

Evaluation of Neutrophil Extracellular Trap Production in Pregnancy

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List of Abbreviations

20α HSD	20α Hydroxysteroid Dehydrogenase
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CBL	Cord Blood Leukocytes
CB	Cord Blood
CD	Cluster of Differentiation
cfDNA	Cell-free Deoxyribonucleic acid
CR	Complement Receptor
DAMP	Damage Associated Molecular Pattern
DNA	Deoxyribonucleic Acid
DPI	Diphenyliodonium chloride
EDTA	Ethylenediaminetetraacetic acid
El LSCS	Elective Lower Segment Caesarean Section
ERK	Extracellular Signal-Regulated Kinase
ET	Extracellular Trap
GBS	Group B Streptococcus (*Streprococcus agalactiae*)
H3	Histone 3
HBSS	Hanks' Balanced Salt Solution
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HVS	High Vaginal Swab
IL	Interleukin
iPTB	Initiated Preterm Birth
IV	Intervillous

KIR	Killer Immunoglobulin-like Receptor
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MB	Maternal Blood
MBL	Maternal Blood Leukocytes
MEK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MNase	Micrococcal Nuclease
mtDNA	Mitochondrial Deoxyribonucleic acid
MPO	Myeloperoxidase
NAC	N-acetyl L-cysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NE	Neutrophil Elastase
NF	Nuclear Factor
NET	Neutrophil Extracellular Trap
NLR	NOD-like Receptor
NMeoSuc-*p*Na	N-methoxysuccinyl-Ala-Ala-Pro-Val *p*-Nitroanilide,
NK	Natural Killer
PAD	Peptidyl Arginine deiminase
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PET	Pre-eclampsia or Pre-eclamptic
PGN	Peptidoglycan
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate

PMN	Polymorphonuclear Leukocyte
PPROM	Preterm Pre-labour Rupture of Membranes
PR	Progesterone Receptor
PTB	Preterm Birth
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
sPTB	Spontaneous Preterm Birth
sPTL	Spontaneous Preterm Labour
STB	Syncytiotrophoblast
STBM	Syncytiotrophoblast Microparticle
T_h	T helper
TNF	Tumour Necrosis Factor
TLR	Toll-like Receptor
TReg	Regulatory T-Cell
vWF	von Willebrand Factor
WHO	World Health Organisation

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1 Abstract

Introduction

During pregnancy there is a unique problem for the maternal immune system in supporting the development of a non-allogenic foetus whilst maintaining an effective immune response to invading pathogens. This is usually achieved through the development of an anti-inflammatory immune phenotype. A novel mechanism of bacterial killing, the formation of neutrophil extracellular traps (NETs), is characterised by the release of an extracellular mesh of DNA, histones and granule proteins. However, this process also leads to local endothelial and epithelial damage, through exposure to NET components, resulting in inflammation. In 40% of cases of spontaneous preterm birth (sPTB), bacterial infection has been implicated and whether NETs play a role in the development of this syndrome is unknown. The aim of this study was to determine whether leukocytes from umbilical cord blood (CBL) and maternal blood (MBL) produced NETs and whether this process is altered in the presence of infection or preterm pre-labour rupture of membranes (PPROM).

Methods

CBL or MBL or were isolated from whole blood by dextran sedimentation. NET production was induced by stimulation with lipopolysaccharide (LPS), a polymer contained within the cell wall of gram-negative bacteria, Lipoteichoic acid (LTA) and peptidoglycan (PGN), both polymers contained within the cell wall of gram-positive bacteria. Phorbol 12-myristate-13-acetate (PMA), is a widely studied inducer of NET formation

and was used as a positive control. NET production was measured by a cell impermeable SYTOX® green assay. In CBL this was supplemented with immunofluorescence to determine the presence and localisation of neutrophil elastase (NE) and the digestion of NET structures with micrococcal nuclease (MNase), with NE concentrations in NET fragments determined.

Results

All of the ligands used stimulated the externalisation of DNA from CBL and MBL in a concentration dependent manner ($p<0.0001$). NET production was confirmed in CBL by visualisation of NE in extracellular structures emitting from the leukocytes. In CBL, there is increased NET formation with PGN stimulation where a maternal GBS infection is present than seen in controls ($p<0.0001$). In MBL from PPROM in the 2nd trimester, the NET response was heightened when compared with MBL at term with LTA, PGN and PMA stimulation ($p<0.05$). Comparisons made between CBL and MBL revealed that CBL are more sensitive to PMA stimulation ($p=0.0032$), whereas MBL are more sensitive to PGN stimulation ($p=0.0392$).

Discussion

Our observations demonstrate that NET formation occurs in CBL and MBL in response to LPS, LTA, PGN and PMA. Further, the level of NET production is altered with GBS infection (PGN) and PPROM (LTA, PGN and PMA). During pregnancy, NETs form part of the host-defence mechanism to infection and dysregulation of this process may be a novel component of sPTB.

2 Introduction

Pregnancy is normally a successful process by which a healthy baby is delivered at term. However in a number of cases pregnancy is associated with complications resulting in early delivery or health complications for the mother and/or the foetus. The cellular and molecular mechanisms involved in normal pregnancy and labour are poorly understood, with many elements still unknown. This makes any diseases affecting this precariously balanced system difficult to treat effectively. Several diseases of pregnancy have a multifactorial aetiology, therefore elucidating mechanisms and determining treatments has been challenging. In fact, today, many treatments that are in use, e.g. for preterm birth (PTB), only delay delivery for the administration of prophylactic assistance for the foetus, for example dexamethasone to help with lung maturity and surfactant levels. Given the lack of new biomarkers and treatments for pregnancy disorders, the need for research into understanding the underlying normal and pathophysiological mechanisms, particularly the interactions between maternal and foetal systems, remains.

The focus of my research is on a novel mechanism, first identified in 2004, whereby neutrophils expel DNA which is decorated with histones and granule proteins, resulting in the formation of an extracellular mesh that can trap and kill bacteria (Brinkmann et al., 2004). Known as neutrophil extracellular traps (NETs), these structures have since also been shown to be involved in a number of autoimmune and infective

disorders and to play a role in the development of pre-eclampsia (PET) (Gupta et al., 2005). The role of NETs in pregnancy and the pathophysiology of PTB, especially where infection is a confounding factor, is not known and is the purpose of my investigation.

2.1 Pregnancy and the Maternal Immune System

A successful pregnancy outcome relies on a number of factors commencing at implantation when the mother's physiological, endocrine and immune systems have to adapt in order to provide a hospitable environment to support the development of the foetus (Thellin and Heinen, 2003, Gomez-Lopez et al., 2014). In particular, the immune system has the uniquely difficult job of recognising a pregnancy and adapting to tolerate the semi-allogenic foetus and to also maintain an effective response to protect the mother from invading pathogens (Aagaard-Tillery et al., 2006). The immunology of pregnancy is of particular importance as many of the disorders associated with pregnancy are linked with an inflammatory response (Aagaard-Tillery et al., 2006, Romero et al., 2007). These diseases include PTB, specifically spontaneous preterm labour (sPTL), preterm pre-labour rupture of membranes (PPROM) and PET. In this section, the development of the placenta and adaptations of the maternal immune system in normal pregnancy are presented before proceeding to consider PTB.

2.1.2 Maternal Immune Adaptations: Controlling Inflammation

The immune system is complex and has two major arms: the **innate immune system,** which mounts non-specific responses to pathogens and the **adaptive immune system** which provides specific responses to previously encountered pathogens (Wood, 2011). Both of these factions have their own subset of leukocytes, each of which has a distinct role to play in the elimination of pathogens. The innate immune system specifically relies upon phagocytic cells to limit the spread of infection through phagocytosis and degranulation (Wood, 2011). Neutrophils at 50-70% (Martini, 2004) are the most numerous of all the leukocytes found in the adult circulation. Monocytes are another component of the innate immune response, where they have an active role in phagocytosis and cytokine production, but only account for 2-8% (Martini, 2004) of leukocytes seen in adults. Monocytes, once they migrate into tissues, can differentiate into macrophages. Natural Killer (NK) cells kill infected cells and non-self-tissue, as well as producing cytokines (Moffett-King, 2002) and have recently been proposed as a link between the innate and adaptive immune systems in pregnancy (Aagaard-Tillery et al., 2006). T and B lymphocytes account for 20-30% (Martini, 2004) of leukocytes and play an important role in the adaptive immune system by modulating the immune system (T lymphocytes) and the production of antibodies (B lymphocytes). The remaining cell types of the immune system include mast cells, basophils (<1%) and eosinophils (2-4%), which all play a role

in allergic responses and the elimination of parasitic and viral infections (Wood, 2011).

Adaptation of the maternal immune system must occur in order for the foetus to be able to develop in the uterus, which culminates in the development of an anti-inflammatory leukocyte profile and an overall level of hyporesponsiveness to foetal tissues (Gomez-Lopez et al., 2014). In particular interleukin (IL)-10 levels remain high (Romero et al., 2007), and this in combination with elevated levels of circulating hormones, particularly progesterone, throughout pregnancy help to maintain the anti-inflammatory profile (Thellin and Heinen, 2003, Robinson and Klein, 2012). The total number of leukocytes in the maternal circulation increases during pregnancy (Romero et al., 2007), which includes increases in the numbers of monocytes, particularly in the first trimester and lymphocytes in the third trimester (Chandra et al., 2012). However, it is unclear whether the proportions of leukocytes within the innate immune system change. It has been suggested that it is the phenotype of the cells that becomes altered, particularly in the case of monocytes and granulocytes (Romero et al., 2007). Neutrophils in particular have impaired chemotactic and phagocytic functions during pregnancy (Chandra et al., 2012). These adaptations are best characterised by a shift from reliance on the innate immune system and pro-inflammatory neutrophils to reliance on the adaptive arm of the immune system (Aagaard-Tillery et al., 2006). Of particular note is the shift in T helper (T_h) cell balance, relying more heavily on T_{h2} cells than

T_{h1} cells, which results in an anti-inflammatory profile (Thellin and Heinen, 2003), however this is a subject of controversy (Chaouat, 2007, Chaouat, 2003). Recent evidence has shown that this view is in fact too simplistic to fully explain the adaptation, particularly in the light of increased production of T_{h1} type cytokine IL-12 in pregnancy (Sacks et al., 2003) and increased roles for regulatory T cells (TReg) and T_{h17} cells (Saito et al., 2010, Gomez-Lopez et al., 2014).

Prior to conception the paternal foetal antigen, which is found in seminal plasma, activates the maternal immune system leading to the creation and proliferation of TRegs (Gomez-Lopez et al., 2014). The maintenance of TReg numbers throughout pregnancy provides continuing maternal tolerance to foetal antibodies (Gomez-Lopez et al., 2014). T_{h17} cells produce the inflammatory cytokine IL-17, and the number of these cells in peripheral blood does not alter during early pregnancy, only decreasing in the third trimester (Saito et al., 2010). However, in decidua T_{h17} cell numbers are elevated when compared to those seen in peripheral blood (Saito et al., 2010). This is particularly interesting as in experiments, IL-17 has been shown to increase both invasion and progesterone secretion in choriocarcinoma cell lines (Saito et al., 2010). As the subject of adaptive immunity in pregnancy is beyond the scope of this project it will not be discussed in any more detail here.

One of the earliest events that promotes tolerance to the foetus is that the syncytiotrophoblast (STB) tissue of the placenta, which comes into

direct contact with the maternal circulation is not recognised by the maternal immune system as foreign. This is because it does not display major histocompatibility complex (MHC) class I or II molecules on the cell surface (Thellin and Heinen, 2003, Broughton Pipkin, 2012). Instead it expresses human leukocyte antigen (HLA)-G, HLA-C and HLA-E, which protect the placenta from degradation by maternal natural killer (NK) cells (Thellin and Heinen, 2003).

NK cells are perhaps the most important cell type for the establishment of pregnancy and are the most numerous leukocyte type in the maternal decidua (70%) (Moffett-King, 2002) compared with around 30% in the maternal endometrium, during the late secretory phase (Moffett and Shreeve, 2015). Decidual NK cells are thought to be distinct to peripheral NK cells due to differences in cell surface markers (Koopman et al., 2003), as they express surface killer immunoglobulin-like receptors (KIRs) which bind with the HLA molecules expressed on the trophoblast. The HLA-C molecule and KIR receptors, each have two different phenotypes and in combination this determines whether the NK cells lose their killer activity (Broughton Pipkin, 2012). This loss of killer activity assists with the development of an anti-inflammatory immune profile and promotes trophoblast invasion (Thellin and Heinen, 2003, Broughton Pipkin, 2012). The combination of KIR and HLA-C phenotypes can also result in the maintenance of killer activity which limits trophoblast invasion and has been shown to cause miscarriage and PET (Broughton Pipkin, 2012).

Inflammation is involved with a great number of processes that occur in the female reproductive tract. These include ovulation, menstruation, implantation and parturition (Romero et al., 2007). During pregnancy it is important that the inflammatory responses are controlled as an exaggerated inflammatory response, whether this is systemic or localised can result in miscarriage, PTB or PET (Romero et al., 2007). This is aided by the adaptation of the maternal endocrine system, whereby the increased levels of progesterone, initially from the corpus luteum and later the placenta also provide a dampening of inflammatory responses (Thellin and Heinen, 2003). In most mammals there is a reduction in the level of progesterone in the maternal circulation prior to the onset of labour (Gomez-Lopez et al., 2014, Nadeem et al., 2016). However, in humans, progesterone levels remain high, although a functional progesterone withdrawal has been suggested (Mesiano, 2004, Nadeem et al., 2016). One particular hypothesis for this is related to the ratio of progesterone receptor (PR) isoforms, PRA and PRB in the myometrium (Mesiano, 2004, Nadeem et al., 2016). Throughout pregnancy PRB levels are high and this results in uterine quiescence, whereas during labour the ratio of PRA:PRB receptors rises. This results in a suppression of the PRB receptors, resulting in uterine contractions (Nadeem et al., 2016). Further to this, intracellular progesterone is being metabolised in the myometrium, by increased levels of 20α hydroxysteroid dehydrogenase (20αHSD). This increase in metabolism results in diminished progesterone concentrations in myometrial cells (Nadeem et al., 2016), which leads to reduced progesterone binding to PRAs. As

progesterone helps to maintain the anti-inflammatory profile of the maternal immune system, this interaction between reduction in progesterone binding and activation of the immune system, leading to increased inflammation results in the initiation of labour (Gomez-Lopez et al., 2014, Nadeem et al., 2016).

At the beginning of labour, there is an influx of neutrophils into the cervix and myometrium, specifically the lower segment, due to increasing IL-8 levels (Thomson et al., 1999). The neutrophils in the myometrium were found to be localised around blood vessels and the decidua (Thomson et al., 1999). It is thought that the release of collagenase and neutrophil elastase (NE) aid the process of tissue remodelling, primarily in the cervix, but also in the myometrium to aid delivery (Thomson et al., 1999). Neutrophils have also been identified in the foetal membranes during labour and have been implicated in membrane rupture (Gomez-Lopez et al., 2017). Macrophages also infiltrate the myometrium, but this occurs prior to the initiation of labour, whereas mast cell numbers reduce in pregnant myometrium (Thomson et al., 1999).

2.1.3 Placental Structure and Function

In the first weeks of pregnancy the blastocyst burrows into the uterine wall and the syncytiotrophoblast (STB) cells begin to invade into the uterine endometrium, making contact with small veins around day 15 (Huppertz and Kingdom, 2012). The STB continues to invade until it makes contact with the maternal spiral arteries where the extravillous

trophoblast tissue replaces and modifies the maternal endothelial cells in order to increase the size of the arteries, although initially the arteries remain plugged by placental tissue (Burton and Jauniaux, 2004, Huppertz and Kingdom, 2012). The placenta develops under hypoxic conditions, as an effective circulation is only established from 10-12 weeks of pregnancy onwards (Burton and Jauniaux, 2004). This is when connections are established with the maternal spiral arteries which allows the establishment of an adequate low volume blood supply to ensure gaseous and nutrient exchange is sufficient to support the developing foetus (Huppertz and Kingdom, 2012). The structure of a placenta at term is displayed in ***Figure 2.1.3***.

The foetus develops in the amniotic cavity, surrounded by amniotic fluid, which up until recently was believed to be sterile (Romero et al., 2007). The amniotic cavity is lined by the amnion and the chorion membranes which are avascular and provide the tensile strength required to contain amniotic fluid. Both amnion and chorion are sites of lipid and cytokine synthesis (Menon, 2008). The maternal endometrium differentiates into decidua which lines the uterus throughout pregnancy, to which the foetal membranes and placenta are attached. The decidua provides nutrition for the embryo in the early stages of development, before the placental blood supply is fully established. The decidua is also actively involved with the synthesis of prostaglandins and cytokines (Menon, 2008).

The only connection that the foetus has to the placenta is via the umbilical cord which consists of two arteries and one vein, and Wharton's jelly to provide structural integrity (Huppertz and Kingdom, 2012). Deoxygenated foetal blood flows through the two umbilical arteries towards the placenta, which spiral round the single centrally positioned umbilical vein, through which oxygenated blood flows back from the placenta to the foetus (Huppertz and Kingdom, 2012). The foetal blood supply branches over the chorionic plate of the placenta, which can be observed in ***Figure 2.1.3***, before extending into tree-like structures, to form cotyledons. The vessels which are very fine spread through the STB of the placenta and maternal blood fills the intervillous (IV) space (Huppertz and Kingdom, 2012). Gaseous, nutrient and waste exchange take place at the maternofoetal interface, where oxygen within the maternal blood in the IV space is exchanged across the very thin villous membranes. A key feature of human placentation is that the maternal and foetal blood supplies rarely mix (Huppertz and Kingdom, 2012).

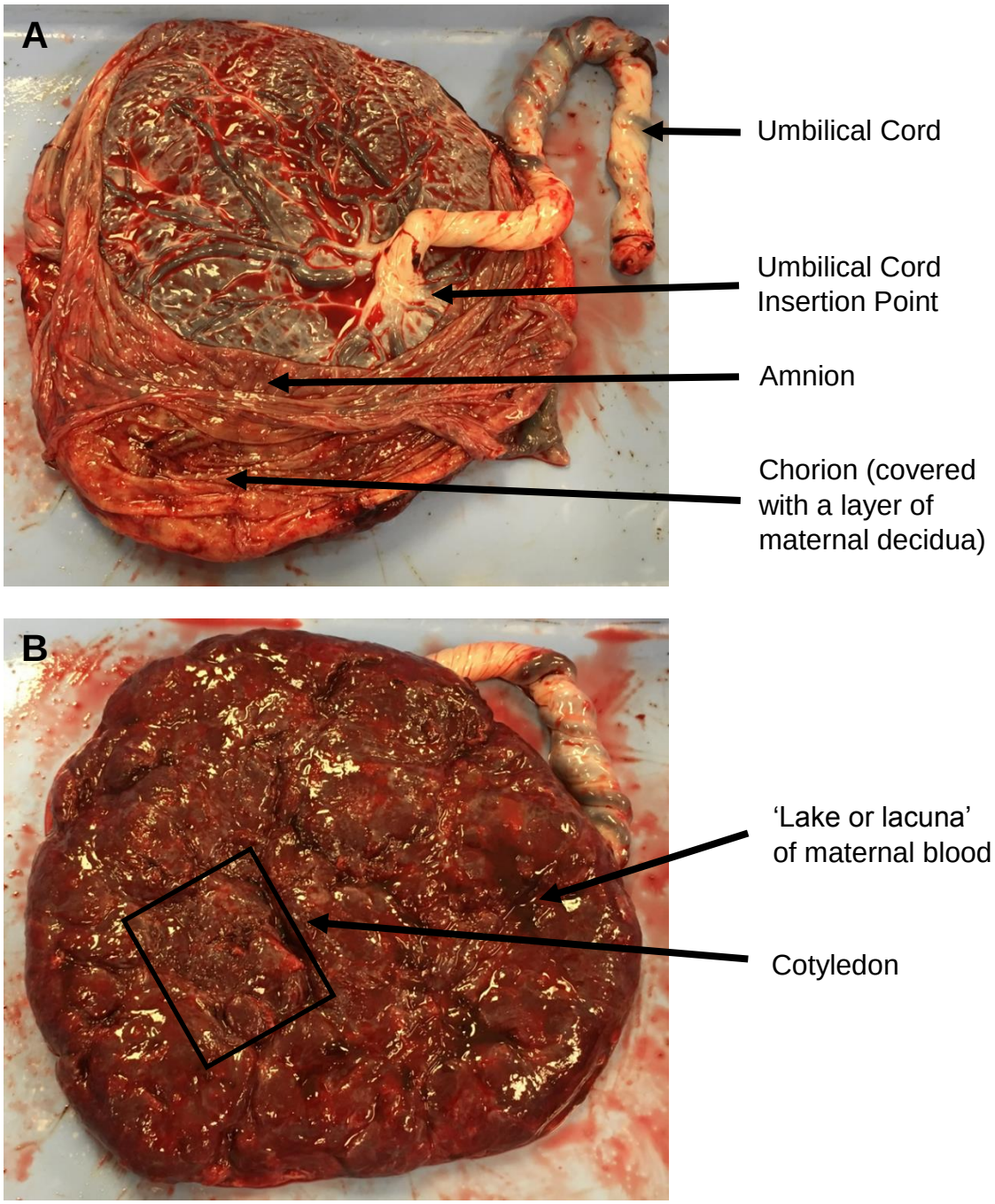

Figure 2.1.3 Structure of the Placenta

The above figure shows the structure of the foetal (A) and the maternal (B) sides of the term placenta. On the foetal side, the chorionic plate and the position of the umbilical cord insertion can be observed, along with the amnion, and choriodecidua. On the maternal side of the placenta the villous tissue, which is formed from syncytiotrophoblast arranges into 12-15 cotyledons which are seen covered in a layer of maternal decidua.

2.2 Preterm Birth

The mechanisms that exist in poor pregnancy outcomes are diverse, with some present even before a pregnancy occurs. In early pregnancy the mother's immune system has undergone adaptations to accommodate the foetus. During labour at term, increased leukocyte trafficking to the uterus is seen, characterised by an influx of neutrophils. Disturbances to this delicate balance result in pregnancy complications, one of which is PTB, the epidemiology and causes of which are discussed below.

2.2.1 Epidemiology of Preterm birth

One of the leading pregnancy complications is PTB, which is defined by the World Health Organisation (WHO) as delivery that occurs before 37 weeks of gestation (Blencowe et al., 2012, Howson et al., 2013). PTB affects 15 million pregnancies worldwide each year, and data in 2010 highlighted that this results in over 1.1 million neonatal deaths (Blencowe et al., 2013). PTB can either be of spontaneous onset (sPTB), which is either caused by sPTL or PPROM or provider-initiated (iPTB) when the neonate requires early delivery, via induction or caesarean section before the natural beginning of labour (Goldenberg et al., 2008). This is usually performed as the result of maternal or foetal health problems, for example PET (Goldenberg et al., 2008) (See **Figure 2.2.1**).

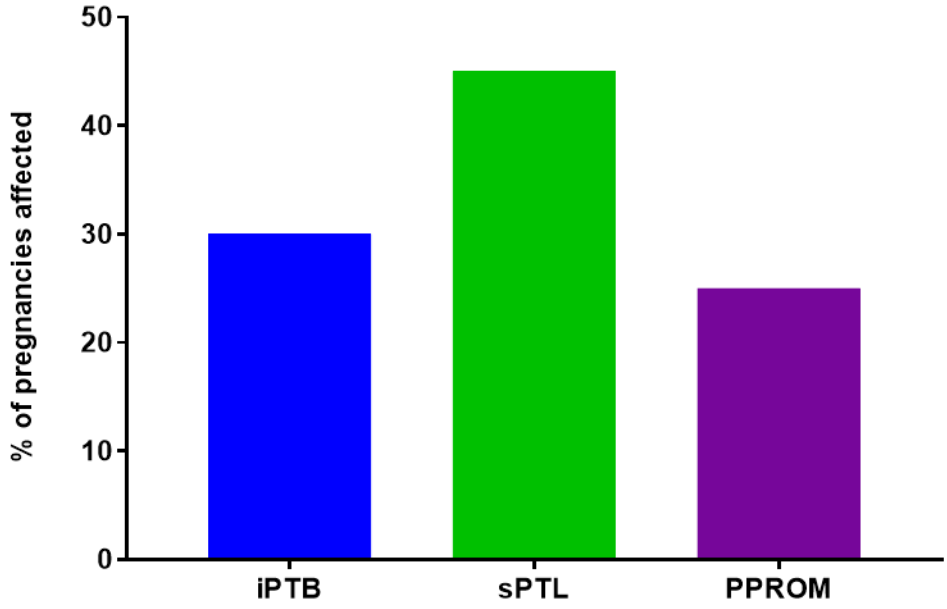

Figure 2.2.1 Mechanisms of Preterm Birth

The proportion of preterm births as a result of either indicated (iPTB) or spontaneous (sPTB) preterm birth. sPTB is split into either spontaneous preterm labour (sPTL) or preterm pre-labour rupture of membranes (PPROM). Figure Adapted from Goldenberg et al. (2008).

Although the causes of sPTB are varied and are still being investigated, there is a genetic component, with sPTL being more common in Caucasian women and PPROM more common amongst Black women (Menon, 2008, Goldenberg et al., 2008). Infection, whether clinical or sub-clinical is a major cause of sPTB, and is implicated in as many as 40% of cases (Menon, 2008, Goldenberg et al., 2008). In fact a precursor of PPROM is often an asymptomatic uterine infection, which often ascends from the vagina into the uterus, resulting in chorioamnionitis or in infection of the previously stable amniotic cavity, which can go on to infect the foetus in 10% of cases (Romero et al., 2007, Goldenberg et al.,

2008). The bacteria most commonly associated with PTB are *Ureaplasma urealyticum*, *Gardnerella vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae,* the vast majority of which are gram-negative organisms (Kenyon et al., 2001), however Group B Streptococcus (GBS – *Streptococcus agalactiae*) has also been implicated. Other infections have also been associated with PTB, including, syphilis and periodontal disease, alongside malaria and human immunodeficiency virus (HIV) (Blencowe et al., 2013). Other causes of PTB include cervical insufficiency, due to a short cervix or previous treatment of cervical abnormalities, smoking, stress and depression (Goldenberg et al., 2008). The common factor among infection, smoking, stress and depression is that there is increased inflammation and increased levels of inflammatory markers seen in the maternal circulation (Goldenberg et al., 2008). This points towards activation of the maternal innate immune system as these factors would activate a non-specific immune response.

One predictor of preterm delivery is having a previous child that was born preterm, a group which nulliparous women will not fall into. Women with a previous preterm delivery are often offered greater screening using foetal fibronectin and cervical length measurements to help predict the timing of prophylactic therapies, although outside of this high risk group there is no screening at all as the current techniques are not specific enough (Menon, 2008). It is of particular note that there are no current treatments that are successful in the prevention of sPTL or PPROM. The most widely used therapy for the delay of delivery in groups at high risk

of PTB is progesterone (Norman et al., 2016), however in a large clinical trial progesterone prophylaxis has been shown to be ineffective in preventing PTB (Norman et al., 2016). Antibiotics are administered in cases where an infection has been identified or one is suspected. However antibiotics do not inhibit the onset of PTB (Kenyon et al., 2001) The administration of antibiotics is thought to confer an advantage to the neonate as this in theory helps to prevent neonatal sepsis, by reducing the likelihood of infections ascending up into the uterus from the vagina (Mercer, 2003). This is particularly important after PPROM as these babies are twice more likely to suffer from neonatal sepsis than those delved after sPTL (Mercer, 2003), although how effective antibiotics are in preventing this bacterial spread is still under debate. In cases of sPTL, tocolytic drugs, such as nifedipine are often used (NICE, 2011), with the aim of delaying labour until drugs can be administered that help development of foetal systems. This includes the use of corticosteroids, such as dexamethasone to aid lung maturation (Mercer, 2003, NICE, 2011) and increasingly magnesium sulphate used to provide neuroprotection in preterm infants (NICE, 2011, Gano et al., 2016). However none of these target the root cause on the initiation of PTB, which as yet is still unknown.

2.2.2 Foetal outcomes of preterm birth

The earlier in gestation that the delivery occurs, the poorer the outcome for the neonate. The Epicure-2 study revealed that only 2% of infants born alive at 22 weeks survive, whereas 77% of infants born alive at 26

weeks survive (Costeloe et al., 2012). For these babies that are born too soon there are a myriad of possible immediate health implications. Interestingly male babies (55%) are more likely to be born preterm than female babies (45%) and male babies are more likely to have poorer outcomes (Blencowe et al., 2013, Lawn et al., 2014). In advanced economies, where neonatal intensive care facilities are available, survival rates of 50% are achieved for preterm infants born at 24 weeks of gestation (Blencowe et al., 2013). When this is contrasted with the developing world, an additional 10 weeks of gestation are required for a survival rate of 50%, which is achieved at 34 weeks (Blencowe et al., 2013); the main difference in these figures stems from the availability of healthcare facilities which can provide advanced neonatal care in the developed world, which are lacking in the developing world (Blencowe et al., 2013).

2.3 Neutrophils

The innate immune response is an important system for its immediate role in responding to infection. Neutrophils, NK cells, monocytes and macrophages are the major cell types associated with the innate immune response, and it is the role of the neutrophils that I will look at in much more detail here.

Neutrophils, of myeloid lineage, are the most numerous of all the leukocytes found within the circulation, accounting for approximately 60% of the total population in adults (Martini, 2004), although this can be as

little as 10% in the foetal circulation (Aagaard-Tillery et al., 2006). They can be distinguished from other leukocytes due to an extremely granular cytoplasm and a multi-lobed nucleus, which means that they are often described as polymorphonuclear leukocytes (PMNs) (Martini, 2004). In the bone marrow, $1–2x10^{11}$ neutrophils are produced each day (Borregaard, 2010) and once released into the circulation, they survive on average for 5.4 days in the circulation (Desai et al., 2016). Activation, depending upon the stimulus, can either shorten or lengthen this survival time (Desai et al., 2016).

Neutrophils are the first cell type recruited in response to infection and rapidly locate to the site of infection or injury by the presence of pathogens or chemoattractant molecules (Borregaard, 2010). Once neutrophils reach this site, they can leave the circulation by attaching to selectins displayed on endothelium and enter tissues by crawling through gaps between the endothelium in the vessel wall to move into the tissue (Borregaard, 2010). Until recently the main focus of neutrophil function had been on their abilities to inactivate and kill bacteria through phagocytosis or to release antimicrobial granule proteins into their surroundings through degranulation (Papayannopoulos and Zychlinsky, 2009). The process of phagocytosis allows the cell membrane of the neutrophil to engulf and encapsulate the bacteria within a phagosome where it can be degraded by proteins contained within the granules and reactive oxygen species (ROS). There are 3 major types of granules present in a neutrophil; these are azurophilic (primary), specific

(secondary) and tertiary granules and these contain a different mixture of proteins (Borregaard, 2010). The proteins contained within azurophilic granules include NE and myeloperoxidase (MPO) which are important antibacterial peptides. Degranulation occurs when the contents of the granules are released into the extracellular fluid where the proteins are able to destroy bacteria which have not been consumed through phagocytosis. A major disadvantage of degranulation is that the granule proteins can travel some distance from the initial stimulus through the circulation and further to this these proteins can cause damage and degradation to self-endothelium and epithelium leading to inflammation (Knight et al., 2012, Saffarzadeh et al., 2012).

2.3.1 Neutrophil Extracellular Traps

In 2004, Brinkmann and colleagues first described an additional function of neutrophils leading to the production of Neutrophil Extracellular Traps or NETs based on their ability to trap and kill bacteria. This process, termed NETosis, had previously been observed, but largely ignored as being an artefact (Takei et al., 1996). Brinkmann et al. (2004) were able to stimulate neutrophils to produce these NET structures using phorbol 12-myristate 13-acetate (PMA), IL-8 and lipopolysaccharides (LPS) from *Staphylococcus aureus, Salmonella typhimurium* and *Shigella flexneri*. They demonstrated that only stimulated neutrophils were able to produce these structures, and that the NET components included histones and the granule proteins NE, MPO, cathepsin G, lactoferrin and gelatinase which were bound to a structural deoxyribonucleic acid (DNA) backbone

(see ***Figure 2.3.1***). Using scanning and transmission electron microscopy to analyse the neutrophils, Brinkmann et al. (2004) were able to determine that NETs consisted of smooth regions which were connected with globular domains, which could aggregate to form thick bundles outside of the neutrophils. It is thought that only approximately 20-25% of PMNs produce NETs, and that NET-forming neutrophils may represent a subset of the neutrophil population, however, this remains to be fully investigated (Fuchs et al., 2007, Yipp and Kubes, 2013).

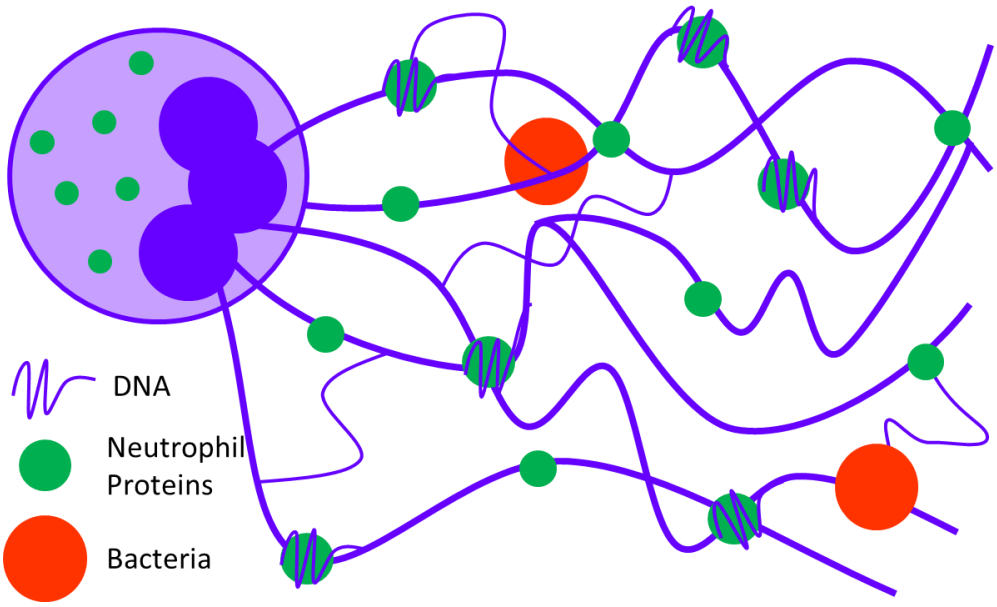

Figure 2.3.1 Schematic Representation of a NET Forming Neutrophil

The structure of NETs consist of DNA, histones and neutrophil granule proteins. The schematic above shows a neutrophil that has formed a NET in response to a bacterial stimuli and those bacteria have become trapped in the NET. Adapted from Miyata and Fan, 2012.

Since the release of this original publication (Brinkmann et al., 2004), research surrounding the release of NETs has exploded and their

presence has been detected in not only bacterial infections, but also in retaliation to fungal (Urban et al., 2006), viral (Schonrich and Raftery, 2016) and parasitic infections (Abdallah and Denkers, 2012). In addition apart from their physiological function, NETs have been observed in thrombosis (Kambas et al., 2012, Miyata and Fan, 2012, Martinod and Wagner, 2014) PET (Gupta et al., 2006a) and autoimmune conditions including, small vessel vasculitis (Kessenbrock et al., 2009), rheumatoid arthritis (RA) (Sur Chowdhury et al., 2014), and systemic lupus erythematosus (SLE) (Wang et al., 2015). This interest has been focused on many different disease states, particularly those characterised by immune activation and especially where these is resultant inflammation. A larger breadth of stimuli have been discovered from those first detected, and these include IL-8, calcium and glucose oxidase (Goldmann and Medina, 2013). Since this first discovery, a number of other immune cell types have been shown to produce extracellular traps (ETs); these are macrophages/monocytes (Chow et al., 2010), mast cells (von Kockritz-Blickwede et al., 2008), eosinophils (Yousefi et al., 2008) and basophils (Yousefi et al., 2015). The pathway leading to the production of NETs was termed NETosis which was initially shown to be a cell death pathway distinct from both apoptosis and necrosis (Fuchs et al., 2007).

Bacteria and fungi however do have mechanisms by which to escape entrapment by NETs. This can be achieved either through the formation of polysaccharides that cause electrochemical repulsion of antimicrobial

peptides or the release of DNase that will digest the chromatin within the structure, breaking the NET apart and releasing the bacteria (Robb et al., 2016). The ability of DNase to digest NETs has been exploited experimentally as a method for removing NETs from the surface of cells to allow for more accurate measurement (Pilsczek et al., 2010, Barrientos et al., 2013).

The pathway resulting in NET formation has not been fully elucidated and is different, depending on the stimulus of NET formation (Yipp et al., 2012, Parker et al., 2012, Hahn et al., 2013). This has led to the proposition of two main pathways for the formation of NETs called **suicidal NETosis** and **vital NETosis** (Yipp and Kubes, 2013) and this naming depends on the ultimate fate of the activated neutrophil. In both pathways there is the resultant production of extracellular strands of DNA which are decorated with histones and granule proteins. However in the suicidal pathway the cell membrane of the neutrophil ruptures to allow for the release of the NETs in contrast to the vital pathway where NETs are released through vesicular trafficking of the structure to the plasma membrane, which allows the membrane to remain intact (Yipp and Kubes, 2013). In essence those neutrophils which undergo the suicidal pathway will not survive the process of NETosis, whereas those that follow the vital pathway can retain some functions such as phagocytosis and crawling as an anuclear neutrophil (Yipp et al., 2012, Peschel and Hartl, 2012). It is not clear whether neutrophils exclusively undergo one pathway or the other during activation and it may be that as activation

increases differing stimuli may push some neutrophils down either track (Pilsczek et al., 2010, Parker et al., 2012). However in a recent publication this naming strategy has been questioned (Desai et al., 2016) for the most part because vital NETosis is an oxymoron which may lead to future confusion surrounding the research in this area. The following section covers the two pathways to NET formation in greater detail.

2.3.2 Suicidal NETosis

Suicidal NETosis is likely employed by chemical agents, cytokines and cellular debris (Gupta et al., 2005, Yipp and Kubes, 2013). The best characterised and most widely utilised inducer of NETs formation is the chemical agent PMA, which initially activates neutrophils via calcium dependent protein kinase C (PKC) (Yipp and Kubes, 2013). This in turn recruits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Parker et al., 2012, Yipp and Kubes, 2013), reactive oxygen species (ROS) (Parker et al., 2012), nuclear factor (NF)-κB and the raf – mitogen-activated protein kinase (MEK) – extracellular signal-regulated kinase (ERK) pathway within the cell. (Yipp and Kubes, 2013). Peptidyl arginine deiminase (PAD) 4 enzyme, which is calcium dependent, requiring an increase in intracellular calcium levels, is responsible for the citrullination of histones within the nucleus and in this post-translational modification, arginine is replaced with citrulline in the histones, particularly histone 3 (H3), which results in the unwinding of DNA (Wang et al., 2009, Yipp and Kubes, 2013, Remijsen et al., 2011). MPO and NE are translocated to the nucleus where there are thought to assist with chromatin

decondensation (Parker et al., 2012, Yipp and Kubes, 2013). Involvement of PAD4 in NET formation depends upon the stimulus (Desai et al., 2016). Different stimuli can activate different isoforms of PKC which can either lead to the activation or inhibition of the PAD4 enzyme (Neeli and Radic, 2013). During this time there are also visible morphological changes to the cell with the nucleus swelling and losing its lobules before the nuclear membrane breaks down allowing the decondensed DNA to mix with the granule proteins (Fuchs et al., 2007). Following this, the plasma membrane then ruptures with DNA and protein mesh extruded from the cell to form the NET (Fuchs et al., 2007).

2.3.3 Vital NETosis

Vital NETosis is employed primarily in response to bacterial activation, with gram-negative bacteria utilising toll-like receptor (TLR) 4 (via platelet interaction) and cluster of differentiation (CD) 11a (Yipp and Kubes, 2013) and gram-positive bacteria through TLR2 and complement receptor (CR) 3 (Yipp et al., 2012). This activation mechanism is not as well characterised as the suicidal response, but again there are morphological changes that can be observed in the cells. In contrast to the suicidal pathway, the nucleus retains its lobed structure throughout and the nuclear membrane does not disintegrate (Yipp and Kubes, 2013). Instead, the neutrophil relies on vesicular trafficking to transport DNA, in vesicles, out of the nucleus where can fuse with and engulf granules (Pilsczek et al., 2010, Yipp et al., 2012). The vesicle then finally fuses with the plasma membrane allowing the NET to be released from the cell,

whilst leaving the plasma membrane intact (Pilsczek et al., 2010, Yipp et al., 2012). During infection, adult neutrophils can release NETs rapidly through this process and it can be completed in as little as 60 minutes (Pilsczek et al., 2010, Yipp et al., 2012). These cells then remain functionally intact, leaving anuclear neutrophils which retain the abilities of chemotaxis, crawling and phagocytosis (Yipp et al., 2012, Peschel and Hartl, 2012). In fact neutrophils appear to be able to utilise all of their abilities simultaneously, and those which have cast NETs crawl through tissues, ensuring widespread coverage throughout the tissue (Yipp et al., 2012).

2.3.4 Mitochondrial DNA in NETs

A recent interesting development is the finding that mitochondrial DNA (mtDNA) can also be utilised for the creation of NETs and its presence has been visualised with the use of anti mtDNA antibodies (Yousefi et al., 2009, Wang et al., 2015). Neutrophils, eosinophils and basophils (Yousefi et al., 2015) have all been shown to release NETs in this manner. Mitochondria are the only intracellular organelle with their own DNA and generate ROS. The utilisation of mtDNA has advantages over the use of gDNA is that cells retain a functioning nucleus, rather than dying or becoming anuclear (Yousefi et al., 2009). It has been suggested that mtDNA may be the first DNA utilised in the formation of NETs, with gDNA following (Wang et al., 2009). In SLE the levels of mtDNA were found to be more pro-inflammatory and a better indicator of disease severity than DNA levels alone (Wang et al., 2015). It is possible that the

release of NETs utilising mtDNA is confined to particular diseases as the results could not be repeated in RA. Mitochondrial DNA is found within the inner membrane of the mitochondria, where ROS are produced, and contains unmethylated CpG DNA repeats which are highly inflammatory TLR-9 ligands (Wang et al., 2015). Circulating mtDNA induces systemic inflammation that can cause profound tissue injury (Wang et al., 2015). An interesting observation is that placental DNA is hypomethylated (Hahn et al., 2014) and as a result may also be an inflammatory stimulus like mtDNA.

2.3.5 Complications of NET production

Under normal physiological conditions, neutrophil activation is tightly controlled (Yipp et al., 2012, Nakazawa et al., 2016) as their activation, while helpful in removing invading pathogens, may lead to the damage or destruction of self-tissue often resulting in inflammation and hypoxia (Knight et al., 2012, Saffarzadeh et al., 2012). The vast majority of damage is caused to endothelial and epithelial cells which may present as damage to arterial, venous and microvascular tissues (Saffarzadeh et al., 2012) as well as in the promotion of thrombus formation (Alhamdi and Toh, 2016). This damage is primarily caused by exposure to decondensed DNA, histones and granule proteins (Saffarzadeh et al., 2012), which have anti-bacterial properties and are present extracellularly subsequent to NET formation. Histones have been shown to increase the membrane permeability and release of IL-8 from endothelial cells and have also been shown to be cytotoxic, with the

ability to activate key inflammatory pathways, which include NFκB, NOD-like receptor (NLR) P3 inflammasome and TLRs (Saffarzadeh et al., 2012, Alhamdi and Toh, 2016). Histones can also lead to the release of von Willebrand factor (vWF) which can result in thrombus formation (Grassle et al., 2014). Histones also stimulate the production of NETs and are released in NET structures, therefore once NET release has begun it can be continued by stimulation with NETs themselves as a damage associated molecular pattern (DAMP) (Alhamdi and Toh, 2016). Histones can also promote coagulation and induce the release of pro inflammatory cytokines from other leukocytes, including IL-6 and tumour necrosis factor (TNF)-α (Alhamdi and Toh, 2016). These effects are not seen with histones when they are linked with DNA and are compacted, as seen in the nucleus; but are seen when histone-DNA complexes are not compacted after PAD4 mediated citrullination results in unwinding of DNA from histones, as is seen in NET formation (Alhamdi and Toh, 2016). When found in low levels in the circulation histones are quickly cleared and as a result are not usually detected, but high levels have been shown in autoimmune disease and recent evidence links them to the pathophysiology of sepsis (Alhamdi and Toh, 2016). NETs are thought to be dismantled by macrophages in a process similar to apoptosis (Farrera and Fadeel, 2013, Nakazawa et al., 2016, White et al., 2016).

2.4 Pregnancy and Neutrophil Extracellular Traps

As alluded to earlier in this introduction, an increase in inflammation has been shown to play a part in the initiation of labour at term. It has also been noticed that throughout the production of NETs, damage is caused to surrounding tissue, particularly endothelium and epithelium through exposure to self DNA, histones and granule proteins (Saffarzadeh et al., 2012). This damage generally results in inflammation which could mean that this is present in the maternal circulation, placenta and foetus. Therefore there is a strong possibility that a rise in inflammation during pregnancy, prior to term may mimic the inflammation seen at term, leading to inappropriate initiation of labour (Gomez-Lopez et al., 2014). Work has been conducted into the involvement of NETs in reproduction including conception, miscarriage (Hahn et al., 2012), PET (Gupta et al., 2005) and in the development of the neonatal innate immune system (Yost et al., 2009, Marcos et al., 2009). With respect to conception, neutrophils via phagocytosis and NETs are thought to play a role in the removal of excess sperm from the female reproductive tract (Alghamdi and Foster, 2005, Hahn et al., 2012). Studies in horses have shown that equine seminal plasma contains a protein which shows similarities to DNase 1. This was able to digest NETs produced in response to the presence of sperm, allowing sperm to evade the traps (Alghamdi and Foster, 2005, Hahn et al., 2012). The absence of the DNase 1 like protein leads to poor rates of conception (Alghamdi and Foster, 2005, Hahn et al., 2012). A recent study in humans also reveals that female leukocytes

produce NETs in response to sperm exposure, leading to a reduction in motility (Zambrano et al., 2016).

2.4.1 Pre-eclampsia and NETs

A role for NETs in the multifactorial disease of pregnancy, PET, which affects 5% of pregnancies (Stein et al., 2013), was demonstrated by Gupta and colleagues between 2005 and 2007. PET is a condition which is characterised by increased blood pressure and proteinuria, which if left untreated progresses to eclampsia. This can result in seizures which can lead to cerebral haemorrhage and maternal death. In very rare cases, HELLP syndrome (Haemolysis, elevated liver enzymes, low platelet count), causes damage primarily to the liver (Gupta et al., 2006b) and reduces the ability of the blood to clot. The only treatment to 'cure' PET is delivery of the foetus and the removal of the placenta, however this can lead to the delivery of extremely premature infants (Gupta et al., 2006b).

Gupta et al. (2005) were able to show that syncytiotrophoblast microparticles (STBM), which are released from the STB directly into the maternal circulation were a stimulant of NET formation and that these small particles (<200nm) could also be immobilised like larger bacteria (>2µm) within NET structures (Gupta et al., 2005), thus leading to activation of the maternal immune system. Further to this, elevated levels of cell-free foetal and maternal DNA have been seen in the maternal circulation alongside raised levels of NE in women diagnosed with PET

(Gupta et al., 2006a, Gupta et al., 2006b). They were also able to visualise an increased concentration of NETs in the larger IV spaces of PET placentae when compared with normal controls in sections of villous tissue. In the normal control placentae the NETs were closely associated with the STB which is the site of STBM release, whereas in the PET placentae they entirely filled the IV spaces. Gupta et al. (2005) surmised that the presence of NET structures within the placenta itself could reduce maternal blood flow through the placenta by restricting erythrocyte movement, leading to placental hypoxia (Gupta et al., 2005, Gupta et al., 2007). As the release of NETs causes damage to endothelial and epithelial tissues due to the exposure to self-DNA, histones and granule proteins the increased level of activation of NETs in PET, this could be causing the damage to the maternal vasculature seen in PET (Gupta et al., 2006a).

In pregnancy, foetal DNA can be readily measured in maternal circulation (identified as potential for risk free methods of prenatal diagnosis). Some of this will be from shedding of STBMs, however there is some element of trafficking of foetal cells into the maternal circulation (this is increased in PET, but occurs before the onset of symptoms (Hahn et al., 2005). An increase in cell free DNA (cfDNA) of foetal origin has also been seen in PTB, but, without the increase in feto-maternal cell trafficking seen in PET; therefore foetal cfDNA could be a useful tool to study abnormal placentation.

2.4.2 NET Formation in the Neonate

In 2009 there was some disagreement between the groups of Yost and Marcos as to the ability of neutrophils from human neonates to form NETs. It had previously been reported that PMNs isolated from premature neonates were different to those isolated from term neonates and adults, stating that their ability to phagocytose and kill bacteria and generate reactive oxygen species was reduced (Yost et al., 2009). Also that PMNs from term and preterm infants have reduced antibacterial functions which lead to complications such as neonatal sepsis which may be due to differences in molecular signalling cascades and impaired calcium signalling (Yost et al., 2009). Yost and colleagues theorised that NETs have an antibacterial function that may be the result of the deficiencies previously seen. In their experiments they saw that PMNs from adults stimulated with LPS for 1 hour formed NETs, whereas those from term and preterm infants did not. The group of Marcos agreed with Yost that there was indeed no response seen with LPS over the 1 hour time point, however they went on to consider the effects seen at increased time points of 2 and 3 hours. They saw that after 2 hours NETs had started to form, with them exhibiting the same level of response as adult neutrophils at 3 hours (Marcos et al., 2009). Therefore NETs can be produced by neonates, but the process of formation takes longer than that seen in adult PMNs (Marcos et al., 2009). However this delay may be important in the development of sepsis allowing bacterial replication to go unchecked (Yost and Zimmerman, 2009). An additional factor is that the proportion of granulocytes, particularly neutrophils present in the

neonatal circulation is lower than that seen in adult blood (Aagaard-Tillery et al., 2006). This needs further evaluation as the differences may be due to reduced neutrophil numbers, rather than a reduced capacity for NET production.

2.4.3 NETs in Chorioamnionitis

In a very recent paper an interesting link has been shown between chorioamnionitis, which as discussed earlier is an infection of the foetal membranes, NETs and PPROM (Gomez-Lopez et al., 2017). The authors were able to demonstrate using immunofluorescence for DNA, NE and H3 that NETs, released from maternal neutrophils, were present in the membranes of women with chorioamnionitis whether delivery was at term or preterm. In contrast, NET structures were absent or present at very low levels in women without chorioamnionitis at term and preterm (Gomez-Lopez et al., 2017). They also noted that there were increased maternal neutrophil numbers infiltrating the membranes in patients with chorioamnionitis than those without (Gomez-Lopez et al., 2017). These observations are particularly exciting in the context of our ongoing work and provide definitive evidence for NETs in PTB with infection.

2.5 Hypothesis and Aims

Many changes occur in both the maternal and foetal circulations as pregnancy is established and the aim of this study is to investigate whether NETs play a role. The specific hypothesis to be tested is that NET formation occurs in cord blood leukocytes (CBL) and maternal blood

leukocytes (MBL) in response to pro-inflammatory mediators, including bacterial surface polymers. Further that NET formation is exacerbated in disorders of pregnancy, including PPROM. The specific aims of this study were:

(i) To develop and validate an assay to measure NET formation in cord blood which could subsequently also be utilised for maternal blood.

(ii) To test a series of ligands to assess NET formation in cord blood and maternal blood leukocytes in response to stimulation in normal pregnancy.

(iii) To compare the NET response in normal pregnancy with that of pregnancy disorders.

(iv) To visualise NET proteins *in vitro* utilising immunofluorescence techniques.

3 Materials and Methods

3.1 Patient Recruitment

This study had ethical approval from Derbyshire Local Research Ethics Committee, study number 09/H0401/90. Written, informed consent for the use of placentae, cord and maternal blood was obtained from pregnant women recruited on either Delivery Suite or Pregnancy Day Care at the Royal Derby Hospital.

3.2 Sample Collection

For this study umbilical cord blood (CB) and maternal blood (MB) were collected and processed as follows.

3.2.1 Umbilical Cord Blood

The majority of the placentae were obtained from normal women undergoing elective lower segment caesarean section (El LSCS) at term (38-41 weeks of gestation). Placentae were also obtained from women with evidence of a GBS infection based on results from a high vaginal swab (HVS) and were undergoing El LSCS at term or preterm (before 37 weeks of gestation).

Immediately following delivery, the umbilical cord of each placenta was clamped and placentae then transferred to the laboratory where blood was collected within 30 minutes of the delivery of the placenta. CB was

collected by venepuncture either directly from the umbilical cord vein or where this was not possible from a vein on the chorionic plate as close as possible to the site of the cord insertion. Veins were identified from arteries, initially by using visual inspection of the chorionic plate and umbilical cord. In the umbilical cord the veins runs down the centre, with the two arteries spiralling around the outside. This was then followed by manual manipulation of the vessels to assess whether the vessel had a rigid, muscular wall which would indicate an artery or a soft, non-muscular wall which would indicate a vein. A 19G needle and 20ml syringe were used to collect up to 60ml of cord blood. The blood was then immediately transferred to vacutainers containing potassium EDTA (BD, Oxford, UK) and gently mixed by inversion to prevent the blood from clotting and used immediately.

3.2.2 Maternal Blood

MB was collected by venepuncture from the antecubital vein of pregnant women who were attending routine appointments at antenatal clinic at 20 weeks and 28 weeks of pregnancy or immediately prior to having an El LSCS. We also sought to recruit patients at any point during their pregnancy where they presented with PPROM. Approximately 10ml of blood was collected using a 21G needle, directly into a vacutainer containing potassium EDTA anticoagulant in order to prevent the blood from clotting. Blood samples were used within 30 minutes of collection. Care was taken to prevent haemolysis during collection by using a large bore needle and mixing the vacutainers gently after filling.

3.3 Mixed Leukocyte Isolation

The same isolation method was used for both CB and MB samples. Red blood cells were removed by sedimentation by mixing 2 parts whole blood, with 1 part of 3% w/v Dextran from *Leuconostoc spp*. (M_r 450,000-650,000 – Sigma Aldrich, Dorset, UK) in 0.9% w/v sodium chloride (NaCl, Sigma Aldrich, Dorset, UK). This mixture was incubated at room temperature for 1 hour in a 15ml Falcon tube where blood volumes were 10ml or less, or a 50ml Falcon tube where blood volumes were over 10ml. During the incubation period, the red blood cells rouleau and sink to the bottom of the tube, leaving the leukocytes suspended in plasma above. This upper fraction was removed using a Pasteur pipette and transferred to a 15ml or 50ml Falcon tube (depending upon the size of the tube previously used) which was then topped up to 15 or 50ml with phosphate buffered saline (PBS – *137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4*). Each tube was centrifuged at 300 x *g* for 10 minutes at room temperature to pellet the cells and the supernatant was removed by aspiration. Residual red blood cells were removed using an osmotic shock process to lyse the cells. This involved resuspending the cell pellet in 5ml (15ml tube) or 10ml (50ml tube) of ice-cold 0.2% w/v NaCl solution followed by an incubation for 2 minutes with gentle agitation. Isotonicity was then restored by adding the same volume of ice-cold 1.6% w/v NaCl to the tube. The tube was then topped up to 15ml or 50ml using PBS and then centrifuged at 300 x *g* for 10 minutes at room temperature to pellet the cells. The resulting cell pellet was then

resuspended in an appropriate volume of assay buffer, depending upon the size of the cell pellet, before cell counting and viability assessments were performed.

3.3.1 Cell Counting and Viability Assessment

Cells were counted using a TC-10 cell counter (BioRad, Hercules, California, USA) and viability assessment performed using the TC-10 cell counter for MBL and a haemocytometer for CBL.

For cell counting, 10µl of cell suspension was added to a TC-10 counting slide and inserted into the cell counter. The number of cells/ml was displayed and then the field of view assessed to ensure that the slide was not overloaded with cells. If this was found to be the case the cell suspension was diluted and the reading repeated. If a viability assessment was to be performed at the same time, a 10µl aliquot of the cell suspension was mixed 1:1 with a 0.4% w/v solution of trypan blue (BioRad, Hercules, California, USA), before 10µl was added to the counting slide. The total number of cells/ml, total number of live cells/ml and viability were displayed and then the field of view assessed to ensure that the slide was not overloaded with cells. If this was found to be the case the cell suspension was diluted and the reading repeated.

It was observed during initial experiments that the TC-10 cell counter could not accurately determine the viability of CBL, providing artificially low readings. When using the haemocytometer to perform a viability

assessment, it was first cleaned with 70% ethanol and allowed to air dry thoroughly. A coverslip was then placed on top of the haemocytometer and adhered using condensation as confirmed by Newton's Rings which can be observed as spherical patterns along the interface where the coverslip meets the haemocytometer. A 10µl aliquot of the cell suspension was mixed 1:1 in a 0.4% w/v trypan blue solution, before 10µl was then added onto the haemocytometer which is drawn under the coverslip using capillary action. A CKX53 light microscope (Olympus, Tokyo, Japan) at 100x magnification was used to visually count the viable and non-viable cells within the counting squares off each corner of the central square. Viable and non-viable cells can be easily distinguished from one another as trypan blue is not able to cross an intact cell membrane. Therefore the non-viable cells take up the trypan blue dye appearing deep blue in colour, whereas the live cells do not take up the stain. The number of viable and non-viable cells counted was determined and the percentage of live cells calculated.

Leukocyte suspensions were then diluted to obtain the appropriate cell concentration(s) for the assay requirements. All calculations were performed on total cell number and samples with viabilities below 90% were not used any further.

3.4 Flow cytometry

Flow cytometry was used to assess the proportions of leukocytes present in the purified cell suspension using a panel of extracellular surface

markers (Beckman Coulter, Marseilles, France). These consisted of CD45 (leukocyte common antigen), CD14 (monocytes), CD66b (granulocytes) and CD16 (neutrophils in conjunction with CD66b) and CD66b. ***Figure 3.4*** shows the excitation and emission spectra for the fluorophores used.

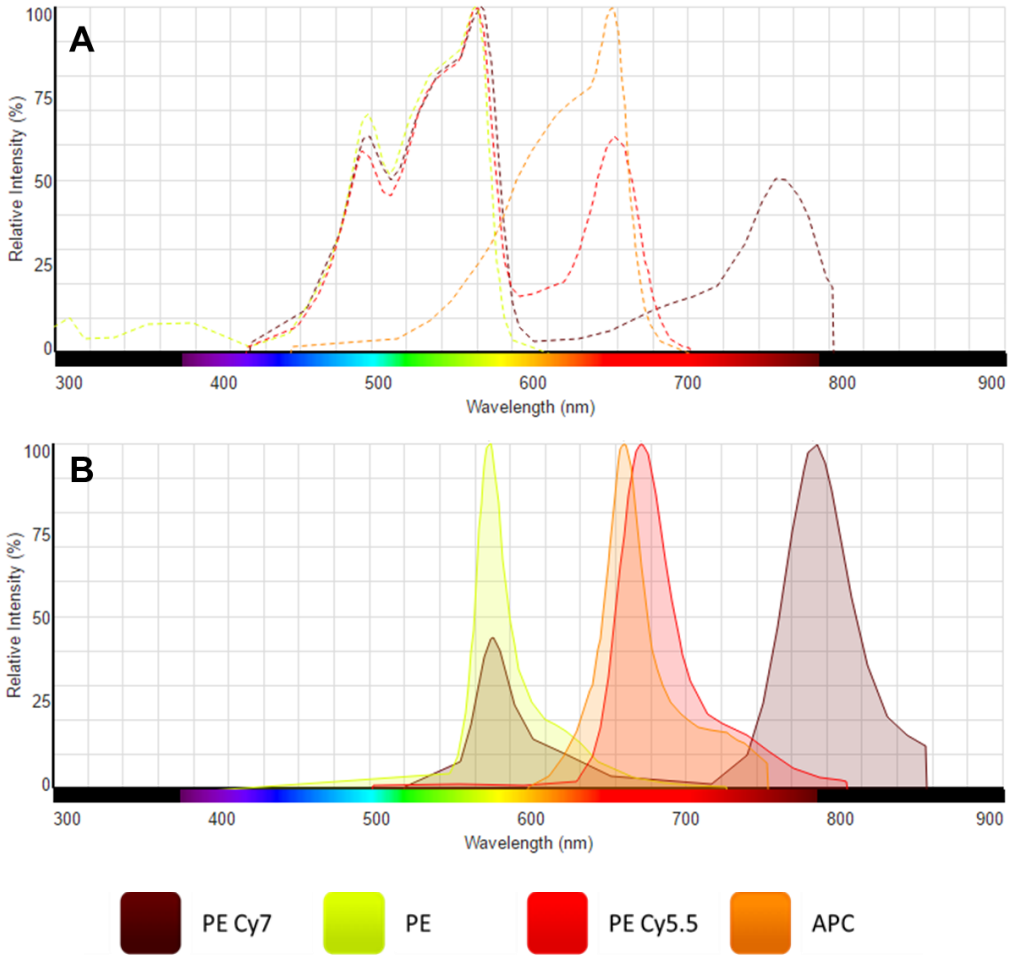

Figure 3.4 Flow Cytometry Spectraviewer

*The excitation (**A**) and emission (**B**) spectra for the fluorophores used for the flow cytometry. From the excitation (**A**) wavelengths observed PE, PE Cy5.5 and PE Cy7 will all be excited using the blue laser and the APC will be excited using the red laser. The emission spectra (B) shows the overlap of the emission wavelengths for each fluorophore, which highlights the need for compensation due to colours bleeding into one another with increasing wavelength. The APC and PE Cy5.5 fluorophores overlap significantly, but as they are excited using different lasers this does not pose a problem. The graphs were produced using the life technologies spectra viewer which can be accessed at https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html#*

Leukocytes were isolated following both the whole leukocyte preparation above and resuspended at $5x10^6$ cells/ml in HBSS (H6648 – Sigma Aldrich, Dorset, UK) containing 1% w/v bovine serum albumin (BSA – Sigma Aldrich, Dorset, UK) to act as a protein carrier. All of the antibodies were removed from the fridge and allowed to equilibrate to room temperature before use. Antibodies were transferred to flow tubes at the volumes shown in ***Table 3.4.*** Cell suspension, 50µl, was then added to each tube and allowed to incubate in the dark at room temperature for 15 minutes. Fixative, 100µl of IntraPrep Reagent 1 (Beckman Coulter, Marseilles, France) was added to each tube and mixed by vortexing before incubating in the dark at room temperature for 20 minutes. PBS, 4ml, was then added to each tube before the cells were pelleted by centrifugation at 500 x *g* for 5 minutes at room temperature. The cells were resuspended in 500µl of 0.8% w/v formaldehyde (Beckman Coulter, Marseilles, France) solution and stored in the dark at 4°C until reading on BD LSR II Flow Cytometer running FACS Diva software (*v8.01, BD, Oxford, UK*). Compensation levels were set to minimise the effects of fluorescence bleeding from one channel to another using the unstained and single fluorophore tubes. Fluorescence minus one tubes, were used as gating controls to aid setting positive and negative gates (Maecker and Trotter, 2006).

Antibody	Fluorescent Tag	Wavelength Ex/Em (nm)	Volume (µl)
CD45	PE Cy7	Ex488/Em770	10
CD16	PE	Ex488/Em575	20
CD14	PE Cy5.5	Ex488/Em692	10
CD66b	APC	Ex633/Em660	10

Antibody	1	2	3	4	5	6	7	8	9	10
CD45	X	√	X	X	X	X	√	√	√	√
CD16	X	X	√	X	X	√	X	√	√	√
CD14	X	X	X	√	X	√	√	X	√	√
CD66b	X	X	X	X	√	√	√	√	X	√

Table 3.4 Flow Cytometry Antibodies and Experiment Set Up

The volumes of each antibody used in the flow cytometry are shown in the table above. An unstained control (tube 1), single fluorophore controls (tubes 2-5), fluorescence minus one controls (tubes 6-9) and a tube with full staining (10), were required for each sample.

3.5 NET Stimulation and Visualisation

A number of techniques were utilised to measure and visualise NET production in mixed leukocytes. These were the SYTOX® green assay, NE activity after nuclease digest and immunofluorescence, which I will describe in detail in the following section.

3.5.1 Leukocyte Stimulation

Leukocytes were isolated following the whole leukocyte preparation above and resuspended in calcium and magnesium free HBSS (H6648 – Sigma Aldrich, Poole, Dorset, UK) or NETs assay buffer, which contains phenol red free Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, UK) with 1% w/v BSA (Cayman Chemical, Ann Arbor, Michigan, USA) and 1mM calcium chloride (Cayman Chemical, Ann Arbor, Michigan, USA) at a cell concentration appropriate for the subsequent experiment. For all of the initial experiments a concentration of $5x10^6$ cells/ml was used. During the cell number validation experiments concentrations of $5x10^6$, $1x10^6$, $5x10^5$ and $1x10^5$ cells/ml were used. The cell concentration was decreased from that used in the initial experiments to identify if lower cell concentrations could be used in the assay. For all subsequent experiments a concentration of $1x10^6$ cells/ml was used, with the exception of some validation experiments, where $5x10^5$ cells/ml was also used. Leukocytes were transferred into each well of the appropriate assay plate. Agonist was added to the cells and incubated at 37°C for 3 hours to allow the extracellular traps to form.

3.5.2 Preparation of Agonists and Antagonists

The leukocytes were treated with the pathogen associated molecular patterns (PAMPs) Lipoteichoic acid from *Staphylococcus aureus* (LTA - Sigma Aldrich, Poole, Dorset, UK), Peptidoglycan from *Staphylococcus aureus* (PGN – Sigma Aldrich, Poole, Dorset, UK), Lipopolysaccharide from *Escherichia coli 026:B6* (LPS – Sigma Aldrich, Poole, Dorset, UK). LTA and PGN are isolated from gram-positive bacteria and LPS is isolated from gram-negative bacteria. Phorbol 12-myristate 13-acetate (PMA) is a well characterised inducer of NET formation and was utilised to validate the assays and further to act as a positive control. LTA and LPS were dissolved in HBSS or NETs assay buffer at concentrations of 10mg/ml and 5mg/ml respectively. PGN, which is insoluble was suspended in HBSS or RPMI at a concentration of 10mg/ml. PMA is insoluble in aqueous solution and was made up at a stock concentration of 1mM in the solvent dimethyl sulfoxide (DMSO). The final assay concentrations used are shown in ***Table 3.5.2*** below and the working concentrations produced were 10 times higher than those shown in the table, due to a 1:10 dilution onto the assay plate (i.e. 10µl agonist and 90µl of cell suspension).

The antagonists N-acetyl-L-cysteine (NAC – Sigma Aldrich, Poole, Dorset, UK) and diphenyliodonium chloride (DPI – Sigma Aldrich, Poole, Dorset, UK) were used in some experiments. NAC was dissolved in HBSS with 0.5% v/v DMSO at a concentration of 30mM, which would give a final assay concentration of 3mM in 0.05% v/v DMSO. DPI was

dissolved in DMSO at a stock concentration of 20mM, which was diluted in HBSS to a working concentration of 100µM in 0.5% v/v DMSO; this would produce a final assay concentration of 10µM in 0.05% v/v DMSO.

PMA (nM)	PMA (nM) extended	LTA (µg/ml)	PGN (µg/ml)	LPS (µg/ml)
1000	1000	100	100	50
500	300	30	30	30
250	100	10	10	10
125	30	3	3	3
62.5	10	1	1	1
31.25	3	0.3	0.3	0.3
15.625	1	0.1	0.1	0.1
0	0.3	0	0	0
	0.1			
	0.03			
	0.01			
	0			

Table 3.5.2 Agonist concentrations used for NET stimulation

The table above shows the concentration range of the agonists used for the stimulation of NETs.

3.5.3 SYTOX® Green assay

For this assay, SYTOX® green nucleic acid stain is used to measure extracellular DNA, as it cannot cross the cell membrane of intact cells. Therefore, it will bind with the DNA extruded from cells producing NETs as well as the nucleus of dead cells and those with compromised membranes. Cells were resuspended in HBSS, with 90µl of cell suspension added to each well of a flat bottomed white opaque 96-well plate. The cells were treated with agonist as detailed above prior to staining. SYTOX® Green nucleic acid stain, 50µM in 1% v/v DMSO (Molecular Probes, Eugene, Oregon, USA) was added to each well to achieve a final assay concentration of 5µM in 0.1% v/v DMSO and incubated at 37°C, 5% CO_2, 95% air, for 10 minutes to allow the dye to bind with the DNA. The plate was then read using a Fluoroscan Ascent FL fluorescent plate reader (Thermo Scientific, Finland) using an excitation wavelength of 485nm and emission wavelength of 520nm. The DNA measured would either be that extruded from the cells during the process of NET formation or the nucleus of dead or dying cells with compromised plasma membranes. Therefore, this assay was used in conjunction with other methods due to the difficulties in distinguishing NET formation from dead cell DNA.

3.5.4 NET digest and NE activity assay

Cells were resuspended in NETs assay buffer at a concentration of $1x10^6$ cells/ml or $5x10^5$ cells/ml. Cell suspension, 900µl, was added to each well of a 24 well tissue culture plate. The cells were treated 100µl of 1mg/ml

LTA, PGN and LPS (assay concentration 100µg/ml) or 150nM PMA (assay concentration 15nM). After the incubation time was completed the media was gently removed from the cells and transferred to 1.5ml centrifuge tubes. The cells were washed carefully twice with 1000µl of assay buffer, which had been warmed to 37°C, with the buffer used also transferred to 1.5ml centrifuge tubes. S7 nuclease (also known as MNase – Cayman Chemical, Ann Arbor, Michigan, USA) was diluted to a concentration of 15U/ml in assay buffer, which had been warmed to 37°C, before 500µl was added to each well and allowed to incubate with the cells at 37°C for 15 minutes to digest the NET DNA fragments and remove them from the surface of the cells. The supernatant containing the NET fragments was then removed from the wells and transferred into 1.5ml centrifuge tubes, to this 10µl of 0.5M EDTA (Cayman Chemical, Ann Arbor, Michigan, USA) was added to chelate the calcium and magnesium ions within the media and cease the activity of the S7 nuclease enzyme. The tubes containing the digested NETs were centrifuged at 300 x *g* for 5 minutes at room temperature to pellet any cellular debris before the supernatant was transferred to a new 1.5ml centrifuge tube. The digest supernatant, culture medium and wash buffer were stored at -20°C until neutrophil elastase levels were determined.

The culture medium, wash solutions, undigested and digested NET supernatants were assayed for NE activity using an assay (Cayman Chemicals, Ann Arbor, Michigan, USA) which utilises a substrate for NE, N-methoxysuccinyl-Ala-Ala-Pro-Val *p*-Nitroanilide (NMeoSuc-*p*Na), that

when cleaved by NE produces a colour change in the solution, colourless to yellow; this is measurable using a colorimetric plate reader at 405nm. An 8 point standard curve was produced of human neutrophil elastase, with a top concentration of 36mU/ml, with 1 in 2 serial dilutions down to the lowest concentration of 0.56mU/ml; the last standard contains no neutrophil elastase. In a 96 well plate 100µl of standard or digested NET supernatant were combined with an equal volume of 500µM NMeoSuc-pNa (3.3% v/v DMSO in PBS) to give a final assay concentration of 250µM NMeoSuc-pNA with a final DMSO concentration of 1.67% v/v. The plate was incubated at 37°C for 1 hour, whilst the substrate cleavage occurred. The plate was read on a Multiskan plate reader (Thermo Scientific, Finland) with absorbance at 405nm with a reference wavelength absorbance of 490nm.

3.5.5 Immunofluorescence

Cells were resuspended in HBSS at a concentration of $5x10^6$ cells/ml or $1x10^6$ cells/ml. Cell suspension, 270µl, was transferred into each well of a 24 well tissue culture plate. The cells were treated with agonist and antagonist as detailed above. The media was then removed from the wells and the cells were fixed using 1ml of a 1:1 ice cold solution of acetone:methanol (Sigma Aldrich, Dorset, UK) and left to incubate for 20 minutes at 4°C. The wells were then washed 3 times with PBS to remove the acetone:methanol. The cells were then blocked against non-specific binding by incubating with 20% v/v goat serum (Sigma Aldrich, Dorset, UK) in PBS for 30 minutes at room temperature. NE primary antibody

(Abcam, Cambridge, UK) was diluted 1:40 in 20% v/v goat serum. The blocking buffer was removed from the wells and then the primary antibody was added and allowed to incubate at 4°C overnight on a rocking platform. The cells were washed 3 times using PBS and then a 1:35 dilution of the FITC conjugated secondary antibody (Sigma Aldrich, Poole, Dorset, UK) added. This was left to incubate at room temperature for 1 hour on a rocking platform. The cells were then washed a further 3 times with PBS, and the final wash was left covering the cells in preparation for visualisation. The cells were observed using a Zeiss Axiovert 25 fluorescence microscope (Olympus, Tokyo, Japan) and bright field images and FITC filter images captured using a DP-70 microscope camera (Olympus) and Cell F software.

3.6 Data and Statistical Analysis

Data analysis is described separately for each technique. In all cases, results are expressed as mean ± standard error of the mean (SEM) or range with significance assumed at $p<0.05$.

3.6.1 Flow Cytometry

Data analysis was performed using Weasel software (version 3.0.2) in order to determine the proportion of cell types present in each population. A region was drawn around the main body of cells within the sample, excluding cell debris and labelled cells. A further region was drawn around the CD45 positive cells and labelled leukocytes. These regions were used to create a gate to ensure that when comparing fluorophores

only CD45 positive leukocytes were included in the analysis. The unstained tube was used to identify the negative cell population and set the positive stained gate using the single fluorescence and fluorescence minus one controls. Quadrants were then used to identify negative, single positive and double positive cells comparing different fluorophores which allowed determination of the cell proportions.

3.6.2 SYTOX® Green Experiments

Baseline correction was applied to all data, whereby the baseline fluorescence intensity was removed from the fluorescence intensity of the treated cells. Data from the PAMPs were further corrected as each showed a level of auto-fluorescence in no cell control samples. Each agonist alone was then normalised against the concentration which showed the highest mean fluorescence intensity; this was selected as the concentration in that sample which achieves the highest response. This was assigned a value of 100% and all other concentrations were expressed relative to this. For LTA, PGN and LPS this is usually, but not always at 300µg/ml and for PMA varies. Samples from patients which did not produce NETs after stimulation with PMA (positive control) were removed from further analysis.

All graphs were produced and statistical analysis performed using GraphPad Prism (version 7.01). Data was assessed for Gaussian distribution using the Shapiro-Wilk normality test and the majority of data points were found to follow a normal distribution and therefore parametric

tests, were selected for statistical analysis. Where curves were fitted, this was achieved using non-linear regression of log [agonist] vs normalised response with a variable slope, using the following formula where **X** is log agonist concentration and **Y** is normalised fluorescence intensity:

[Y=100/(1+10^((LogEC50-X)*HillSlope))]

This formula was chosen to fit the curves on the normalised data in order to constrain the top of the curve at 100% which would provide a more accurate EC_{50} for each patient group. Statistical analysis was accomplished either using one-way analysis of variance (ANOVA) with a Bonferroni post-test or a two-way ANOVA with a Bonferroni post-test, depending upon the number of variables to be assessed.

3.6.3 NET Digest and NE Activity Assay

The reference wavelength absorbance was subtracted from the sample absorbance readings. A standard curve was plotted of concentration vs. mean absorbance, before a linear trendline was fitted to data with R^2 values above 0.95 considered acceptable. The equation of the line was used to interpolate the neutrophil elastase concentrations of the samples. The samples were compared using a two-way ANOVA with a Bonferroni post-test.

4 Results

4.1 CBL Population Analysis

Flow cytometry was performed to identify the leukocyte sub-populations in the purified preparations isolated from cord blood to determine the proportion of each cell type present. The results showed that the isolation procedure produced a leukocyte rich preparation with measurable sub-populations of the differing leukocytes as represented in ***Table 4.1*** below, for n=3 samples. Neutrophils were found to be the most abundant cell type in the preparation accounting for 36.3 ± 2.71% of all leukocytes present.

Cell Type	Cord Blood (%) *Mean (±SEM)*
Granulocytes	43.5 (±4.14)
Neutrophils	36.3 (±2.71)
Mononuclear	33.5 (±3.84)
Monocyte	10.9 (±1.03)
NK Cells and Macrophages	12.19 (±5.16)

Table 4.1 Proportions of cell populations identified by flow cytometry

In the figure below, the gated leukocytes are shown and the granulocytes, monocytes and lymphocytes can be distinguished by forward scatter and side scatter profiles, with the granular leukocytes scattering more light (see ***Figure 4.1 A***). The difference between stained and unstained

leukocytes can be clearly seen for each fluorophore used, with a rightward shift in the fluorescence intensity profile (see ***Figure 4.1 B***). Quadrant plots of related fluorophores are shown in ***Figure 4.1 C***, these represent cells which show either no staining, single fluorophore or double fluorophore staining. This clearly shows the total population of leukocytes (CD45), along with monocytes (CD16), granulocytes (CD66b) and neutrophils (CD16 and CD66b).

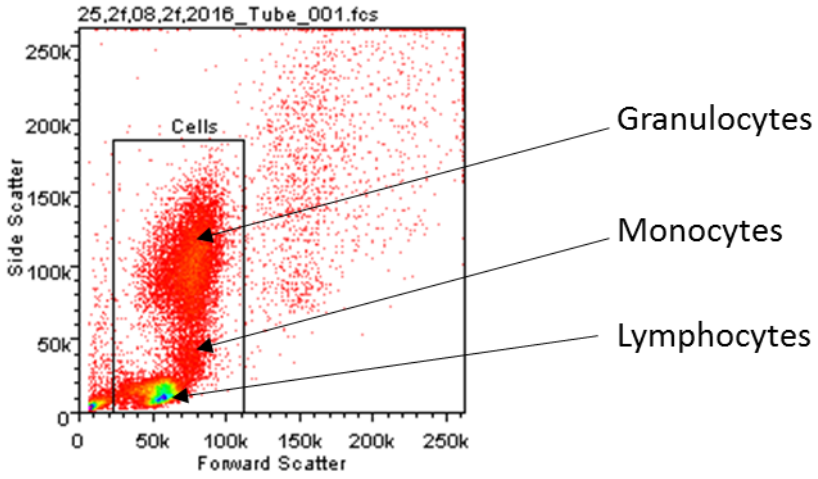

Figure 4.1 A Forward Scatter and Side Scatter of CBL

The size and granularity of CBL was assessed using forward and side scatter. From this information the CBL cell types can be roughly distinguished with granulocytes being the largest and most granular cells, with lymphocytes the smallest and agranular. Data are displayed as single dots, representing a cell those areas that are red in colour are the least dense, through to blue which are the most dense.

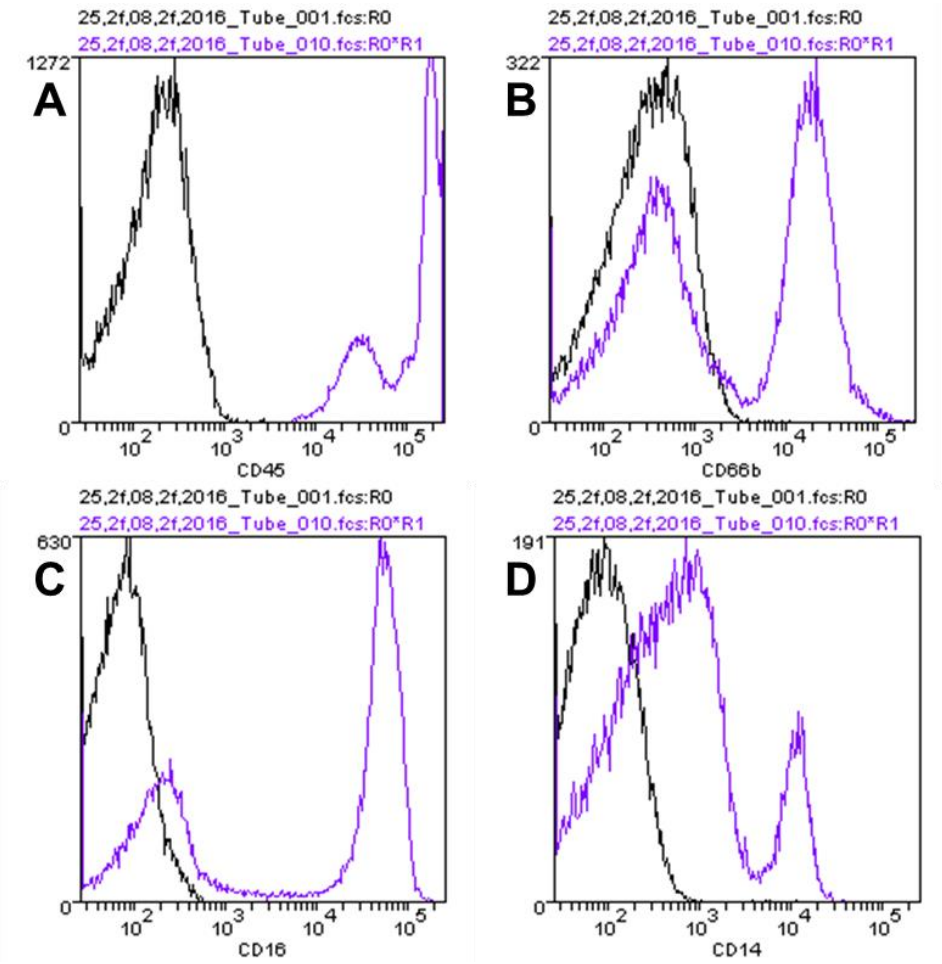

Figure 4.1 B Fluorescence profile of stained and unstained cells.

CBL were isolated and stained with cell surface markers for CD45, CD66b, CD16 and CD14 (purple lines); these were then compared with unstained controls (black lines). The rightward shift in the curves indicates increased fluorescence intensity in stained CBL compared with unstained CBL.

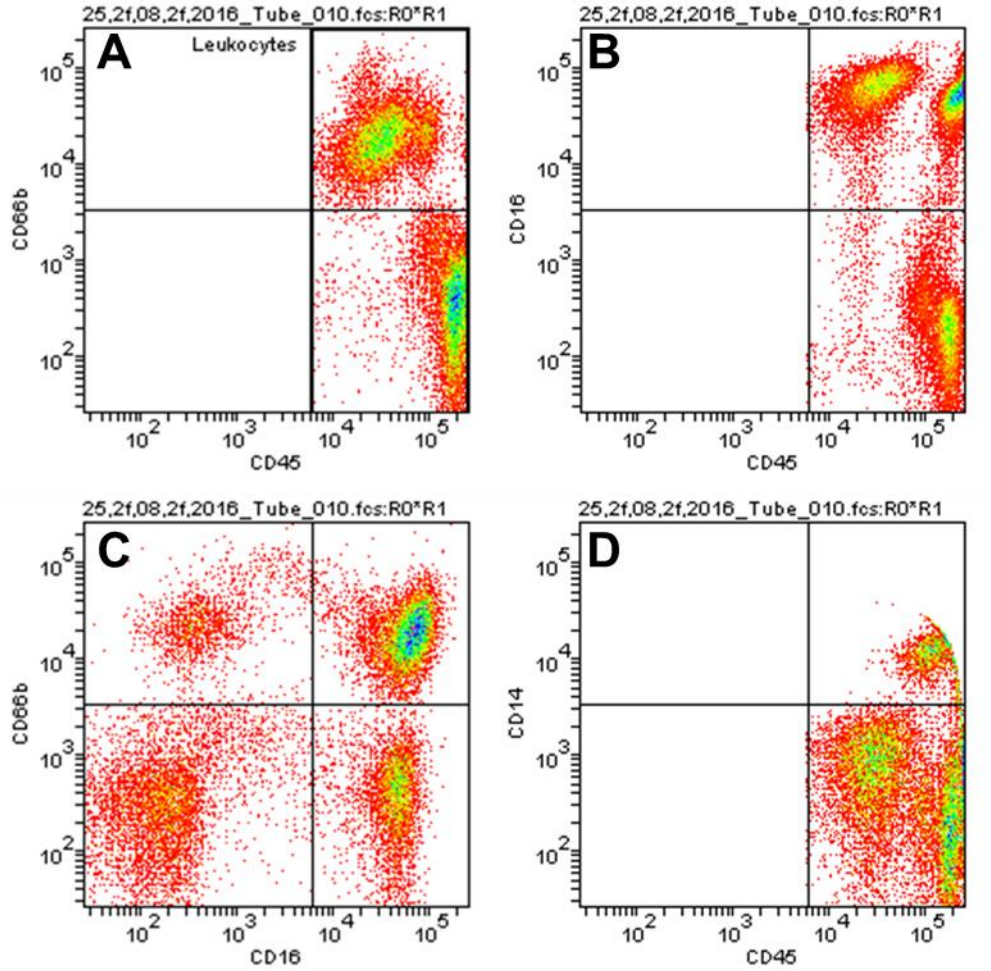

Figure 4.1 C Quadrant Scatter Plots

Quadrant plots of CBL stained with cell surface markers. Each plot shows the staining of a pair of surface markers. The bottom left square shows cells that are negative for both stains, with the top right square showing those which are positive for both stains. The top left and bottom right show single staining for the fluorophore noted on the axis. Data are displayed as single dots representing a cell. Those areas that are red in colour are the least dense, through to blue which are the most dense.

4.2 Assay Validation

4.2.1 Initial SYTOX® Green experiments

Initial experiments were performed to assess the effects of the NETs inducer PMA and LTA in the presence and absence of antagonists DPI, an NADPH oxidase inhibitor and NAC, a scavenger of ROS. Both of these compounds were utilised as inhibitors of downstream signalling pathways knows to be implicated in NET formation. Using $5x10^6$ cells/ml, with increasing concentrations of PMA or LTA there was an increase in the amount of extracellular DNA measured with the SYTOX Green nucleic acid stain. These experiments were performed in parallel with undergraduate project student Victoria Ankrah.

It can clearly be seen in ***Figure 4.2.1*** (panel **A**) that the addition of DPI and NAC ($p<0.0001$) significantly reduced the response to PMA by attenuating the fluorescence measured by SYTOX® green. In comparison, with LTA (***Figure 4.2.1***, panel **B**) the addition of the antagonists NAC and DPI does not have any noticeable effect on the increase in fluorescence measured with increasing LTA concentrations. In this set of experiments, the concentration used for PMA stimulation was deemed to be too high based on the observation that values at the bottom of the concentration-response curve produced around 50% of the maximal response. This prompted further experiments where the concentration of PMA was extended to cover the full concentration range (0.001-1000nM – 5 log units) of PMA activation.

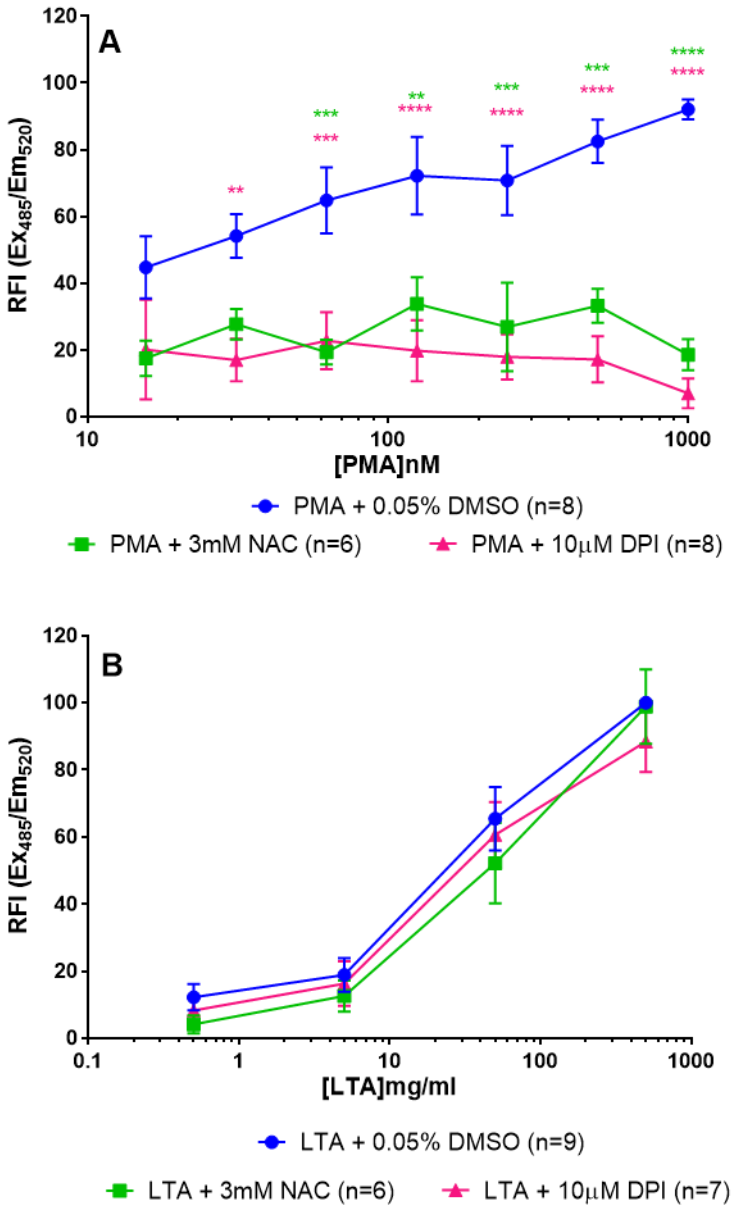

Figure 4.2.1 PMA and LTA with NAC and DPI

CBL were isolated from normal pregnancy at term and incubated with LTA or PMA for 3 hours in the presence of antagonists NAC (3mM) and DPI (10μM). NET production by PMA is attenuated in the presence of NAC and DPI ($p<0.0001$), whereas LTA stimulated CBL were unaffected. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test ($p<0.005$ (**), $p<0.0005$ (***) and $p<0.0005$ (****)).

4.2.2 Validation of PMA Concentration

The PMA concentration used in the assay was increased from the initial range of 15.625 – 1000nM (1:1 dilutions) to 0.01 – 1000nM (half log unit dilutions). Increasing this concentration range, and in particular the lower PMA concentrations used allowed for the production of a full PMA concentration-response curve, which is shown in ***Figure 4.2.2*** below. The segmented line shown on the graph (***Figure 4.2.2***) highlights the lowest concentration used in the previous concentration range. Based on these observations, for the remainder of the experiments (with the exception of the assay buffer validation) the concentration range of 0.1 to 100nM was selected as this covered the full dose-response seen with PMA.

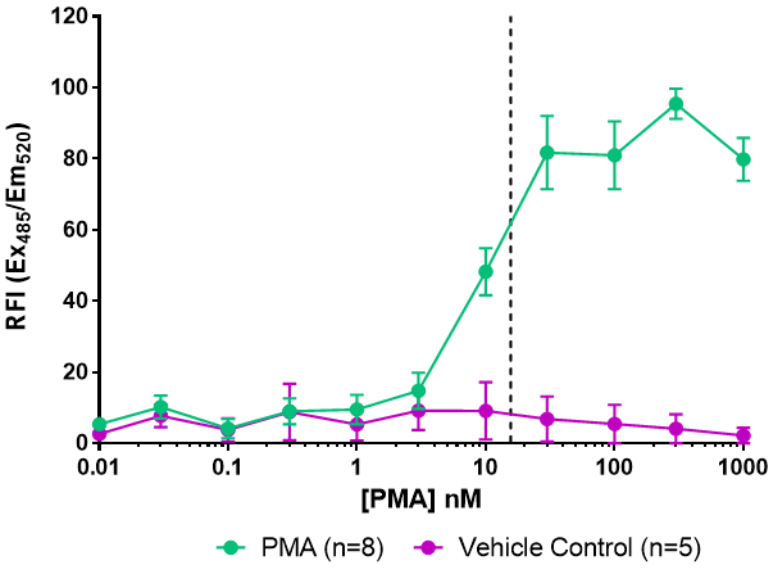

Figure 4.2.2 Extended PMA Dose-Response Curve in CBL

CBL (5x10^6/ml) were isolated from normal pregnancy at term and incubated with PMA for 3 hours. The lowest PMA concentration that had been used in previous experiments is represented by the dashed vertical line on the graph. Data presented as mean ± SEM.

4.2.3 Cell Number and Assay Buffer Validations

In some of the early experiments the baseline control samples had a high level of fluorescence when stained with SYTOX® green. Therefore, further validation was performed to determine the appropriate cell concentration and buffer to be used in the SYTOX® green assay. The original assay protocol was established by a post-doctoral researcher working in the group, and the conditions were re-evaluated after the initial experiments performed. Cell concentrations ($5x10^6$/ml, $1x10^6$/ml, $5x10^5$/ml and $1x10^5$/ml) were tested with the two different assay buffers. These were HBSS and RPMI 1640 medium containing 1% w/v BSA and 1mM $CaCl_2$ (RPMI). In order to assess changes in the shape of the curves at the different cell concentrations the response was normalised against the highest PMA response at each of the cell concentrations.

In HBSS, the response seen at $5x10^6$ cells/ml was found to be significantly different to the response seen at the other cell concentrations $1x10^6$ cells/ml (p=0.0153), $5x10^5$ cells/ml (p=0.0227) and $1x10^5$ cells/ml (p=0.0009). This can be seen in ***Figure 4.2.3*** (Panel **A**), below where there is a clear leftward shift when comparing $5x10^6$ cells/ml with all other concentrations. There is no significant difference observed between the remaining cell concentrations. The response seen in the CBL was erratic when using $5x10^6$ cells/ml resuspended in RPMI buffer with no clear dose-response relationship discernible, hence was not plotted. There was a clear dose-response relationship observed at the other 3 cell

concentrations tested (***Figure 4.2.3*** – Panel **B**). Similarly to the HBSS there was a significant leftward shift observed when comparing $1x10^6$ cells/ml with $5x10^5$ cells/ml ($p=0.0059$) and $1x10^5$ cells/ml ($p=0.0032$). Interestingly, comparing the response of PMA in the different assay buffers, when $1x10^6$ cells/ml were used, a 10 fold lower concentration of PMA produces the same effect when cells are resuspended in HBSS when compared with those in RPMI.

A cell concentration of $1x10^6$ cells/ml, resuspended in HBSS was considered to be optimal for this assay as it provided an adequate signal window (vehicle control subtracted from highest response – ***Figure 4.2.3 B***) with a discrete range of results when compared with the higher cell concentration and the comparable RPMI suspended cells. These conditions were used for all SYTOX® green assays going forwards.

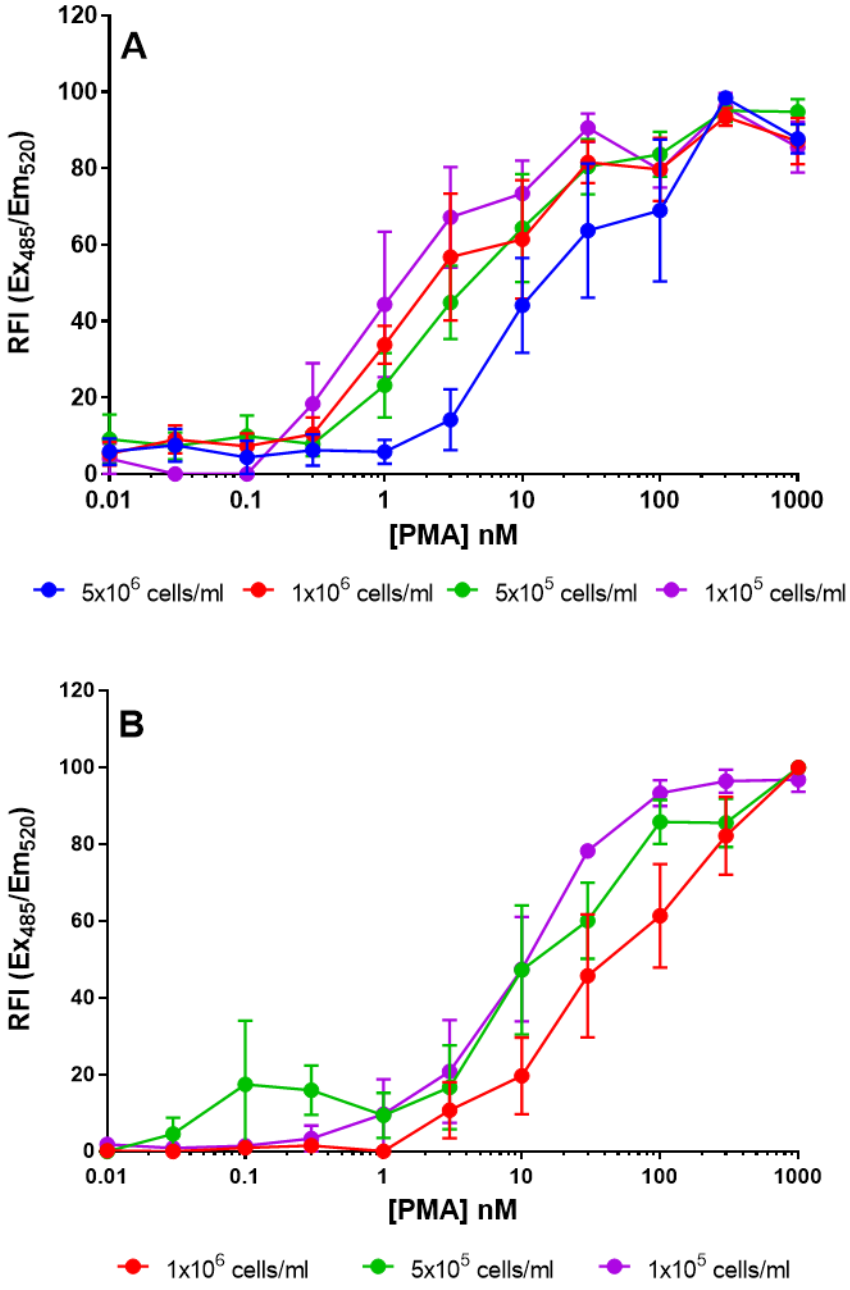

Figure 4.2.3 A Effect of Cell Concentration and Assay Buffer in CBL

*CBL were isolated from normal pregnancy at term and incubated with PMA for 3 hours at a number of different cell concentrations ($5x10^6$, $1x10^6$, $5x10^5$ and $1x10^5$ cells/ml) in either HBSS (**A** – n=4) or RPMI (**B** – n=3) assay buffer. Data presented as mean ± SEM.*

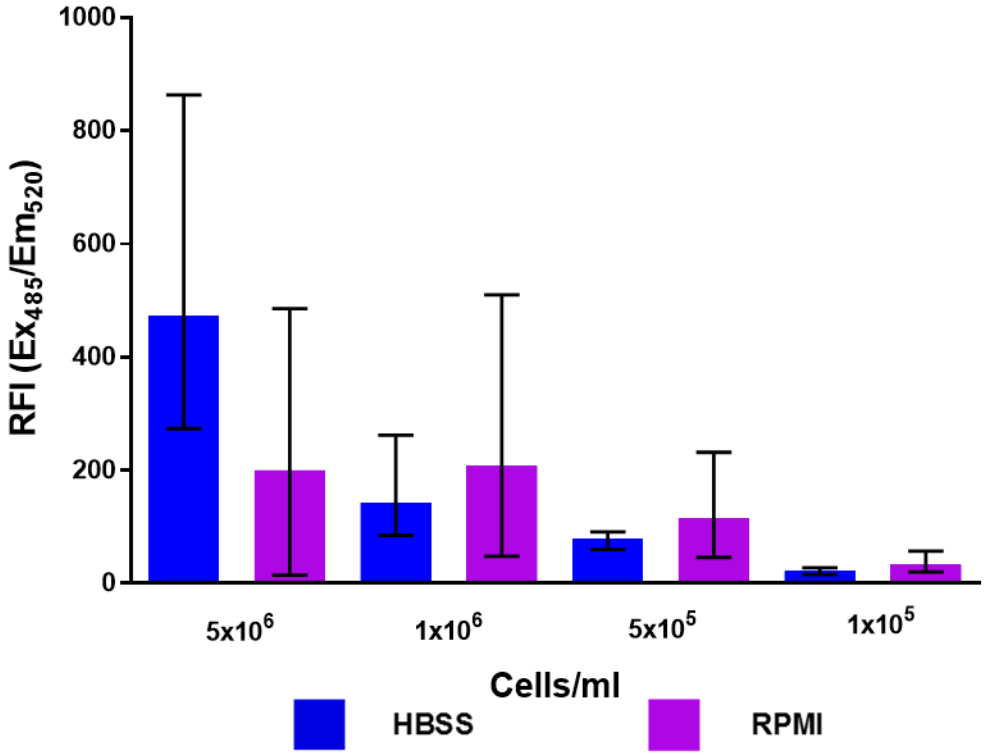

Figure 4.2.3 B Signal Window in CBL

CBL were isolated from normal pregnancy at term and incubated with PMA for 3 hours at a number of different cell concentrations ($5x10^6$, $1x10^6$, $5x10^5$ and $1x10^5$ cells/ml) in either HBSS (n=4) or RPMI (n=3) assay buffer. Data are presented as mean ± range.

4.2.4 NE Assay

NE activity assay was conducted on culture media, washes and NET fragments that had and had not been digested with S7 nuclease to determine the neutrophil elastase concentrations present in these samples. This set of experiments also considered whether any neutrophil elastase was lost during the washing and digestion process at 2 different cell concentrations, $1x10^6$/ml and $5x10^5$/ml. These cell concentrations were chosen as the kit recommended using a minimum of $1x10^6$ cells/ml, however in previous assays that had been performed at $5x10^6$ cells/ml

the stimulation was inconsistent in the assay buffer (RPMI 1640, with 1% BSA and 1mM $CaCl_2$), therefore a lower cell concentration was also selected of $5x10^5$ cells/ml.

CBL, (n=3) were treated with PMA (15nM) or control for 3 hours prior to digestion with MNase. From the results displayed in ***Figure 4.2.4 A*** an example standard curve is shown. As in early experiments culture medium and digested NETs were assayed and a standard curve produced both with and without EDTA as this is only present in the digested samples. This will therefore provide a much more accurate standard curve to interpolate values from.

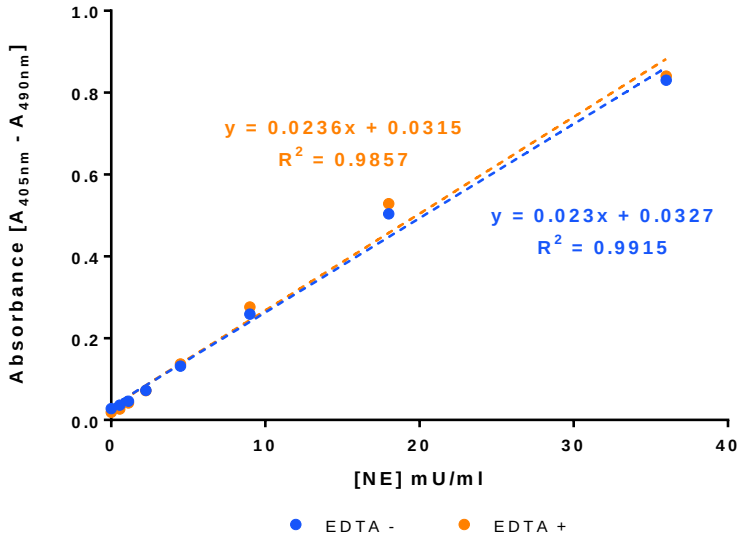

Figure 4.2.4 A Standard Curve of Neutrophil Elastase Activity

NE standard was diluted from 36mU/ml to 0.62mU/ml and incubated with NE substrate for 1 hour in duplicate. Concentrations was plotted against mean absorbance and a linear trend line fitted. Concentrations in the test samples were interpolated from the standard curve.

Figure 4.2.4 B shows the NE concentrations determined in culture medium, wash medium, undigested and digested NETs for treated and untreated samples. At $1x10^6$ cells/ml there was 0.74 (±0.21) U/ml NE in the culture medium of control samples compared with 2.58 (±0.27) mU/ml NE in the PMA treated samples and at $5x10^5$ cells/ml there were 0.19mU/ml (±0.12) in the control culture medium and 0.63 (±0.09) mU/ml in the PMA treated media. These levels are most likely to have been caused by degranulation in the neutrophils, which although not significantly different have a trend to be higher in treated than untreated samples. There was no evidence of NET disruption during the washing steps prior to digestion as there was minimal NE measured in any of these samples and all levels were consistent with the background control. In the undigested NET samples, only low levels of NE were measured of 0.57 (±0.31) mU/ml and 1.68 (±0.54) mU/ml in control and PMA treated CBL respectively at $1x10^6$ cells/ml and 0.37 (±0.28) mU/ml and 0.45 (±0.26) mU/ml in control and PMA treated samples at $5x10^5$ cells/ml. In the digested NET samples there was 16.13 (±1.07) mU/ml of NE in PMA treated samples at $1x10^6$ cells/ml compared with 2.70 (±1.36) mU/ml in control samples. At $5x10^5$ cells/ml there was 6.43 (±0.52) mU/ml of NE in PMA stimulated cells and 0.92 (±0.38) mU/ml in control cells. At both cell concentrations tested there was a statistically significant difference between PMA treated and control digests ($p<0.0001$) and also between undigested and digested NET samples in PMA treated CBL ($p<0.0001$).

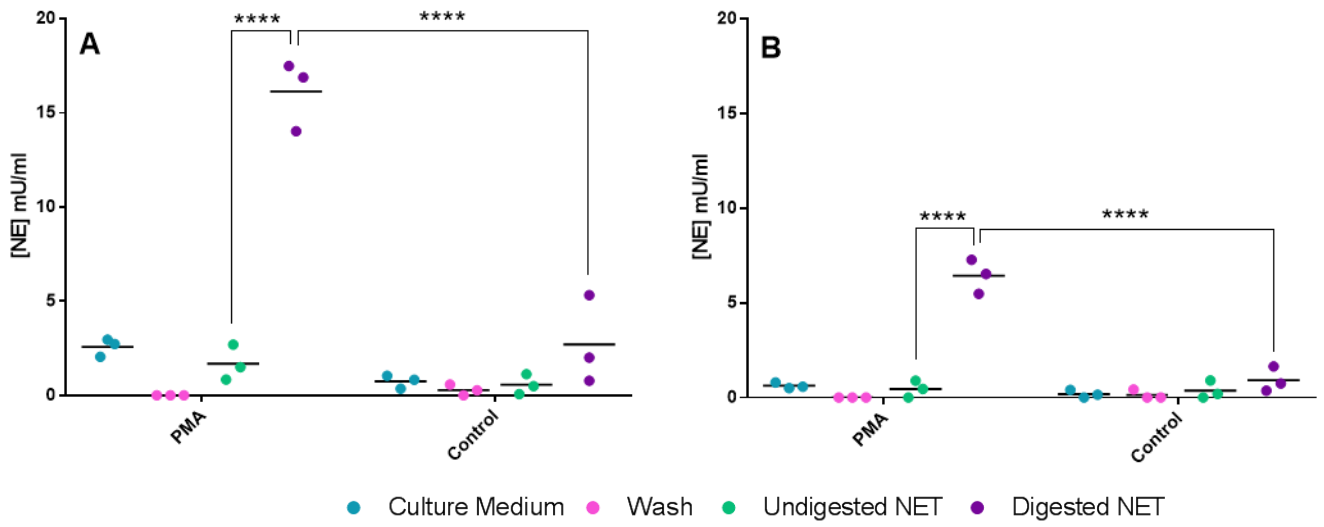

Figure 4.2.4 B Validation of the Neutrophil Elastase Activity Assay

*CBL were stimulated with PMA (15nM) or control for 3 hours before the NET structures were digested with MNase (n=3). NE concentrations were measured in culture medium, wash buffer, undigested and digested NETs were assayed to determine NE concentration. Panel **A** shows the NE concentrations when 1x10^6 cells/ml were used and Panel **B** shows the NE concentration when 5x10^5 cells/ml were used. In both cell concentrations statistically significant differences were observed between control and PMA treated digested NETs (p<0.0001) and between PMA treated digested and undigested NETs (p<0.0001). Data are presented as mean and individual data points. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test. (p<0.0001 (****)).*

4.3 NET Formation with PAMPs in CBL

The following section will cover the assays used for the measurement and visualisation of NETs in CBL in response to PAMPs. I will firstly cover the results obtained from the SYTOX® green assay, followed by immunofluorescence and NE activity assay. This will be followed by a comparison of NET formation in CBL from term neonates with preterm neonates and CBL from term neonates where the mother did not have a known GBS infection with those that did have a known GBS infection.

4.3.1 SYTOX® green assay

The effects of the bacterial products LTA, PGN and LPS on the SYTOX® green assay were evaluated to confirm that these agents produced a reliable and consistent NET response in CBL collected and isolated from term placentae following elective caesarean section. Utilising the gram-positive bacterial ligands LTA and PMA (***Figure 4.3.1 A***) there was increasing DNA measured with increasing concentration. This indicated that more NETs are being formed with increasing agonist concentration. This increase was found to be statistically significant over the concentration range tested ($p<0.0001$). The same was true when NET formation was stimulated using the gram-negative ligand LPS (***Figure 4.3.1 B***) and chemical stimulant PMA ($p<0.0001$). An interesting observation when viewing the concentration-response of the PAMPs is that once the top of the maximum response has been reached, the fluorescence intensity measurement begins to fall. LTA in particular shows the largest reduction. As shown in ***Table 4.3.1***, there were no

demographic differences between the subjects in each of the treatment groups

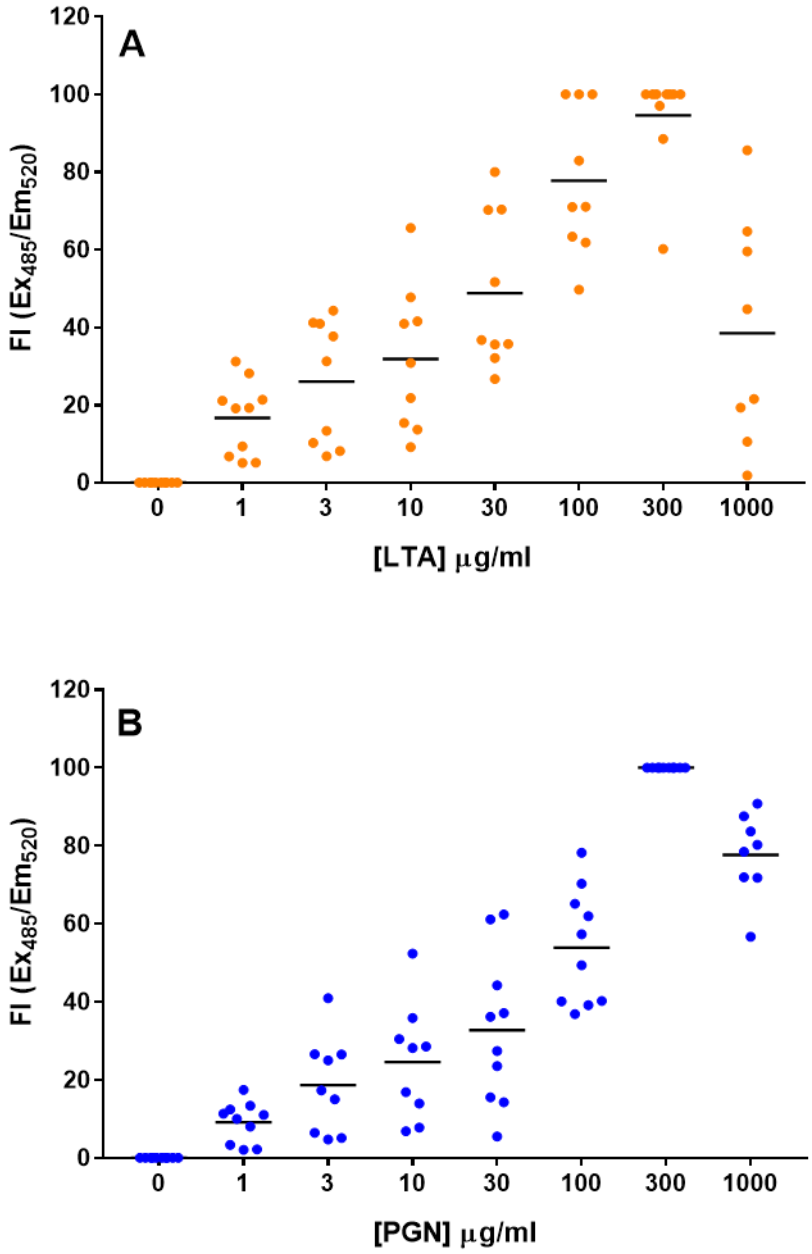

Figure 4.3.1 A NET Formation in CBL Stimulated with LTA and PGN

*CBL were isolated from normal pregnancy and incubated with LTA (n=10) or PGN (n=10) for 3 hours. With both LTA (**A**) and PGN (**B**) stimulation there was increasing levels of DNA measured, with increasing agonist concentration (p<0.0001). Data presented are mean and individual data points and statistical analysis was performed using a one-way ANOVA with Bonferroni post-test.*

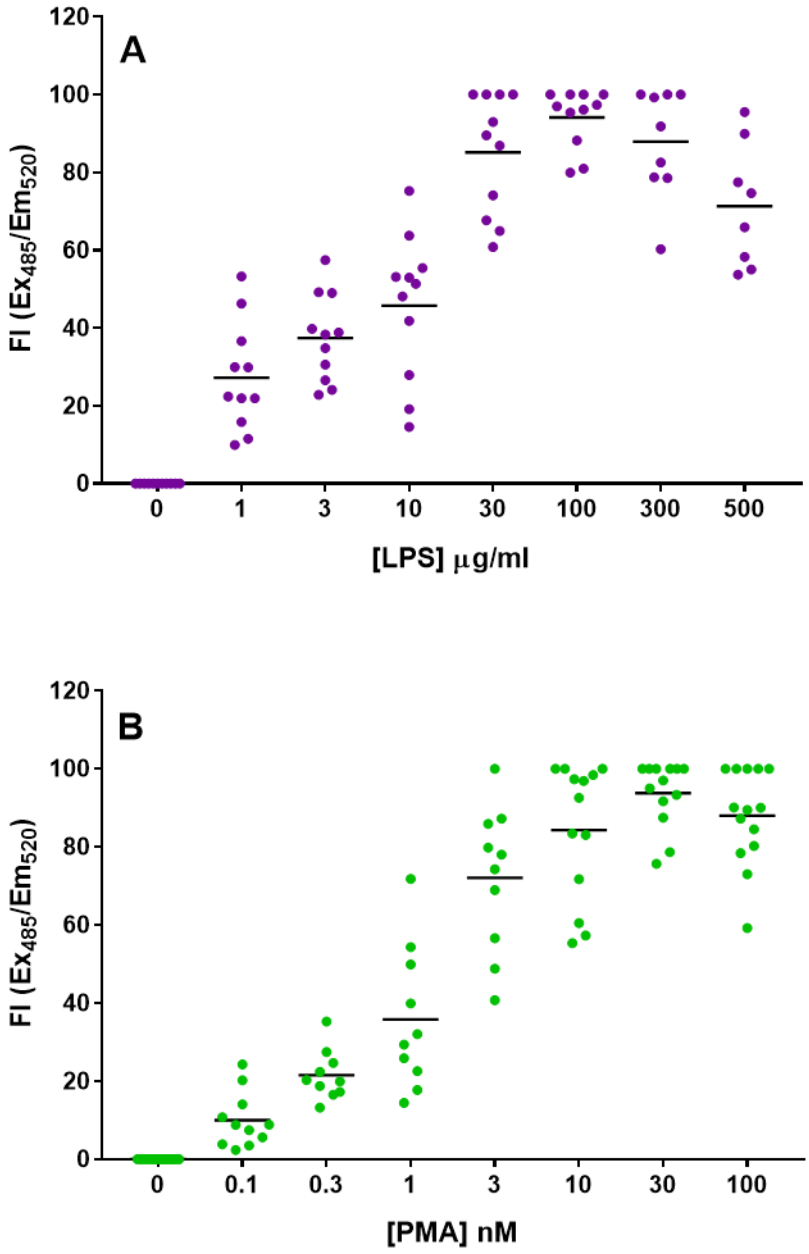

Figure 4.3.1 B NET Formation in CBL Stimulated with LPS and PMA

*CBL were isolated from normal pregnancy and incubated with LPS (n=11) or PMA (n=15) for 3 hours. With both LPS (**A**) and PMA (**B**) stimulation there was increasing levels of DNA measured, with increasing agonist concentration ($p<0.0001$). Data presented are mean and individual data points and statistical analysis was performed using a one-way ANOVA with Bonferroni post-test.*

Group	PMA	LTA and PGN	LPS	PTB	GBS
n	15	10	11	3	3
Maternal Age (Mean ±SEM)	31 ±0.96	32 ±0.95	30 ±1.1	30 ±1.7	29 ±2.4
Gravida (Mean ±SEM)	2.9 ±0.44	2.7 ±0.33	3.0 ±0.62	2.7 ±0.67	2.3 ±0.33
Parity (Mean ±SEM)	1.5 ±0.41	1.1 ±0.28	1.5 ±0.58	1.3 ±0.33	1.3 ±0.33
Situation at time of collection	El LSCS	EL LSCS	EL LSCS	EL LSCS	sPTB
Gestational age at collection (days) (Mean ±SEM)	273 ±0.79	274 ±0.41	272 ±1.00	243 ±7.1	274 ±1.00
Gestational age at collection (weeks)	39+0	39+1	38+6	34+5	39+1
Sex of baby	4 Male 11 Female	2 Male 8 Female	4 Male 7 Female	2 Male 1 Female	1 Male 2 Female
Weight of baby (g) (Mean ±SEM)	3410 ±94	3579 ±77	3276 ±11	2450 ±320	4075 ±95

Table 4.3.1 Demographics of CBL samples collected for PAMP experiments

A demographics table illustrating the characteristics of the samples used in the study for the PAMP experiments with CBL and also for the GBS and PTB patient groups. The PTB group had a significantly lower gestational age and birth weight to all other groups ($p<0.0001$), and the birth weight of the GBS group was significantly higher than the LPS group ($p=0.0430$). Statistical analysis was performed using a one-way ANOVA with Bonferroni post-test.

4.3.2 Immunofluorescence

Immunofluorescence was performed to allow for the visualisation of NET structures outside the cells by using an antibody to human NE which was counter stained with a FITC conjugated secondary antibody. This allowed visualisation of the NE present in the strand like extracellular trap structures protruding from the cells. These were particularly evident when compared with unstimulated (control) leukocytes (n=3) where the NE is mainly localised in the cytoplasm of the cells (see ***Figure 4.3.2***). This staining supports the data obtained in the SYTOX green assay and that the increase in DNA fluorescence is due to the production of NETs. Controls where the primary antibody was omitted to assess for non-specific staining of the secondary antibody. This revealed a low level of non-specific staining on the cells, but there was no staining of extracellular fibres.

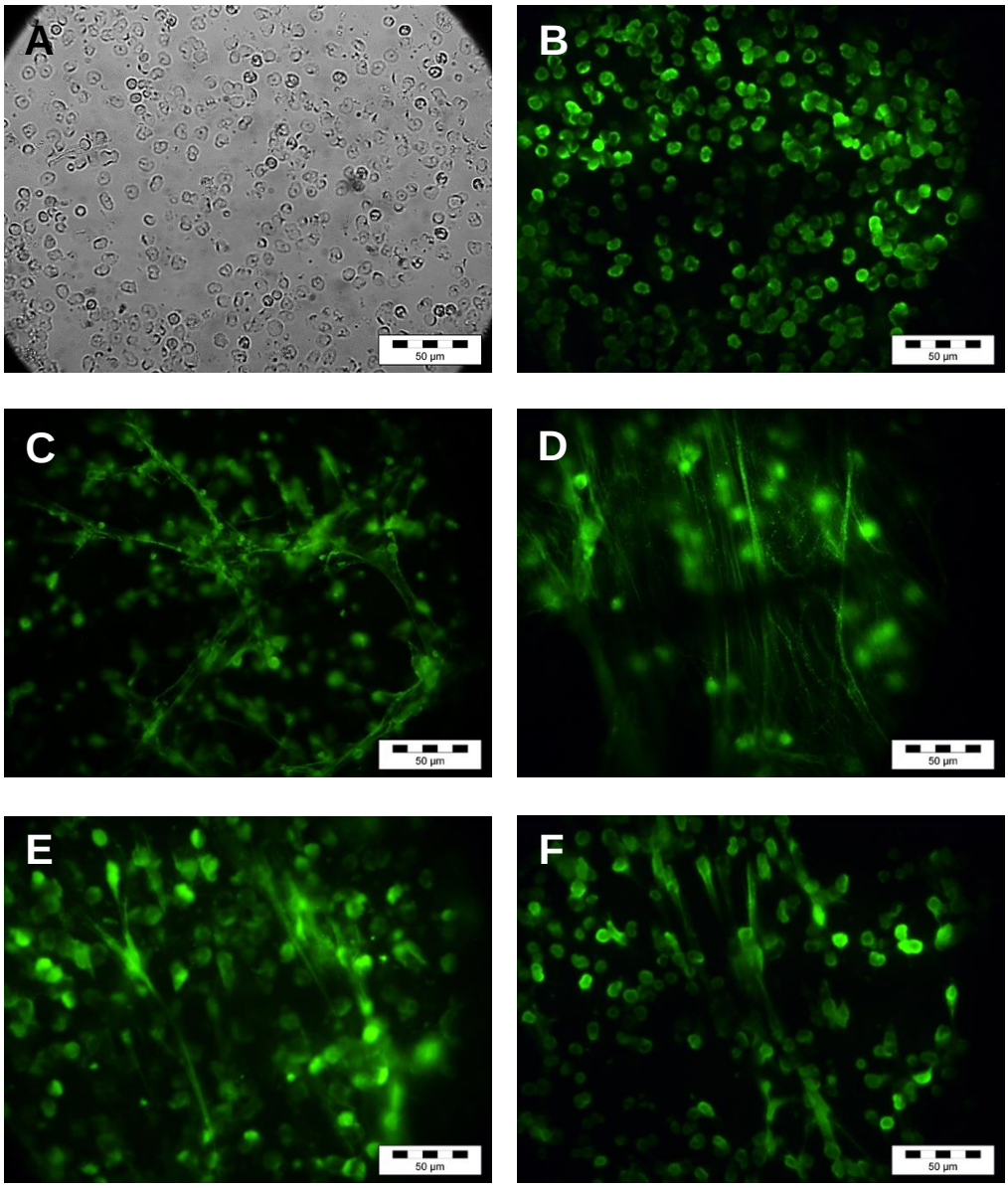

Figure 4.3.2 Immunofluorescence of NET structures in CBL

CBL were isolated from normal pregnancy at term (n=3) and incubated with LTA (300µg/ml), PGN (300µg/ml), LPS (100µg/ml) or PMA (100nM) for 3 hours. Cells were then fixed and stained with a NE elastase antibody and a FITC conjugated secondary antibody. The figure shows a bright field image of CBL (A) followed by fluorescent images of NE (FITC) staining in control (B), PMA (C), LTA (D), PGN (E) and LPS (F). In the control leukocytes there is NE staining which appears to be localised in the cytoplasm. When compared with the treated cells, strand like structures can be seen protruding from the cells, which indicate the formation of NETs.

4.3.3 NE Activity

The PAMPs used in the previous assay LTA, PGN and LPS were all tested, using PMA as a positive control. There was no discernible response to LTA and LPS using this assay method. The culture medium (n=6), digested and undigested samples (n=6) were assayed. Unfortunately only 3 of the 6 samples tested showed positive results in the assay to the positive control PMA or any of the other PAMPs after MNase digestion. It was noted that the experiments for the 3 samples that did not work were performed using a different batch of MNase than those which did respond, therefore it is likely that this is down to reduced e of the enzyme. The 3 samples that did not work were therefore not displayed on the graph.

As shown in ***Figure 4.3.3*** (n=3 shown), both PGN and PMA-treated CBL there was a statistically significantly higher amount of NE measured in digested NETs (9.60 ±3.95 mU/ml PMA and 6.77 ±2.97 mU/ml PGN) when compared with undigested NETs (p=0.0008 PMA and p=0.0198 PGN). With PMA stimulation, the level of NE in digested NETs was significantly different to digested control NETs (p=0.0073), but this difference was not maintained with PGN. This may be due to the fact that one of the patients used for this assay, which did show a response to PMA had much lower results than the other two patients and it was later discovered that this patient was GBS positive and had gestational diabetes.

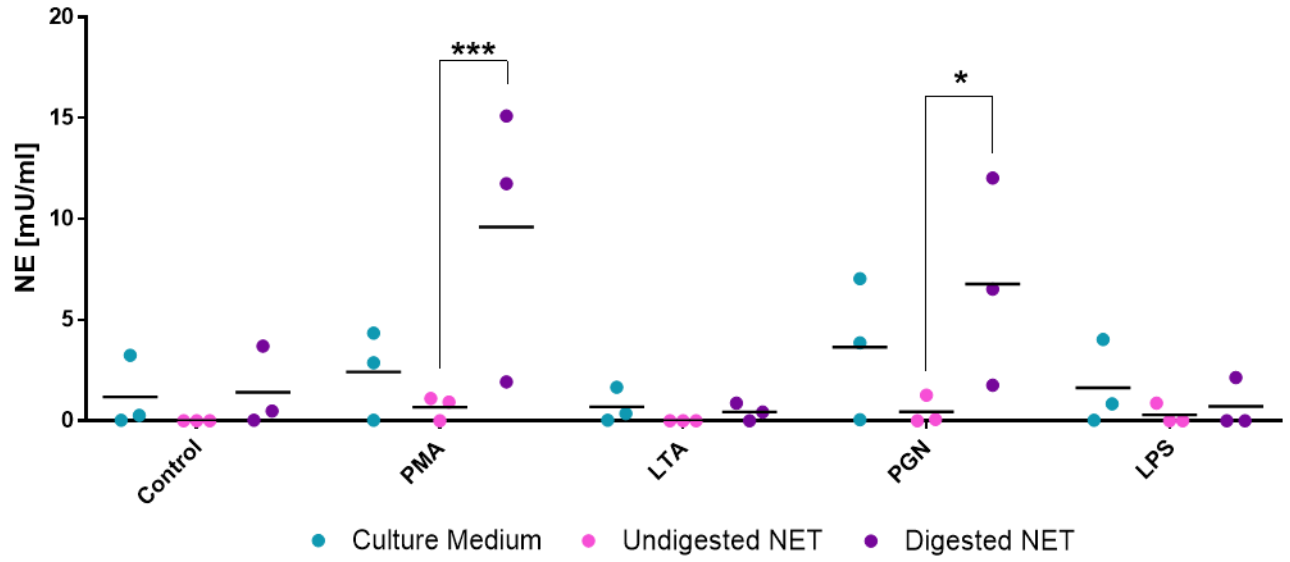

Figure 4.3.3 NET Assay Using CBL Stimulated with PAMPs

CBL were stimulated with PMA (15nM), LTA (100µg/ml) PGN (100µg/ml) and LPS (100µg/ml) for 3 hours before the NET structures were digested with MNase and assayed for NE activity (n=3). NE concentrations were measured in culture medium, digested and undigested NETs. PGN and PMA had positive responses in the assay with the NE in digested samples statistically significantly greater than in undigested samples (p=0.0008 PMA and p=0.0198 PGN). Data presented are mean (black line) with individual data points and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test (p<0.05 (*) and p<0.0005 (***)).

4.3.4 Preterm Birth

CBL were purified and treated from placentae collected from deliveries which occurred preterm (gestational age range 32+5 – 36+0 weeks) and compared with CBL from neonates that delivered at term (gestational age range 38+0 – 39+5 weeks). The demographics table (Table 4.3.1) reveals that babies born preterm had a significantly lower gestational age and birth weight when compared with babies born at term ($p<0.0001$). There was no significant difference observed in NET formation between the two groups when stimulated with LTA, PGN and LPS (***Figure 4.3.4 A***, ***Figure 4.3.4 B*** – *Panel* ***A*** and ***Table 4.4.1***). There was however a statistically significant rightward shift of the concentration response curve in the CBL from PTB patients compared with the term patients ($p=0.0057$) when stimulated with PMA. (***Figure 4.3.4 B*** – *panel* ***B*** and ***Table 4.4.1***).

Group	LTA (µg/ml)	PGN (µg/ml)	LPS (µg/ml)	PMA (nM)
Term	37.66	26.2	7.91	4.36
Preterm	29.8	60.9	1.08	3.47

Table 4.4.1 EC_{50} values of MBL with differing gestational age

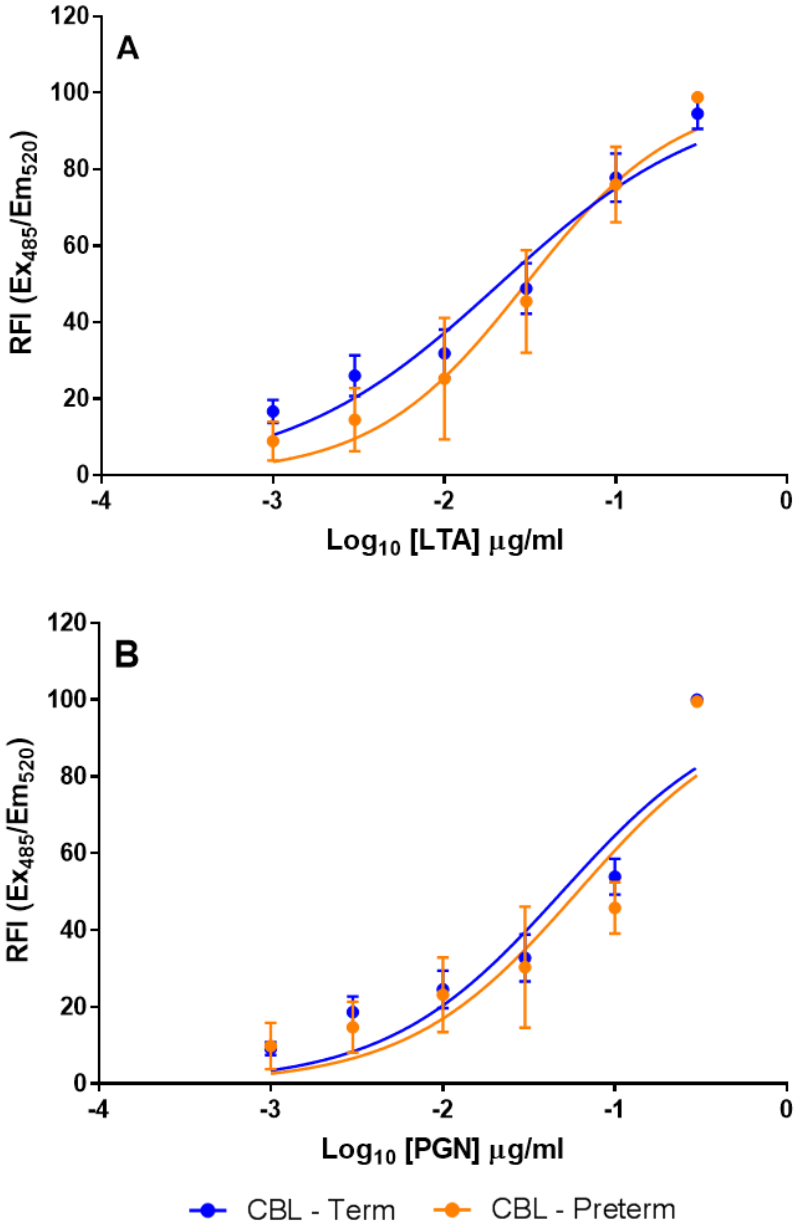

Figure 4.3.4 A LTA and PGN induced NET formation in CBL

*CBL were isolated from normal pregnancy at term (n=10) and preterm birth (n=3) and incubated with LTA or PGN for 3 hours. With LPS (**A**) and PMA (**B**) stimulation there was no difference in NET formation, when comparing these two groups. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.*

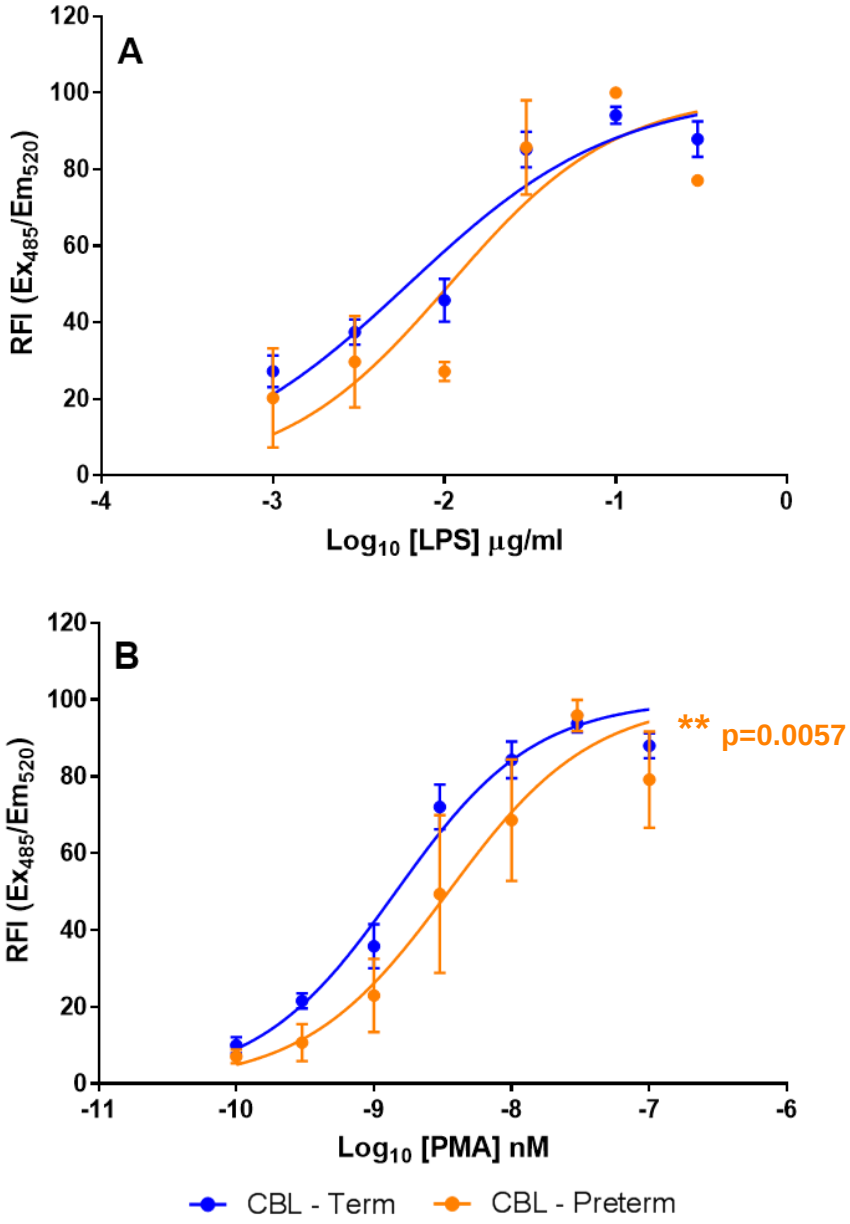

Figure 4.3.4 B LPS and PMA induced NET formation in CBL

*CBL were isolated from normal term pregnancy (n=11 LPS and n=15 PMA) and preterm birth (n=3) and incubated with LPS or PMA for 3 hours. With LPS (**A**) stimulation there was no difference in NET formation. With PMA (B) stimulation there was a significant rightward shift in the concentration response curve of PTB compared with term (p=0.0057), although there were no individual differences at any concentration. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.*

4.3.5 Group B Streptococcus (GBS)

GBS is often an asymptomatic infection within the female reproductive tract which can cause severe infection of the neonate after delivery. A comparison was made to see whether there was any difference in the response of mothers who tested positive for GBS on a HVS when compared with ladies who were not known to have GBS infection. The gram-positive bacterial ligands LTA and PGN had contrasting results (see ***Figure 4.3.5 A*** and ***Table 4.3.5***). LTA showed no significant difference between the two groups whereas PGN revealed a statistically significant difference ($p<0.0001$), with CBL where there is a maternal GBS infection displaying a greater level of NET formation when stimulated with the same concentration of PGN. There was also no significant difference seen when the same comparison was performed after stimulation with LPS and PMA (see ***Figure 4.3.5 B*** and ***Table 4.3.5***). The demographics table (***Table 4.3.1***) highlights that there were no differences between the patient groups, with the exception of a significantly higher birth weight in the GBS group, when compared with LPS stimulated samples ($p=0.0320$)

Group	LTA (µg/ml)	PGN (µg/ml)	LPS (µg/ml)	PMA (nM)
Term	37.6	26.2	7.91	4.36
Term + GBS	16.5	12.9	6.13	2.40

Table 4.3.5 EC_{50} values of CBL with and without maternal GBS infection

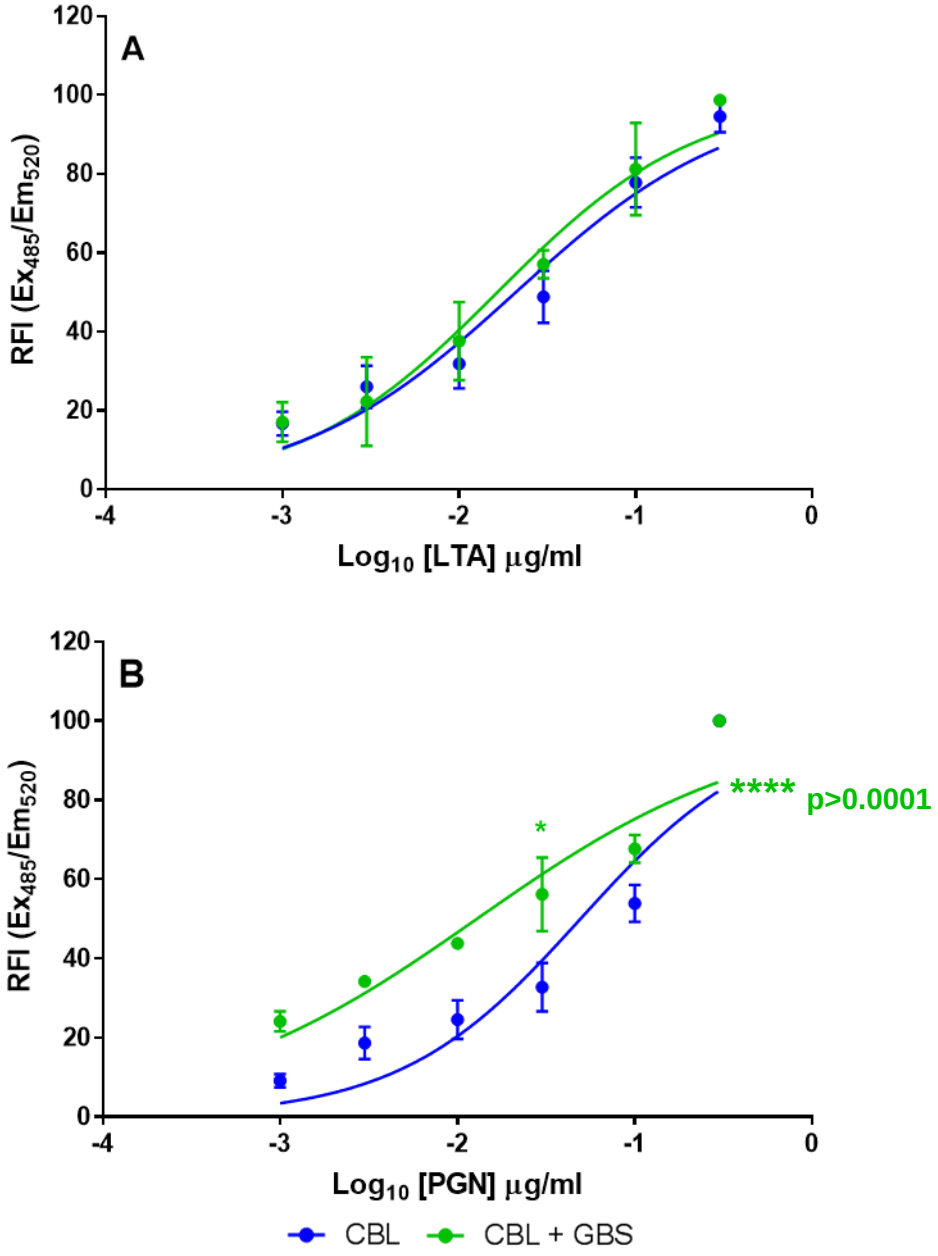

Figure 4.3.5 A LTA and PGN induced NET formation in CBL

*CBL were isolated from normal pregnancy (n=10) and pregnancy with maternal GBS infection (n=3) and incubated with LTA or PGN for 3 hours. With LTA stimulation (**A**) there was no difference in NET formation, whereas with PGN stimulation (**B**) there was a statistically significant shift in the curve (p<0.0001), with a greater response seen in GBS at the same concentration as normal pregnancy. Data presented are mean ± SEM and statistical analysis was performed using a one-way ANOVA with Bonferroni post-test (p<0.05 (*)).*

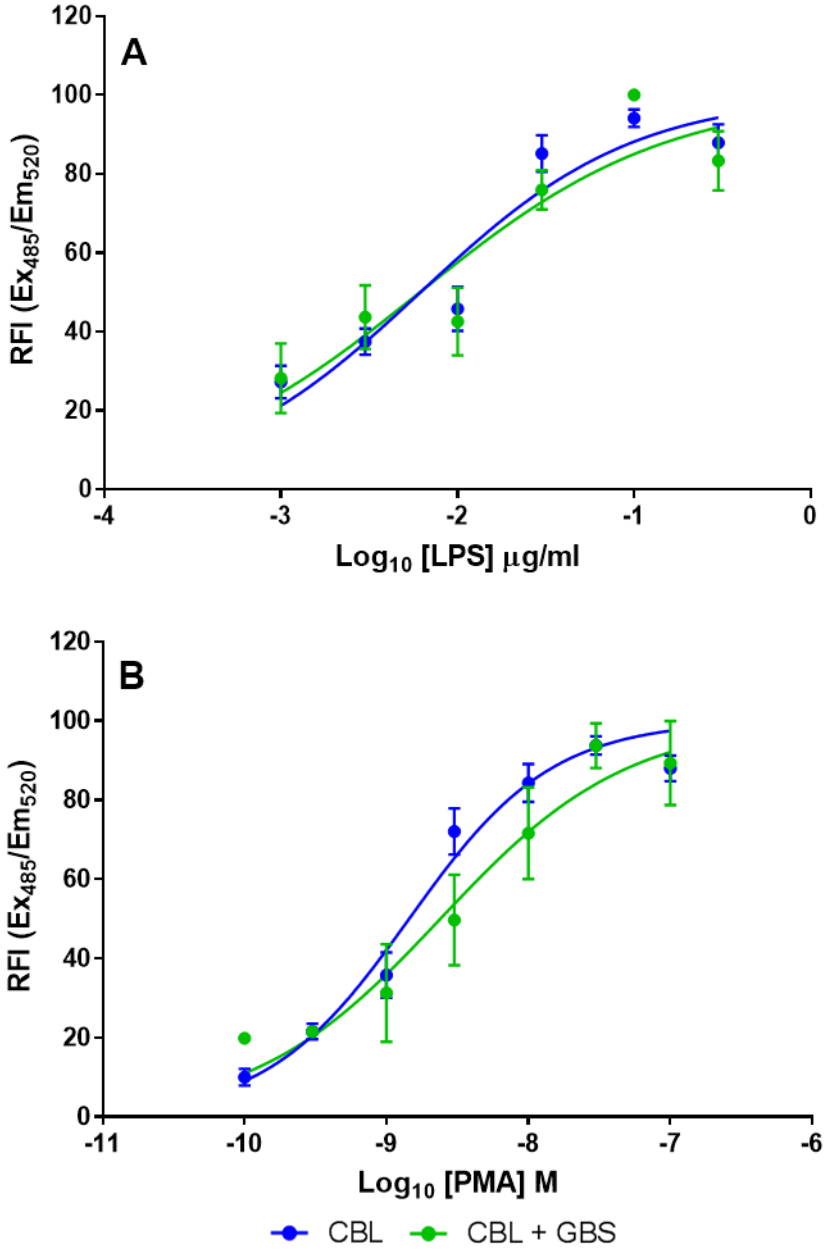

Figure 4.3.5 B LPS and PMA induced NET formation in CBL

*CBL were isolated from normal pregnancy (n=11 LPS and n=15 PMA) and pregnancy with maternal GBS infection (n=3) and incubated with LPS or PMA for 3 hours. With LPS (**A**) and PMA (**B**) stimulation there was no difference in NET formation, when comparing these two groups. Data presented are mean ± SEM and statistical analysis was performed using a one-way ANOVA with Bonferroni post-test.*

4.4 NET Formation with PAMPs in MBL

As a comparison, further experiments were also conducted to assess NET formation in isolated leukocytes from maternal blood, using the PAMPs LTA, PGN and LPS, alongside the positive control PMA. MB samples were collected (n=29) from patients at varying stages of gestation and clinical presentation, represented in the demographics table (***Table 4.4***). Larger variations in the gestational age range noted in the PPROM groups, was primarily due to the fact that women presenting with PPROM can do so at any point during their pregnancy. Whereas women in the remaining groups were recruited when attending routine scan or clinic appointments at 20 and 28 weeks of gestation or for elective caesarean section at 38-40 weeks. The fate of the pregnancy is collated in the demographics table, where available, although not all of the women had delivered at the time of the compilation of this thesis.

Group	2nd Trimester	3rd Trimester	Term	2nd Trimester PPROM	3rd Trimester PPROM
n	5	6	4	4	5
Maternal Age (Mean ±SEM)	25 ±2.9	30 ±2.8	31 ±2.6	33 ±3.0	29 ±1.3
Gravida (Mean ±SEM)	1.8 ±0.58	1.8 ±0.31	2.3 ±0.25	3.3 ±1.30	3.2 ±0.66
Parity (Mean ±SEM)	0.4 ±0.4	0.5 ±0.34	1.3 ±0.25	2.3 ±1.3	0.5 ±0.34
Situation at time of collection	28 week clinic appointment	20 week scan	Prior to El LSCS	PPROM	PPROM
Gestational age at collection (days) (Mean ±SEM)	140 ±0	196 ±1	273 ±0	171 ±9.5	230 ±12
Gestational age at collection (weeks)	20+0	28+0	39+0	24+3	32+6
Latency period, if PPROM (days) (Mean ±SEM)	Not applicable	Not applicable	Not applicable	30 ±15	17 ±9
Sex of baby (if delivered)	None delivered	None delivered	1 Male 3 Female	4 Male 0 Female	4 Male 1 Female
Weight of baby (g) (Mean ±SEM)	None delivered	None delivered	3244 ±320	1206 ±139	2043 ±501

Table 4.4 Demographics of MBL Samples Collected for PAMP Experiments

A demographics table illustrating the characteristics of the samples used in the study for the PAMP experiments with MBL. The 2nd Trimester PPROM group had a significantly lower birth weight when compared with term (p=0.0127), and the gestational ages of all groups were significantly different from one another (p<0.0001). Statistical analysis was performed using a one-way ANOVA with Bonferroni post-test.

4.4.1 Gestational Age

In order to assess the impact of gestational age on the level of externalised DNA as measured by SYTOX green fluorescence a comparison was made between the term, 3rd trimester and 2nd trimester groups. In LTA, PGN, LPS and PMA there were no statistically significant differences between these groups (***Figure 4.4.1 A*** and ***Figure 4.4.1 B***), which indicates that with these compounds gestational age alone does not have an impact on NET production (see ***Table 4.4.1*** for EC_{50} values).

Group	LTA (µg/ml)	PGN (µg/ml)	LPS (µg/ml)	PMA (nM)
Term	37.6	26.2	7.91	4.36
3rd Trimester	55.6	18.8	5.99	1.79
2nd Trimester	33.2	7.59	4.49	3.46

Table 4.4.1 EC_{50} Values of MBL with Differing Gestational Age

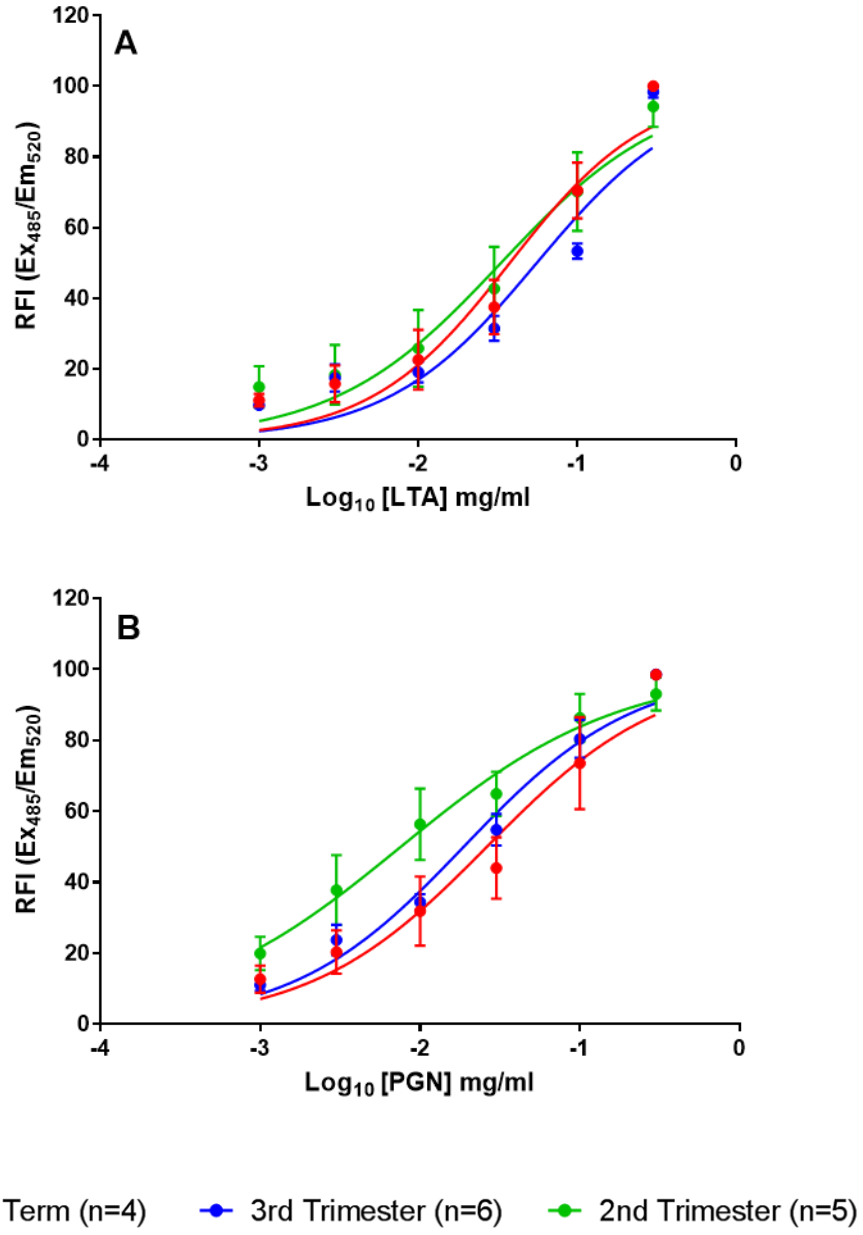

Figure 4.4.1 A LTA and PGN Induced NET Formation in MBL from Normal Pregnancy at Varying Gestation

*MBL, collected from normal pregnancies at 20 weeks (n=5), 28 weeks (n=6) or term (n=4), were stimulated with LTA and PGN for 3 hours to induce NET formation. With LTA (**A**) or PGN (**B**) stimulation there was no difference in NET formation, when comparing these three groups. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.*

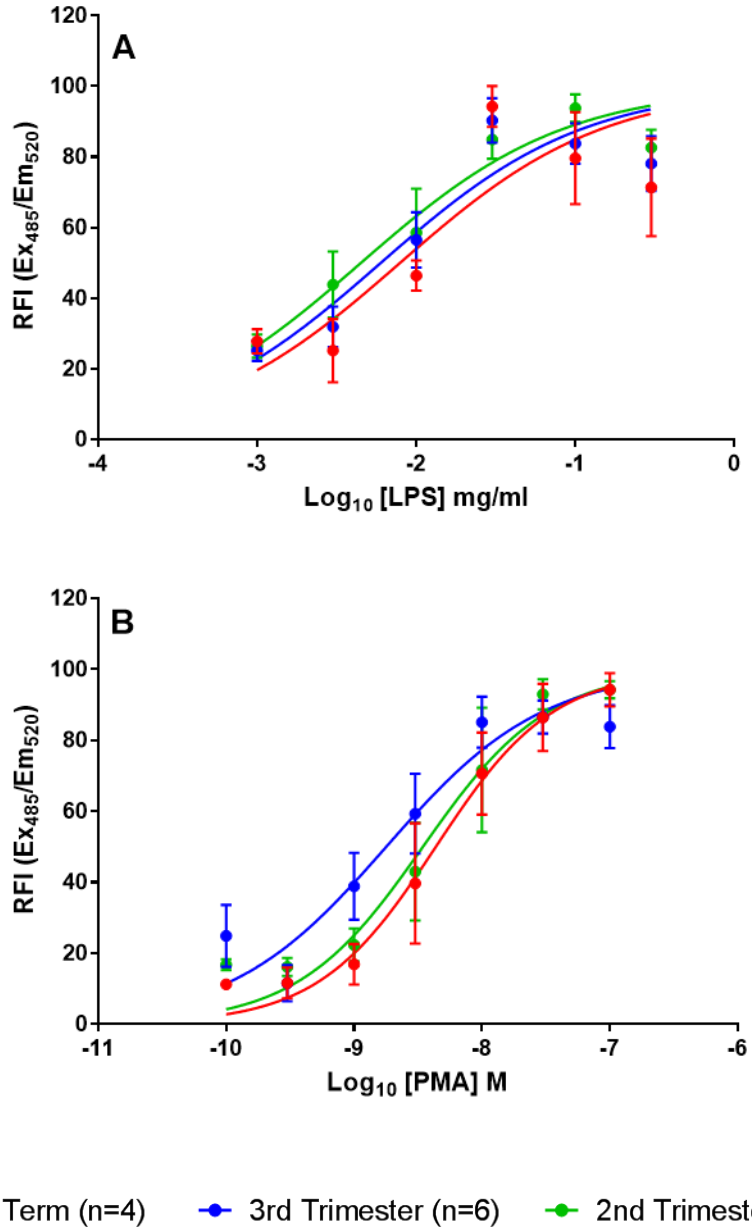

Figure 4.4.1 B LPS and PMA Induced NET Formation in MBL from Normal Pregnancy at Varying Gestation

*MBL, collected from normal pregnancies at 20 weeks (n=5), 28 weeks (n=6) or term (n=4), were stimulated with LPS and PMA for 3 hours to induce NET formation. With LPS (**A**) or PMA (**B**) stimulation there was no difference in NET formation, when comparing these three groups. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.*

4.4.2 PPROM vs Term

In order to identify differences in response based on both PPROM and gestational age together, comparisons were made between 2nd trimester PPROM group, 3rd trimester PROM group and term group (See ***Table 4.4.2*** for EC_{50} values). There were no significant differences identified between the 3rd trimester PPROM and term groups for any of the agonists tested. Further to this, in LPS (***Figure 4.4.2 B*** – panel **A**) there was also no significant difference observed between the term and 2nd trimester PPROM groups. This indicates that for LPS, the clinical presentation of PPROM does not affect the level of NET production in MBL. There was a significant leftward shift in the dose-response curve seen with LTA ($p<0.0001$) and PGN ($p<0.0001$) when comparing 2nd trimester PPROM and term (***Figure 4.4.2 A***). With PMA there was no overall difference between the 2nd trimester PPROM and term curves (***Figure 4.4.2 B*** – panel **B**), but there was a significantly higher level of fluorescence observed in the 2nd trimester PPROM group at 1nM and 3nM PMA ($p=0.0332$ and $p=0.0002$).

Group	LTA (µg/ml)	PGN (µg/ml)	LPS (µg/ml)	PMA (nM)
Term	37.6	26.2	7.91	4.36
3rd Trimester PPROM	53.6	30.5	7.13	5.27
2nd Trimester PPROM	3.6	3.80	17.9	0.98

Table 4.4.2 EC_{50} Values of MBL with Differing Gestational Age and PPROM

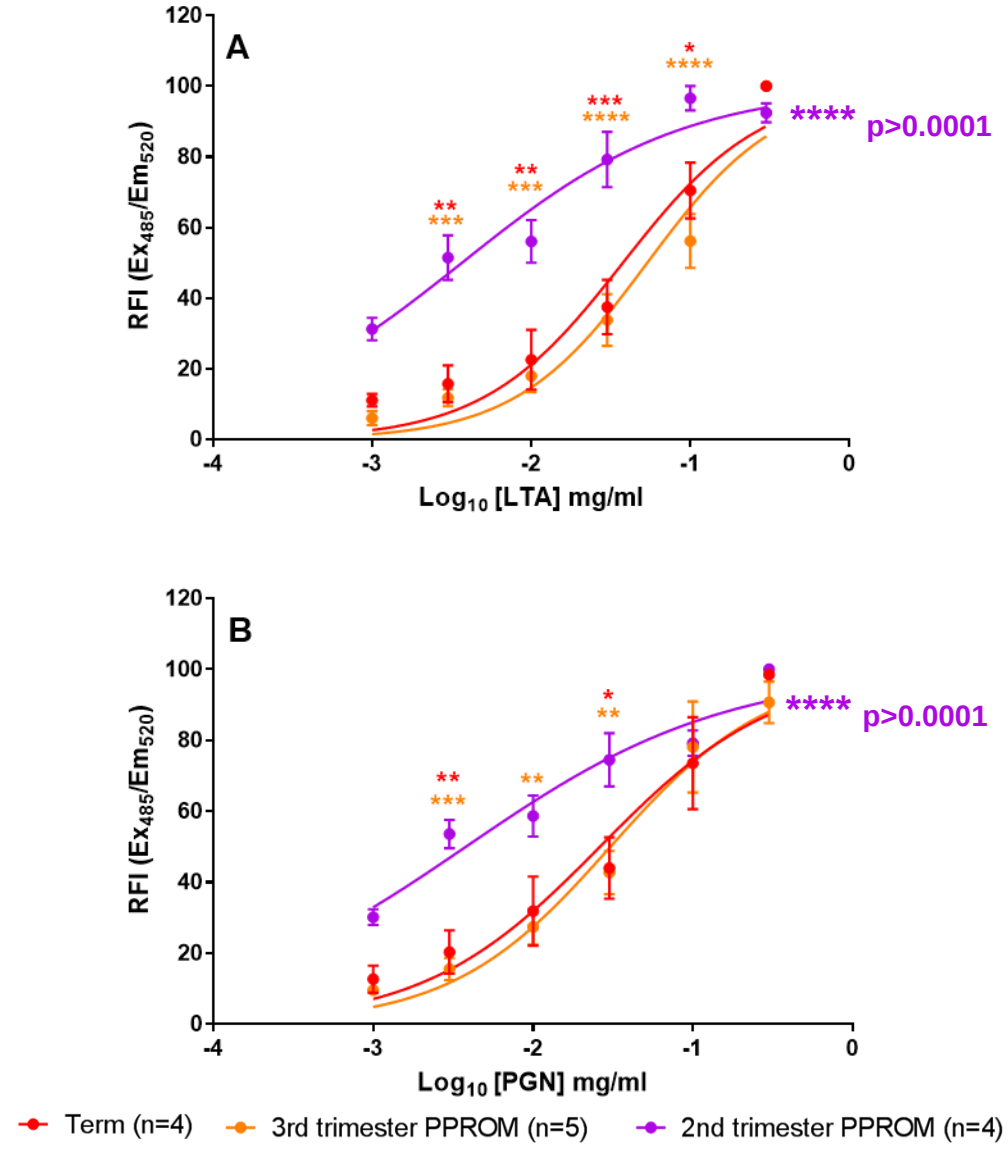

Figure 4.4.2 A LTA and PGN Induced NET Formation in MBL from Normal Term Pregnancy or with PPROM at Varying Gestational Age

*MBL, collected from normal pregnancies at term, or with PPROM at varying gestational age were stimulated with LTA and PGN for 3 hours to induce NET formation. With LTA (**A**) or PGN (**B**) stimulation the NET response seen in the MBL from PPROM in the 2nd trimester (n=4) were statistically significant (p<0.0001) to MBL after PPROM in the 3rd trimester (n=5) and MBL from normal pregnancy at term (n=4). Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test (p<0.05 (*), p<0.005 (**), p<0.0005 (***) and p<0.0005 (****)).*

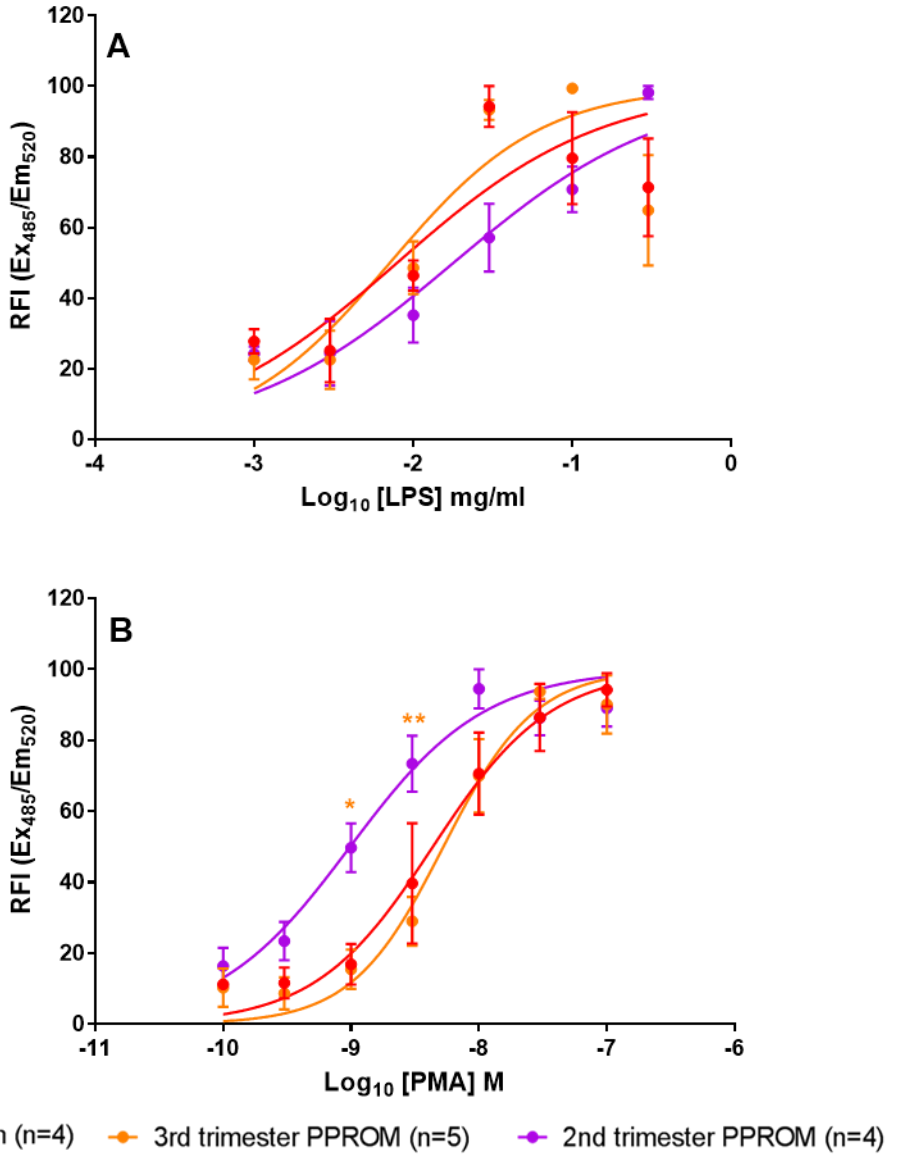

Figure 4.4.2 B LPS and PMA Induced NET Formation in MBL from Normal Term Pregnancy or with PPROM at Varying Gestational Age.

*MBL, collected from normal pregnancies at term (n=4), or with PPROM at varying gestational age were stimulated with LPS and PMA for 3 hours to induce NET formation. With LPS (**A**) or PMA (**B**) stimulation there was no overall difference between the data sets. Although with PMA stimulation there was a statistically significant difference between PPROM in 2nd (n=4) and 3rd (n=5) trimesters at 3nM and 1nM PMA concentrations. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test (p<0.05 (*), p<0.005 (**)).*

4.4.3 Comparing PPROM and Normal with Gestational Age

Comparisons were then made to assess the effect of PPROM on the intensity of NET fluorescence to determine whether there were any differences in MBL as a result of PPROM at a similar gestational age.

There were no significant differences identified between the 3rd trimester PPROM and 3rd trimester groups for LTA, PGN or LPS (***Figure 4.4.3 A*** and ***Figure 4.4.3 B*** – Panel **A**). In PMA, there was no difference in the overall curve between 3rd trimester and 3rd trimester PPROM, but a significantly higher level of fluorescence was measured at 3nM PMA in the 3rd trimester group ($p=0.0474$).

When comparing 2nd trimester and 2nd trimester PPROM groups there was a significant leftward shift in the concentration response curve in the 2nd trimester PPROM group, when compared with the 2nd trimester group when stimulating with LTA (***Figure 4.4.3 C*** – panel **A**; $p<0.0001$). There were no significant differences observed with PGN and PMA stimulation (***Figure 4.4.3 C*** – panel **B** and ***Figure 4.4.3 D***).

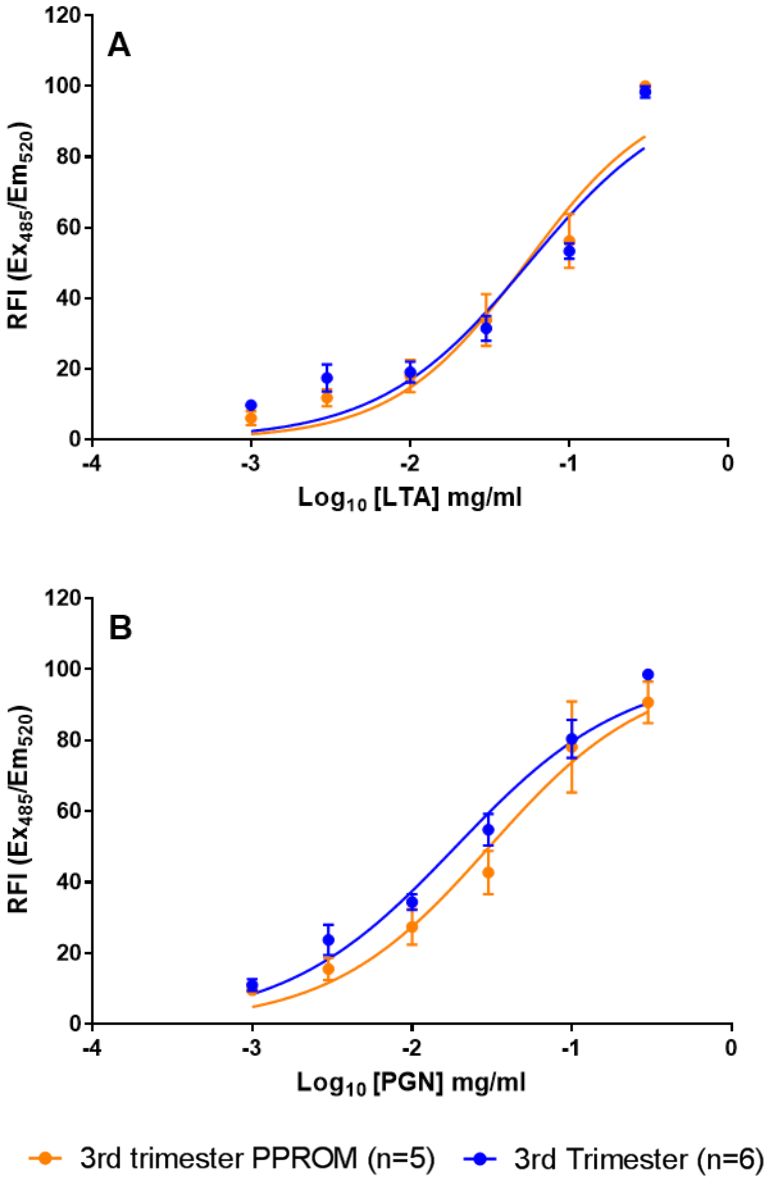

Figure 4.4.3 A LTA and PGN Induced NET Formation in MBL from Normal Pregnancy and PPROM in the 3rd Trimester

*MBL, collected from 3rd trimester normal (n=6) or PPROM (n=5) pregnancies, were stimulated with LTA or PGN for 3 hours to induce NET formation. With LTA (**A**) and PGN (**B**) stimulation there was no difference in NET formation, when comparing these two groups. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.*

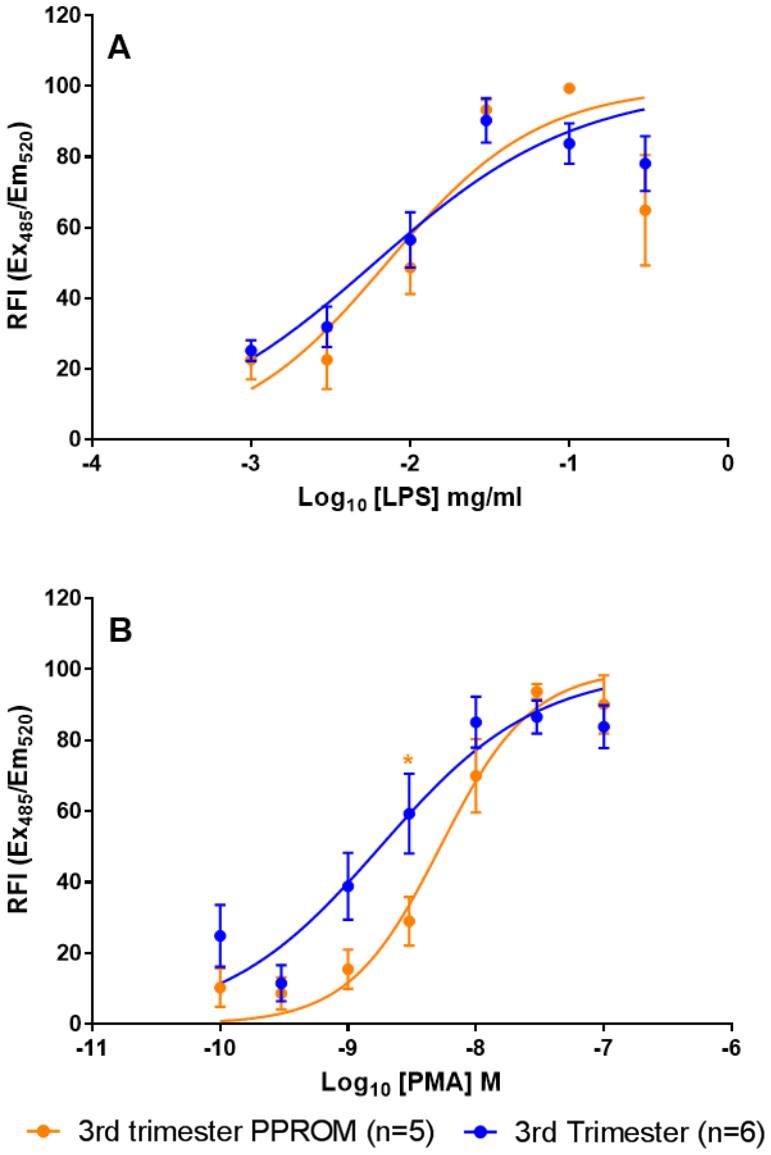

Figure 4.4.3 B LPS and PMA Induced NET Formation in MBL from Normal Pregnancy and PPROM in the 3rd Trimester

*MBL, collected from 3rd trimester normal (n=6) or PPROM (n=5) pregnancies, were stimulated with LPS or PMA for 3 hours to induce NET formation. With LPS (**A**) or PMA (**B**) stimulation there was no overall difference between the data sets. Although with PMA stimulation there was a statistically significant difference between PPROM and normal pregnancy in the 3rd trimester at 3nM PMA concentration. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test (p<0.005 (**)).*

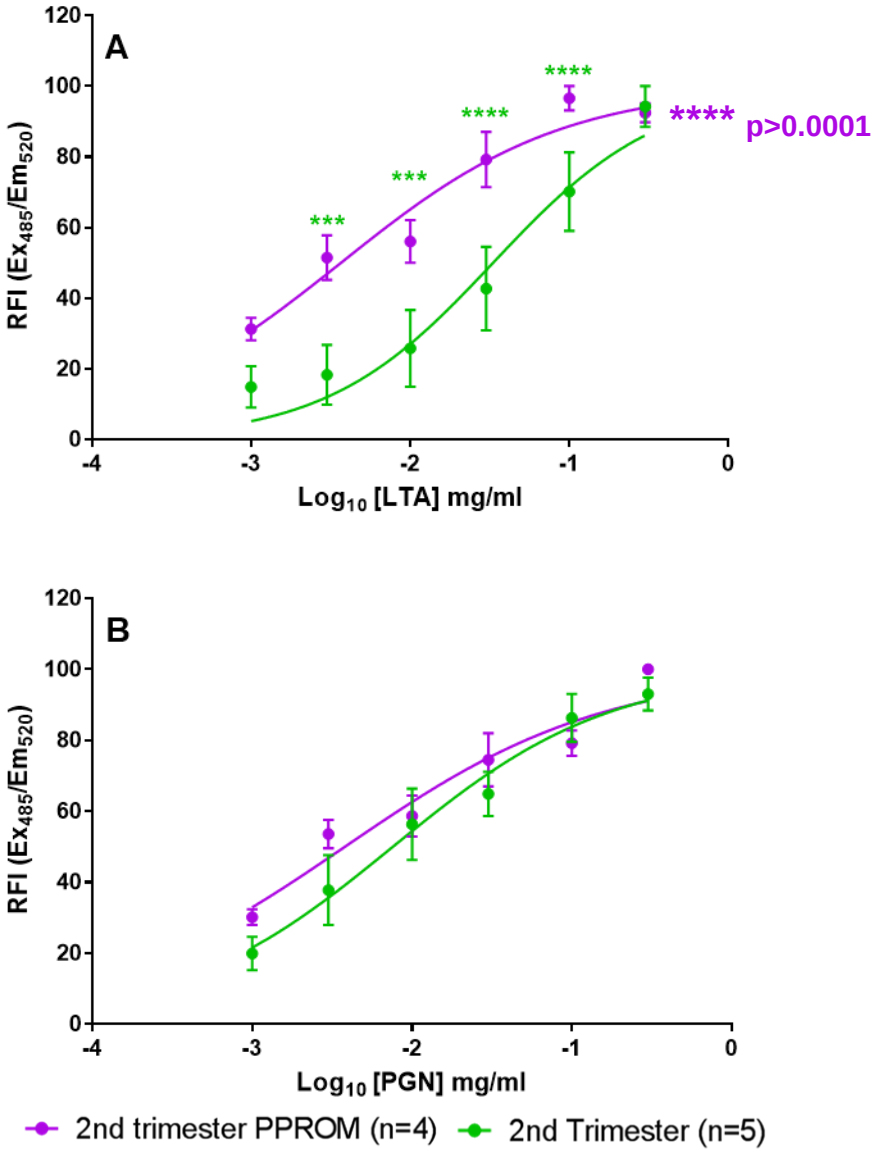

Figure 4.4.3 C LTA and PGN Induced NET Formation in MBL from Normal Pregnancy and PPROM in the 2nd Trimester

*MBL, collected from 2nd trimester normal (n=5) or PPROM (n=4) pregnancies, were stimulated with LTA or PGN for 3 hours to induce NET formation. With LTA (**A**), stimulation the NET response seen in the MBL from normal and PPROM pregnancies in the 2nd trimester were statistically significant different to one another (p<0.0001). No significant differences were observed between the two groups with PGN (**B**) stimulation. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test (p<0.0005 (***) and p<0.0005 (****)).*

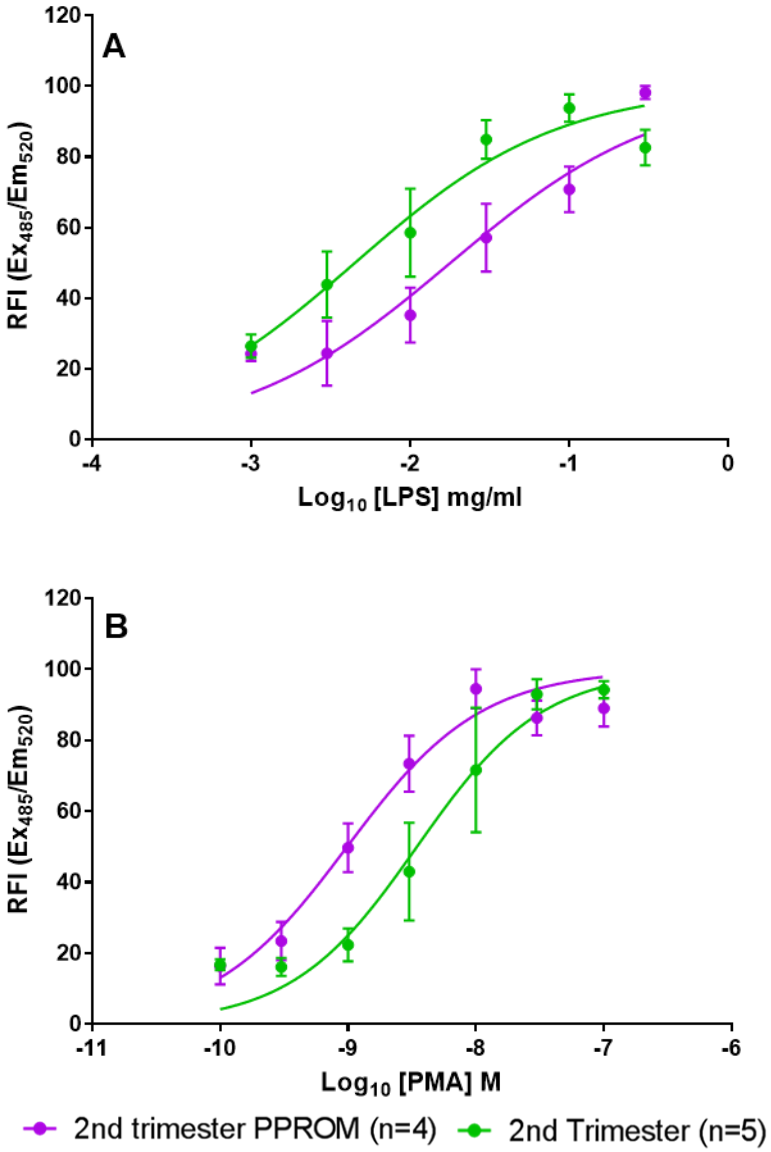

Figure 4.4.3 D LPS and PMA Induced NET Formation in MBL from Normal Pregnancy and PPROM in the 2nd Trimester

*MBL collected from 2nd trimester normal (n=5) or PPROM (n=4) pregnancies, were stimulated with LPS or PMA for 3 hours to induce NET formation. No significant differences were observed between the two groups with LPS (**C**) or PMA (**B**) stimulation Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.*

4.5 Comparing NET Response in CBL and MBL

CBL and MBL collected and purified at term were both treated with PAMPs over the same concentration range. Therefore comparisons were drawn between the ability of CBL and MBL to produce NETs to determine if both sets of leukocytes produced comparable NETs when stimulated with PAMPs. With LTA (***Figure 4.5 A*** – *panel* ***A***) and LPS (***Figure 4.5 B*** – *panel* ***B***) there was no significant difference between MBL and CBL after stimulation for 3 hours. However, opposing significant differences were seen with PGN and PMA (see ***Table 4.5*** for EC_{50} values). In PGN (***Figure 4.5 A*** – *panel* ***B***), MBL had a greater level of NET production when stimulated with the same concentration of PGN, than CBL as measured by SYTOX® green fluorescence (p=0.0392), as illustrated by a leftward shift in the concentration response curve. Conversely, in PMA CBL had a greater level of NET production than MBL when stimulated with the same concentration (p=0.0032).

Group	LTA (µg/ml)	PGN (µg/ml)	LPS (µg/ml)	PMA (nM)
MBL	21.0	49.4	6.18	1.44
CBL	37.6	26.2	7.91	4.36

Table 4.5 EC_{50} Values of MBL and CBL

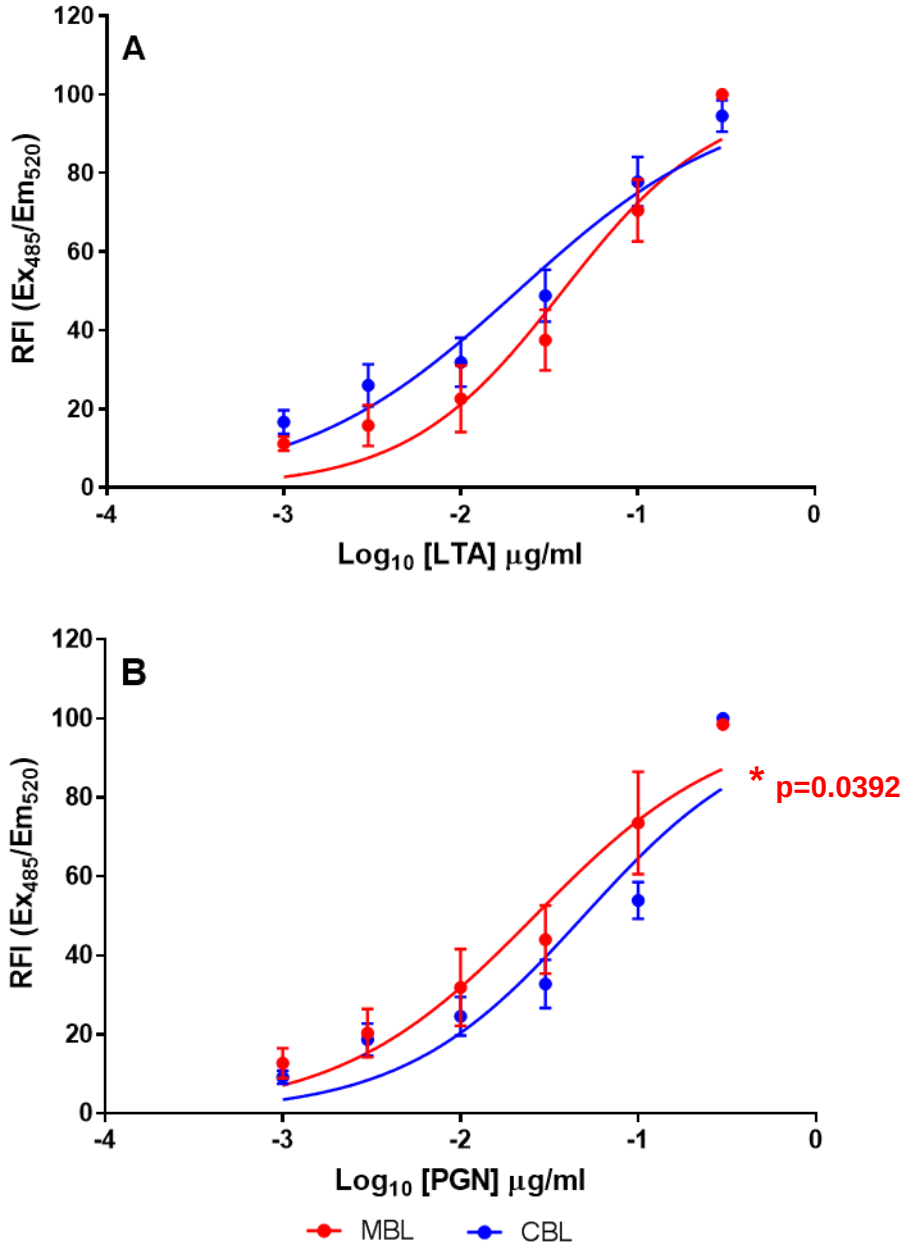

Figure 4.5 A Comparison of LTA and PGN Induced NETs in CBL and MBL

*MBL (n=4) and CBL (n=10) were isolated and incubated with LPS or PMA for 3 hours. With LTA stimulation (**A**) there was no difference in NET formation, whereas with PGN stimulation (**B**) there was a statistically significant shift in the curve, with MBL showing a greater response than CBL (p=0.0392), although there were no individual differences at any concentration. Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test.*

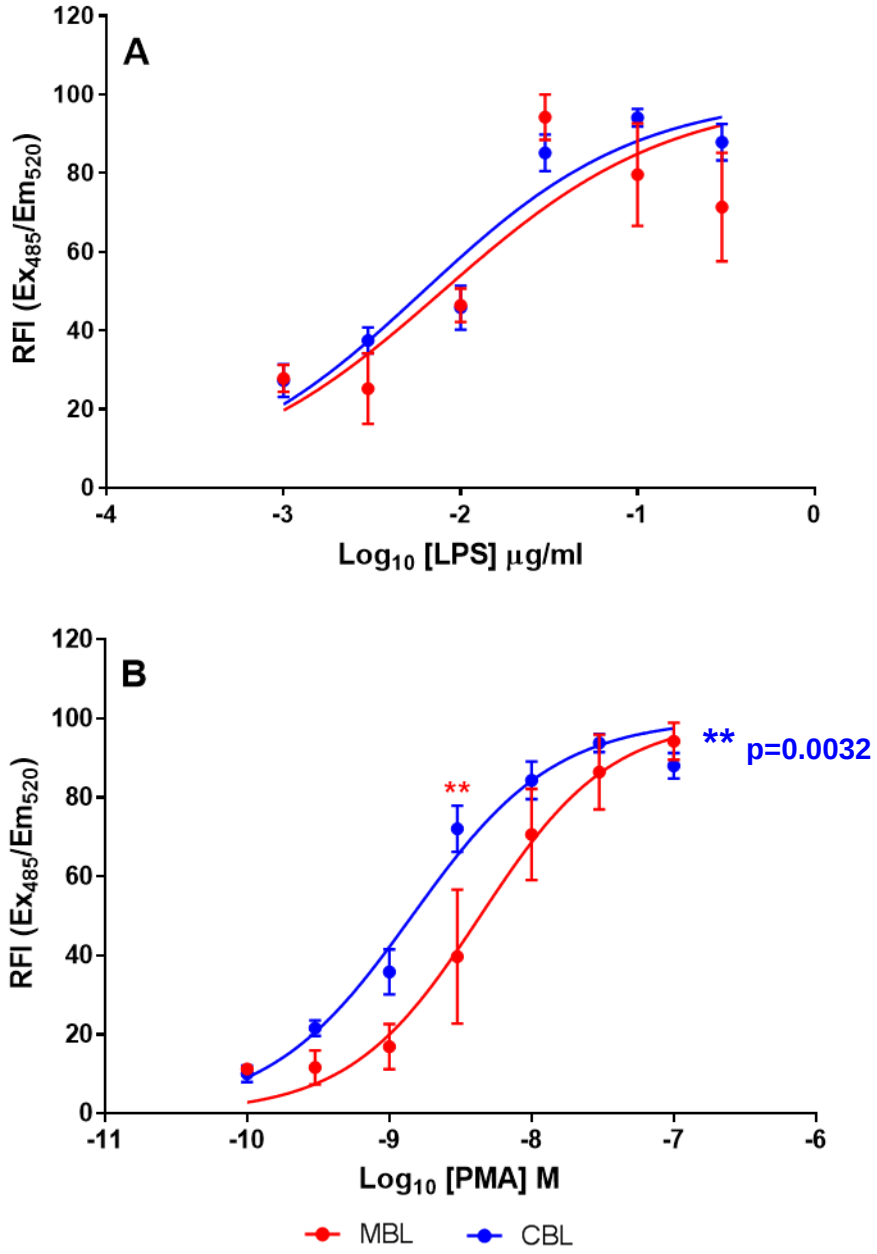

Figure 4.5 B Comparison of LPS and PMA Induced NETs in CBL and MBL

*MBL (n=4) and CBL (n=11 LPS and n=15 PMA) were isolated and incubated with LPS or PMA for 3 hours. With LPS stimulation (**A**) there was no difference in NET formation, whereas with PMA stimulation (**B**) there was a statistically significant shift in the curve, with CBL showing a greater response than MBL at the same concentration (p=0.0032). Data presented are mean ± SEM and statistical analysis was performed using a two-way ANOVA with Bonferroni post-test (p<0.005 (**)).*

5 Discussion

Pregnancy is unique in that it requires the tolerance of a part-self foetus whilst maintaining an effective maternal immune response. The aim of this study was to determine whether NETs play a role in pregnancy and whether they could be formed in CBL and MBL by PAMPS, LTA, PGN and LPS. Further to this was to assess the effect of pregnancy disorders, which were PPROM (MBL), PTB and GBS (CBL) as NETs are stimulated by bacteria.

5.1 Flow Cytometry

The cell proportions observed with the flow cytometry analysis revealed some discrete differences between CBL and the ranges reported in adults. The breakdown of leukocyte cell types in the neonate has not been extensively studied and the composition changes drastically over the first few days after delivery. This was particularly evident in the granulocytes, in particular neutrophils with there being fewer in CBL than adult blood, this may be because only around 10% of foetal cells are leukocytes (Aagaard-Tillery et al., 2006). The proportion of monocytes remained consistent with that expected. An unusual finding was that there were a number of $CD16^{+}$ cells that did not stain for either CD66b or CD14 which would indicate that they are neither neutrophils nor monocytes. The other cell type that expresses CD16 is the NK cells, however in adult circulation these represent a small proportion of the leukocytes observed. The representative proportions of leukocytes needs a more careful assessment utilising a greater number of patient

samples and perhaps also utilising additional surface markers to identify NK cells, for example CD56 (Moffett-King, 2002, Aagaard-Tillery et al., 2006, Moffett and Shreeve, 2015), to better distinguish between cells of different types.

5.2 Validation of NET Assays

NETosis is a strategy employed by neutrophils to capture and kill bacteria as an addition to their other weapons of phagocytosis and degranulation (Brinkmann et al., 2004). There are two pathways that have been identified as leading to NETosis and these are the vital pathway and the suicidal pathway (Yipp et al., 2012). In the initial experiments it was demonstrated that CBL could be stimulated by PMA and LTA to produce NETs. Further, that the response seen with PMA stimulation could be attenuated by NAC and DPI, whereas the response to LTA remained unchanged. DPI and NAC block distinct parts of the lytic NETosis pathway, though which PMA induces after activation of PKC, which helps to explain the lack of NET formation along the full concentration range used. The reduction in NET formation, by inhibiting NADPH oxidase (DPI) or scavenging ROS (NAC), agrees with the published literature (Fuchs et al., 2007, Yipp et al., 2012), suggesting suicidal NETosis. The lack of inhibition of NET formation induced by LTA, is also in agreement with other studies (Henneke et al., 2005, Yipp and Kubes, 2013) in that it utilises the vital NETosis pathway, as gram-positive bacteria activate neutrophils through TLR-2, and CR3 and NADPH oxidase and ROS are not part of the molecular mechanism (Yipp et al., 2012).

The early experiments performed raised questions regarding the concentration range for PMA selected for this set of experiments. This was addressed by performing further validation which allowed determination of an extended concentration range of 0.1-100nM to be used in subsequent experiments. This is consistent with the concentration-range used in a previous study where increasing levels of NET DNA were observed from 0.5-50nM PMA concentrations (Brinkmann et al., 2004).

One of the defining features of NETs in the production of extracellular DNA lattices and it is important to distinguish DNA generated by NETs from other forms of cell death. In studies of NETs, one method utilised is the digestion of NET structures using bacterial nucleases. This allows tests for DNA or NET proteins in the digest supernatant, without the confounding factors of degranulation, cells and cell debris. By digesting NET structures utilising MNase (also called S7 nuclease), which is the same protein secreted by *Staphylococcus aureus* to escape from NET structures (Fuchs et al., 2007) allowed for the measurement of NE contained within NET structures alone. As the NE levels observed were significantly greater in digested than undigested samples this showed that the NE present in the digests was down to the activity of the S7 nuclease breaking down the NETs into fragments and releasing them from the surface of the cell. Again this agrees with the literature as NE and DNA have been measured in digested NETs (Fuchs et al., 2007).

5.3 PAMPs Induce NETs in CBL and MBL

The study set out to determine whether NETs could be formed by CBL and MBL in response to inflammatory mediators, specifically PAMPs. The ligands assessed were LTA, PGN (*Staphylococcus aureus*; gram-positive) and LPS (*Escherichia coli*; gram-negative) which are all bacterial surface polymers and were used to mimic the presence of a bacterial infection. In gram-negative bacteria, LPS is known to be the primary activator of immune response and is well conserved between different organisms (Henneke et al., 2005). In gram-positive bacteria the situation is not so clear cut, with LTA and PGN molecules differing between organisms (Henneke et al., 2005). The observations that all of these stimulants could induce NET formation was evaluated by SYTOX® green assay and confirmed in CBL by immunofluorescence. This agreed with previous studies which have shown that LPS (Brinkmann et al., 2004) can induce NETs and LTA and PGN can activate neutrophils (Henneke et al., 2005) and that *Staphylococcus aureus*, from which these compounds are isolated is a potent inducer of NETs (Pilsczek et al., 2010). Previous studies have also shown that NETs from different neutrophils link together to form a large mesh, which we also observed with PMA and LTA stimulation in particular when performing immunofluorescence. Our observations contradict one another with the results of the NE activity assay, whereby NET formation could not be measured in LTA and LPS, whilst revealing high NE concentrations with PGN and PMA. The lack of response seen to LPS agrees with the work of Pieterse et al. (2016), who observed that with this sub-type of LPS

(from *Escherichia coli* 026: B6) in particular, supernatants did not contain NE after nuclease digest. They did however also note that with the presence of platelets all of the LPS species that they tested were able to produce NETs. This effect was likely as TLR-4 interaction through platelets has been reported to be the mechanism of action of LPS stimulation. The nuclease concentration that was recommended in the assay kit (15U/ml) was higher than that used in other studies of 500mU/ml (Fuchs et al., 2007) and 5U/ml (Pieterse et al., 2016). The conditions of the assay will need to be further validated, with particular attention paid to MNase concentration.

Comparisons were made between CBL and MBL at term in order to evaluate whether there were any differences between the NET formation in CBL and MBL. At term there was no difference in the NET response between CBL and MBL treated with LTA and LPS, however CBL were more sensitive to PMA stimulation, whereas MBL were more sensitive to PGN stimulation. This is particularly thought-provoking as it both agrees and disagrees with the published literature (Yost et al., 2009, Marcos et al., 2009). Neonatal neutrophils were described to have impaired NET formation when compared with adult neutrophils as they did not release NETs (Yost et al., 2009). The work presented here does not agree with this finding as NETs were measured after treatment of CBL with all agonists. A further study revealed that neonates could indeed produce NETs but did so after an extended time point, 3 hours (Marcos et al., 2009), which agrees with the findings displayed here. However, neither

of these findings offer an explanation as to why MBL are more sensitive to PGN and CBL to PMA. It may mean that CBL have some deficiency in NET production compared with adult neutrophils when presented with gram-positive bacterial pathogens. This may point towards an explanation of why gram-positive bacteria, including GBS, pose such a problem to neonates as our data show that CBL produce fewer NETs than MBL at equivalent concentrations of PGN. If as detailed in Yost et al., (2009) NET formation is delayed with gram-positive bacteria, when compared with adults, this could allow the development of neonatal sepsis, whereby neonatal leukocytes can produce an inflammatory response, but are not able to clear the infection (Henneke et al., 2005) in GBS for example. Worryingly, GBS can be particularly dangerous to neonates who rapidly contract the infection during delivery, where it can lead to death and long term morbidity. The reason for the enhanced response seen in CBL compared with MBL with PMA warrants further investigation as there is no simple explanation. PMA activates NET formation via PKC and activates a number of intracellular signalling pathways, including ROS (Yipp and Kubes, 2013). Many of these pathways are associated with enzymes which are reliant on intracellular calcium concentrations. However evidence reveals that neonatal neutrophils have a reduced production of ROS and reduced calcium signalling (Yost et al., 2009). This in itself is a paradox because previously demonstrated neonatal impairments would suggest that the PMA response should be reduced in CBL, where in fact our data show it is increased.

Interestingly, comparisons were also made between term CBL with and without a maternal GBS infection, and the observation that CBL where mothers had GBS had a greater level of NETs than those without GBS. In fact the response seen is similar to that it in term MBL. GBS infection was chosen as it is often seen as an asymptomatic infection in the mother as are many infections which lead to preterm birth (Romero et al., 2007). More specifically it allowed for the comparison between normal pregnancy and asymptomatic maternal infection. Continuing with the theme of infection, a role for NETs in PPROM specifically has been recently suggested with the discovery that in chorioamnionitis infections, NETting maternal neutrophils are present in the foetal membranes (Gomez-Lopez et al., 2017). These are thought to be responsible for the initial lesion occurring at the site of membrane rupture, which subsequently results in weakening and eventual failure of the membranes (Gomez-Lopez et al., 2017).

There have been virtually no studies on the link between maternal infection and PTB and to the best of my knowledge this is the first study that has stimulated the production of NETs in MBL and compared both differing gestational age, but also PPROM. Previous studies conducted in pregnancy have evaluated the presence of NETs in placental tissues or for the presence of NET components in maternal blood, including cfDNA and NE (Gupta et al., 2006a, Gupta et al., 2006b).

5.4 NETs in sPTB

The observation that the NET response between CBL from neonates delivered at term and those born preterm does not differ was initially unexpected. After all, the evidence in the literature states that infection is associated with 40% of all preterm births (Goldenberg et al., 2008) and NETs are produced in response to infection (Brinkmann et al., 2004). As the number of samples were low in both the preterm group (n=3) and were from a gestational age group that showed no changes in MBL it would be prudent to increase both patient numbers and the gestational age range from which samples are collected. This in itself can pose its own challenges as placentae from mothers delivering preterm is often sent to pathology for histological analysis. However, it would allow for the subdivision between different types of preterm birth, in particular between PPROM and sPTL as these are likely to have different mechanisms and as a result may display differing NET formation.

Early in pregnancy the maternal immune system has to adapt to the challenges of pregnancy which allows the mother to retain an effective response to invading pathogens, but to also host the non-allogenic foetus. The reduction of NET formation in MBL in the second trimester when compared with third trimester and term after LPS stimulation is intriguing. This may suggest that as pregnancy progresses, pathways for the formation of NETs may become altered in preparation for labour.

LTA shows an increased NET production in PPROM compared with normal pregnancy in the second trimester. The resulting reduction in the concentration of agonist required to activate the cells suggests that the cells are more sensitive to activation and therefore may be primed due to an asymptomatic maternal infection or that the MBL in these women have a lower level of tolerance to activation.

5.5 Critics of NETs

A number of groups have begun to bring into question the validity of NETosis and whether such a system would ever be advantageous to the host as a process to deal with infectious pathogens. Yousefi and Simon in particular (2016) even go as far as describing NETosis as 'Nature's suicide bomber' due to the destructive potential to self-tissues. This has been shown to be the case in autoimmune disease including RA and SLE, but is not necessarily the case with bacterial, viral and fungal infections where they play a role in infection clearance; although inflammation will result in tissue damage in both scenarios. Part of their argument centres around the vast majority of the work being conducted using *in vitro* studies based on isolated neutrophils and the comparative lack of *in vivo* work (Yousefi and Simon, 2016). Centred on part of this argument is distinguishing differences between NET formation and NETosis as two different entities, with only NETosis resulting in cell death. Overall, the evidence supports NET formation as a method of bacterial arrest and killing, especially in those studies that have been conducted on tissue and have shown NETs *in situ*. In PET, NETs have

been seen in IV spaces of the placenta (Gupta et al., 2005) and in the skin in response to *Staphylococcus aureus* infection (Yipp et al., 2012). Although I do agree that the terminology surrounding NET formation needs to be refined to avoid any ongoing confusion, especially with the research explosion surrounding NETs.

5.6 Limitations of the Study

A mixed population of leukocytes isolated form cord or maternal blood was used to complete this study. As this is not a suspension of a single-cell type the NET response seen may not solely be down to the presence of neutrophils within the sample, 20-25% of which produce NETs (Fuchs et al., 2007, Yipp and Kubes, 2013). Other cell types which have been demonstrated to produce extracellular traps (ETs) are monocytes (Chow et al., 2010), mast cells (von Kockritz-Blickwede et al., 2008) eosinophils (Yousefi et al., 2008) and basophils (Yousefi et al., 2015) which will also be present in the sample. Of further note is that the DNA present within the NET structures may be of nuclear or mitochondrial origin as both have been observed in NET structures (Yousefi et al., 2008, Yousefi et al., 2015). SYTOX® green cannot distinguish between mtDNA and gDNA and therefore the NETs produced in response to the PAMPs may be formed from mtDNA, gDNA or a mixture of the two. It is thought that neutrophils preferentially utilise gDNA, although can release NETs containing mtDNA, and that eosinophils and basophils in particular rely on mtDNA (Yousefi et al., 2008, Yousefi et al., 2009, Yousefi et al., 2015). The limitation of selecting a vital nucleic acid stain that cannot cross an

intact cell membrane is that it will cross compromised membranes of dead and dying cells. Therefore measurements taken will not only include the extracellular DNA lattices that make up the NETs, but also the genomic DNA of cells undergoing apoptosis or necrosis. Additional techniques that identify apoptosis and necrosis could be used to alleviate this concern. In respect to mtDNA, other studies have utilised dyes that are specific for nuclear DNA and mtDNA in order to distinguish between the two, Yousefi et al., (2009, 2015) utilised Hoechst 333342 which stained the nucleus and MitoSox which stained the extracellular DNA within NETs released from neutrophils and basophils, thought to contain mtDNA.

The results presented in this thesis are based around assessment of DNA and NE levels. There are however a variety of neutrophil proteins that could be measured to reveal NET formation. Of these proteins, we have attempted to assess histone and myeloperoxidase presence and localisation utilising immunofluorescence and histone and DNA complexes using ELISA. Unfortunately the antibodies selected for the immunofluorescence need to be further evaluated to produce consistent results. The DNA and histone ELISA did not perform as expected, however with the results that we have observed after MNase digest, utilising digested NETs, as we used for the measurement of NE, for this purpose should provide better results and also allow for measuring more discrete NET components. This may include more specific antibodies, for example citrullinated H3 is a hallmark of NET formation (Yipp et al., 2012,

Martinod and Wagner, 2014) or dual capture sandwich enzyme-linked immunosorbent assay (ELISA) to measure NET complexes (Sur Chowdhury et al., 2014), for example NE and DNA.

The results presented in this dissertation are from NET production that has been stimulated *in vitro*, therefore this may not be a true representation of the picture *in vivo*. NETs have been shown to be produced in tissue in the placenta of patients presenting with preeclampsia (Gupta et al., 2005). This was further backed up by the presence of cell-free foetal and maternal DNA (Gupta et al., 2006a) and raised maternal NE levels (Gupta et al., 2006b). Further to this, in a rheumatoid arthritis (RA) study (Sur Chowdhury et al., 2014) raised levels of NE were seen in the serum, along with DNA-histone and DNA-MPO complexes which were suggestive of NET formation. Therefore a more accurate way to consider differences in NET production in disease states is to look for markers of NET formation and epithelial and endothelial damage resultant of NETs, rather than to try and stimulate NET production *in vitro* which would have better future clinical application.

The trigger sources used to assess NET formation were PAMPs of bacterial origin. Therefore the aim was to mimic bacterial infection and induce NET production. However this leaves out a great number of substances that can also stimulate NETosis, including platelets and IL-8. NETs have a perpetuity around them in that NET components can also stimulate NET production, such as histones and DNA (Alhamdi and Toh,

2016) and therefore during activation the stimulant may shift from the initial stimulus to a NET driven stimulus. This could mean that the mechanism of activation could change from that seen with the initial stimulus, this could prove difficult to account for when assessing the NET formation capabilities of different agonists.

An implication of the study surrounds sample collection as it can be extremely difficult to identify and collect samples from patients who have pregnancy complications. In the case of PTB, samples cannot always be collected because in many cases, the placenta is often sent to pathology for histological analysis, especially where the cause of preterm birth is unknown or if the neonate is unwell. From the samples that we can collect, the lower the gestational age, the smaller the placenta which can make collecting CB especially difficult. In the same way MB can also be difficult to collect, especially if clinical blood samples are not required, additional blood collections are not well tolerated, particularly when the mother is unwell.

5.7 Further Work

The work presented has provided evidence for NET formation in pregnancy and has opened avenues for future investigations. Increasing the numbers within each of the patient groups would increase the confidence in the statistical analysis performed. Further to this it would allow for the results to be broken down by the gender of the foetus and the ethnicity of the mother and neonate. These are both important factors

as it has been shown that male foetuses are more susceptible to preterm delivery, accounting for 55% (Blencowe et al., 2013) and that black women are more likely to give birth preterm, whilst Asian women are the least likely (Goldenberg et al., 2008). It would also allow for evaluation of other pregnancy conditions afflicted by inflammation, which often result in preterm birth including IUGR and PET. Further to this, utilising more advanced imaging techniques, such as high content screening would allow for identification of morphological changes induced by NET formation at a sub-cellular level and allow for simultaneous detection of neutrophil protein directly involved. This could provide a signature of NETs associated with disease and help to provide increasing knowledge of the activation pathways of the PAMPs.

Interestingly in this study, in MB there were quantifiable differences between PPROM, especially at early gestation and normal pregnancies. This is of particular importance in advent of the development of diagnostic screening tests as MB can be easily collected during pregnancy whereas CB cannot. A recent study in RA revealed that NET fragments containing DNA, NE, MPO and DNA-MPO complexes could be detected in peripheral blood serum (Sur Chowdhury et al., 2014). Therefore further work could focus away from stimulating MBL and CBL to identifying hallmarks of NET production and aligning these with markers of inflammation and endothelial and epithelial cell damage such as IL-8 and soluble thrombomodulin. Perhaps alongside those which have already been quantified as having predictive potential in PTB including foetal

fibronectin (Goldenberg et al., 2001) and matrix metalloproteinase-9 (Goldenberg et al., 2005) This would best be accomplished by recruiting a large population of women who can be sampled at various points in their pregnancy when clinical blood samples are obtained, this would allow the identification of changes in markers during the course of the pregnancy and also as maternal status could be followed up and delivery recorded. The combination of all this information would allow identification of women who later had problems and differences could then be determined based on outcomes. A subset of patients that would be of particular interest to study would be those suffering from auto-immune conditions, particularly type III hypersensitivity, which includes RA and SLE, where NETs have been shown to be implicated in disease development. RA would be of particular interest as it tends to improve during pregnancy (Ostensen and Villiger, 2007, Saito et al., 2010). The ability to compare the NET response seen in pregnancy with sterile inflammation which is seen in RA (mediated by DAMPs) to the inflammation seen in infection (mediated by PAMPs), would be particularly worthwhile.

A perhaps greater question, which has not yet been addressed is that the process utilised to dismantle and remove NET structures has not been identified, although is thought to be performed by macrophages (Farrera and Fadeel, 2013, Nakazawa et al., 2016, White et al., 2016). Therefore caution must be exercised when assessing differences in NET formation in disease states, as to whether any differences identified are due to

aberrant NET formation or a failure of the body to clear NETs that have been formed, or perhaps a combination of both. Further work needs to be completed by the scientific community at large to fully understand the molecular mechanisms behind both NET production and NET clearance.

A functional study of the effects of NET fragments on isolated myometrium or placental and myometrial arteries would provide useful information on any changes in tone or contractility and evaluate damage caused by exposure to NET fragments. A variety of DNases could be utilised to produce NET fragments of varying sizes, which could be applied to the tissue in a contractility bath or vessels in a myograph. The tissue could also be utilised to assess changes in RNA expression and protein levels in response to NET treatment to assess the extent of endothelial and epithelial tissue damage. This could provide invaluable information regarding the effect NET formation has on the vessels of the placenta and female reproductive tract. As high levels of NETs have been identified in the placenta with PET (Gupta et al., 2005) damage to the surrounding tissue is inevitable and this could be compared to PTB.

5.8 Conclusions

NETs are an important part of the innate immune response to infection, but their production can lead to local tissue damage. This can result in inflammation, which in the case of pregnancy is not desirable due to the maternal immune system adopting an anti-inflammatory phenotype prior to the onset of labour. Increased levels of inflammation outside of this

process may lead to PTB. From the data obtained I was able to prove the hypothesis that NET formation occurs in CBL and MBL in response to bacterial surface polymers. The data also shows that NET formation is exacerbated in MBL where PPROM has occurred, in response to PMA, PGN and PMA, but not LTA. Therefore NETs may play a key role in the development of PTB. However further work is required to provide a definitive role for NETs within this process.

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7 Appendix 1

The following 6 pages display the patient information sheets and consent forms for the collection of cord blood and maternal blood.

Derby Hospitals NHS
NHS Foundation Trust

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Patient Information Sheet

Studies of Human Pregnancy and Preterm Birth

You are being invited to take part in a research study of the University of Nottingham being carried out in Derby. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

1. What is the purpose of the study?

The switch from pregnancy to labour and how the uterus contracts for delivery is not understood. In some cases, labour may begin too early when the baby is not ready to be born (preterm). Preterm birth is delivery occurring before 37 completed weeks of pregnancy and affects about 8% of all pregnancies in the UK. Preterm labour is a factor in approximately 40% of all preterm deliveries. Two other pregnancy-specific disorders preeclampsia (high blood pressure with protein in the urine) and intra-uterine growth restriction (IUGR), where babies are born small for their size, may also lead to preterm birth. To date, there are still no effective drugs to treat preterm birth due to preterm labour nor any reliable markers that allow these disorders to be monitored throughout pregnancy.

Here at the Royal Derby Hospital, we are carrying out research into term and preterm birth with the aim of improving our understanding of the causes of preterm birth. We hope that new information from our studies will pave the way for the future development of new drugs and new ways designed to reduce the incidence of preterm delivery. The three projects we are involved with are as follows:

Project 1: Inflammation in pregnancy: The uterus produces many important proteins which play a role in pregnancy and labour. It is thought that infection or inflammation of the female reproductive tract may activate the process of labour too early by causing uterine contractions through molecules produced by the placenta and cord blood.

Project 2: Lipid-protein interactions in the pregnant human uterus. In recent years, there has been great interest in how certain fats affect pregnancy. We are studying how these fats (also known as lipids) present in the uterus (womb) influence labour. In addition, we know that the location of these lipids within cells may also indirectly activate pathways that control the production of proteins in the uterus.

Project 3: Vascular problems in pregnancy: During pregnancy, blood flow to the baby provides food and oxygen essential to baby's growth and development. In some 10% of cases, poor blood flow to the placenta (afterbirth) may mean that the baby does not develop or grow properly, a condition known as intra-uterine growth restriction (IUGR). In addition, 1-2% of pregnancies result in preeclampsia, a leading cause of high blood pressure in pregnancy, which may endanger both mother's and baby's health if left undiagnosed. The aim of our project is to study the properties of the blood vessels in the uterus and placenta of women with preeclampsia or a growth-restricted pregnancy to identify whether blood vessel function is changed in these disorders compared with vessels of women with a normal pregnancy.

2. Why have you been invited?

We are recruiting patients from four different groups. You have been chosen as somebody who falls into one of the four following groups: (a) normal non-labouring, term pregnancy (b) labouring pregnancy, or you have been diagnosed with (c) a growth-restricted pregnancy, (d) preeclampsia. The 'normal pregnancy' group is a control group and including patients from this group will allow us to compare normal versus potentially difficult pregnancies. The groups will be divided further into term or preterm. We are recruiting patients for all three projects and it is likely that your tissue and cord blood samples will be used for any one of these 3 projects. We expect to recruit 3000 women in five years. There will be no direct benefit to you from this study but your participation may significantly aid our understanding of pregnancy and birth thus enabling us to make advances in the field of female and fetal health.

3. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form.

You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. Any stored tissue samples that can still be identified as yours will be destroyed if you wish.

4. What will happen to me if I take part?

Should you decide to take part, if you will be undergoing a caesarean section, you may be asked to provide a myometrial (uterine muscle) biopsy which will be taken at caesarean section from the site of the operation. The biopsy is ~2g in weight and the size of a thumb tip. We will also ask you if we can take your placenta for research. In the case of a normal delivery, we will ask you to consider providing a placental and cord blood sample only after your baby has been delivered. Cord blood samples collected will not be available for any clinical needs such as subsequent treatment of your baby or other investigations. All samples will be taken after delivery of the baby. There is no further follow-up in relation to our study. This investigation is laboratory-based therefore samples will be taken to the lab and analysed using a variety of experimental techniques.

We would also wish to store any unused tissue and blood samples obtained from you for future unspecified research. Samples will only be used for studies approved by a research ethics committee. If however, you change your mind later about having your tissues stored for future research, then you can withdraw at any time without providing a reason. This should be done by contacting the Chief Investigator (contact details at bottom of this sheet).

5. What are the other possible disadvantages and risks of taking part?

There is minimal risk from taking the uterine muscle biopsy over and above that associated with the caesarean section. Taking the sample at caesarean section will not harm your baby, nor prolong the operation and will not affect the normal healing of your uterus. Nor will be there be any effect on the structure or function of your uterus afterwards, your future fertility or any risk to baby as the biopsy will be taken after delivery of your baby. If it is felt by the clinician in charge of your care that taking a uterine muscle biopsy is not appropriate, the sample will not be taken. Your wellbeing and that of your baby is paramount. There is no risk to you or your baby from the use of placental samples.

6. What are the possible benefits of taking part?

There is no direct benefit to you or your baby in taking part in this study. However the information obtained from using your uterine muscle biopsy and placenta may enable us to identify possible new pathways that will improve our understanding of uterine function.

7. What happens when the research study stops?

The data collected will be analyzed and prepared for scientific publication. We would hope to expand the study to understand more about the functions of the human uterus in normal and abnormal conditions. Participants will not be identified in any report, presentation or publication.

8. Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. If you consent to take part in the research, any of your medical records may be inspected by researchers directly involved with the project for purposes of analysing the results and all details will remain strictly confidential. They may also be looked at by people from regulatory authorities to check that the study is being carried out correctly.

9. Who is organising and funding the research?

This study is being funded through various sources including the Wellcome Trust, the Medical Research Council, British Heart Foundation, Action Medical Research and the University of Nottingham. The research is sponsored by the University of Nottingham.

10. Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Derbyshire Research Ethics Committee.

Complaints:

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Raheela Khan 01332 724664; raheela.khan@nottingham.ac.uk). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or details can be obtained from the Royal Derby Hospital Patient Advice and Liaison Service, Uttoxeter Road, Derby, DE22 3NE, dhft.contactpals@nhs.net, tel: 0800 783 7691).

Contact for further information:

Dr Raheela N Khan 01332 724664; email: raheela.khan@nottingham.ac.uk
Mr Daniel Hay 01332 724842; email: daniel.hay@nottingham.ac.uk

For further information on the research of the group:
http:\\www.nottingham.ac.uk/research/groups/reproductivephysiology/index.aspx

Thank you for taking the time to read this.

RNK – V4/PTB 01/2014

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Derby Hospitals NHS
NHS Foundation Trust

CONSENT FORM: Pregnancy
(Version 3 – 01/2014)

Title of Study: Studies of human pregnancy and preterm birth

REC ref: 09/H0401/90

Name of Researcher: Dr Raheela Khan

Name of Participant: _____ **Please initial box**

1. I confirm that I have read and understand the information sheet version number 4 dated 01/2014 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐

3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐

4. I understand and agree to a myometrial ☐ placental ☐ and cord blood sample to be taken for research investigations. ☐

5. I agree to the storage of uterine, placental and cord blood samples for any future, as yet, unspecified research studies relating to the study of pregnancy and pregnancy related disorders. ☐

6. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent (if different from Principal Investigator)	Date	Signature
Name of Principal Investigator	Date	Signature

3 copies: 1 for participant, 1 for the project notes and 1 for the medical notes

RNKV3 PTB Consent Form

Derby Hospitals NHS
NHS Foundation Trust

The University of Nottingham

Patient Information Sheet: Blood samples

Studies of human pregnancy and preterm birth

You are being invited to take part in a research study of the University of Nottingham being carried out in Derby. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

1. What is the purpose of the study?

The switch from pregnancy to labour and how the uterus contracts for delivery is not understood. In some cases, labour may begin too early when the baby is not ready to be born (preterm). Preterm birth is delivery occurring before 37 completed weeks of pregnancy and affects about 8% of all pregnancies in the UK. Preterm labour is a factor in approximately 40% of all preterm deliveries. Two other pregnancy-specific disorders preeclampsia (high blood pressure with protein in the urine) and intrauterine growth restriction (IUGR), where babies are born small for their size, may also lead to preterm birth To date, there are still no effective drugs to treat preterm birth due to preterm labour nor any reliable markers that allow these disorders to be monitored throughout pregnancy.

Here at the Royal Derby Hospital, we are carrying out research into term and preterm birth with the aim of improving our understanding of the causes of preterm birth. We hope that new information from our studies will pave the way for the future development of new drugs and new ways designed to reduce the incidence of preterm delivery. The two projects we aim to recruit to are:

Project 1: Inflammation in pregnancy: The uterus produces many important proteins which play a role in pregnancy and labour. It is thought that infection or inflammation of the female reproductive tract may activate the process of labour too early by causing uterine contractions through molecules produced by the placenta and cord blood.

Project 2: Vascular problems in pregnancy: During pregnancy, blood flow to the baby provides food and oxygen essential to baby's growth and development. In some 10% of cases, poor blood flow to the placenta (afterbirth) may mean that the baby does not develop or grow properly, a condition known as intra-uterine growth restriction, (IUGR). In addition, 1-2% of pregnancies result in preeclampsia, a leading cause of high blood pressure in pregnancy, which may endanger both mother's and baby's health if left undiagnosed. The aim of our project is to measure factors produced by the mother and found in her blood to identify whether preeclampsia or a growth-restricted pregnancy are linked to levels of these molecules compared with normal pregnancy.

2. Why have you been invited?

We are recruiting patients from four different groups. You have been chosen as somebody who falls into one of the four following groups: (a) normal non-labouring, term pregnancy (b) labouring pregnancy, or you have been diagnosed with (c) a growth-restricted pregnancy, (d) preeclampsia. The 'normal pregnancy' group is a control group and including patients from this group will allow us to compare normal versus potentially difficult pregnancies. The groups will be divided further into term or preterm. We are recruiting patients for both projects and it is likely that your blood sample will be used for either project. We expect to recruit 350 women in five years. There will be no direct benefit to you from this study but your participation may significantly aid our understanding of pregnancy and birth thus enabling us to make advances in the field of female and fetal health.

3. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form.

You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. Any stored samples that can still be identified as yours will be destroyed if you wish.

4. What will happen to me if I take part?

Should you decide to take part, we will ask you to provide a single blood sample (20mls which is less than 2 tablespoons) which will be taken from your arm. There is no further follow-up in relation to our study. This investigation is laboratory-based therefore the blood sample will be taken to the lab and analysed using a variety of experimental techniques.

We would also wish to store any unused blood samples obtained from you for future unspecified research. Samples will only be used for studies approved by a research ethics committee. If however, you change your mind later about having your tissues stored for future research, then you can withdraw at any time without providing a reason. This should be done by contacting the Chief Investigator (contact details at bottom of this sheet).

5. What are the other possible disadvantages and risks of taking part?

There is no extra risk to you from taking an additional blood sample. Taking the sample will not harm your baby

6. What are the possible benefits of taking part?

There is no direct benefit to you or your baby in taking part in this study. However the information obtained from using your blood sample may enable us to identify possible new pathways that will improve our understanding of uterine function

7. What happens when the research study stops?

The data collected will be analyzed and prepared for scientific publication. We would hope to expand the study to understand more about pregnancy in normal and conditions. Participants will not be identified in any report, presentation or publication.

8. Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. If you consent to take part in the research, any of your medical records may be inspected by researchers directly involved with the project for purposes of analysing the results and all details will remain strictly confidential. They may also be looked at by people from regulatory authorities to check that the study is being carried out correctly.

9. Who is organising and funding the research?

This study is being funded through various sources including the Wellcome Trust, the Medical Research Council, British Heart Foundation and the University of Nottingham. The research is sponsored by the University of Nottingham.

10. Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Derbyshire Research Ethics Committee.

Complaints:

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Dr Raheela Khan 01332 724664; raheela.khan@nottingham.ac.uk). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or PALs. Details can be obtained from the Royal Derby Hospital.

Contact for Further Information

Dr Raheela N Khan 01332 724664; bleep 1208; email: raheela.khan@nottingham.ac.uk

Mr Daniel Hay 01332 724842; email: daniel.hay@nottingham.ac.uk

For further information on the research of the group:
http://www.nottingham.ac.uk/research/groups/reproductivephysiology/index.aspx

Thank you for taking the time to read this and for taking part in this study.

The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

CONSENT FORM: Blood sample
(Version 2- 12/02/2014)

Title of Study: Studies of human pregnancy and preterm birth

REC ref: 09/H0401/90

Name of Researcher: Dr Raheela Khan

Name of Participant: **Please initial box**

1. I confirm that I have read and understand the information sheet version number 2 dated 02/2014 for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis. ☐

3. I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records and to collect, store, analyse and publish information obtained from my participation in this study. I understand that my personal details will be kept confidential. ☐

4. I understand and agree to a blood sample to be taken for research investigations. ☐

5. I agree to the storage of any used sample for any future, as yet, unspecified research studies relating to the study of pregnancy and pregnancy related disorders ☐

6. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent (if different from Principal Investigator)	Date	Signature
Name of Principal Investigator	Date	Signature

3 copies: 1 for participant, 1 for the project notes and 1 for the medical notes

RNK/V2-PTB Bloods PTB Consent Form