped and the plate was read at 450nm (Figure2).

	STD	2	3	4	5	6	7	8	9	10	11	12
A	1000pg/ml	sample	sample	sample	sample	sample	STD	sample	sample	sample	sample	sample
B	500	CA=1/500 DA=1/500					STD	CA=1/1000 DA=1/1000				
C	250						STD					
D	125						STD					
E	62.5						STD					
F	31.25						STD					
G	15.625						STD					
H	DIL	DIL	DIL	DIL	DIL	DIL	DIL	DIL	DIL	DIL	DIL	DIL

Figure 2 : Indicating the ELISA layout. Where columns 1-6 were coated at 1/500 and columns 7-12 were coated at 1/1000. Detecting antibody was also added at 1/500 for columns 1-6 and 1/1000 for columns 7-12. CRP standard (STD) was added to columns 1 and 7 and were serially diluted until row G. the same samples were added to both sections. The last row contained diluent (H)

2.5 Determination of the ELISA detection range

Once the optimum capture and detecting antibody concentrations were determined, plates were pre-coated at 1/1000 and stored at -20°C.

Upon usage, the pre-coated plate was washed then blocked for an hour, followed by another wash. Thereafter, eight Foetal Cord serum (FCS) samples (4 term and 4 preterm) were diluted at 1/30000 and 1/90000, using the 1/10 assay diluent. These samples were firstly diluted at 1/100 and then further diluted to the respective dilution factors (Figure 3).

FCS samples that had much higher concentrations of CRP were diluted at 1/90000. Maternal samples were highly concentrated and needed to be diluted to 1/300000 in order to fall within the range of detection. Stock aliquots of the samples were stored at 1/100 in small volumes.

	1	2	3	4	5	6	7	8
A	STD	STD		166B			166B	
B	STD	STD		167B			167B	
C	STD	STD		169B			169B	
D	STD	STD		189B			189B	
E	STD	STD		67B			67B	
F	STD	STD		69B			69B	
G	STD	STD		70B			70B	
H	STD	STD		71B			71B	

Figure 3: CRP STD curves were assayed in replicas (columns 1 and 2). The FCS samples were assayed in triplicate. Columns 3-5 contained FCS diluted at 1/30000, whereas columns 6-8 contained FCS diluted at 1/90000

2.6 General method for the optimized in-house ELISA for CRP.

Pre-coated plates were lightly rinsed with wash buffer upon being thawed. A volume of 200µl of blocking agent was added and incubated at room temperature for an hour on a shaker. The plate was rinsed five times with wash buffer, followed by the addition of the CRP standard and the samples to their respective dilution factors (FCS at 1/30000 and Maternal at 1/300000). These antigens were then incubated overnight at 4°C (Figure 4).

Thereafter, the plate was rinsed and 50µl per well of detecting antibody (1/1000) was added and incubated for an hour on a microtitre plate shaker at room temperature. The plate was rinsed several times with Wash Buffer, followed by the addition of 50µl of TMB substrate and incubated in a dark drawer, whilst colour change was monitored for approximately 20-30 minutes. Stop solution was added and the plate was read at 450nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD		166B			sample			sample			
B			sample			sample			sample			
C			sample			sample			sample			
D			sample			sample			sample			
E			sample			sample			sample			
F			sample			sample			sample			
G			sample			sample			sample			
H	0		sample			sample			sample			

Figure 4: ELISA plate layout for CRP analysis. Sample 166B was run with all the ELISAs and used as an internal control to ensure consistency

2.7 Calculation of results

A standard curve was used to determine the amount of CRP in the samples. Standard curves were generated by plotting the average O.D (450nm) obtained from the standard concentration on the horizontal axis (X) versus the corresponding CRP concentration (pg/ml) on the vertical axis (Y) using Excel. The polynomial equation was used in order to calculate the CRP concentration within the samples. Sample values were calculated by substituting the X value with the O.D values in the standard curve equation and multiplying by the dilution factor. These values were then subtracted by the value of the blank and then converted to ng/ml.

2.8 Statistical analysis

Statistical analysis was done using One Way Analysis of Variance (ANOVA) on a Sigma plot statistical programme. The Holm-sidak test was used to determine the significant difference between the groups being compared. Significance was determined based on whether the *P*-value was ≤ 0.05 and the standard error of the mean (SEM) was used to determine how far the average concentration was distributed from the rest of the data set in a particular group.

The FT maternal and FCS samples were the control groups with which PT maternal and FCS were compared in this study. The average concentration of CRP was determined in each sample and then grouped in order to investigate possible trends or significant associations between CRP levels and pregnancy outcome.

CHAPTER 3

RESULTS

3.1 Maternal demographics and pregnancy outcomes

Information regarding these factors were notarised and tabulated (Table 1). Not all participants responded to all of the questions asked. Mothers who failed to complete the questionnaire were excluded from the study, thereby reducing the sample size to 195. Due to sample losses and/or insufficiencies, the total number of samples reported here include 46 full term maternal (FT-Mat), 90 full term foetal cord serum (FT-FCS), 70 preterm maternal (PT-Mat) and 79 preterm foetal cord (PT-FCS) samples. Of these complementary sets of maternal and foetal cord serum samples constituted 40 full term and 60 preterm.

3.1.2 Maternal Age

Based on patient history gathered from 180 mothers, the data showed that most mothers were between the ages of 20-35 years old. The highest percent of the sample population (39.08%) were mothers between the ages of 20-25 years who delivered at PT, followed by 32.26% of mothers between the age of 26-30 years who delivered at FT (Table 1). The smallest fraction came from mothers < 20 years old who delivered preterm. There was no visible trend with regards to the various age groups and pregnancy outcome.

3.1.3 Maternal weight

In many instances, anthropometric measurements such as weight and height are used to assess health risk. The majority (27.84%) of mothers who delivered at FT were within the range of 56-60 kg, which is considered to be the normal and healthy body weight of the average female. However, the highest percentage of the population (39.13%) was mothers who delivered PT and weighed more than 65kg. The lowest percentage were underweight mothers who delivered FT (11.39%) and PT (7.61%) (Table 1).

3.1.4 BMI

The majority of mothers in the group (53.76% and 67.61% for FT and PT mothers, respectively) had an ideal BMI. However, 37.63% of mothers who delivered at FT were considered overweight, followed by 26.76% of mothers who delivered at PT. Smaller percentages of the group were underweight and obese. The smallest percentage came from underweight mothers, 1.07% and 2.82% for FT and PT mothers, respectively (Table 1).

3.1.5 Maternal Level of Education

Of the 165 mothers who supplied information on their education, 55.05% of FT mothers and 66.67% of PT mothers reported a primary level of education. This was followed by smaller percentages for mothers with secondary, tertiary and no level of education. More mothers with tertiary education delivered PT infants than mothers with no education (Table1).

3.1.6 Parity

The majority of mothers reported that their current pregnancy was their first pregnancy, with 37.62% delivering FT and 40.45% delivering PT. For the remaining FT mothers, 25.74% reported parity of 4^{th} and subsequent child, followed by 20.79% for 2^{nd} child and lastly 15.84% for mothers who delivered their 3^{rd} child. The reverse was noted for the PT group of mothers where the majority (22.47%) of mothers delivered their 3^{rd} child, 21.35% delivered their 2^{nd} child and 15.73% delivered their 4^{th} and subsequent child (Table1).

3.1.7 Maternal Medical History

Among the medical conditions reported by these mothers, 21.21 % of the FT and 26.88% of PT mothers reported a history of recurrent UTI's, while 14.56% FT and 14.13% PT mothers reported a past STI. A minority (2%) of the population of mothers had diabetes mellitus, while 7.76% FT and 11.95% of PT mothers reported inherited heart disease (Table 1).

3.1.8 Infant Birth Weight

Of the mothers who delivered FT infants, 82.61% gave birth to babies with a normal birth weight (NBW). The rest of the babies born at term (17.39%) had low birth weight (LBW). Twenty–two percent (22%) of the mothers who gave birth prematurely, delivered a baby with a normal weight. However, the majority of babies born preterm (64.04%) had LBW. Unlike the mothers who delivered PT, none of the mothers who carried until term delivered a baby with very low (VLBW) or extremely low birth weight (ELBW) (Table 1).

Table 1: Maternal demographics and risks factors associated with PTD

Categories	Full Term	Preterm	
Maternal age	**n (%)**	**n (%)**	**N**
< 20	8 (8.6)	5 (5.75)	
20 - 25	20 (21.5)	34 (39.08)	
26 - 30	30 (32.26)	24 (27.59)	
31 - 35	27 (29.03)	12 (13.8)	
36 >	8 (8.6)	12 (13.8)	
Sub total	93	87	180
Maternal BMI (kg/m²)			
Underweight > 18.5	1 (1.07)	2 (2.82)	
Ideal (18.5 - 24.9)	50 (53.76)	48 (67.61)	
Overweight (25 - 29.9)	35 (37.63)	19 (26.76)	
Obesity (< 30)	7 (7.53)	2 (2.82)	
Sub total	93	71	164
Level of maternal education			
Primary	49 (55.05)	62 (66.67)	
Secondary	24 (26.97)	18 (19.35)	
Tertiary (University)	6 (6.74)	7 (7.53)	
No education	10 (11.24)	6 (6.45)	
Sub total	89	93	165
Parity			
First pregnancy	38 (37.62)	36 (40.45)	
2nd child	21 (20.79)	19 (21.35)	
3rd child	16 (15.84)	20 (22.47)	
4th and subsequent	26 (25.74)	14 (15.73)	
Sub total	101	89	190
Maternal weight (kg)			
< 50	9 (11.39)	7 (7.61)	
51 - 55	14 (17.72)	13 (14.13)	
56 - 60	22 (27.84)	20 (21.74)	
61 - 65	20 (25.31)	16 (17.39)	
> 65	14 (17.72)	36 (39.13)	
Sub total	79	92	171
Infant birth weight			
Normal birth weight ≥ 2500g	76 (82.61)	20 (22.47)	
Low birth weight < 2500g	16 (17.39)	57 (64.04)	
Very low birth weight < 1500g		11 (12.36)	
Extremely low birth weight < 1000g		1 (1.12)	
Sub total	92	89	181
Maternal medical history			
Recurrent urinary tract infections	21 (21.21)	25 (26.88)	
n=	99	93	192
Sexually transmitted diseases	15 (14.56)	13 (14.13)	
n=	103	92	195
Diabetes mellitus	0	2 (2.17)	
n=	103	92	195
Inheritary heart disease	8 (7.76)	11 (11.95)	
n=	103	92	195

*N indicates overall totality of the PT and FT within a category whereas n indicates the total number of mothers who answered the question

3.2 Determining CRP concentrations using ELISA

The checkerboard ELISA experiments were used to determine optimum dilution factors for antibodies in order to determine the CRP concentration within the samples. Optimum concentrations for coating and detecting antibodies were shown to be 1/1000. Serum samples dilution factors were determined as 1/30000 for FCS and 1/300000 for maternal serum samples, with the exception of highly concentrated FCS samples that were further diluted to 1/90000. The data obtained from the ELISA would be substituted into the polynomial equation to equate the concentration of CRP in the sample.

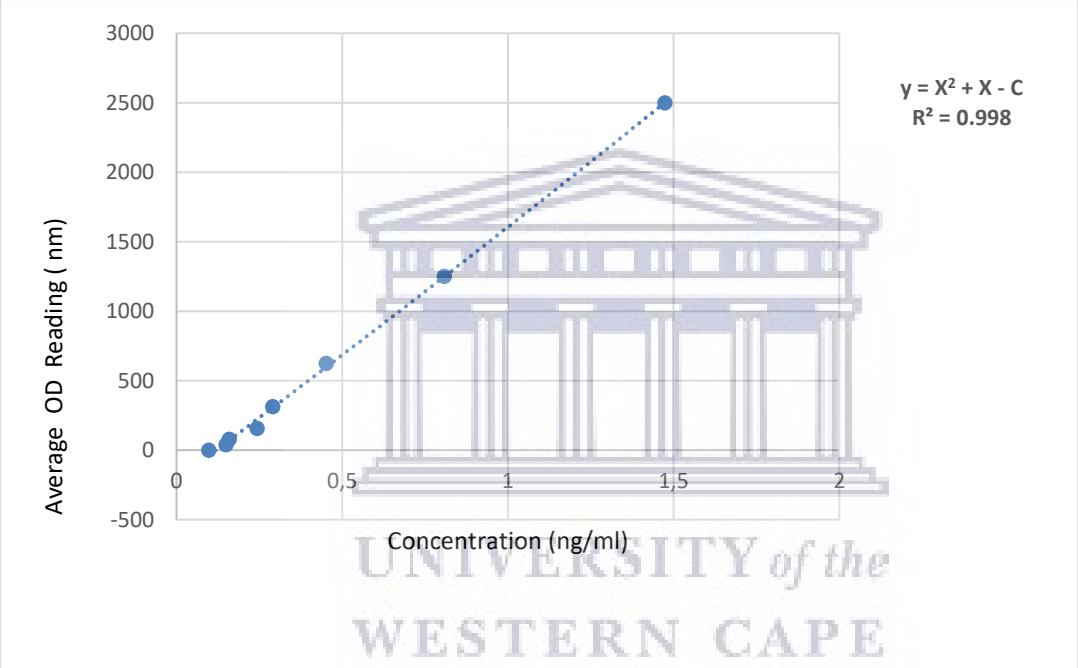


Figure 5: Illustrates the standard curve used to calculate the concentration of CRP in samples

3.3 CRP detection in Maternal and Foetal cord serum samples

3.3.1 DAS ELISA

Using the in-house ELISA, maternal and FCS samples were analysed. When FT (control group) and PT maternal serum samples were compared, no statistical significant difference (P=0.300) was observed between the mean FT maternal CRP concentration (75.94 (±142.85)ng/ml) and mean PT maternal CRP concentration (56.96 (±11.39)ng/ml) (Table 2). No statistically significant difference was observed (P=0.944) between the mean CRP concentration of the FT-FCS (3.99 (±1.47)ng/ml) and the PT-FCS (4.16 (±1.93)ng/ml). Maternal CRP concentrations were significantly higher than FCS mean CRP concentrations. CRP values for many of the FCS samples were below detection (Figure 6).

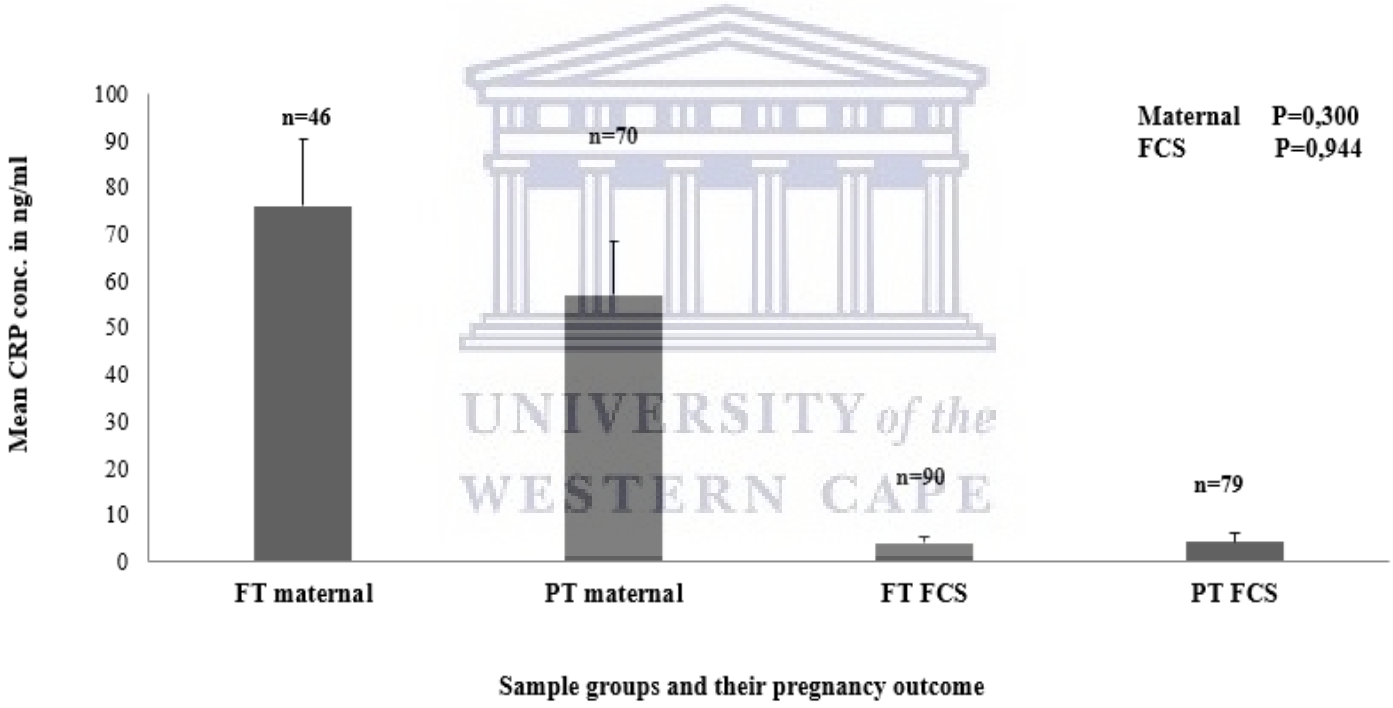


Figure 6: The correlation between pregnancy outcome and the mean CRP concentration in the maternal serum and Foetal Cord serum (FCS). FT represent Full term maternal samples and PT maternal represent the preterm samples. The Full-term groups (control groups) were compared to PT group. *P* - value for the difference between FT and PT maternal groups was P=0.300 and the difference for the PT and FT-FCS groups was P=0.944.

3.4 Validation of the in-house ELISA

An internal positive control (PT-FCS sample 166B) was used to validate the consistency between all assays. If any variation to the OD reading on 166B (0.2nm) was observed, the assay would be repeated. Samples were tested in triplicate and average OD readings were compared. If the standard curve and average 166B OD readings were not consistent and differed, the assay was repeated.

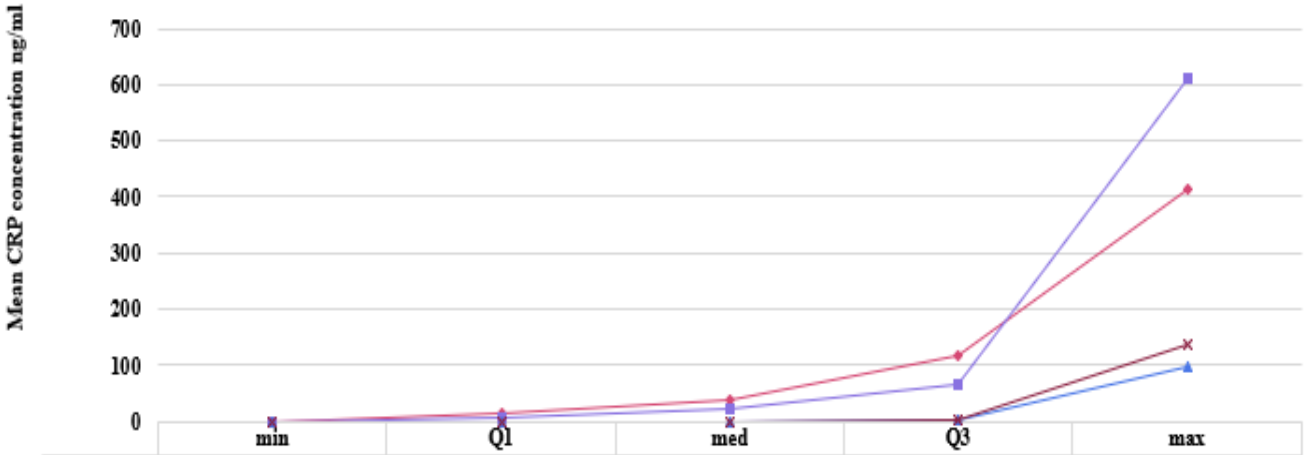
3.5 Comparison of CRP with Pregnancy outcomes

3.5.1 Maternal Serum analysis

The results do not typically confine to a normal distribution of data as there are many outliers resulting in large standard deviations. Descriptive statistics showed that the median CRP concentrations for FT and PT mothers were 36.175ng/ml and 22.770ng/ml, respectively. Both sample groups had a mode of 0ng/ml. The PT maternal data indicated a much higher maximum value than that of the FT maternal samples. However, FT maternal data indicated a higher 1st quartile, median and 3rd quartile values compared to the PT maternal data set (Figure 7).

3.5.2 Foetal Cord Serum analysis

CRP was undetectable in approximately 24% of PT-FCS samples and 37.77% of FT-FCS samples. Therefore, the modal CRP concentration within the FCS sample group was 0ng/ml. The remainder of the FCS samples tested positive for the CRP antigen. The median values for the FT and PT-FCS were 0.214.44ng/ml and 0.417.25ng/ml, respectively. Median CRP concentrations calculated have shown to be much less than the mean FCS CRP concentrations. FT and PT-FCS line graphs appear to be similar, with the exception of the PT-FCS data set which had a higher maximum value than the FT-FCS data set (Figure 7).



	min	Q1	med	Q3	max
FT-Mat	0	13,62748	36,17464	115,9636	412,2643
PT-Mat	0	7,9855	22,77045	65,11841	612,1815
FT-FCS	0	0	0,21444	1,10846	96,22392
PT-FCS	0	0,00159	0,41725	0,9345424	137,0609

Figure 7 : The trend in mean CRP concentration within the different sub-groups. The graph illustrates the concentration of CRP at the minimum, first quartile (Q1), median, third quartile (Q3) and maximum values. Full team maternal (FT-Mat), Preterm maternal (PT-Mat), Full term foetal cord serum (FT-FCS) and Preterm foetal cord serum (PT-FCS)

3.6 Association of C-Reactive protein levels with maternal risk factors

3.6.1 Influence of maternal age on mean CRP concentrations

3.6.1.1 Maternal samples

FT mothers in the age group < 20 years had the highest mean CRP concentration, namely 113.99 (±45.67)ng/ml followed by FT mothers in the age group 31-35 years with 94.10 (±25.89)ng/ml whereas the age group 20-25 years had the lowest CRP concentration of 31.08 (±11.45)ng/ml. Furthermore, in age groups 20-25 years and > 36 years, the mean PT maternal CRP concentrations of 60.48 (±1.3)ng/ml and 4.01 (±0.18)ng/ml respectively, appeared to be slightly higher than in the FT maternal groups. No visible trend was observed between the mean concentrations of CRP and maternal age between FT and PT mothers, nor was a statistically significant difference observed between the age groups and pregnancy outcome (Figure 8).

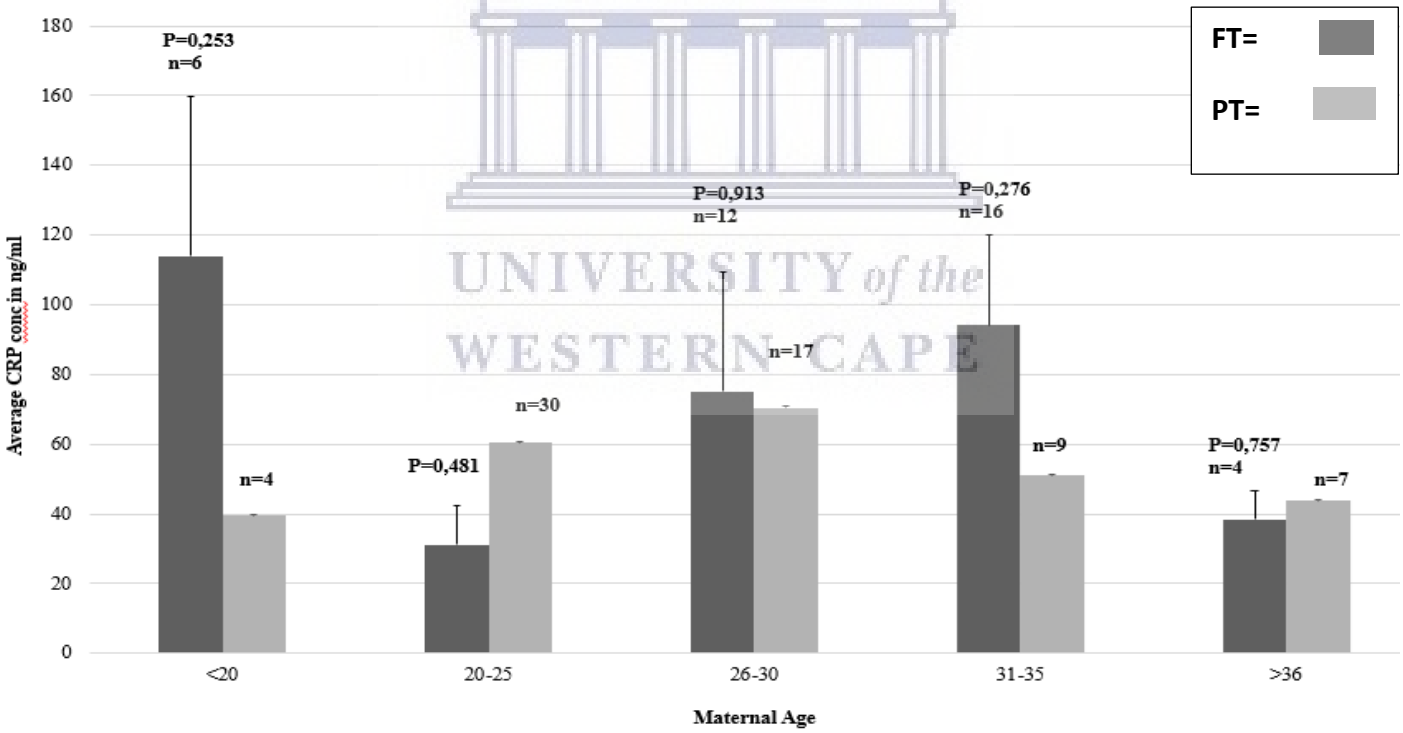


Figure 8: The correlation between the different maternal age groups in years and the mean maternal CRP concentration for the specific pregnancy outcomes

3.6.1.2 Foetal Cord Samples (FCS)

The PT-FCS samples within the 26-30year-old maternal age group had the highest CRP concentration (8.61 (±6.54)ng/ml) compared to the other age groups (< 20 years, 20-25 years, 31-35 and > 36 years). The lowest CRP concentration, 0.48 (±0.23)ng/ml, was observed in the 31-35year group who delivered PT, while those who delivered FT in this age group showed the highest CRP concentration (7.65 (±0.69)ng/ml) of all the age groups. No visible trend was observed between the maternal age and the mean CRP concentration in the FCS nor were any significant differences demonstrated. Mothers who delivered at FT in the age groups of < 20 years, 20-25 years and 31-35 years had higher CRP concentrations in the FCS than mothers of the same age groups who delivered PT (Figure 9).

When observing the graphs of the maternal age factor for maternal and FCS sample groups, both FT maternal and FT-FCS for the age groups < 20 years and 31-35 years were higher than the PT cases. The PT group for the mothers older than 36 years showed higher CRP concentrations than the FT groups in both FCS and maternal serum cases (Figures 8 and 9).

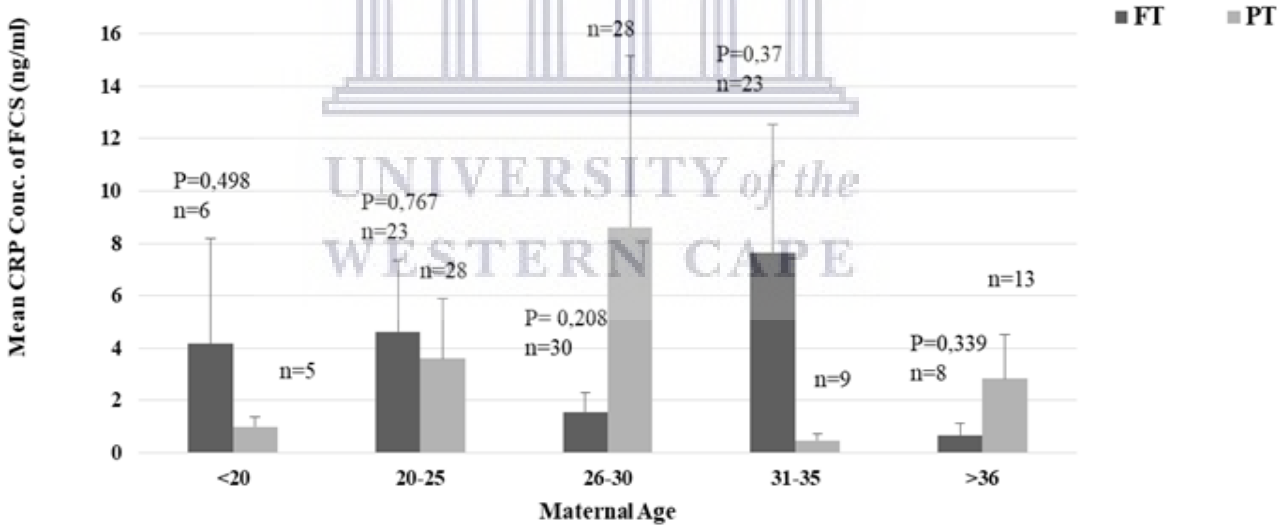


Figure 9: The relationship between different maternal age groups and mean foetal cord serum (FCS) CRP concentrations for the specific pregnancy outcome. *P*-values represent the difference in FCS CRP concentration between (FT) full term and preterm (PT) samples within a particular age group.

3.6.2 Maternal weight and mean CRP concentrations

3.6.2.1 Maternal CRP

No significant difference in CRP concentrations were observed between the various maternal weight categories and pregnancy outcome with the exception of the 56-60kg group (Figure 10). FT Maternal weight groups < 50kg and 56-60kg had higher mean CRP concentrations, (172.11 (±59.83)ng/ml and 103.99 (±39.67)ng/ml respectively) compared to the other weight groups (Figure 10). The FT maternal 56-60kg weight group also had a significantly higher mean CRP concentration (103.99 (±39.67)ng/ml) in comparison to the PT maternal group (35.75 (±9.73)ng/ml), despite the smaller sample size.

The lowest mean CRP concentrations were observed in the 51-55kg weight range, with 21.80 (±8.19)ng/ml and 42.46 (±13.17)ng/ml for FT and PT mothers respectively. PT maternal CRP concentrations of 72.48 (±42.48)ng/ml and 78.61 (±38.80)ng/ml for maternal weight groups 61-65kg and >65kg, respectively, were higher than that of the FT maternal concentrations of 39.13 (±27.15)ng/ml and 73.40 (±21.18)ng/ml for weight groups 61-65kg and >65kg, respectively (Figure 10).

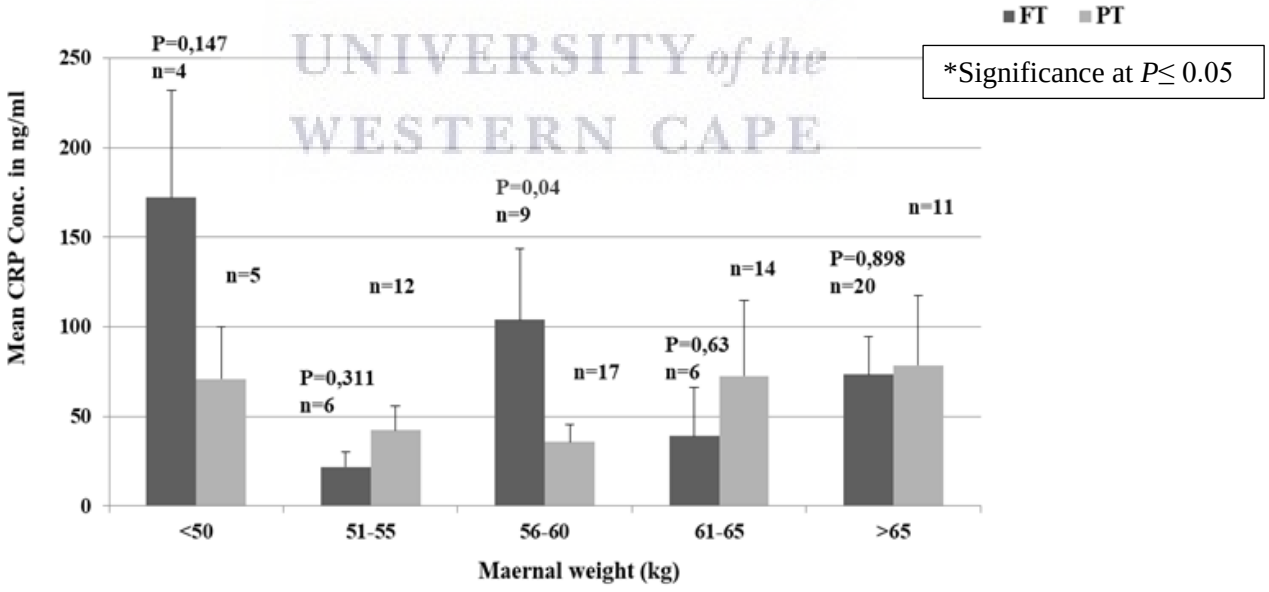


Figure 10: Maternal weight groups and mean maternal CRP concentrations for the specific pregnancy outcome. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group.

3.6.2.2 FCS CRP

The mean CRP concentration of the FT-FCS (23.29 (±12.08)ng/ml) was much higher than in the PT- FCS (7.01 (±5.11)ng/ml), for mothers weighing <50kg. FCS from mothers who delivered PT and weighed 51-55kg, 61-65kg and > 65kg had higher CRP concentrations (2.03 (±1.64) ng/ml), 2.74 (±1.50) ng/ml) and 14.05 (±12.36) ng/ml, respectively) than the FCS from mothers who delivered at FT (0.71 (±0.27)ng/ml, 0.64 (±0.30)ng/ml and 2.03 (±1.03)ng/ml., respectively). However, these differences were not significant (Figure 11). The CRP concentrations from mothers who weighed < 50kg were the highest in both FT- maternal and FT-FCS samples (Figures 10 and 11).

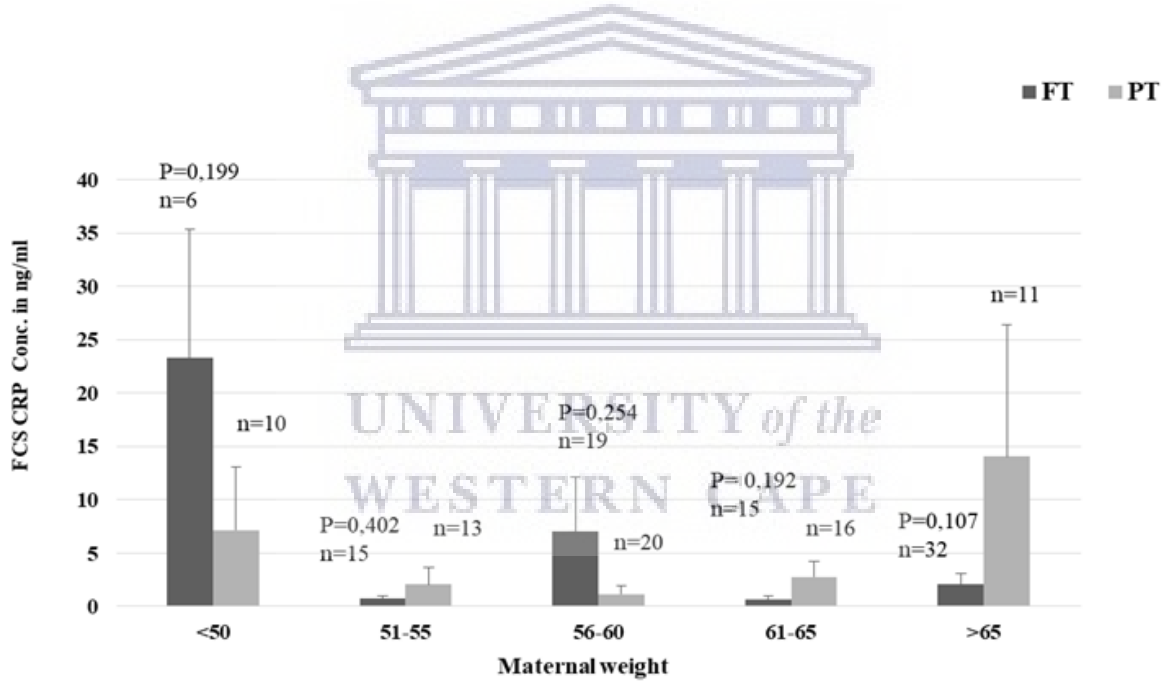


Figure 11: Maternal weight groups and their mean foetal cord serum CRP concentration for the specific pregnancy outcome. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group.

3.6.3 Maternal BMI and CRP

3.6.3.1 Maternal CRP

Mothers who delivered FT showed higher CRP concentrations (67.47 (±16.08)ng/ml and 113.9 (±35.49)ng/ml in the Ideal and Overweight BMI, respectively) in comparison to the PT maternal groups (41.14 (±7.18)ng/ml and 77.26 (±42.96)ng/ml for Ideal and Overweight BMI groups, respectively). The Underweight (not shown) and Obesity groups had a small sample number and thus no applicable comparison could be drawn. No statistically significant difference was observed between FT and PT mothers in the Ideal (P=0.097) and Overweight (P=0.52) BMI groups (Figure 12).

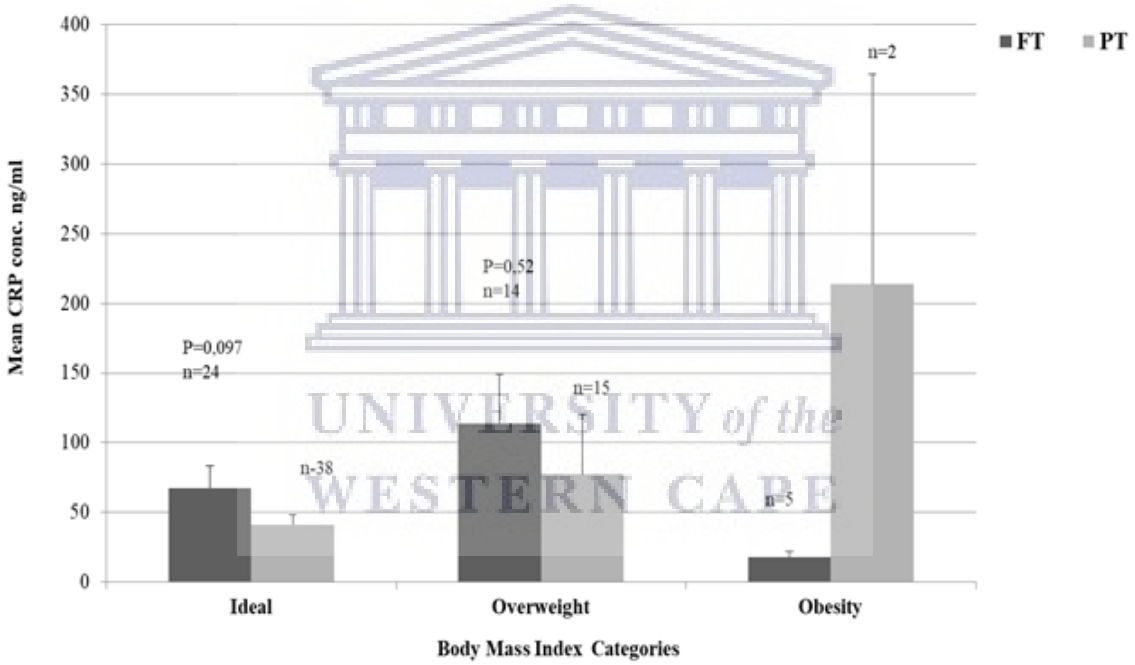


Figure 12: The relationship between maternal BMI groups and mean maternal CRP concentrations for the specific pregnancy outcomes. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group.

3.6.3.2 FCS CRP

The FCS samples showed a similar pattern to the data from the maternal serum sample set, whereby the mean FT-FCS CRP concentration (6.39 (±2.63)ng/ml) was higher than the PT-FCS (3.29 (±1.64)ng/ml) samples in the Ideal BMI group (Figure 13).

The FCS CRP concentrations from the Overweight mothers were the lowest of the BMI groups, namely 1.06 (±0.622)ng/ml and 1.53 (±0.91)ng/ml for FT-FCS and PT-FCS, respectively. While CRP values from samples in the Obesity group in both PT maternal (213.87 (±150.46) ng/ml and FCS 68.76 (±68.30)ng/ml were the highest in the BMI category (Figures 12 and 13).

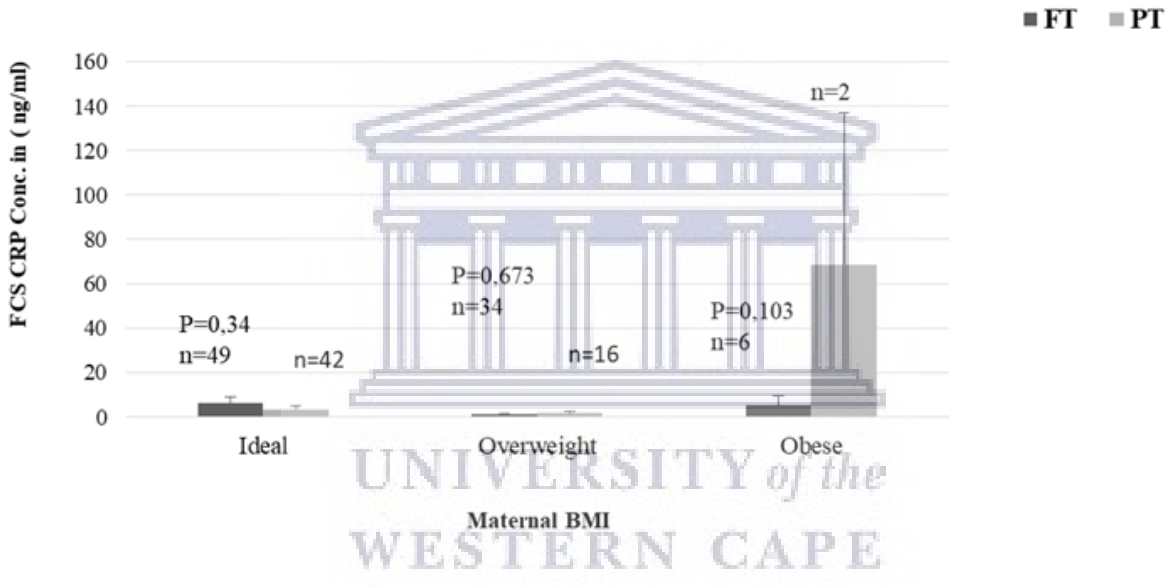


Figure 13: The relationship between the maternal BMI groups and mean FCS CRP concentration for the specific pregnancy outcomes. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group

3.6.4 Maternal Level of Education and CRP levels

3.6.4.1 Maternal CRP

A statistical significant difference was seen between the mean CRP concentration of FT and PT mothers (p=0.02) with a primary level of education. FT mothers with primary education had a higher mean CRP concentration (81.65 (±19.47)ng/ml) compared to PT mothers (36.86 (±6.96)ng/ml).

No statistical significant difference was observed between FT and PT mothers with secondary (P=0.948), tertiary (P=0.372) and no formal education (P=0.652). However, the highest mean CRP concentrations were observed in the group with no formal education with a mean concentration of 128.43 (±102.17)ng/ml and 204.09 (±105.82)ng/ml for FT and PT mothers, respectively. The lowest mean CRP concentration 21.37 (±3.98)ng/ml was seen in the FT maternal group with tertiary education (Figure 14).

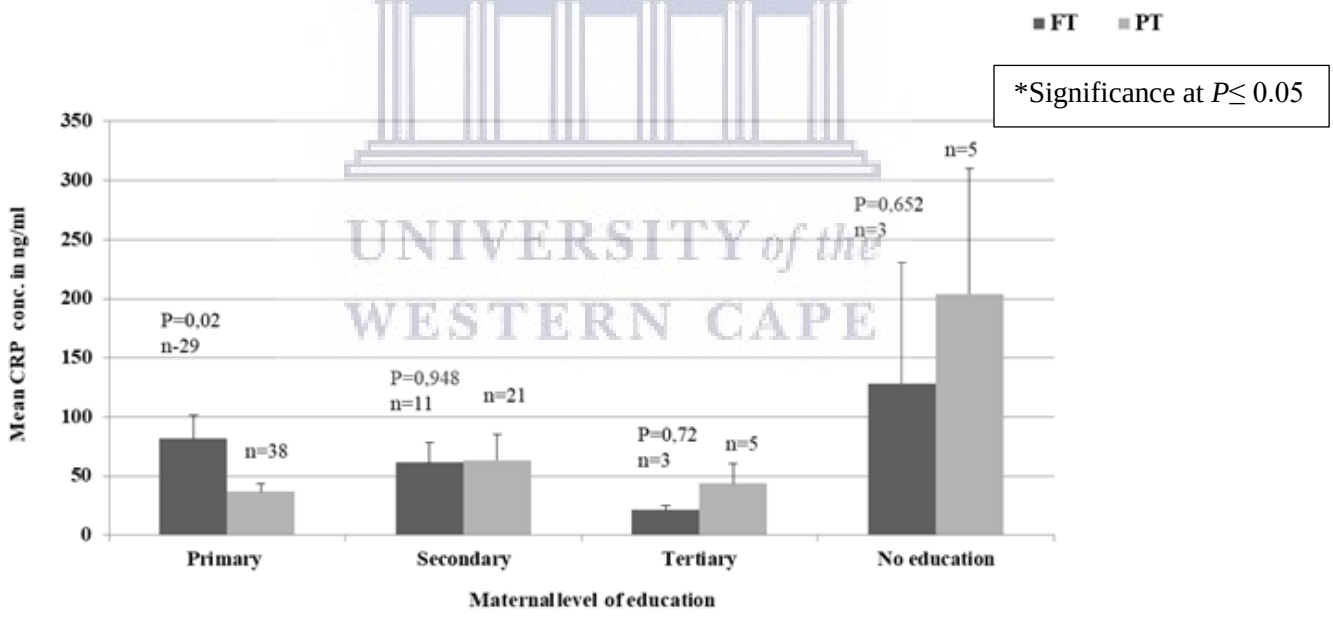


Figure 14: The relationship between the different levels of maternal education and mean maternal CRP concentrations for the specific pregnancy outcomes. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group

3.6.4.2 FCS CRP concentration

The FT-FCS concentration (4.33 (±2.07)ng/ml) was higher than the PT-FCS (1.85 (±0.66)ng/ml) for mothers with a primary level of education as well as for those mothers with no formal education (9.49 (±8.15)ng/ml for FT versus 7.33 (±6.95)ng/ml for the PT). The PT-FCS CRP concentration (7.82 (±6.8)ng/ml) was higher than the FT-FCS CRP concentration (0.75 (±0.29)ng/ml) in mothers with a secondary education, with a slight increase in PT mothers with tertiary education (4.73 (±4.49)ng/ml) compared with mothers who delivered at FT (4.5 (±4.2)ng/ml). However, the FCS from mothers with no formal education who delivered at FT had the highest CRP concentration 9.49 (±8.15)ng/ml in the category, while the highest CRP values from PT-FCS samples (7.82 (±6.8)ng/ml) were seen in the mothers with secondary education. Educational levels showed no significant differences between the FCS CRP concentrations and pregnancy outcomes (Figure 15).

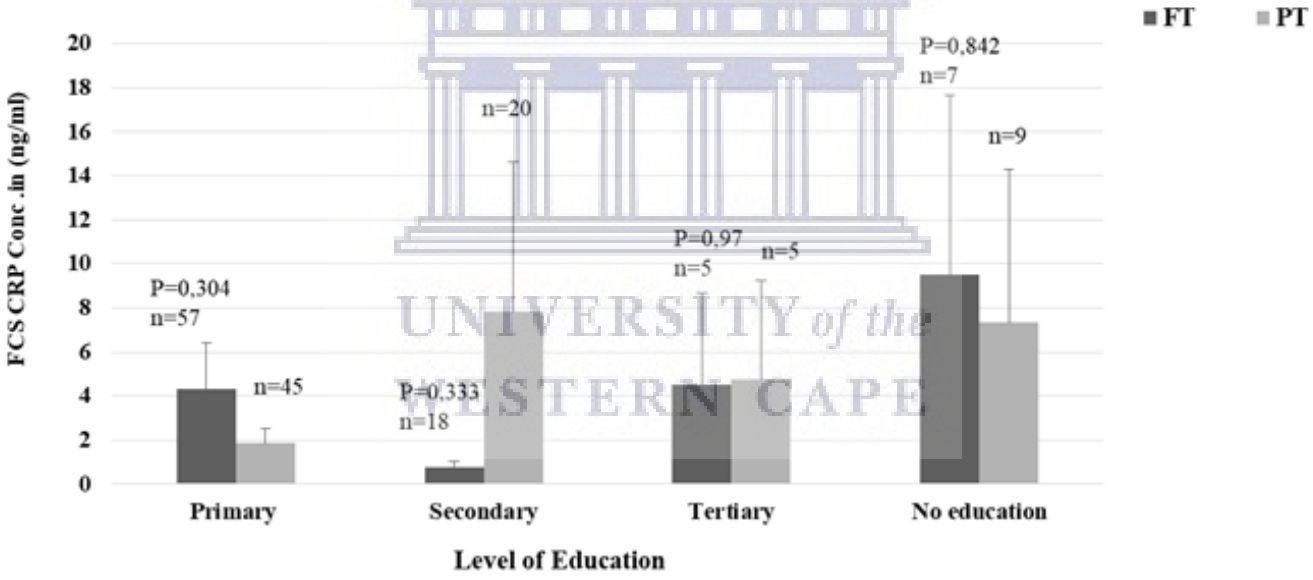


Figure 15: The relationship between the different levels of maternal education and mean foetal cord serum CRP concentrations for the specific pregnancy outcomes. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group

3.6.5 Parity and Mean CRP concentration

3.6.5.1 Maternal CRP concentration

FT mothers who delivered their 1st child and 2nd child had higher mean CRP concentrations than their PT counterparts (Figure 16). However, FT mothers who delivered their 3rd child and 4th or subsequent child respectively, had a lower mean CRP concentration (27.40 (±10.98)ng/ml and 104.36 (±35.67)ng/ml) compared to the PT mothers who delivered their 3rd child and 4th and subsequent children (52.59 (±17.27)ng/ml and 110.78 (±19.47)ng/ml, respectively).

Mothers who prematurely delivered their 4th or subsequent child had the highest mean CRP concentration within the parity category. No statistically significant difference was observed in CRP levels between the various parity groups (Figure 16).

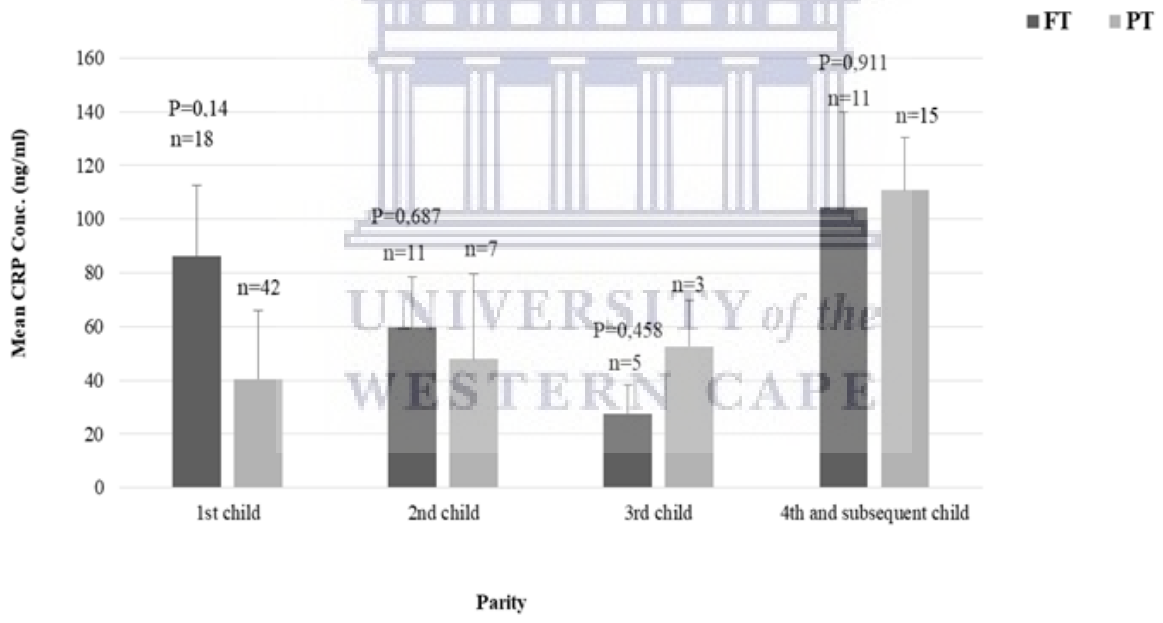


Figure 16: The influence of parity on maternal CRP concentrations in different pregnancy outcomes. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group

3.6.5.2 FCS CRP concentration

The FT-FCS was higher than the PT-FCS in parity groups 2nd, 3rd or 4th and subsequent child, with PT-FCS from mothers who delivered their first born, showing the highest CRP concentration in the category (11.01 (±6.69)ng/ml). No statistical difference was observed between the FCS CRP levels and pregnancy outcomes with regard to parity (Figure 17).

Although FT mothers who delivered their first child were shown to have a higher CRP level than PT mothers, (Figure 16) their FCS showed the lowest CRP concentration (0.62 (±0.31) ng/ml, Figure 17).

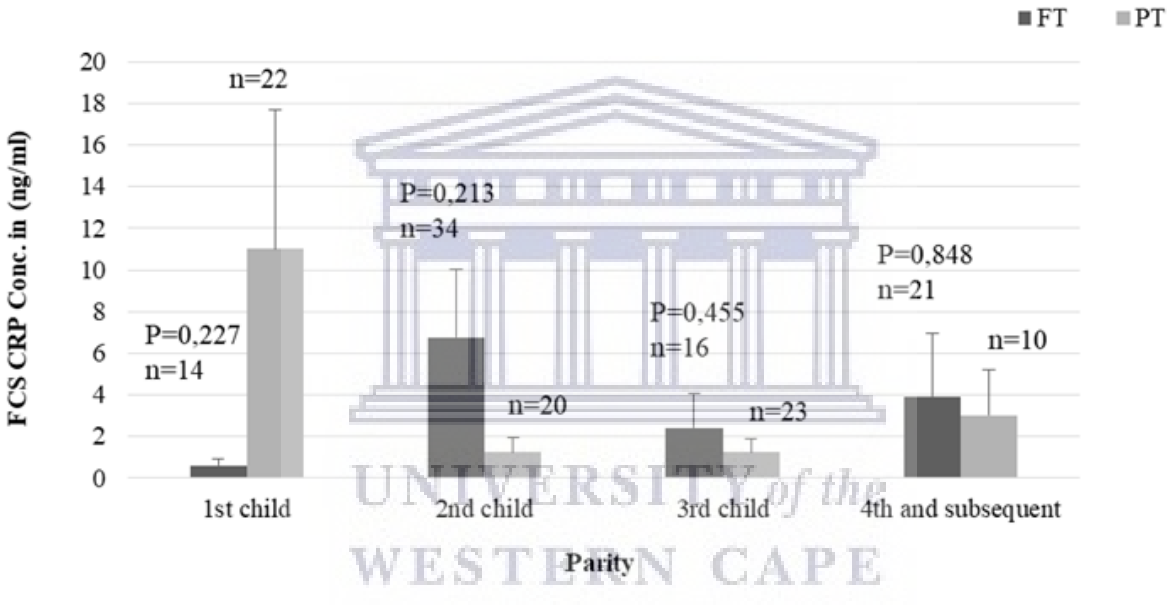


Figure 17: The mean FCS CRP concentrations for the specific pregnancy outcomes according to parity. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group.

3.6.6 Maternal infection and CRP concentration

3.6.6.1 Maternal CRP concentration

Mothers with a history of recurrent UTIs, who delivered at FT had a higher mean CRP concentration (75.56 (±28.33)ng/ml) than mothers who delivered at PT (45.59 (±13.40) ng/ml).

This tendency was also observed amongst mothers who had a history of a STI. Mothers who had delivered at FT had a CRP concentration of 75.46 (±42.82)ng/ml compared to mothers who delivered at PT (22.43 (±7.0)ng/ml), although no statistical significant difference was observed in mean CRP concentrations between PT and FT mothers (Figure 18).

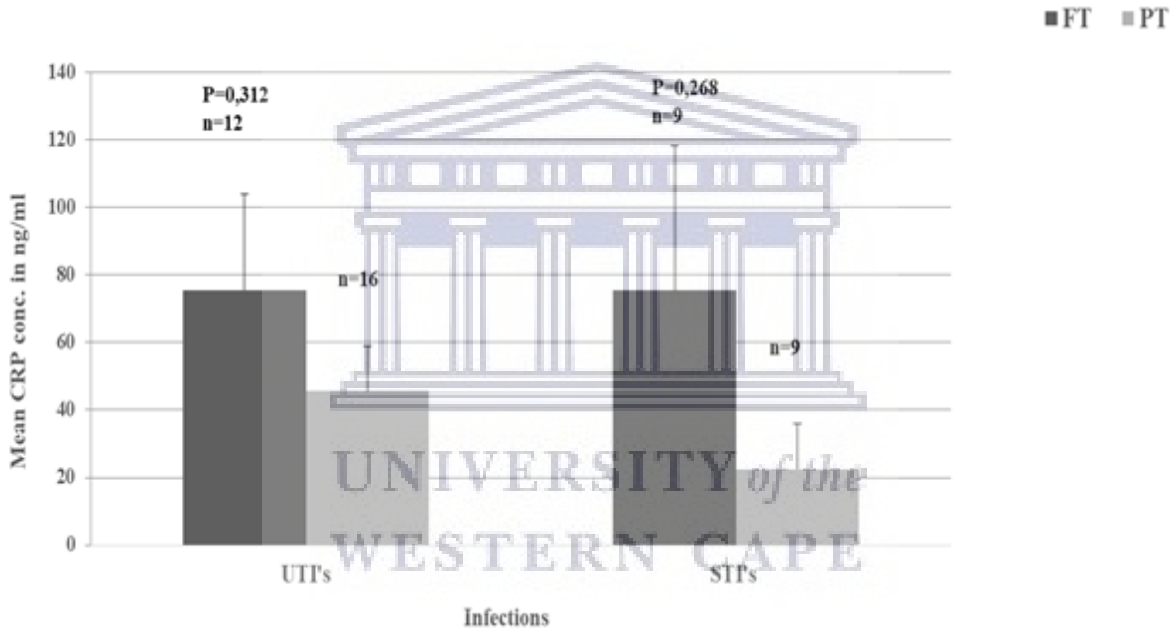


Figure 18 : The relationship between maternal infection history and mean maternal CRP concentrations with pregnancy outcomes. *P*-values represent the difference in CRP concentrations between (FT) full term and preterm (PT) samples within a particular group.

3.6.6.2 FCS CRP concentration

The FCS of mothers who delivered at FT and had a history of recurrent UTIs had the highest CRP concentration (7.8 (±5.67)ng/ml) within the category, followed by the FT-FCS of mothers with a history of STI (4.38 (±3.84)ng/ml). The lowest FCS mean CRP concentrations were seen in mothers with a history of STI (Figure 19) who delivered prematurely (0.185 (±0.071)ng/ml).

Both FT maternal (Figure 18) and FT-FCS CRP (Figure 19) concentrations were higher than PT samples, when comparing the history of maternal infections and pregnancy outcomes, but no significant difference was observed (Figure 19).

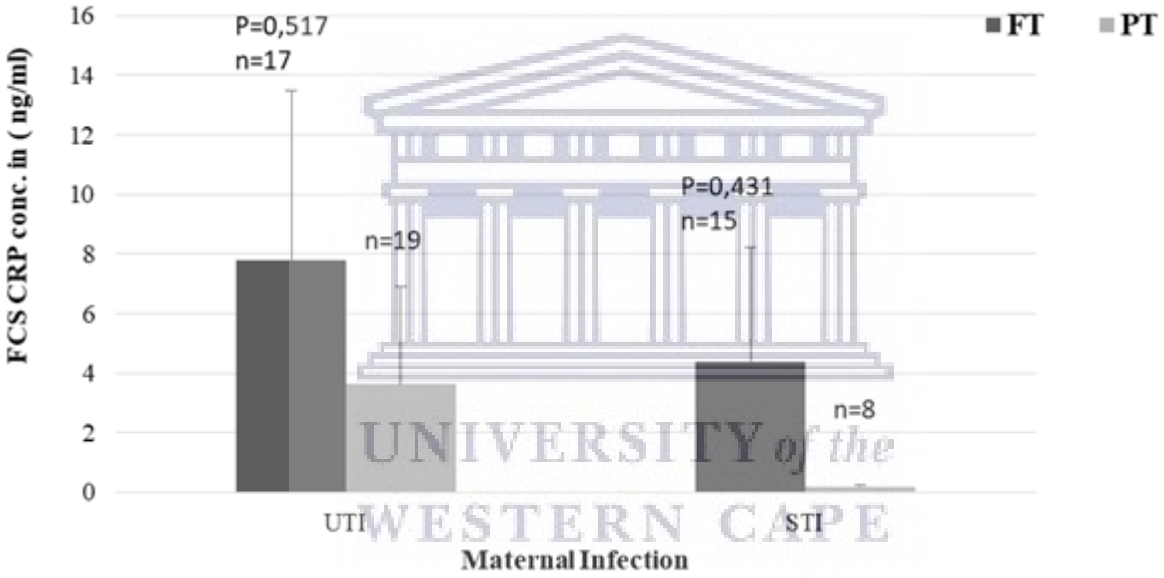


Figure 19: The relationship between maternal history of infection and mean FCS CRP concentrations with pregnancy outcomes. *P*-values represent the difference in CRP concentrations between (FT) full term and preterm (PT) samples within a particular group.

3.6.7 CRP and Infant Birthweight

3.6.7.1 Mean Maternal CRP

PT maternal mean CRP concentrations (89.85 (±3.86)ng/ml) appeared to be slightly higher than FT maternal mean CRP concentrations (79.29 (±8.34)ng/ml) for infants with normal birth weight (NBW) (Figure 20). No statistical significant difference (*P*= 0.788) was recorded for FT and PT mothers who delivered a baby with NBW.

FT mothers who delivered a baby with low birth weight (LBW) were seen to have a higher mean CRP concentration (68.09 (±28.72)ng/ml) than PT mothers who delivered a baby with LBW (39.64 (±22.13)ng/ml). No statistical significant difference (*P*=0.232) was seen between the mean CRP concentrations of the FT and PT of mothers who delivered a baby with LBW (Figure 20).

Although more FT mothers delivered LBW infants than PT mothers, babies who were born with very low birth weight (VLBW) or an extremely low birth weight (ELBW) only occurred within the PT group (Figure 20). The highest mean CRP concentration (83.03 (±6.64)ng/ml) was observed in the PT group of mothers who delivered VLBW infants. Only one mother delivered an ELBW infant with a birth weight of < 1000g which was included in the VLBW group.

No significant difference was noted between CRP levels of NBW and LBW infants in both FT (*P*=0,745) and PT (*P*=0.299) maternal groups.

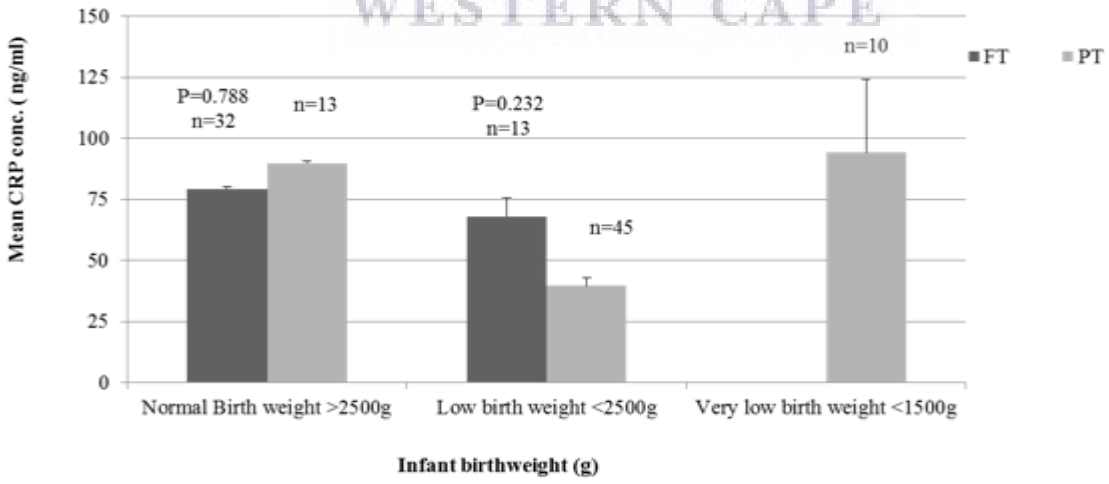


Figure 20 : The relationship between infant birth weight and mean maternal CRP concentrations for pregnancy outcomes. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group

3.6.7.2 Mean CRP in FCS

No statistical significant difference (*P*=0.694) was seen between the mean CRP concentrations of FT-FCS (2.28 (±0.94)ng/ml) and PT-FCS (1.52 (±0.86)ng/ml) of the babies with NBW or between the mean CRP concentration of FT-FCS (13.76 (±7.67)ng/ml) and PT-FCS (5.77 (±3.26)ng/ml) of the infants born with LBW (*P*=0.275) although FT-FCS from the LBW group had the highest mean CRP concentration of 13.76 (±7.67)ng/ml and the PT-FCS group of babies with NBW had the lowest CRP concentration of 1.52 (±0.86) ng/ml in the category.

A similarity was observed in the FT maternal and FCS CRP concentrations where both groups had higher CRP concentrations in the LBW group (Figures 20 and 21). In the NBW group, the FT-FCS group had a higher CRP concentration than the PT-FCS group (Figure 21).

Infants born FT with LBW showed the highest mean CRP concentration while in the PT-FCS group the highest CRP concentrations were seen in the LBW, followed by VLBW and then NBW. No significant difference was noted between CRP levels of NBW and LBW infants in both FT (*P*=0,160) and PT (*P*=0.213) FCS groups.

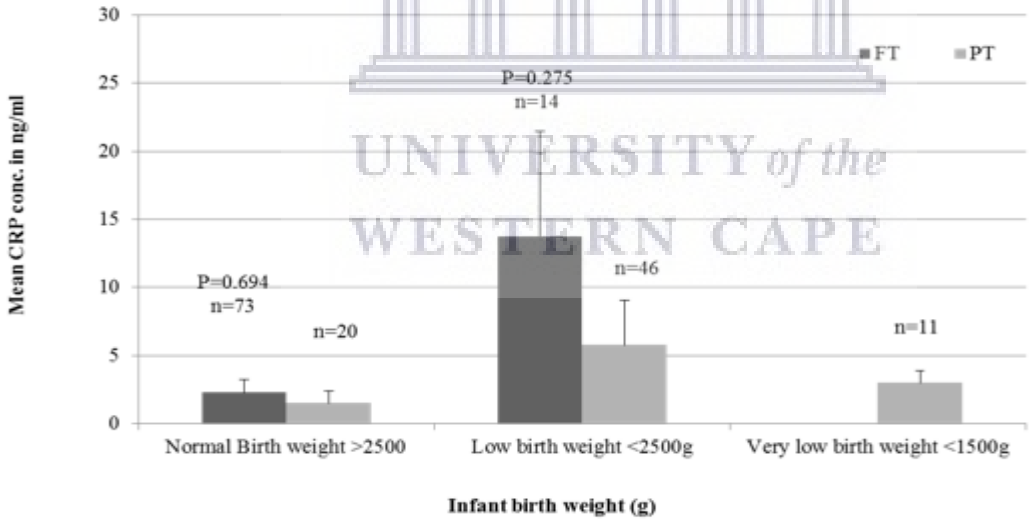


Figure 21: Infant birth weight and mean FCS CRP concentrations for the specific pregnancy outcomes. *P*-values represent the difference in CRP concentration between (FT) full term and preterm (PT) samples within a particular group

CHAPTER 4

DISCUSSION

During the first few weeks of a normal pregnancy, a mild systemic inflammatory reaction occurs in pregnant women when the implantation and development of the placenta takes place (von Versen-Hoeynck *et al.,* 2009), resulting in an increase of inflammatory markers such as CRP. There is a gradual increase in CRP throughout pregnancy (Belo *et al*., 2005), with some cases reporting a reduction during the mid-phase of pregnancy as the mother adapts to and tolerates the foetal antigens (Mor *et al*., 2011). An elevation or spike in CRP is again observed during labour, when the uterus is activated (Romero *et al.,* 2006; Erlebacher 2013).

Elevated CRP is widely used to provide evidence of subclinical infection or inflammation in women with symptoms of preterm labour and other pregnancy complications namely intrauterine infection, preeclampsia, miscarriage, PPROM, chorio-amnionitist and IUGR in women who have delivered preterm (Yoon *et al.,* 1996; Tjoa *et a*l., 2003; Pitiphat *et al*.,2005; Sorokin *et al*., 2010; Maguire *et al*., 2015; Vecchié *et al*., 2018). CRP finds application in reproductive medicine and neonatology to assess the risk for foetal growth restriction and neonatal complications such as PTD, LBW and small size for gestational age. Elevated maternal CRP values may also be indicative of a maternal inflammatory reaction to an underlying infection or a low-grade systemic inflammation (Ansar and Ghosh 2013).

Most studies are based on the finding that CRP increases as gestational age increases (Picklesimer *et al*,. 2008) and that at an upper quartile value of CRP, the risk of PTD increases. Additionally, the spike in pro-inflammatory cytokines responsible for the birthing process, along with the elevation of CRP, was reported to be much higher in PT cases in comparison to the concentration of these cytokines at FT for the relative gestational age (Ghezzi *et al*,. 2002; Belo *et al*., 2005; Pitiphat *et al*., 2005; Sorokin *et al*., 2010; Moghaddam Banaem *et al*., 2012).

The primary findings of this study yielded results where higher FT maternal mean CRP concentrations were observed, compared to the PT maternal mean concentrations. However, no statistically significant difference was seen between the different pregnancy outcomes (P= 0.300). Another study confirmed similar results where mothers with elevated CRP levels had decreased odds of pregnancy loss or spontaneous abortion (Boggess *et al*., 2005). The FCS

in this study showed a similar trend to the maternal serum analysis, where the FT-FCS levels were higher than the PT-FCS, but with no statistically significant difference (P=0.900).

Many studies of CRP in pregnant mothers have included mothers either at risk for, or already diagnosed with, an adverse pregnancy condition such as preeclampsia, PPROM and infection (Pitiphat *et al*., 2005; Moghaddam Banaem *et al*., 2012; Maguire *et al*., 2015; Kesrouani *et al*., 2015). These studies had similarities in parameters and results, although several other aspects were contradictory (Tjoa *et al.*, 2003; Hastie *et al.*, 2011; Moghaddam Banaem *et al*., 2012; Khairy *et al.*, 2012).

The parameters examined in the present study included maternal age, maternal weight, BMI, level of education, parity, infant birth weight and infection, all of which are reported to directly or indirectly influence the CRP concentration.

Factors that appeared to influence CRP concentrations and pregnancy outcomes in this study were the maternal weight and level of education categories, while the other proposed risk factors showed no association with the CRP levels nor pregnancy outcome. These results are supported by epidemiological data implicating certain social and environmental influences rather than genetic variation in pregnancy outcomes (Picklesimer *et al*., 2008).

Studies of the negative implications obesity has on pregnancy, have shown that although a high pre-pregnancy weight improved the chances of a healthy infant birth weight (Arrowsmith, Wray and Quenby 2011; Hirshberg, Levine and Srinivas 2014; Sharifzadeh *et al.*, 2014), it was also associated with late foetal death, while large babies born to overweight mothers required delivery induction (Sebire *et al*., 2001).

A significant difference was observed in this study between mothers delivering FT and PT in the 56-60kg weight group. It is not clear why the FT group had a higher CRP level but since they were found to have no underlying medical conditions, and since a higher CRP concentration can be beneficial to sustaining a pregnancy, we speculate that being of ideal weight, the elevated CRP levels in these mothers maintained the pregnancy to FT or that the elevated levels were due to the activation of the uterus during the birthing process.

Being overweight or obese has been associated with uterine quiescence or a suppression of myometrial activity. There is a suggestion that obesity is linked to the reduction in myometrial contractility. Reasons why overweight women may have higher CRP levels include induction of labour due to prolonged pregnancy (Arrowsmith, Wray and Quenby 2011) and a lengthy

labour period (Hirshberg, Levine and Srinivas 2014). These reasons could possibly explain the higher CRP levels in the FT maternal and FT-FCS, as opposed to the PT maternal and PT-FCS groups.

In the present study, no significant correlation was found between pregnancy outcomes of the various BMI and CRP concentrations in mothers and the FCS, just as in the Cohen *et al* (2014) study which showed that CRP was influenced by pregnancy status (whether normal, extra uterine or delayed abortion) and not age, BMI or gravidity.

As previously mentioned, the effects of a high or low BMI are similar to the effects experienced by mothers that are under or overweight. Mothers with high BMIs have a higher risk of their infant being born abnormally large or in some cases, induction of delivery whereas mothers with a low BMI have a greater chance of PTD as well as other adverse pregnancy effects such as LBW (Mokuolu, Abdul and Adesiyun 2002; Sharifzadeh *et al.,* 2014). The adverse pregnancy effects in mothers with high BMI are reported to be due to the build-up of lipids in the placenta and the infiltration of macrophages as well as reduced blood flow to the foetus resulting in hypoxia and inflammation of the placenta (Wilson and Messaoudi 2015).

Many of the mothers would be considered overweight if maternal weight was the only anthropometric measurement taken into account. When BMI is factored in, most of the mothers were considered proportional to their height. These two factors were investigated separately and yielded different results with regard to CRP levels in the mothers.

The other factor that showed a significant result was the effect of maternal education. CRP levels differed significantly when mothers with different levels of education were compared. Although the highest mean CRP concentration came from the group of mothers with no formal education, mothers with tertiary education had very low levels of CRP. FCS on the other hand showed no significance or trend when different levels of education were compared.

The literature supports the findings of this study namely, the correlation between mothers with low or no formal education and an increased CRP levels and adverse pregnancy outcomes (Ernst *et al.,* 2011). Mothers with a lack of formal education are more likely to be unaware of how their actions/behaviour or surroundings may affect their pregnancy. Unemployment in combination with an unplanned pregnancy have been associated with increased levels of maternal stress, which, in turn, can affect changes in hormones and neuropeptides such as prolactin, progesterone and oxytocin, all of which are implicated in the timing of the delivery

(Olson *et al.,* 2016; Bülbül *et al.*,2018). The findings of this study showed that even though maternal CRP levels were elevated in mothers who lacked education, it did not increase their risk of PTD.

Of the other parameters that were investigated in the study, no association between pregnancy outcomes and maternal age, infant birth weight, parity, and a history of STIs and recurrent UTIs were observed in the present study, nor were any statistically significant differences observed between mean CRP concentrations for FT and PT mothers.

Advanced maternal age has been associated with adverse reproductive outcomes, such as infertility, impaired foetal growth, stillbirth, chromosomal and congenital abnormalities, PTD and even multiple births (Menon 2008; Frederiksen *et al*., 2018). These associations have been linked to possible deterioration of the uterus vascular and placental function (Gillman *et al*., 2004). Women that are older and pregnant also have a greater risk of an adverse pregnancy outcome and therefore tend to stress more about losing their baby (Bülbül *et al*., 2018). Studies have shown that the aging process in the ovaries increases the rate of meiotic non-disjunction causing miscarriage and chromosomal abnormalities, as well as placental dysfunction and co-morbidities that occur with the aging process (Frederiksen *et al*., 2018). Age did not show any significant effect on pregnancy outcomes of mothers older than 36 years in this study, even though PT mothers have been reported to have a slightly higher mean CRP concentration than FT mothers (Pitiphat *et al.,* 2005). The highest CRP concentration found in the youngest age range (< 20years), may be attributed to the high CRP values from the two underweight and one overweight mother. More information would be needed in order to more clearly establish the effects of CRP levels in younger mothers (< 20years) on pregnancy outcome.

The inverse relationship between the maternal and FCS CRP concentrations observed in the 20-25 and 26-30 year age groups in this study, could be explained by other findings which showed that CRP either gets transferred or accumulates in the foetus, independent of the amount within the maternal serum (Fink *et al.,* 2019) or that the placenta produces and releases its own CRP into the maternal circulation (Malek *et al.,* 2006). The high CRP concentration in the maternal serum could possibly have stressed the foetus resulting in an activated immune response, which increased CRP levels within the foetus. In the study conducted by Cohen *et al* (2014) the mean concentration of CRP at the various maternal ages did not affect the outcome of the pregnancy, nor was there a trend between maternal age and pregnancy outcome within this study population.

Intra uterine growth restriction due to elevated CRP has been associated with lowered birth weight at FT (Ertas *et al.*,2010). In this study, no significant difference was seen between PT and FT groups when comparing concentrations of maternal CRP and foetal cord CRP with the infant's birth weight.

Although more mothers delivered NBW infants at FT than PT, those mothers who delivered PT infants with a NBW had a higher mean CRP concentration than the mothers who delivered FT, while the mean CRP concentration was higher in mothers who delivered FT infants with LBW than mothers who delivered PT infants with LBW. One of the FT mothers had a high CRP concentration and reported a history of recurrent UTIs, but no definite reason can be proposed for the other mothers with high CRP levels. Of the three mothers from the PT group with the highest mean CRP value, two of the mothers were < 20 years old, one of whom also had a history of recurrent UTIs. The slightly elevated CRP in FT cases with NBW could possibly be explained by CRP and other cytokines acting as an advantage for attempting to prolong the pregnancy (during a potential PT case) and allowing for growth of the foetus (in situations where the foetus might be underweight). The need for an immune reaction to sustain the pregnancy in times of stress remains evident, in cases of relatively higher CRP during extreme PT.

The FCS data showed a relatively similar trend where mothers who delivered an infant at FT with a LBW had the highest CRP concentration in the group followed by the concentration of PT-FCS with LBW. This could possibly be due to one mother having an abnormally high CRP concentration. The highest CRP levels of the FT-FCS for the infants with LBW belonged to mothers that had a history of recurrent UTIs, who had high levels of CRP as well.

According to previous studies, mothers with infections have highly elevated CRP levels which potentially increase the risk of PT birth (Goldberg *et al.*, 2008; Nakishbandy and Barawi 2014). In addition, infant CRP levels were reported independent of infection and shown to be at its highest at 48 hours after birth (Perrone *et al*., 2018; Macallister *et al*., 2019). Chronic infection such as human immunodeficiency virus 1 (HIV-1) and hepatitis B can lead to higher cytokine concentrations in the foetal cord blood and change foetal immune responses (Yockey and Iwasaki 2018). The majority of mothers with a history of STI in the study had HIV, followed by gonorrhoea, candidiasis and one case of trichomoniasis. Mothers who reported prior STIs most likely treated their infection resulting in a reduced immune response. For example, anti-retroviral therapy is shown to reduce systemic inflammation and immune action in patients

(Hileman and Funderburg 2017). An earlier study revealed that mothers who have completed a course of antibiotics intra partum, showed significantly lower levels of CRP in the infant (Macallister *et al*., 2019). This could explain why the foetus did not have highly elevated CRP levels during this perilous period. Another study found that the CRP levels in the PT foetal cord blood was lower than the baseline value during infection in FT cord blood and that an increase in CRP only occurred 2 -3 days after birth (Hofer, Müller and Resch 2013).

The present study's results revealed that mothers who experience a history of recurrent UTIs had a much higher CRP concentration than those with a history of STIs. A study carried out by Peltola, Mertsola and Ruuskanen (2006) confirmed that bacterial infections tend to produce a higher concentration of white blood cells and CRP than viral infections. One could possibly associate the number of UTI cases with a higher inflammatory response, due to the fact that UTIs are mainly bacterial in origin as opposed to viral UTIs. When comparing patient data, it was found that the FT mothers with the highest CRP concentrations contracted recurrent UTIs, had no education and were underweight, thereby demonstrating that a combination of factors along with infection may influence the maternal CRP concentration. However, whether or not they reported a history of recurrent UTIs or STIs, their pregnancy outcomes did not differ significantly.

There is much inconsistency regarding the association of parity with PTD as well as with CRP levels. Sacks *et al* (2004) found no significant differences in parity and CRP levels, nor has an association been found between the influence of parity and CRP levels with PTD (Ernst *et al*., 2011; Cohen *et al.,* 2014). A number of authors reported a significant and direct association with parity and CRP levels in women with an association between low parity and spontaneous PTD (Mokuolu, Abdul and Adesiyun 2002; Shah 2010; Nazmi *et al.,* 2008), while Shaikh *et al* (2011) showed an association between high parity and PTD.

Although in this study, no visible trend was observed when comparing CRP concentrations of mothers who delivered their children at FT and PT, mothers who delivered their first-born child FT had a higher mean CRP level compared to the PT mothers. This could possibly have been influenced by the high CRP concentrations found in samples from two mothers who were relatively normal and healthy with the exception of bad oral hygiene reported in the questionnaire. Evidence exists for the association of bad oral hygiene and STI resulting in elevated inflammatory cytokines during pregnancy (Sharma *et al.,* 2009). Likewise, the high CRP concentration in a mother who reported a STI may have strongly influenced the high CRP levels reported in mothers who delivered PT and had three children.

Furthermore, the FCS analysis showed a different pattern where FT-CRP of mothers who delivered their 2nd, 3rd and 4th and subsequent child had higher concentrations of CRP than PT-FCS, whereas mothers who delivered their first child at PT had the highest FCS CRP concentration. With no statistically significant difference between the parity groups and pregnancy outcome, we cannot clearly associate parity with maternal CRP concentration nor the FCS CRP concentration with the pregnancy outcome, although it appears that women with great parity tend to be older and more likely to be in lower socioeconomic classes.

The differences experienced from one pregnancy to another have been reported by many mothers including the presence or absence of severe morning sickness (Sostre, Varma and Sostre 2008). This response during the first pregnancy could possibly be an elevated immune response to the new physiological changes to the mother's body or a heightened reaction could have occurred during the second and subsequent pregnancy. These physiological changes occur throughout a women's life as she ages and therefore each pregnancy experienced will not be identical (Louik *et al.,* 2006).

Numerous studies have been conducted on the use of FCS to either assess the health (genetic disorders and blood analysis) of the baby or the use of the umbilical components, such as haematopoietic stem cell transplantation/cord banking (Food and Drug Administration, 30 July 2014). In this study, the FCS samples provided insight into the delivery process, where it was possible to determine whether the FCS CRP was elevated or not at the time of birth and if the outcome of the pregnancy showed any variation.

Many of the FCS samples were below the level of detection and the FCS samples that were positive for CRP could possibly have been taken at a time when the integrity of the placenta was diminished or the immunological privilege discontinued during delivery. Depending on the foetal requirements, permeability of the placenta may alter towards cytokines at various stages of pregnancy, as well as during chorio-decidual infection and the onset of labour (Aaltonen *et al.*,2005; Fink *et al.*,2019).

FCS CRP is produced by the foetus within the placenta and an elevation of CRP in umbilical cord blood is caused by a neonatal infection (in some cases) or other clinical pathologies such as chorion-amnionitis (Hofer, Müller and Resch 2013; Malek *et al.*, 2006). In the study by Bartkeviciene *et al* (2015), the authors found a close relationship between the inflammatory markers in mothers and foetal inflammatory response syndrome (FIRS). Intra uterine infections have been associated with the elevation of CRP in mothers eliciting FIRS and in turn increasing proinflammatory cytokines in the umbilical blood (Bartkevičienė *et al.,* 2015). Small amounts of CRP are able to cross the placenta (Hofer, Müller and Resch 2013). Only a small fraction of inflammatory cytokines are capable of transplacental movement such as; IL-8 (59kda with a 67 kda subunit), IL-1β and IL-6 (21-28Kda) (Zhang and Chen.2002 ;Tanaka, Narazaki and Kishimoto, 2014; Kitano *et al.*, 2019), all of which have a relatively smaller molecular mass than CRP with a molecular mass of 106kd (Ghezzi *et al*., 2002).

There is a lack of data on human placental CRP transference, and that which exists is often conflicting. When uterus contractions are initiated, white blood cells infiltrate the maternal tissue, placental and foetal membranes. These white blood cells (macrophages and neutrophiles) allow the progression of labour and are involved with the production of cytokines in the respective tissues. Furthermore, receptors for the various cytokines are expressed in the placenta thus, both sources and targets of cytokines are present in the placenta (Farina and Winkelman 2005; Raghupathy 2013).

The lower CRP levels in PT infants may be due to the PT infant's liver not being able to produce sufficient CRP at the time and since infant weight has been associated with CRP production, infants who weigh < 2500g were shown to produce a smaller rise in CRP (Ishibashi *et al.*, 2002). Another possibility could be that CRP is responding as an anti-inflammatory mediator eliciting an immune-suppressive function that protects the foetal allograph (Szukiewicz 2012).

As pointed out earlier, most of the evidence points to CRP being highly elevated during infection or various medical complications such as, PPROM, preeclampsia or PTD. The converse was observed in this study where the control groups had a higher mean CRP concentration than the PT groups. This phenomenon was seen in the majority of risk factors investigated. The increased CRP levels may be attributed to the physiological changes in mothers that tend to be beneficial to the growth and survival of the infant. This is supported by reports of reduced CRP levels in PT cases, (Chiesa *et al.*, 2011). Higher CRP levels in FT-FCS may also be attributed to the size or gestational age of the foetus, as developed/ functional infant livers are able to produce more CRP than PT infants. High CRP levels do not necessarily indicate early onset neonatal sepsis or bacteraemia, but could be associated with high immature neutrophil and procalcitonin levels in umbilical cord blood (Kitano *et al*.,2019).

It has been suggested that cytokine function (physiological and pathophysiological) may be modified by ethnicity (Menon 2008). The results of Menon (2008) could possibly illustrate an inflammatory corrective balance within the immune systems of black African mothers that might differ from one population to another. However, this is negated by epidemiological literature stating that race and ethnicity are governed by certain social and environmental influences rather than by variation in the genetics of the population per se (Picklesimer *et al*., 2008). CRP could mediate an anti-inflammatory response by modulating neutrophil recruitment. The study by Belo *et al (*2005) indicated that CRP levels vary greatly between mothers throughout a normal pregnancy with some mothers showing increased CRP levels and others showing decreased levels.

These findings suggest that the relation between maternal CRP levels and PTD is strongly confounded by maternal socioeconomic and health-related factors such as educational level, medical history and maternal weight.

STRENGTHS AND LIMITATIONS OF THIS STUDY

What makes this study different is that the CRP concentrations were much lower than most studies conducted on CRP, with some studies reporting concentrations as high as 16mg/L (Huang *et al*., 2020) or 8mg/L (Pitiphat *et al*., 2005), and others reporting lower values such as 4.5mg/L (Ernst *et al.,* 2011), 1.53mg/ml (Maguire et al., 2015) or 1.86µg/ml (Vecchie *et al.,* 2018).

There is no standardised study with which to compare all the variables. For example, in some studies the site from which the sample was taken differed. Placental and decidual cell cultures yield higher concentrations of CRP than sera and blood (Rewatkar *et al*., 2018), while other studies make use of amniotic fluid, which might yield a lower concentration, depending on the time of gestation and intact membranes (Moghaddam Banaem *et al*., 2012; Kesrouani *et al*., 2016). In addition, the way in which the CRP concentration is calculated may also differ from study to study for example, Hastie *et al* (2011) transformed CRP concentrations using $\log^2$ to normalise the distribution.

Importantly, the majority of studies conducted on CRP were done in developed countries in the Northern hemisphere (Pitiphat *et al*., 2005; Ernst *et al*., 2011; Vecchie *et al*., 2018; Huang *et al*., 2020), unlike this and other studies from underdeveloped, poorly resourced countries in the sub-Saharan region which are over-populated, with a high unemployment rate, plagued by parasites, HIV/AIDS and malaria outbreaks, famine and unfavorable basic services such as access to adequate educational opportunities, medical care and living conditions.

The samples used in the present study came from a population of black Rwandan mothers, many of whom lack education and are unemployed, or live in rural settlements without proper sanitation. Numerous families are still affected by the civil war and genocide that took place with a number of victims still struggling with some degree of post-traumatic stress disorder (PTSD). While the country is burdened with many challenges, maternal mortality, reproductive health and the increase in PTD tend to be less prioritised. The collaboration between the teaching hospital and the University of the Western Cape made it convenient to obtain these valuable samples from a population of mothers in Butare.

Among the limitations of this study are the fact that many samples were omitted from analysis due to incomplete patient history. Due to factors beyond control, such as the removal of samples from storage in the laboratories by third parties without warning, some samples were

lost and there was also a lack of corresponding samples (maternal and foetal cord blood from the same patient) which resulted in an unequal number of complementary samples. It is possible that the small sample size may have affected the categorisation into subgroups of variables thereby affecting the statistical analyses.

Other limitations of the study include insufficient patient information with regard to the type of birth or the duration of the labour and delivery, which could also possibly have influenced the cytokine levels. Since samples were collected post-delivery only, there was no base line value of CRP during pregnancy with which to compare the CRP concentration. Having a baseline value would have assisted in determining a causality relationship between the CRP levels and the risk for adverse pregnancy outcomes.

It was not possible to investigate gravidity as a risk factor and the effects of previous induced abortions and spontaneous abortions due to numerous mothers giving contradictory information about the stage of their termination.

Lastly, maternal samples were taken 48 hours after birth and FCS at birth. Maternal blood samples were collected after delivery to avoid inconvenience to the mother. However, it can be conceded that collecting the samples 48 hours after delivery might not be ideal, considering that metabolic and healing processes differ from one person to the next and need to be taken into account, when interpreting the cytokine concentrations (Ganeshan and Chawla 2014; Buck, O'Sullivan, and Pearce 2015). However, in the infant, CRP levels were previously shown to increase 48 hours post-delivery (Perrone *et al.*,2018) and since all maternal samples were collected at 48 hours without exception, the 48-hour sample collection was considered acceptable since they were all collected within the same time period.

CONCLUSION

CRP has been widely researched as a biomarker in the diagnosis of many medical conditions and yet there is still uncertainty as to whether it could be considered as an effective predictive marker for high-risk pregnancies.

Based on the results of this study, it would appear that elevated CRP concentration was not a helpful predictive biomarker for adverse pregnancy outcomes, more specifically for PTD.

These results are not consistent with the hypothesis of the study that elevated CRP levels are correlated with the risk of PTD. There are several elements that affect CRP concentration and taking into account the sensitivity of the acute phase marker it appears that CRP has to be used in conjunction with other markers to correctly determine the risk of PTD.

RECOMMENDATIONS

Multiple blood samples taken at different trimesters (before, during and after pregnancy) will allow for the trend of the CRP concentrations throughout the pregnancy to be tracked, thus indicating whether these fluctuations are natural or sporadic while also explaining the reasons behind it.

Although no statistical significance was observed in the present study regarding CRP and pregnancy outcome, further studies are needed in order to determine causality of the CRP levels and whether the use of an additional biomarker could distinguish between infected and uninfected infants and strengthen the investigation into predicting high risk pregnancy within this and other population groups.

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APPENDIX

APPENDIX TO CHAPTER 2 (A)

Consent Form for Participation In Research Project

Title of Project: **The of Role of C - Reactive Protein as a marker for Preterm Delivery**

Names of Researchers: **Vermeulen MP; Africa CWJ**

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 (BLOCK CAPITALS)	Date	Signature

……	….……	…….
Name of person taking consent	Date	Signature

……	……	……
Name of researcher	Date	Signature

THANK YOU FOR AGREEING TO PARTICIPATE IN THIS RESEARCH

Health Risk Assessment Questionnaire

APPENDIX TO CHAPTER 2 (B)

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?	**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) How long has it been?	(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 diabetic? 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	**14.** Do you have easy access to medical or dental care?

(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	Yes / No
15. Do you have medical insurance? Yes / No	**16.** (i) How often do you have a drink containing 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