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Declaration/Permission

I confirm that this thesis represents original work and has not been submitted as an exercise for any other degree or qualification at this or any other university. This thesis is composed of work which is entirely my own. I confirm that the library may lend or copy this work upon request.

A handwritten signature in black ink, appearing to read 'Matthew McGovern', is written over a horizontal line.

Dr Matthew McGovern

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Abbreviations

4I group: European Society for Paediatric Research section on neonatal Infection, Inflammation, Immunology and Immunisation

$\gamma\delta 1$: gamma delta 1

$\gamma\delta 2$: gamma delta 2

AEDF: absent end-diastolic flow

AKI: acute kidney injury

ANZNN: Australian and New Zealand Neonatal Network

AR: androgen receptor

ASC: apoptosis-associated speck-like protein containing a CARD

ASGNI: Australasian Study Group for Neonatal Infections

BTK: Bruton's tyrosine kinase

CDC: Centre for Disease Control and Prevention

CLD: chronic lung disease

CNS: central nervous system

CONS: coagulase negative staphylococci

CP: cerebral palsy

CPAP: continuous positive airway pressure

CPR: cardiopulmonary resuscitation

CR3: complement receptor 3

CRIB: Clinical Risk Index for Babies

CRP: c-reactive protein

CSF: cerebrospinal fluid

Ct: cycle threshold

CVS: cardiovascular system

E2: 17- β Estradiol

ELBW: extremely low birth weight

ELCS: elective caesarean delivery

ELISA: enzyme-linked immunosorbent assay

EMCS: emergency caesarean delivery

EOS: early-onset sepsis

EPO: erythropoietin

ER- α : estrogen receptor alpha

ER- β : estrogen receptor beta

FiO₂: fraction of inspired oxygen

FSC: forward scatter

GBS: group B streptococcus

Haem: haematological

ICAM-1: intracellular adhesion molecule-1

IFN: interferon

IKK- γ : inhibitor of nuclear factor kappa-B kinase subunit gamma

IL: interleukin

IL-1RA: interleukin 1 receptor antagonist

IPPV: intermittent positive pressure ventilation

IRAK-1: Interleukin-1 receptor-associated kinase 1

IVH: intraventricular haemorrhage

LBW: low birth weight

LOS: late-onset sepsis

LPS: lipopolysaccharide

Mac-1: macrophage 1 antigen

MFI: mean fluorescence intensity

mRNA: messenger RNA

miRNA: microRNA

MYD88: Myeloid differentiation primary response 88

NAC: no-amplification control

NICHD: National Institute of Child Health and Human Development

NLRP3: NOD-, LRR- and pyrin domain-containing protein 3

NTC: no-template control

NTISS: Neonatal Therapeutic Intervention Scoring System

NEC: necrotising enterocolitis

NEOMOD: Neonatal Multiple Organ Dysfunction score

NeonIN: Neonatal Infection surveillance Network

NI: neurodevelopmental index

NICE: National Institute for Health and Care Excellence

NICU: neonatal intensive care unit

NK: natural killer

NP: nasal prongs oxygen

nSOFA: neonatal-specific sequential organ failure assessment score

Pam3Csk4: Pam3Cys-Ser-(Lys)4. Trihydrochloride

PBA: phosphate buffered alanine

PBS: phosphate buffered saline

PDA: patent ductus arteriosus

Pg: progesterone

PPHN: persistent pulmonary hypertension

PROM: prolonged rupture of membranes

pSOFA: paediatric sequential organ failure assessment score

PVL: periventricular leukomalacia

qRT-PCR: quantitative reverse-transcriptase polymerase chain reaction

qSOFA: quick sequential organ failure assessment score

SVD: spontaneous vaginal delivery

RDS: respiratory distress syndrome

REDF: reversed end-diastolic flow

Resp: respiratory

ROP: retinopathy of prematurity

RSV: respiratory syncytial virus

SIRS: systemic inflammatory response syndrome

SNP: single nucleotide polymorphism

SOFA: sequential organ failure assessment score

SSC: side scatter

TLR: Toll-like receptor

TNF: tumour necrosis factor

TPN: total parental nutrition

TTMI: Trinity Translational Medicine Institute

UAC: umbilical arterial catheter

UVC: umbilical venous catheter

VLBW: very low birth weight

VON: Vermont-Oxford Network

WHO: World Health Organisation

Abstract

Introduction: Preterm neonates are at high risk of sepsis and sepsis-related complications. Despite the prominence of sepsis in neonatal practice, there is no consensus definition and the role of organ dysfunction in screening for neonatal sepsis remains unknown. Male preterm neonates are at especially high risk of sepsis compared to females. A combination of genetic, hormonal and immunological factors are thought to underlie this male disadvantage. There is ample evidence suggesting clinically important differences in innate immune response between the sexes in older children and adults though similar studies in preterm neonates are lacking.

Methods: Preterm neonates <1500 grams and healthy term controls were enrolled, and immune studies were compared between the sexes. TLR2 and CD11b expression were quantified on peripheral blood neutrophils and monocytes using flow cytometry, ELISA was used to quantify pro- and anti-inflammatory cytokines and the effect of female sex hormones on these aspects of immune response was examined. miRNA profiles were examined and expression of X-linked genes with immune function along with genes involved in the inflammasome were quantified with PCR. A literature review was undertaken to examine the current approaches to defining neonatal sepsis and clinical measurements on patients enrolled in this study were used to examine the value of organ dysfunction in preterm neonates both during and outside of sepsis.

Results: Term and preterm female neonates displayed more robust cytokine, CD11b and TLR2 responses following immune stimulation compared to males. Estrogen and progesterone showed promise as immunomodulators with significant effects on the expression of many cytokines. Males had higher levels of organ dysfunction and had poorer clinical outcomes compared to females. Literature review highlighted the weakness of current approaches to defining neonatal sepsis and our complementary clinical study showed that organ dysfunction has prognostic value in screening for neonatal sepsis.

Conclusion: Male neonates have impaired immune responses and have greater levels of organ dysfunction compared to females of equivalent gestation. Female sex hormones have potentially important effects on innate immune response and suggest that antenatal steroid and antenatal progesterone may provide opportunities for immunomodulation.

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Chapter 1: Introduction

1.1. Premature neonatal birth

Fetal development progresses in a clearly programmed manner *in-utero* and, under normal circumstances, this development will continue until a mother reaches between 37-42 weeks of gestation and delivers a healthy neonate. A baby born between 37-42 weeks gestation is considered a “term” neonate. A premature or “preterm” neonatal birth is defined as birth of a baby before 37 completed weeks of gestation. There are many reasons why a baby may be born prematurely: some may be intentionally delivered early for medical reasons, in other instances maternal ill-health may induce spontaneous premature labour, in the majority of cases however the reasons for spontaneous premature delivery are unknown ¹. The major consequence of premature birth is that the biologically programmed development of the fetus is interrupted and a neonate born under these circumstances may be ill-equipped for life ex-utero. By 23-24 weeks of gestation all major organs required for life are present within the fetus, though these organ systems will undergo significant maturation in the remaining months of pregnancy. Those neonates born at lower gestations have therefore missed out on weeks or months of growth and development in-utero and are at higher risk of organ dysfunction and ill-health than term neonates. Birth between 23-24 weeks is generally considered the “edge of viability” ², a term which refers to the fact that very few neonates born below this gestation survive past the early neonatal period, and those born between 23-24 weeks gestation require significant medical intervention after delivery ³. In general, there is a strong inverse relationship between the gestation of delivery and the degree of ill-health a premature neonate will suffer with those of lower gestation being sicker than more mature neonates ⁴.

1.1.1. Definitions and epidemiology

Premature neonatal delivery affects over 10% of neonates born worldwide ⁵ and there is evidence that the burden of premature birth is increasing internationally ^{6,7}. In recent decades there has been a significant improvement in premature neonatal outcomes due largely to advances in obstetric and neonatal care ⁸. Within Ireland it is

estimated that approximately 1 in 4 admissions to a tertiary neonatal unit is due to prematurity⁹ and between 7.2-12.9% of neonatal intensive care unit (NICU) admissions are of babies weighing <1500 grams⁹⁻¹¹. These trends are characteristic of those worldwide with risk of NICU admission increasing with lower birth weight and gestational age¹².

Premature neonatal births are most commonly subclassified based on the World Health Organisation (WHO) classification system for both gestation⁵ and weight¹³ (Table 1.1). Prognosis is much poorer in neonates of lower gestation and birth weight so both are important as they allow neonatologists to plan and deliver care in the early neonatal period and provide important prognostic information on long-term outcomes.

Birth weight	Classification
<2500 grams	Low birth weight (LBW)
<1500 grams	Very low birth weight (VLBW)
<1000 grams	Extremely low birth weight (ELBW)

Table 1.1: The WHO classification of premature neonates based on birth weight

1.1.2. Health service implications of premature delivery

With premature neonatal birth becoming more common there is an increasing need for the provision of specialised neonatal services. This specialised care is expensive however, and requires not only specialised medical equipment and medications, but also the provision of medical and nursing staff specifically trained in the area.

While premature neonates may need significant medical intervention after delivery and are at risk of adverse clinical outcomes there have been significant advances in the care of premature neonates. Mortality has improved greatly among premature neonates in recent decades, in particular at the extremes of gestation¹⁴. Additionally, reports from international organisations such as the Australian Cerebral Palsy (CP) Register have shown that a reduction in CP diagnosis among ex-premature neonates

has been a significant contributor to an overall reduction in the incidence of CP ¹⁵. Despite the continuing improvements in premature neonatal outcomes, survivors remain medically-complex and are at higher risk of health problems in later years ¹⁶. Collectively, the changes in neonatal care and outcomes which we have witnessed in recent decades have had, and continue to have, far-reaching implications for the planning and delivery of healthcare services worldwide ^{17,18}. With greater survival to hospital discharge comes the need for greater after-hospital care and improved screening for longer-term organ dysfunction which, at present, is likely under-recognised ¹⁹. Thus, improving outcomes in neonatology, particularly in those born extremely premature, may be seen as a “moving target” ⁴ where the profile of surviving neonates is changing constantly and health services must continually adapt to meet their needs.

1.1.3. The impact of premature delivery on parents

The most direct impact of premature neonatal birth is clearly on parents and families, but this can be difficult to quantify. A study by Irish Premature Babies, a charity organisation, found that the impact on families is considerable and extends beyond the often-difficult initial weeks of life in hospital ²⁰. Loss of earnings, the added costs of travel, food and childcare while in NICU and medical costs contribute to significant financial pressure on families ²⁰. In addition, up to 45% of premature babies in Ireland will require readmission to hospital after leaving the neonatal unit ²⁰, reflecting the continued medical difficulties these families must deal with after discharge. Families are also faced with the fact that community therapy and disability services in Ireland are often under-resourced and they are therefore faced with long waiting lists for much-needed assessments and services. This not only generates stress for parents but means that many parents will opt to access therapy privately ²⁰, adding to existing financial hardship. Lastly, while it is often recognised that parents undergo considerable hardship while their children are in hospital, the after effects of this life-changing event are often not acknowledged. The report from Irish Premature babies found that as many as 45% of parents felt they had post-traumatic stress after their time in the NICU and psychological support was often not available to parents when

needed²⁰. In combination the findings of this report suggest that parents undergo considerable financial, medical and psychological hardship both during and after their child's time in NICU.

1.2. Clinical implications of premature birth

1.2.1. Neurodevelopmental implications of premature delivery

One of the commonest early neurological complications of premature neonatal birth is intraventricular haemorrhage (IVH) a condition which is more common with lower gestation, especially those who are critically unwell^{21,22}. This involves haemorrhage into the CNS and is classified as grades I-IV with increasing severity²³, terminology which was originally described in VLBW neonates by Papile *et al.* in 1978²⁴. Though IVH is most often identified incidentally on routine neuroimaging and is usually asymptomatic at the time of onset it predispose to a number of later neurological complications including CP, global developmental delay and hydrocephalus^{25,26}.

Periventricular leukomalacia (PVL) is a form of neonatal white matter injury most common in neonates born <30 weeks gestation²⁷. The pathophysiology of PVL is complex and is thought to be due to a combination of hypoxic and inflammatory injury which may occur prenatally or postnatally²⁷. Lower birth weight and prematurity are both major risk factors for the development of PVL²⁸, as are neonatal circulatory abnormalities such as hypotension²⁸, and perinatal inflammatory events such as chorioamnionitis²⁹ and early-onset neonatal sepsis (EOS)³⁰. While the neurological manifestations of PVL in the early neonatal period are often mild or subclinical²⁸, PVL is a major precursor to later CP and developmental delay³¹.

Retinopathy of prematurity (ROP) is a disorder of ocular vascularisation characterised by abnormal angiogenesis secondary to postnatal changes in a variety of environmental factors including vascular endothelial growth factor, oxygen and insulin-like growth factor I³². ROP is among the leading causes of blindness worldwide and prematurity and lower birth weight are the strongest risk factors for its development³³.

Neurodevelopmental outcome is strongly influenced by both gestational age and birth weight and while the incidence of severe deficits in late preterm neonates has sharply declined in recent decades, the incidence among those of extreme prematurity remains largely unchanged ³⁴. Neurodevelopmental deficits may manifest in many ways ranging from isolated, mild deficits to complex and severe disability which will cause lifelong impairment. CP, cognitive impairment, sensory processing disorders, visual and co-ordination abnormalities, speech and language delay, and emotional and behavioural disorders may all result from premature birth ³⁵. Neonates born <33 weeks are three times more likely to have complex abnormalities involving multiple developmental areas compared to neonates born at term ³⁶. While estimation of the global incidence of neurological impairment in survivors is difficult due to differences in measurement and reporting in published studies, systematic review and meta-analysis suggests that up to 16.9% of very preterm and VLBW neonates will develop some form of cognitive delay ³⁷.

1.2.2. Respiratory complications of premature delivery

A fully functioning neonatal respiratory system requires 4 key components: a developed series of conducting airways, a vascular network, a mature alveolar network to facilitate gas exchange, and adequate surfactant in the terminal airways ³⁸. Lung development *in-utero* progresses through a well-defined series of developmental stages, namely: the embryonic, pseudoglandular, canalicular, saccular and alveolar stages ³⁹. Premature delivery interrupts this process meaning that the capillary network and alveolar sacs are under-developed and there is inadequate surfactant production. Collectively these result in inadequate gas exchange and respiratory support, including mechanical ventilation, is often required.

Respiratory distress syndrome (RDS) is the most common medical complication of prematurity and is among the most common causes of neonatal mortality in the developing world ⁴⁰. RDS is a condition characterised by increased work of breathing and inadequate gas exchange secondary to surfactant deficiency ⁴¹. Surfactant is produced by type II pneumocytes and acts to decrease alveolar surface tension, prevent airway collapse and help in recruiting distal airways. Premature airways

contain fewer type II pneumocytes resulting in increased alveolar surface tension and atelectasis. RDS classically presents shortly after delivery with incidence and severity increased in more premature neonates ⁴². Corticosteroids may be administered antenatally to promote lung development in threatened preterm labour and is one of the only effective preventative interventions for RDS ⁴³. Depending on severity, RDS may require a combination of ventilatory support or exogenous surfactant administration after delivery. Most very preterm neonates will require some form of ventilatory support which may be of prolonged duration and may itself predispose the neonate to further complications such as ventilator-associated lung injury ⁴⁴ and pneumothorax ⁴⁵.

Persistent pulmonary hypertension (PPHN) occurs when normal haemodynamic transition fails to occur, and pulmonary vascular pressures fail to fall. While traditionally viewed as a disease of term infants, it is increasingly recognised in preterm neonates secondary to lung hypoplasia or sepsis and the prevalence of PPHN in extremely preterm neonates is increasing ⁴⁶. Hypoxia and increased cardiovascular strain may result and there is increasing recognition that preterm PPHN may have a role in the later development of the chronic lung disease (CLD) ⁴⁷. CLD is generally defined as the requirement for oxygen therapy beyond 28 days of life or beyond 36 weeks of corrected gestation ⁴⁸. CLD is characterised by poorly formed pulmonary vasculature and hypoplastic alveoli ⁴⁸ which results in the ongoing need for respiratory support. While itself being a complication of premature delivery, CLD is a risk factor for longer-term neonatal morbidity such as prolonged hospitalisation, recurrent infection and adverse neurodevelopmental outcome ⁴⁹.

With improved understanding of neonatal respiratory disease and improved long-term survival of premature neonates it is becoming clear that former preterm adults display a unique pulmonary phenotype characterised by airflow obstruction, eosinophilic infiltration and lung parenchymal destruction ^{50,51}. While the exact pathophysiology involved in developing this form of lung disease is poorly understood it is known that severity of CLD is an important predictor of severity ⁵².

1.2.3. Cardiovascular complications of premature delivery

Cardiovascular transitioning, the process whereby the fetal circulation adapts to *ex-utero* life, involves lung expansion, a reduction in pulmonary vascular pressure, an increase in systemic vascular pressure and closure of fetal vascular channels ⁵³. Transitioning in premature neonates is complicated by many factors including immaturity of the cardiovascular system, placental factors, timing of cord clamping, initial resuscitation and underlying maternal disease states ⁵³. Due to a combination of these factors premature neonates may develop low blood pressure, compromised cerebral autoregulation, elevated pulmonary pressures, persistence of the ductus arteriosus and poor myocardial contractility ⁵³. The level of cardiovascular intervention required varies between premature neonates but is heavily influenced by gestational age ⁵⁴. Hypotension is the commonest early cardiovascular complication of prematurity and has been associated with IVH and later adverse neurodevelopmental outcome ^{55,56}. Measuring perfusion in premature neonates is also complex as many of the traditional clinical parameters are poorly representative of perfusion in the premature population ⁵⁷, and while mean arterial blood pressure and point-of-care echocardiography are the most frequently used no true “gold-standard” exists ⁵⁷.

Under normal circumstances a reduction in circulating prostaglandins, downregulation of prostaglandin receptors and alterations in oxygen tension cause the ductus arteriosus to close in the initial 72 hours of life ⁵⁸. In the premature neonate however, closure may not occur and a patent ductus arteriosus (PDA) results. Incidence of PDA increases with lower gestation and birth weight with approximately 30% of ELBW neonates being affected ⁵⁹. Small PDAs may be asymptomatic however where the PDA is large there may be significant left-to-right shunting resulting in pulmonary over-circulation and cardiac compromise. Respiratory failure, myocardial remodelling and systemic hypoperfusion are all potential side-effects ⁶⁰. Aside from the direct cardiorespiratory effects, many short- and long-term complications have been associated with the presence of a PDA including renal injury ⁶¹, necrotising enterocolitis (NEC) ⁶², failure to thrive and IVH ⁶³. In addition to the acute cardiac issues described previously, it is now recognised that former preterm neonates have cardiac dysfunction extending into adolescence and adult life ⁶⁴. The association between increased blood pressure in adulthood and preterm birth is well-established

^{65,66}, but there is now increasing focus on other forms of cardiovascular disease such as traditional atherosclerotic cardiac disease ⁶⁷, metabolic syndrome ⁶⁸ and the abnormal structure of the ex-preterm myocardium ⁶⁹.

Given the complexity of neonatal haemodynamics it is unsurprising that acute illness can compromise cardiovascular function and septic neonates may therefore manifest both systolic and diastolic cardiac dysfunction ⁷⁰. The aetiology of sepsis-related cardiac dysfunction in neonates is complex though similar to adults, immune dysfunction is thought to contribute ⁷¹. Clinically this may result in systemic hypoperfusion with hypotension being one of the commonest manifestations ⁷². Septic neonates also develop higher pulmonary pressures during sepsis ⁷³ and PPHN may result in premature infants. Given the diverse clinical sequelae, the treatment of sepsis-related cardiovascular complications is complex in preterm neonates, often requiring multiple therapeutic agents ⁷². Notably, sepsis-induced cardiac dysfunction may be sub-clinical in some paediatric patients ⁷⁴. This is also likely to be the case in neonates who manifest subtle cardiovascular changes such as heart rate variability early in the disease ^{75,76}. Mortality during sepsis is higher in neonates with higher troponin T levels and more significant cardiac dysfunction on echocardiography ⁷⁰, suggesting that measurements of cardiac dysfunction may be of prognostic value in septic preterm neonates.

1.2.4. Renal complications of premature delivery

Acute kidney injury (AKI) is the predominant early renal complication in premature neonates with almost half of very preterm neonates affected ⁷⁷. In addition to the inherent susceptibility of the immature kidney to injury, many of the medications and medical interventions which may be necessary in the care of the preterm neonate have potentially nephrotoxic effects ^{78,79}. Monitoring and treating AKI is also challenging in premature neonates as creatinine levels in the early postpartum period may be influenced by maternal factors ⁸⁰, renal replacement therapy is challenging and the outcome of such interventions unclear ⁸⁰. Similar to many of the other organ systems we have discussed, ex-premature neonates are at risk of long-term renal dysfunction in their adolescent and adult years ⁸¹.

1.2.5. *Haematological complications of premature delivery*

Anaemia is the most common haematological complication in premature neonates with most extremely preterm neonates requiring at least 1 red cell transfusion during their inpatient stay⁸². Anaemia of prematurity is multifactorial with inadequate iron stores, low circulating erythropoietin (EPO), increased red cell catabolism and recurrent phlebotomy all contributing⁸². Thrombocytopaenia may occur in sick premature neonates or secondary to maternal or placental factors⁸³ with the risk of thrombocytopaenia having an inverse relationship with gestational age⁸⁴. Aside from the deleterious effects of both anaemia and thrombocytopaenia it is important to note that premature neonates may also be at higher risk of adverse outcomes following transfusion⁸⁵ and this is a particular concern for red cell transfusion which may increase oxidative stress⁸⁶.

1.2.6. *Gastrointestinal and metabolic complications of premature delivery*

Most premature neonates will require some form of nutritional support after delivery. In late-preterm neonates this may be short duration intravenous fluid therapy, in those born extremely preterm this may constitute prolonged total parental nutrition (TPN) in combination with gradual introduction of enteral feeds and nutritional supplementation for the initial months of life. This early nutritional support is one of the cornerstones of premature neonatal care as nutritional status is thought to influence important short- and long-term health outcomes^{87,88} and premature neonates are known to be at high risk of failure to thrive⁸⁹.

NEC affects between 2-5% of VLBW neonates with a case fatality rate of >20%⁸⁷. NEC is characterised by intestinal necrosis with rapid clinical deterioration. While the pathophysiology is not fully understood, intestinal dysbiosis, nutrition and immaturity of both the intestinal mucosa and immune response are thought to contribute⁹⁰. Though relatively uncommon, affected neonates are often profoundly unwell and are at high risk of adverse short- and long-term gastrointestinal and neurological complications⁹¹⁻⁹³.

Due to their poor nutritional reserves at birth, premature neonates are also at high risk of a variety of other, less immediate metabolic complications. Hypoglycaemia, for

example, is commonly observed among premature neonates in the early neonatal period ⁹⁴. Premature neonates are also at risk of metabolic bone disease ⁹⁵ requiring vitamin supplementation as prophylaxis in the months after delivery and have been noted to have a high incidence of thyroid dysfunction, though the significance and management of this remains controversial ⁹⁶.

1.3. Organ dysfunction in premature neonates

Many scoring systems exist for grading the severity of organ dysfunction in adults and older children ^{97,98}. These scoring systems have 4 main functions: to provide an accurate and reproducible measure of ill-health; to identify patients who are not responding to therapy or require escalation of care; to monitor response to medical intervention and to provide prognostic information on likely outcome ⁹⁸. More recently, organ dysfunction scores have been adopted as “early warning scores” ⁹⁹ to detect deterioration in high risk patients and as screening tools for sepsis in adult populations ¹⁰⁰. The adoption of these scores in this manner is logical as adults, older children and term neonates who are healthy will not have clinical signs of organ dysfunction. The use of such scores is more complicated in premature neonates who frequently have organ dysfunction present from birth secondary to organ immaturity ¹⁰¹ or due to the various complications discussed previously. This organ dysfunction may manifest in many ways such as the requirement for oxygen and pressure support in RDS secondary to lung immaturity or the requirement for intravenous nutrition early in life secondary to gut immaturity. While certainly meeting the criteria for organ dysfunction, these manifestations are often within what might be expected as “normal” challenges for a premature neonate of a particular gestation ¹⁰². This baseline need for medical support makes defining and screening for organ dysfunction complex in the premature neonatal population. Several scores are available for estimating illness severity in neonates which record information on birth history and organ dysfunction ¹⁰³⁻¹¹³. Despite the range of scores available few are extensively validated, many have been examined only in the early neonatal period and most focus only on predicting either mortality or neurodisability only ¹¹⁴. While changes in behaviour and vital signs are frequently the earliest indications of clinical deterioration

in neonates, to date neonatal organ dysfunction scores have not been widely incorporated into the routine NICU care of premature neonates. This is despite the fact that records of such scores are routine in many paediatric and adult ICUs ^{115,116}. Additionally, while the detection of organ dysfunction is now acknowledged as a core piece of defining and screening for sepsis in adults and older children ^{117,118}, this idea has not yet been thoroughly investigated in neonatal populations. As such, there is clearly a need for studies evaluating baseline organ dysfunction in premature neonates and investigating potential organ dysfunction scores as screening tools in sepsis.

1.4. Neonatal Sepsis in premature neonates

1.4.1. *Epidemiology and risk factors for neonatal sepsis*

Infection and sepsis are the leading cause of neonatal mortality worldwide ¹¹⁹. Incidence is estimated to be approximately 22/1000 live births with an estimated case fatality rate of between 11-19% ¹²⁰. While mortality is greatest in developing countries, sepsis remains a problem worldwide. Premature neonates are at particularly high risk of neonatal sepsis due to an immature immune system ¹²¹ and a variety of factors unique to the perinatal period ¹²² which place neonates at especially high risk compared to older children and adults. Broadly speaking, neonatal sepsis can be divided into EOS and late-onset neonatal sepsis (LOS) depending on the time of onset after birth. This division is important as EOS is thought to represent sepsis secondary to maternal infection and therefore presents in the first 48-72 hours after birth; whereas LOS is thought to represent postnatally-acquired sepsis presenting after 48-72 hours ¹²³. Although clinical manifestations of both forms of sepsis are similar, the organisms responsible, risk factors, populations affected, and clinical outcomes are very different ¹²⁴. Risk factors associated with EOS are related to susceptibility in the perinatal period such as maternal sepsis, maternal colonisation with pathogens and prolonged or premature rupture of membranes ¹²⁴. Risk factors associated with LOS relate to postnatal susceptibility such as the presence of multiple comorbidities, presence of medical devices and nutritional factors ¹²⁵. Despite their different aetiologies, prematurity is a risk factor for both ^{126,127}, reflecting the particular susceptibility of premature neonates to all forms of neonatal infection. The reasons

for this are complex and include the fact that premature neonates often have many of the risk factors for both forms of sepsis, for example, a neonate may be delivered early due to maternal sepsis and may later have multiple invasive procedures and a medically complex inpatient course. Additionally, premature neonates have deficits in almost all areas of innate and adaptive immunity ¹²¹, further increasing their risk. Despite significant advances in neonatal sepsis management in recent decades ¹²⁸, neonatal sepsis remains a leading cause of neonatal morbidity and mortality ¹¹⁹. Premature neonates are at especially high risk of all forms of sepsis and with early premature neonatal mortality decreasing, sepsis will likely become an even greater health problem among survivors.

1.4.2. *Outcomes of neonatal sepsis*

Sepsis is an important outcome in neonatology not only because of the high mortality but also because there is considerable associated morbidity in survivors ¹²⁴. The complications associated with sepsis are diverse and may be immediate, such as death or organ dysfunction ¹²⁹, or may only become obvious after resolution of the sepsis episode, such as neurodevelopmental delay ^{72,124}. Many factors affect the clinical outcome after sepsis such as the infecting organism ¹³⁰⁻¹³², presence of comorbidities ^{133,134}, presence of organ dysfunction ^{129,135,136}, gestational age ^{137,138} and number of sepsis episodes suffered ¹³⁹. Non-septic premature neonates are already at higher risk of brain injury than term neonates ¹⁴⁰. In addition, premature neonates develop more organ dysfunction ¹⁴¹ and are at higher risk of sepsis than those born at term ¹⁴². As such, premature neonates represent a population that are predisposed to both sepsis and sepsis-related morbidity in the short- and long-term.

1.4.3. *Difficulties in defining and screening for neonatal sepsis*

Despite the significant contribution of sepsis to neonatal morbidity and mortality there is no unified definition of neonatal sepsis ¹⁴³. Instead many approaches to defining neonatal sepsis exist which differ from each other in fundamental ways. A combination of clinical, microbiological and laboratory data are used within these

definitions though the exact criteria employed are variable ¹²³. Attempts have been made to produce unified definitions within paediatrics ^{144,145} but these do not provide neonatal-specific criteria, thereby ignoring factors such as circumstances of delivery and prematurity which combine to make sepsis diagnosis so challenging in neonates. The result is that there is no standard approach to diagnosing neonatal sepsis across NICUs, a fact which has important clinical implications for patient management and treatment. Additionally, reporting of research involving potentially septic neonates becomes problematic as investigators use different criteria to identify patients of interest, making results difficult to interpret or generalise.

Early recognition and treatment of is the cornerstone of modern sepsis management ¹⁴⁶. Time to first dose antibiotics has become a metric of quality within sepsis management ¹⁴⁷ as shortening this time interval is known to improve outcome ¹⁴⁸. Due to the frequency of organ dysfunction in septic patients it has been suggested that identification of new-onset organ dysfunction should form the basis of sepsis screening ¹⁴⁹. At present there are no validated screening criteria in neonates with suspected sepsis with clinicians largely relying on their knowledge of the patient's history and their clinical acumen do decide on investigation and treatment. Similar to the lack of a consensus definition, the lack of sepsis screening criteria is clearly detrimental to neonatal sepsis care as single-point clinical assessment is very subjective in neonatal patients ¹⁵⁰.

The Sepsis-3 group in adult medicine formed to address these exact concerns within the adult population ¹⁰⁰. They formed an expert group, undertook extensive literature review and convened a Delphi process to identify optimal criteria for use in defining and screening for sepsis. These criteria were then clinically validated and currently represent a new benchmark in sepsis care. The criteria produced are clinically focused and highlight the importance of new-onset organ dysfunction in defining and screening for sepsis. These criteria can be easily applied at the bedside and have now been widely adopted in clinical practice, including acute hospitals within Ireland ¹⁵¹. A similar initiative is clearly needed within neonatology as, at present, sepsis definitions are heterogenous and screening criteria non-existent.

1.5. Immune implications of premature delivery

1.5.1. *Lymphocytes*

Premature neonates have reduced absolute lymphocyte numbers in comparison to term neonates ¹⁵². In addition, the subpopulations of lymphocytes are known to differ between term and preterm neonates at birth ¹⁵² and while many subpopulations show similar trends in the months after delivery others, such as NK cells, appear to be over-represented in premature neonates ¹⁵²⁻¹⁵⁴. These differences extend beyond the neonatal period with ex-preterm children having decreased lymphocyte numbers compared to their non-preterm peers ¹⁵². Aside from the effect of premature delivery itself, several factors in the peripartum period may affect lymphoid cell development. Glucocorticoids, which are frequently given to mothers with threatened premature labour, are known to decrease circulating lymphocyte numbers in premature neonates when administered both antenatally ¹⁵⁵ and postnatally ¹⁵⁶. Neonates developing CLD appear to have fewer CD3+, CD4+ and CD19+ lymphocytes ¹⁵³ though it has been suggested that these lymphocytes are more active in neonates developing CLD ¹⁵⁷. In combination these data suggest that premature neonates are somewhat lymphopaenic at birth and have different lymphocyte subpopulations compared to term neonates. These differences, which may be due to a combination of prematurity and other perinatal factors, persist as these children get older, reflecting that ex-premature neonates have an altered immune phenotype.

1.5.2. *Neutrophils*

Although preterm neonates appear to have fewer circulating neutrophils than term neonates ¹⁵⁸, the effect of extreme prematurity on absolute neutrophil count is not well-studied. Growth-restricted preterm neonates and neonates born to mothers with pregnancy-induced hypertension appear to be at increased risk of neutropaenia ¹⁵⁹, while glucocorticoid administration is known to markedly increase neutrophil counts in neonates when administered antenatally ¹⁶⁰. Premature neonatal neutrophils also appear to have impaired chemotaxis and migration. L-selectin is reduced in premature neonatal neutrophils compared to term neonates ¹⁶¹ and the endothelium of premature infants has reduced expression of P-selectin ¹⁶² suggesting a diminished

ability to initiate endothelial adhesion. Migration is further compromised by lower expression of macrophage 1 antigen (Mac-1) (CD11b/CD18) on the surface of premature neonatal neutrophils ¹⁶¹. It remains unclear whether the phagocytic ability of neutrophils is reduced in premature neonates compared to term neonates with some sources suggesting poorer phagocytosis among preterms under certain circumstances ¹⁶³ and others finding no difference ¹⁶⁴. It has been suggested however that neonates of all gestations have reduced phagocytic activity early in life when compared to adults ¹⁶⁵, though this effect may be organism- and stress-dependant ¹⁶⁶. The formation of neutrophil extracellular traps also appears to be reduced in premature neonatal neutrophils ¹⁶¹ suggesting an impaired ability to kill pathogens.

In combination this data suggest that premature neonates have a lower number of circulating neutrophils which are functionally immature compared to term neonates and adults. This immaturity is reflected by the fact that prophylactic administration of GM-CSF, while increasing neutrophil counts in small for gestational age premature neonates does not reduce sepsis incidence or mortality ¹⁶⁷.

1.5.3. *CD11b*

CD11b is a member of the integrin family of transmembrane receptors and is expressed on the surface of many immune cells including monocytes, macrophages, granulocytes and natural killer cells ¹⁶⁸. It is bound non-covalently with CD18 on the surface of these cells to form complement receptor 3 (CR3), also known as Mac-1, a member of the $\beta 2$ integrin family. While many ligands have been reported for CR3, the main ligands which bind to CR3 are iC3b, C4b, intracellular adhesion molecule-1 (ICAM-1) and fibrinogen ¹⁶⁹. The $\beta 2$ integrin family of receptors mediate movement of leucocytes into lymphoid organs and inflamed tissues by allowing firm adhesion of leucocytes to endothelium and subsequent extravasion. It has been suggested that the primary function of CR3 is in the “crawling” action of leucocytes on the endothelial surface prior to extravasion ¹⁷⁰. CR3 also plays an important role in the phagocytosis of both non-opsonised bacteria and those coated in iC3b ¹⁷¹. It has also been proposed that CR3 may have an important role in regulating the immune response through both pro- and anti-inflammatory activity ¹⁶⁹, distinct from its role in leucocyte movement

and phagocytosis. CR3 expression on antigen-presenting cells has been shown to affect T cell activity potentially limiting inflammatory response, whereas activated CR3 on the surface of monocytes has been shown have a pro-inflammatory effect ¹⁶⁹. The relationship between CD11b expression and Toll-like receptor (TLR) signalling is complex with evidence that CR3 may have both a pro- and anti-TLR signalling effects depending on the context, though this relationship is not well-understood ¹⁷². The complex immunological role of CR3 is reflected by the fact that patients with absent or defective CR3 have markedly impaired leucocyte recruitment and phagocytosis ¹⁷³, a profound susceptibility to recurrent bacterial infection ¹⁷⁴ and have an increased susceptibility to autoimmune conditions ¹⁷⁵.

Neonatal neutrophils are known to have reduced CR3 compared to adults ¹⁷⁶. It has been suggested that an increased density of immature neutrophils with lower CR3 expression among neonates may partially explain these differences ¹⁷⁷. Additionally, neutrophil CR3-related adhesion is reduced in neonates compared to adults ¹⁷⁸ as is CR3 mobilisation from intracellular stores ¹⁷⁹ suggesting a functional deficit in comparison to older patients. These deficits may persist for weeks after birth even among term neonates with CD11b expression being lower in neonatal neutrophils after stimulation when compared to adults ¹⁸⁰. CR3 has been identified as an essential component of the host immune response to group B streptococcus (GBS) ¹⁸¹, the organism which is most commonly responsible for EOS. While normally lower in premature neonates compared to term neonates, CD11b has been shown to be higher in the first week of life among premature neonates who later develop CLD ¹⁸², suggesting a role in the inflammatory pathogenesis of premature neonatal lung disease. Neutrophil CR3 has been shown to directly correlate with gestational age ^{183,184} with levels below 30 weeks being comparable to those of patients with inherited deficiencies of $\beta 2$ integrins ¹⁸⁴. Given that neonates have low levels of circulating pathogen-specific antibodies and impaired opsonin production, it has been suggested that much of the phagocytic function of innate neonatal cells is dependent on the opsonin-independent phagocytic activity of the CD11b/CD18 complex ¹⁸⁵. Given the immaturity of this response in term and especially preterm neonates it may partially explain their profound susceptibility to infection in the early months of life ¹⁸⁵.

Due to the key role it plays in early innate immune response, CD11b is currently under investigation as a biomarker of neonatal sepsis. To date CD11b has shown promise in the early detection of sepsis either alone ¹⁸⁶⁻¹⁹⁰ or in combination of other inflammatory markers ^{191,192}. Further study is required to validate CD11b as a biomarker of sepsis in the neonatal population and at present it is unclear what effect prematurity has on its sensitivity and specificity as a diagnostic marker.

1.5.4. *Toll-like receptors*

TLRs are a family of pattern recognition receptors present on many cells in the innate immune system. There are 10 TLRs expressed in human cells ¹⁹³ and these receptors recognise pathogen-specific carbohydrates, lipoproteins and nucleic acids ¹⁹⁴. TLRs are present in immune and non-immune cells of many tissues within the body and the ligands recognised by TLRs are common to many microorganisms. This therefore provides many tissues with the ability to mount an immune response to a wide variety of pathogens, a fact that makes TLRs one of the most important components in the innate immune response ¹⁹⁵. Some TLRs are present on the cell surface, while others are present intracellularly, localising to the endosome ¹⁹⁴. TLRs are thought to act as dimers and after ligand binding initiate an intracellular signalling cascade that results in the production of inflammatory cytokines, chemotactic factors, type I interferons and antimicrobial peptides ¹⁹⁴.

The baseline expression of TLRs in term neonatal immune cells is comparable to that of adults ^{196,197} though data on the ability of term neonates to upregulate TLRs in response to infective stimuli is somewhat more variable ¹⁹⁷⁻¹⁹⁹. While TLRs are expressed in healthy term neonates, the production of TLR-mediated effector molecules appears reduced in neonates compared to adults ²⁰⁰. While TLR expression is not well-studied in this population, preterm neonates have exhibited reduced monocyte TLR4 expression compared to both term neonates and adults ²⁰¹, though TLR2 expression is thought to be similar to term neonates ²⁰².

In addition to differences in TLR expression, several molecules important in TLR-signalling are also diminished in neonatal immune cells compared to adults. CD14,

which acts as an accessory protein in TLR4 response to endotoxin, is lower in neonatal serum ¹⁹⁶ and has diminished endotoxin-responsiveness ¹⁹⁹ when compared to healthy adults. Myeloid differentiation primary response 88 (MYD88), the adapter which is used most frequently to initiate TLR signalling is lower in both term neonatal neutrophils ²⁰³ and monocytes ¹⁹⁹; and premature neonatal monocytes, than in healthy adults ²⁰². The expression of both Interleukin-1 receptor-associated kinase 1 (IRAK-1), a kinase which binds the death domain of TLR-bound MYD88, and inhibitor of nuclear factor kappa-B kinase subunit gamma (IKK γ), which plays a downstream role in activating NF κ B, are lower in neonatal whole blood compared to adults ²⁰⁴. Pro-inflammatory cytokine production is significantly diminished in response to TLR activation in premature neonates compared to adults ^{201,202}. The addition of neonatal serum impairs TLR signalling in adult monocytes, suggesting either the presence of a TLR inhibitor in neonatal serum or the absence of factors which enhance TLR signalling ¹⁹⁶.

TLR2 is of particular interest in premature neonatal immunity as animal models have suggested that TLR2 is crucial for the neonatal immune response to GBS ²⁰⁵ and that its production is increased early in coagulase negative staphylococci (CONS) sepsis ²⁰⁶. GBS and CONS represent the commonest causes of EOS and LOS respectively in the neonatal population. TLR2 has also been suggested as a potential contributor to GBS-related neuronal injury ²⁰⁷. Additionally, immature innate response with altered TLR2, TLR4, MyD88, TRAF6 and NF κ B mRNA expression in intestinal mucosa have been implicated in the pathogenesis of NEC ²⁰⁸, further highlighting the importance of TLR signalling in premature neonatal outcomes.

1.5.5. Cytokines

Cytokines are small proteins secreted by immune cells that affect nearby cells bearing the appropriate receptor ²⁰⁹. Cytokines have a diverse range of pro- and anti-inflammatory actions and, in the setting of infection, the transcription and secretion of cytokines are one of the key effects of immune cell TLR signalling and cell surface receptor activation ²¹⁰. Due to their ability to regulate inflammation, cytokines have been recognised as crucial determinants of the initial response to sepsis; the

development of sepsis-related organ dysfunction ²¹⁰; and, crucially to premature neonates, the later sequelae of sepsis-related inflammation ²¹¹. Cytokine responses *in-utero* are generally weighted towards the Th-2 responses to allow fetal development and maintenance of pregnancy ²¹². There has been increasing recognition that loss of this pro- and anti-inflammatory balance may be associated with preterm labour, with a systematic review suggesting that IL-6 may be elevated in spontaneous-onset premature delivery ²¹³.

The fetal bias in cytokine production towards a Th-2 response appears to persist in term neonates after delivery with reduced pro-inflammatory and increased anti-inflammatory cytokine production ²¹⁴. Cytokine expression changes throughout childhood ²¹⁵ with some cytokines, such as TNF- α and IFN- γ , having clear age-related increases and other having a more complex relationships with increasing age ²¹⁵. Data on postnatal serum cytokine levels in healthy, uninfected premature neonates are sparse and somewhat conflicting. A study by Lusyati *et al.* in 2013 found that IL-1Ra, IL-2, IL-8, IL-12, IL-13, IL-15, IL-17, IFN γ , TNF α , IL-6 and IL-10 were lower in premature neonates compared to those of older gestation whereas IL-1b, IL-4, IL-5, IL-7 and IFN- α did not differ between groups ²¹⁶. An earlier study by Matoba *et al.* found that IL-1 and IL-18 were lower in premature neonates than those born at term and IL-2, IL-4, IL-5, IL-8, IL-10 and TNF- α were higher in premature neonates ²¹⁷, though notably infected neonates were not excluded from this study, infection being a factor known to affect postnatal cytokine profiles in premature neonates ²¹⁸. The effect of growth restriction on cytokine expression in the premature population is uncertain as there are few studies in this area and those that exist differ greatly in methodology and study populations ²¹⁹.

While there is limited data on “normal” cytokine levels in the premature population, it is clear that their cytokine response is distinct from that of older children and adults. This is important clinically as deranged cytokine expression has been associated with the pathogenesis of many important neonatal diseases. Elevated pro-inflammatory cytokines in cord and neonatal blood have been associated with later brain injury among premature neonates ^{220,221}. Elevated levels of IL-6, IL-8 and TNF- α are associated with mortality in term and premature neonates with sepsis ²²². A

variety of cytokines are raised in NEC and of particular note is that IL-1 β has been shown to correlate with disease severity and survival ²²³, suggesting that deranged cytokine expression is key to the pathogenesis and outcome of NEC. Respiratory inflammation is known to contribute to the development of CLD and IL-1, IL-6, IL-8, IL-10 and TNF- α have all been identified as contributors to the inflammatory lung damage responsible for CLD ²²⁴. In addition to their role in modulating neonatal immune response, measurement of serum cytokines have been suggested as potential biomarkers in neonatal sepsis ^{225,226}, though data on their specificity and sensitivity in premature neonates is lacking.

1.5.6. *Inflammasome*

The inflammasome is a multi-protein complex which forms in the cytoplasm of cells involved in innate immune system in response to a variety of cellular stimuli ²²⁷. Several inflammasomes exist, among the best-studied of which are the NLRP3 and AIM-2 inflammasomes. Inflammasome formation results in the accumulation and activation of caspase-1 which then cleaves the inactive forms of the pro-inflammatory cytokines IL-18 and IL-1 β along with inducing a form of inflammatory cell death known as pyroptosis ²²⁸. Though important contributors to innate immunity, the pro-inflammatory response which results from inflammasome activation has potentially adverse neurological effects with elevated IL-1 β linked to the pathogenesis of acute brain injury ²²⁹. In addition, the potent inflammatory action of inflammasomes have been suggested to contribute to organ dysfunction in sepsis, making them attractive candidates for new drug development ²³⁰.

Experimental data on inflammasome activity in the premature population is lacking at present, with much of the existing research focusing on the role of the inflammasome in preterm labour and hypoxia-induced brain injury in term neonates. It has however been suggested that inflammasome activation may mediate brain injury and subsequent disability in preterm infants with IVH ²³¹ and IL-18 levels in cord blood correlate with later brain injury in premature neonates ²³². In addition, IL-18 levels are potentially higher in the serum of extremely premature neonates ²³³,

suggesting a dysregulated inflammasome response in the more immunologically immature preterm neonates.

1.5.7. *Complement*

The complement system is a family of plasma proteins which have important immunological actions in promoting inflammation, phagocytosis and attacking the cell membranes of invading organisms ²³⁴. There are 3 pathways of complement activation: the classical, alternative and lectin pathways which converge to initiate cleavage of C3 ²³⁵. Complement proteins usually circulate in their inactivated form and are one of the earliest immune defences encountered by pathogens after they have bypassed normal epithelial barriers, responding to a variety of stimuli ²³⁴. The importance of complement in the immune response is highlighted by the increased susceptibility to invasive infections and autoimmune conditions seen in those with complement deficiencies ^{236,237}. Complement levels appear to increase with both gestational age and weight ²³⁸, and data suggests immaturity in the activity and regulation of the complement system in premature neonates compared to term neonates ^{239,240}. In addition to the role of complement in immune defence it is thought to have important roles in pregnancy and labour with derangements in the complement system having been implicated in preterm delivery ²⁴¹.

1.6. **Sexual dimorphism in preterm clinical outcomes**

Throughout life the incidence of many diseases differs between males and females. For example, it is generally accepted that autoimmune conditions are more common among women ²⁴² and men are disproportionately affected by ischaemic heart disease ²⁴³. The severity, prognosis and outcomes of many diseases also differ between the sexes, with males often having poorer disease outcomes, reflected in the shorter lifespan of males and overall greater mortality of males across a variety of major diseases ²⁴⁴. These findings have led to the “male disadvantage” hypothesis where it has been suggested that males are at inherently higher risk of poor health outcomes due to a combination of social and physical factors ²⁴⁵. These differences in outcome are particularly evident in the premature population where male sex is a major risk factor for adverse outcome. The biological reasons for these differences are

poorly understood but it has been suggested that a combination of genetic, hormonal and immunological factors contribute ²⁴⁶. In the sections that follow we will briefly review the differences in short- and long-term outcomes between male and female neonates.

1.6.1. *Sexual dimorphism in short-term outcomes*

Sepsis is the leading cause of neonatal mortality worldwide ¹¹⁹. It is well-established that male sex is a risk factor for neonatal sepsis ²⁴⁷. On a more granular level however male sex is associated with increased risk of almost all forms of commonly encountered neonatal infections including community-acquired sepsis ¹³⁷, GBS sepsis ²⁴⁸ and sepsis secondary to drug-resistant or atypical bacteria ²⁴⁹. In addition, sepsis related mortality in males is higher than females ²⁵⁰⁻²⁵². The incidence of NEC does not appear to differ between male and female premature neonates though there is some evidence that males have poorer outcomes following the onset of NEC. Male neonates with NEC have an increased risk of intestinal perforation ²⁵³ and are more likely to develop more severe NEC than females ²⁵⁴. Male sex has also been suggested to be a risk factor for mortality among neonates affected with NEC ²⁵⁵.

Much of the early management of NICU is directed towards respiratory care and male sex has been associated with higher risk of a variety of respiratory illnesses. These range from relatively benign conditions such as transient tachypnoea of the newborn ²⁵⁶ to life-threatening emergencies such as pulmonary haemorrhage ²⁵⁷ and pneumothorax ²⁵⁸. RDS is the most common respiratory diagnosis in premature neonates and male sex is very strongly associated with the risk of RDS ²⁵⁹ and males also appear to be at higher risk of mortality from RDS ²⁶⁰. Clinical study has also shown different sensitivity between the sexes to surfactant replacement in RDS with authors even suggesting that higher surfactant doses are required in male neonates ²⁶¹.

Structurally there appears to be no difference in the incidence of congenital heart disease between males and females though functionally males appear to require more cardiovascular support in the transitioning period. This is reflected by LBW male

neonates having lower mean blood pressures on day of life 1 than females ²⁶², and ELBW males being more likely to require inotropic support for hypotension in the first week of life ²⁶³. Evidence in PPHN is mixed with affected preterm males seeming to have higher mortality overall ^{264,265} but females having poorer survival in cases where extra-corporeal membrane oxygenation is required ^{265,266}.

Preterm male infants have a higher incidence of neuroradiological abnormalities such as IVH, white matter injury, PVL and other major cranial abnormalities than females ^{257,267}. It has also been suggested that premature males have a higher risk of adverse neurological outcome even in the absence of severe IVH or PVL ²⁶⁸ or in instances where neuroimaging is entirely normal ²⁶⁹.

1.6.2. *Sexual dimorphism in long-term outcomes*

Even at a population level, male sex has long been acknowledged as a risk factor for CP ^{267,270}. Within the preterm population specifically, males are at higher risk of long-term cognitive, behavioural and neurological complications ^{257,271}. Follow-up studies of premature neonates at high risk of perinatal hypoxia found that males have poorer clinical outcomes ^{272,273} and lower cerebral white matter volumes on subsequent neuroimaging ²⁷⁴. The EPICURE ²⁷² and EXPRESS ²⁷⁵ studies were large prospective cohort studies of extremely preterm neonates. Follow-up of study participants at 30 months revealed that boys enrolled in the EPICURE study were more likely to have CP and impaired cognitive function ²⁷⁶, and boys with delayed myelination from the EXPRESS study had poorer cognitive and language outcomes ²⁷⁷. Males in the EPICURE study also had more pronounced cognitive, language, educational and motor disability at 6-year follow-up ²⁷⁸⁻²⁸⁰. Male vulnerability to adverse neurological outcome appears to be even more pronounced with decreasing gestational age and birth weight. Males born <32 weeks have poorer cognitive, motor and language scores on Bayleys III scoring at 12 months corrected age ²⁸¹, and extremely preterm males are at especially high risk of perinatal brain injury along with later neurological and cognitive impairments ²⁷⁹.

To date, sex differences in long-term cardiovascular outcomes of former preterm neonates have not been extensively evaluated though there is some evidence that males have an unfavourable cardiac profile. Adiposity is increased in former preterm adults and this is especially so in male former preterm infants who weigh significantly more and have increased BMI compared to non-preterm controls ²⁸². Additionally, healthy former preterm males are also known to have reduced exercise capacity compared to healthy male controls in adulthood ²⁸³.

Former preterm males appear to be at higher risk of long-term respiratory morbidity with males having higher rates of CLD, oxygen dependency and chronic respiratory insufficiency ^{257,284-288}. Although surfactant therapy successfully reduces mortality of RDS in both sexes, male preterms affected by RDS and CLD continue to show a higher incidence of morbidity ^{284,287,289}. Various indices of respiratory function display a marked gender disparity in neonates. Maximal expiratory flow at functional residual capacity tends to be higher in preterm females at hospital discharge compared to males and respiratory resistance is lower in preterm females ²⁹⁰. Also, when corrected for size, females have higher expiratory flow rates compared to males and this difference persists throughout life ²⁹¹. Respiratory Syncytial Virus (RSV) is a virus commonly causing lower respiratory tract infections in preterms and male sex is known to be an independent risk factor for severe RSV bronchiolitis, potentially indicating that palivizumab prophylaxis may be clinically warranted more frequently in males ^{292,293}. It has been noted that the male disadvantage in respiratory outcomes persists despite the advent of antenatal steroids and timely administration of surfactant ²⁹⁴ and it has been suggested that gender-specific differences in lung development contribute to this disparity ²⁹⁵.

1.7. Sexual dimorphism in the immune system of premature neonates

In addition to the differences in clinical outcome between the sexes, there is a marked difference in immune response between males and females throughout life ²⁹⁶. While the immune system continues to mature throughout childhood and adolescence it is becoming clear that sex differences in immune response are present from birth which may have important implications for premature neonates ²⁴⁶. Within

the neonatal population these differences manifest mainly as an increased male susceptibility to sepsis ²⁴⁷. While the aetiology of sex differences in immune development are poorly understood it has been hypothesised that a combination of sex hormones, genetics, microbiome and early environmental exposures may contribute ²⁹⁶.

1.7.1. *Sex-differences in immune function begin in-utero*

Data from placental histology, amniotic fluid, maternal serum and umbilical cord blood provide a window into the *in-utero* environment and suggest that differences exist in the immune response of males and females even before delivery. The carriage of a male fetus is associated with a more pro-inflammatory state in maternal serum with significant differences in several pro- and anti-inflammatory cytokines depending on fetal sex ²⁹⁷. Maternal serum during pregnancies also appears to be more anti-oxidative in female pregnancies ²⁹⁸. This is also reflected in the fact that the placentas of male preterm neonates are more likely to have histological evidence of lymphoplasmacytic cell infiltration ²⁹⁹. A study on mice exposed to early prenatal stress found that the placentas of male mice had higher levels of IL-6 and IL-1 β than females and the male mice displayed more hyperactivity than females ³⁰⁰. This pro-inflammatory disposition among males is confirmed in human studies where umbilical cord blood in males exhibits higher IL-6 and IL-1 β following endotoxin stimulation ³⁰¹ and IL-1RA is higher in the amniotic fluid of term females ³⁰². Female umbilical cord blood has lower TNF- α and IL-6 compared to males ²⁹⁸ and male cord blood has higher IgE levels ³⁰³. While total white cell percentages are similar in umbilical cord blood of male and female pregnancies ³⁰⁴, females tend to have more CD4+ T cells and fewer CD8+ T cells compared to males, a trend that continues throughout life ²⁹⁶. Expression of IRAK1, a protein involved in TLR signalling is higher in the umbilical cord blood of female neonates ³⁰⁵. Male pregnancies also appear to be more sensitive to the inflammatory effects of maternal obesity with higher cytokine production and macrophage activation in murine placental specimens compared to females ³⁰⁶.

Much of the recent clinical data on the impact of maternal immune response in pregnancy on childhood development has been in the area of psychiatry where there

is compelling evidence that maternal inflammation is associated with later psychiatric illness and behavioural disorders among offspring³⁰⁷⁻³⁰⁹. Males appear to be more sensitive to maternal inflammation in-utero with a higher incidence of psychosis in later life when mothers had bacterial infection during pregnancy³¹⁰ and an increased risk of depression among males with a maternal pro-inflammatory cytokine profile³¹¹. In addition, maternal c-reactive protein (CRP) during pregnancy correlates to electrophysiological patterns that place neonates at risk of later psychosis among males³¹². These findings are supported by data from animal models which show that female mice have higher anti-inflammatory factor expression in the brain compared to males following induced maternal inflammation³¹³.

1.7.2. Postnatal sex-differences in immune function

Many of the existing human studies on sex differences in neonatal immune profile utilise blood taken from umbilical cords. This is problematic as umbilical cord blood is immunologically distinct from blood samples obtained postnatally³¹⁴ and is therefore not representative of the neonatal immune system as it functions after birth. Therefore, much of our current understanding of the sexual dimorphism in neonatal immunity comes from animal models of tissue inflammation. Similar to antenatal data, many animal studies suggest that males have a predominantly pro-inflammatory phenotype after delivery. In mice who underwent a lipopolysaccharide (LPS) challenge in the neonatal period, myeloperoxidase, an enzyme present in granulocytes and a marker of inflammatory activity, was more active in the hippocampus of males compared to females, a change which persisted into adulthood³¹⁵. Following E coli infection female neonatal rats have higher cerebellar IL-6 than males³¹⁶. In mouse models of induced ischaemic stroke males had increased myeloid cell infiltration and greater microglial expression of TNF α in areas of injury compared to females³¹⁷. A rat model of neonatal systemic inflammatory response syndrome (SIRS) found that neonatal males had a higher neutrophil concentrations in alveoli compared to females³¹⁸. Additionally, the spleen of males showed a predominately TH-1 weighted response to endotoxin challenge with increased IL-2 and TNF- α production, whereas females had a predominantly TH-2 weighted response with decreased IL-2, TNF- α and IFN- γ

compared to controls ³¹⁸. A piglet model of neonatal intestinal immunity showed that females had greater numbers of regulatory T cells in the intestinal mucosa, had a greater serum antibody response to challenge, and greater IgA production within mesenteric lymph nodes compared to males ³¹⁹. In humans the level of colostrum immunoglobulin may also be affected by sex with IgA and IgM both higher in the colostrum of female pregnancies ³²⁰ suggesting increased immune protection in breastfed females. These differences in the passage of humoral immunity are supported by clinical findings that breastfed premature female neonates have lower incidence of acute, severe respiratory infection after discharge from hospital compared to males ³²¹.

1.7.3. *The postnatal effect of sex hormones*

Estrogens and progesterone (Pg) are required in high concentrations to maintain pregnancy and increase with each trimester ³²². Late in the third trimester the levels of estrogen and Pg in maternal circulation are several times that of non-pregnant women ³²³. Sex hormones are known to affect immune response and it has been hypothesised that sex hormones are key to promoting immune tolerance during pregnancy ³²⁴, and may contribute to sex-differences in immune response after birth ²⁴⁶.

The effects of sex hormones on maternal immunology is well described with hormones influencing immune cell migration, differentiation and activity ³²⁴. The role of hormones in fetal immune system development and neonatal immune response is less well-understood. While clinical studies are lacking there is sufficient scientific evidence to suggest that hormones play a potentially important role, though most of this work is on brain tissue in animal models of hypoxic brain injury. There is considerable evidence of differences in brain injury between males and females and the immunomodulatory effect of estrogen and Pg have been suggested as significant contributors to this difference ³²⁵. 17- β Estradiol (E2) has been shown to have a neuroprotective effect in induced excitotoxic brain injury in rats by downregulating pro-inflammatory genes including IL-1 β and IL-6 in brain tissue ³²⁶, a finding which is supported by evidence that estrogen modulates inflammatory signalling through action on NF κ B ³²⁷. Pg has a neuroprotective effect on brain damage in a neonatal

mouse model of induced cerebral hypoxia with reduced microglial activation at sites of injury and a reduction in the size of brain lesions which was sex-specific with smaller lesions in males ³²⁸. It has also been shown that Pg reduces hypoxia-induced blood-brain barrier damage in neonatal rats ³²⁹, and reduces COX-2 ³³⁰, IL-1 β ³³⁰, TNF- α ³³¹ and NF κ B ^{331,332} in the brain tissue of neonatal rats with induced hypoxic brain injury. Of particular interest is the finding that a combination of estrogen and Pg reduced pro-inflammatory cytokine expression in rat brains in a model of induced neonatal hypoxia and was found to be more effective than either hormone in isolation ³³³, though these findings have not been replicated elsewhere.

Estrogen receptor alpha (ER- α) and beta (ER- β) are both expressed on mononuclear cells from umbilical cord with expression of ER- β being higher than that of adult cells ³³⁴. Pg receptor has not been identified in neonatal immune cells though it is now recognised that progesterone may act through the glucocorticoid receptors (GR) ³³⁵ or through non-genomic means such as membrane progestin receptors (mPRs) or progesterone receptor membrane component 1 (PRMC1) ^{336,337}. Accordingly, mPRs have been identified in neonatal cord blood mononuclear cells and expression is higher than in adults mononuclear cells ³³⁴. Similarly, GR has also been identified in umbilical cord blood of premature neonates ³³⁸ and higher GR expression in cord blood of premature neonates is associated with less RDS and less nosocomial infection ³³⁹.

Despite the potentially important role of sex hormones in neonatal immune response, there is limited data on sex differences in neonatal hormone concentrations. Additionally, most studies use maternal blood samples, amniotic fluid or umbilical cord blood ³⁴⁰ which, while giving an excellent idea of the *in-utero* steroid environment, do not shed light on the hormone concentrations after delivery. Many of the existing studies use radioimmunoassay for hormone analysis which has since been supplanted by mass spectrometry as the gold standard technique ³⁴¹ leading to questions about the accuracy of results from older studies. Lastly, very few studies divide hormone results by sex meaning that our understanding of sex-differences in neonatal hormone concentrations is limited. The most complete summary of sex hormone levels in pregnancy and the peripartum period is the systematic review of Kuijper *et al.* ³⁴⁰. While direct comparison of results between studies included in this review is not

possible, it does appear that the sex hormone profiles of males and females are distinct with females having higher levels of estrogens and Pg, and males having higher androgen concentrations. Concentrations of both estrogens and Pg rise throughout pregnancy and are highest late in the third trimester. Given that premature delivery interrupts the supply of placental hormones, preterm neonates miss this “peak” of hormone concentration. The effect of this on immune development is unknown but given the potential of hormones to affect the immune system and the relative immaturity of preterm immune responses it seems likely that there may be important repercussions to this.

1.7.4. *The role of the X chromosome and microRNAs*

Separate from the immune modulating role of sex hormones, females may have an immune advantage conferred by their XX genotype ³⁴². Many genes with important immune functions are coded on the X chromosome. These include TLR7, TLR8, cytochrome b-245 beta polypeptide, androgen receptor (AR), CD40, IKK- γ and IRAK-1 ^{343,344}. Females inherit both a maternal and paternal copy of the X chromosome, whereas males inherit a maternal copy only. To avoid over-expression of proteins coded by the X chromosome females initiate a process of X chromosome inactivation whereby each cell in the body will inactivate a random copy of the X chromosome, a process which means that women essentially exhibit mosaicism for the X chromosome ³⁴⁵. The advantage this process confers is clear when considering single nucleotide polymorphisms (SNPs) or X-linked disease where a male inheriting an affected X chromosome will express the mutant protein in all cells whereas a female will express it in only half of her cells, potentially limiting any deleterious effects ³⁴⁵. Additionally, females may display X-chromosome skewing whereby if one X chromosome confers a positive or negative attribute cells may be environmentally challenged, resulting in expression skewed towards the favourable X chromosome in blood, bone marrow and immune organs ³⁴⁵. There is also a process whereby 15% of X-linked genes escape the silencing effect of X inactivation in females meaning that certain genes may be expressed at higher levels in females, and in the case of genes involved in immune response this may have an important bearing on the ability to respond to infection ³⁴⁶.

Even in non-disease states the ability to produce proteins from both maternal and paternal X chromosomes provides potential diversity in cellular responses, which in the case of infection and inflammation may allow better adaptation to infectious challenges ³⁴⁴.

The X chromosome is also rich in microRNA (miRNA) accounting for approximately 10% of the 800 documented miRNAs within the human genome ³⁴⁷. Many of the miRNAs encoded on the X chromosome have important functions in immune defence ³⁴⁷. Conversely the Y chromosome does not encode any miRNA. It is thought that the expression of X-linked miRNA may vary between males and females according to the methods discussed above and since a single miRNA may have several targets it has been hypothesized that sex-differences in miRNA expression may have a cascade-like effect with minor differences in miRNA expression having large differences in gene regulation ³⁴⁸.

1.7.5. Sex differences in neonatal immune response: areas requiring further study

While the data discussed above provides an excellent insight into the ways in which neonatal immunity differs between males and females there are clearly gaps in our knowledge. The first of these is that many clinical and laboratory studies have not report results by sex, therefore limiting our insights into the potentially important differences in neonatal immunity between the sexes. Another important deficit in scientific investigation is the lack of study on postnatally acquired samples in neonates. At present much of our human data is from umbilical cord blood which, while easily obtainable, does not mirror the more clinically important postnatal immune system. Compounding this lack of postnatal human studies is the profound lack of any immunological study on postnatal sex-differences in premature neonates, a vulnerable population in whom it is reasonable to assume even minor differences in immunity between the sexes can have clinically significant implications.

While the immune differences discussed above will affect the ability of neonatal males to prevent and fight infection, there may also be implications for survivors of neonatal sepsis. Within adult critical care patients it is now recognised that a

syndrome of dysregulated immune response may develop in sepsis survivors characterised by defects in innate and adaptive immunity with persistent inflammation beyond the initial infective period ³⁴⁹. Such patients may have chronic organ dysfunction and despite recovering from the initial sepsis episode may go on to have poor clinical outcomes ³⁵⁰. This phenomenon is not yet widely described in the neonatal population but given that the development of CLD ³⁵¹, neuropsychiatric disorders ³⁵² and CP ³⁵³ have been linked to persistent neonatal inflammation, it is likely that a similar syndrome is present in the neonatal population. Given the stark differences in immune response between male and female neonates it is therefore reasonable to be concerned about the impact that sex may have on persistent inflammation and any resulting long-term clinical sequelae.

1.8. Conclusion

Premature neonates are at high risk of sepsis and organ dysfunction in the neonatal period and these clinical findings are likely due in part to immaturity of underlying immune responses. Despite the importance of neonatal sepsis, there is no consensus definition and organ dysfunction is not well-explored as a potential screening tool in high-risk neonates. Important differences in clinical outcomes exist between male and female premature neonates and these are likely due to a combination of hormonal, immune and genetic factors. Data from animal models and study of older humans has informed much of our current understanding of the aetiology of sex-differences in clinical outcome in the premature population, though direct clinical and experimental observations from the premature population are lacking.

1.9. Hypothesis

Male preterm neonates are at higher risk of sepsis and organ dysfunction following delivery compared to females, and sex-differences in immune function and hormone status may contribute to the differences in sepsis outcomes that we see clinically.

1.10. Aims

Aim 1: Review current approaches to defining sepsis within the neonatal population.

Rationale: At present numerous definitions of neonatal sepsis exist. This is problematic in terms of standardising clinical practice and reporting research findings. A consensus definition of neonatal sepsis is required and would improve the care of term and premature neonates of both sexes. A review of the current approaches to defining neonatal sepsis is required to inform any future work on a consensus definition of neonatal sepsis.

Objectives: To review the definitions of neonatal sepsis currently in use by neonatal surveillance and research networks internationally.

Deliverables: Publication of a review paper outlining current approaches to neonatal sepsis definitions and comparing these to the standards recently set by the Sepsis-3 group.

Aim 2: Effect of sex and infection on outcome (NEOMOD scoring).

Rationale: Recent consensus definitions on sepsis in adult populations have identified organ dysfunction as crucial in screening for and defining sepsis. Sex differences in organ dysfunction during sepsis are not well-studied in the preterm population and could provide novel insights on which organ systems are most severely affected in each sex.

Objectives: To record organ dysfunction scores at baseline in preterm patients and subsequently during each suspected and confirmed sepsis episode.

Deliverables: Objective measurement of organ dysfunction during times of infection in male and female preterm infants.

Aim 3: Measuring neonatal inflammatory response and sepsis outcomes by sex.

Rationale: Sex differences in immune response are not well-studied in the preterm population. Understanding how the components of innate and adaptive immunity

differ between male and female preterm infants will contribute to a better understanding of why preterm males have poorer outcomes.

Objectives: To examine cell surface markers, cytokines, miRNA and inflammasome gene expression in male and female preterm infants and compare these to healthy term controls.

Deliverables: Examination of these innate immune factors in male and female term and preterm neonates.

Aim 4: Effect of sex steroids on immune function.

Rationale: The effect of steroid hormones on immune response is not well studied in neonates. Investigating the ability of sex steroids to modulate immune response to inflammatory stimuli will improve our understanding of preterm immunity and identify potential therapeutic targets for future study.

Objectives: To measure the effect of estrogen and progesterone on innate cell activity, cytokine responses and the inflammasome following in vitro immune activation.

Deliverables: Data on the ability of sex steroids to modulate immune responses in males and female term and preterm neonates.

Chapter 2: Materials and Methods

2.1. Ethical approval

This project was approved by the ethics committees of The Coombe Women and Infant's University Hospital, Holles Street National maternity hospital and the Rotunda Hospital from July 2017 to July 2020 (appendix i).

The Coombe Women and Infant's University Hospital, Dublin:

This is a tertiary neonatal centre located in the south of Dublin city with over 7,000 births annually. The Coombe provides care for premature neonates of all gestations born both within the Coombe and referred from other centres nationally.

The National Maternity Hospital, Holles Street, Dublin:

This is a tertiary neonatal centre located in the North of Dublin city with over 7,000 births annually. Holles Street provides care for premature neonates of all gestations born both within Holles Street and referred from other centres nationally.

The Rotunda Hospital, Dublin:

This is a tertiary neonatal centre located in the North of Dublin city with over 7,000 births annually. The Rotunda provides care for premature neonates of all gestations born both within The Rotunda and referred from other centres nationally.

2.2. Consent and data protection

Families of eligible participants were approached for consent either shortly before or shortly after delivery of their baby. A discussion was held with the parents regarding the nature of the research project and the implications of participation. A written patient information leaflet and consent form were then given to parents in all cases (appendix ii & iii). Parents were given time to read the information provided and make a decision regarding participation. If the parents had any further questions these were addressed before formal written consent was obtained.

All consent forms were GDPR compliant and were stored in a locked office in the Coombe Women and Infant's University Hospital. The clinical and laboratory data which was collected during this study was anonymised and stored on a password-protected computer.

2.3. Clinical Data

All participants had clinical data collected as part of this study. Baseline demographic data was collected on the sex, gestation, birthweight, mode of delivery, antenatal steroid administration and initial resuscitation. Data were then collected on a number of important outcomes for each participant: number of sepsis episodes, NEC, results of cerebral imaging, time to discharge, time to full enteral feds, duration of ventilation, CLD and survival to discharge (appendix iv). Each patient also had serial organ dysfunction scores performed during their inpatient stay in the early postnatal period and during every episode of confirmed and suspected sepsis (appendix v).

2.4. Study population

2.4.1. Premature neonates

Premature neonates were recruited across 3 clinical sites within Dublin. Premature neonates were eligible for inclusion if their birth weight was <1,500 grams. Premature neonates were not eligible for inclusion if there was a history of maternal substance abuse or confirmed serious congenital abnormalities (genetic, metabolic, cardiac).

2.4.2. Healthy term neonatal controls

Peripheral blood was collected in healthy term controls recruited in the Coombe Women and Infant's University Hospital within the first 48 hours after delivery. Term controls were eligible for participation if they were having routine phlebotomy performed on the postnatal ward and did not have any clinical or laboratory evidence of infection or serious congenital abnormalities (genetic, metabolic, cardiac).

2.4.3. Healthy term cord blood controls

Cord blood was collected at the time of delivery in term infants born via caesarean section from uneventful pregnancies.

2.4.4. *Healthy adult controls*

Blood was collected from healthy adult controls (age range: 26-34) with no history of any inflammatory disorders. These samples were used to standardise laboratory protocols before neonatal samples were collected.

2.5. Biological sample collection

Blood samples were collected on all patients and controls in sodium citrate tubes. The maximum volume collected was 1ml. Samples were transported to the Trinity Translational Medicine Institute (TTMI) on St James' Hospital campus for processing immediately after collection. Figure 2.1 provides a flow diagram of how samples were processed after collection. Data were also collected on the routine phlebotomy samples collected during the inpatient stay of the premature neonates. This included full blood count (FBC), CRP, urea and electrolytes, blood cultures and blood gas analysis results. These blood samples, which formed part of the routine care of these premature neonates, were processed locally in the laboratory of the respective participating hospital.

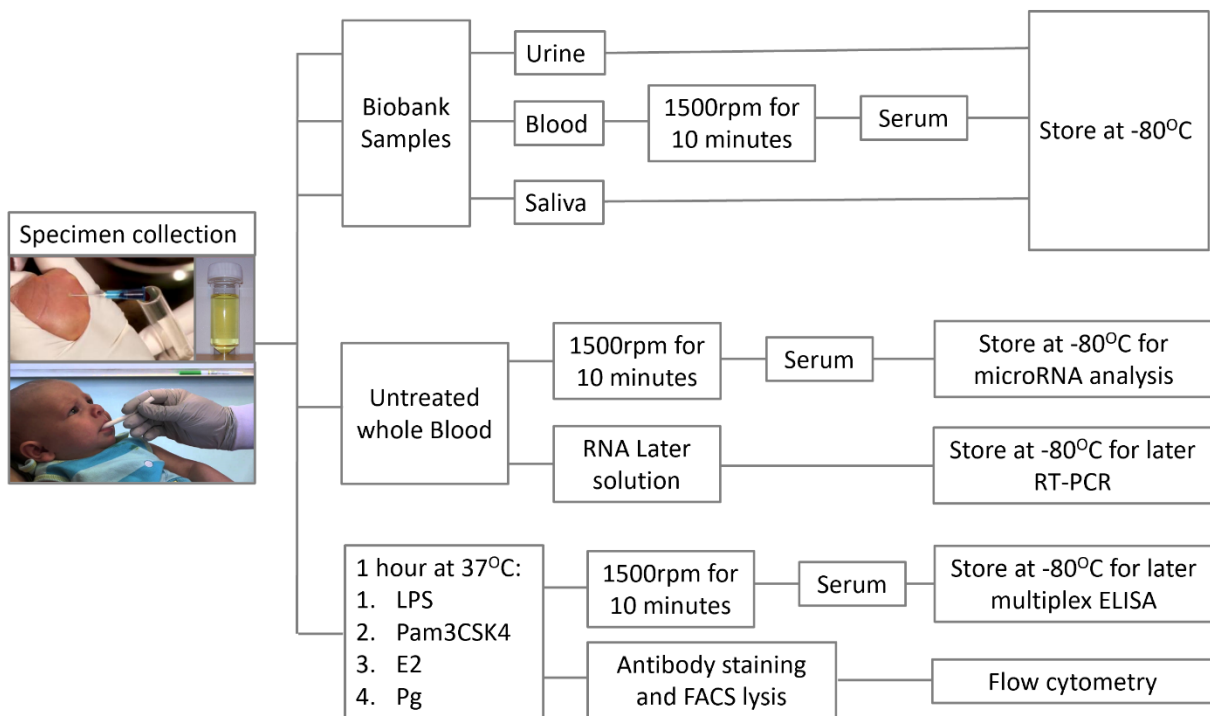


Figure 2.1: Flow diagram of biological sample processing after collection

After collection samples were processed immediately. Urine, saliva and untreated serum were stored in a biobank for future analysis. Untreated whole blood samples were also processed for microRNA analysis by extracting serum and for RT-PCR by the addition of RNAlater® before storage at -80°C. Whole blood was also treated for 1 hour with immune stimulants (endotoxin and Pam3CSK4) and hormones (estradiol and progesterone) and then prepared for either flow cytometry or had serum extracted and stored for later multiplex ELISA. LPS: lipopolysaccharide; Pam3CSK4: Pam3CysSerLys4; E2: 17 beta estradiol; Pg: progesterone.

2.6. Biological sample processing

2.6.1. Whole blood processing

Whole blood samples were processed for one of the following after collection: 1.) white blood cell activity and enumeration by flow cytometry; 2.) cytokine levels by multiplex enzyme-linked immunosorbent assay (ELISA); 3.) miRNA expression; 4.) gene expression by quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

Samples for microRNA analysis were immediately centrifuged at room temperature (Thermo Scientific, Hereaus Fresco 17) at 1,500 RPM for 10mins. Serum was then extracted and stored at -80°C for later analysis. In cases where qRT-PCR was to be undertaken, 350ul of whole blood was immediately aliquoted into eppendorfs with 1.3ml of RNeasy lysis solution. This was then vortexed and stored at -80°C for future analysis. ELISA and flow cytometry samples were treated with a variety of hormones and pro-inflammatory stimulants after collection. Samples were treated with Pg (10nM), E2 (10nM), Pam3Cys-Ser-(Lys)4 trihydrochloride (Pam3CSK4) (TOCRIS bio-technique, Abingdon, UK) (5ng/ml) and LPS (E.coli 0111:B4: SIGMA Life Science, Wicklow, Ireland) (10 ng/mL) alone and in combination. Samples were then incubated at 37°C for 1 hour. Samples processed for multiplex ELISA were then centrifuged at room temperature (Thermo Scientific, Hereaus Fresco 17) at 1,500 RPM for 10 minutes. Serum was then extracted and stored at -80°C for future analysis.

2.7. Flow cytometry

Flow cytometry measures the scatter of light as cells pass through a laser in suspension allowing measurements to be taken on cell size (forward scatter or FSC) and granularity (side scatter or SSC). Additionally, cells may be labelled with fluorescent antibodies specific to cell surface markers allowing identification of individual cell populations and relative quantification of cell surface marker expression based on the fluorescence of these antibodies. Within our population of interest flow cytometry was used to quantify the expression of CD11b and TLR2 on the surface of monocytes and neutrophils. Flow cytometry was also used to identify the following

populations of lymphocytes: natural killer (NK), CD4+, CD8+, CD19+, gamma delta 1 ($\gamma\delta 1$) and gamma delta 2 ($\gamma\delta 2$).

Whole blood samples were incubated at 37°C for 1 hour with both pro-inflammatory stimuli (Pam3Csk4 and LPS) and hormones (E2 and Pg) both alone and in combination. Following incubation, 100ul whole blood samples were incubated with a dead cell stain (100 μ L; Fixable Viability Dye eFlour 506, Invitrogen, California USA), diluted to working concentration (1/1000) in phosphate buffered saline (PBS). To study monocytes and neutrophils, the following fluorochrome-labelled monoclonal antibodies were diluted in phosphate buffered alanine (PBA) and added to each sample of whole blood: CD14-PerCp, CD15-PeCy7, CD16-FITC, CD66b-PB, TLR2-APC and CD11b-PE (Table 2.1). 40ul of this antibody mixture was added to each 100ul blood sample. To study lymphocytes the following fluorochrome labelled monoclonal antibodies were diluted in PBA and added to a separate, untreated 100ul sample of whole blood: CD3-PB, CD4-PeCy7, CD8-PerCp, CD19-APC, CD56-APC/Cy7, $\gamma\delta 1$ -FITC, $\gamma\delta 2$ -PE (Table 2.2). 40ul of this antibody mixture was added to 100ul of whole blood. Following addition of the antibody mixture, each blood sample was incubated in the dark for 15 minutes. 1 ml of BD FACS™ lysing solution (BD biosciences) was added to each sample and samples were incubated in the dark for 15 minutes. Cells were then pelleted by centrifuging at 1,500 RPM for 7 minutes and supernatant was removed. Samples were then vortexed and FACS lysis was repeated. Cells were then washed twice using PBA and fixed in 500ul of 1% paraformaldehyde. Cells were then pelleted by centrifuging at 1,500 RPM for 7 minutes and the resulting cell pellet was resuspended in 100ul of PBA and analysed using a BD FACSCanto™ II flow cytometer (Figure 2.2).

Antibody	Tag	Manufacturer	Volume
CD14	PerCp	BioLegend	2.5 ul
CD15	PeCy7	BioLegend	2.5 ul
CD16	FITC	BioLegend	2.5 ul
CD66b	PB	BioLegend	2.5 ul
TLR2	APC	Invitrogen	2.5 ul
CD11b	PE	BD Biosciences	10 ul
PBA			27.5 ul

Table 2.1: Antibody mixture used for study of monocytes and neutrophils.

Antibody	Tag	Manufacturer	Volume
CD3	PB	BioLegend	2.5 ul
CD4	Pe/Cy7	BioLegend	10 ul
CD8	PerCp	BioLegend	2.5 ul
CD19	APC	BioLegend	2.5 ul
CD56	APC/Cy7	BioLegend	2.5 ul
$\gamma\delta 1$	FITC	Invitrogen	2.5 ul
$\gamma\delta 2$	PE	BioLegend	2.5 ul
PBA			25 ul

Table 2.2: Antibody mixture used for study of lymphocytes.



Figure 2.2: BD FACSCanto™ II flow cytometer used for flow cytometry analysis.

2.7.1 Identification of cell populations and cell surface antigen expression

Data produced by the BD FACSCanto™ II flow cytometer were then exported for analysis using FlowJo v10 software (FlowJo LLC, Oregon USA). Cell populations were isolated based on their SSC and FSC, dead cells were excluded, and doublets were removed (Figure 2.3). Cell populations were further subdivided based on cell surface marker expression with a gating strategy adapted from Prabhu et al.³⁵⁴. Lymphocytes were isolated based on their size and granularity and the following lymphocyte populations were delineated based on cell surface antigen expression: NK cells (CD3-/CD56+), $\gamma\delta 1$ (CD3+/ $\gamma\delta 1$ +), $\gamma\delta 2$ (CD3+/ $\gamma\delta 2$ +), B cells (CD3-/CD19+), CD4+ T cells (CD8-/CD4+), CD8+ T cells (CD8+/CD4-) (Figure 2.4). Neutrophils and monocytes were isolated based on size and granularity and delineated from each other based on CD66b positivity (granulocytes CD66b+, monocytes CD66b-) (Figure 2.4). CD14 and CD16 expression was used to distinguish between classical (CD14+/CD16-), intermediate (CD14+/CD16+) and non-classical (CD14dim/CD16+) monocytes (Figure 2.5). A minimum of 10,000 events were recorded for each blood sample. Both CD11b and TLR2 expression were quantified via mean channel fluorescence (MFI). MFI is a measure of the average fluorescence intensity emitted by all cells in a selected population and is therefore a representation of the number of receptors expressed on the surface of each cell. Lymphocyte numbers are expressed as a percentage of the total lymphocyte population present. All samples were analysed by the same researcher (MMG) to minimise variability.

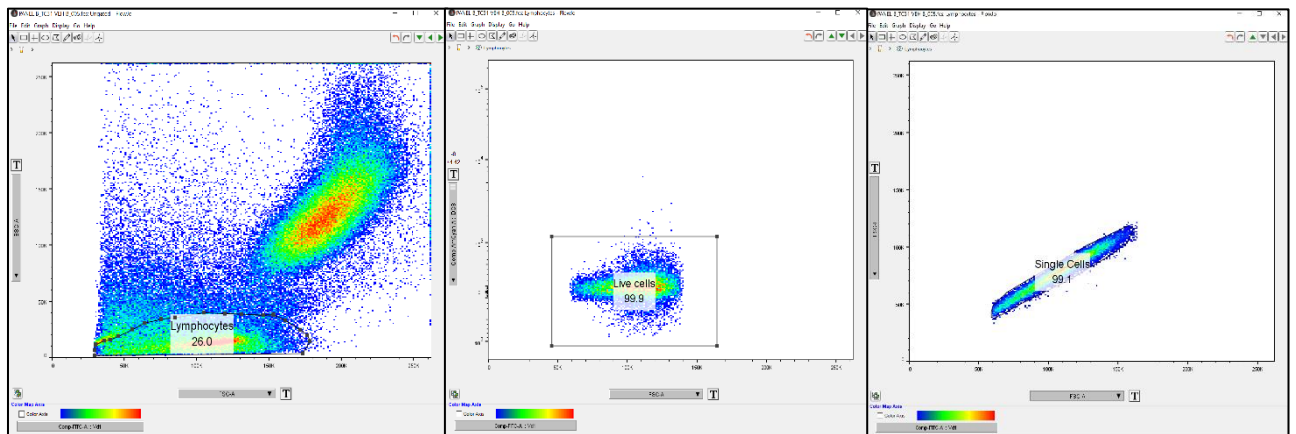


Figure 2.2: Removal of dead cells and doublets in cell populations of interest.

FSC and SSC was used to identify cell populations of interest. Cells expressing dead cell stain were then excluded and doublets removed, leaving only live, single cells of the cell population of interest.

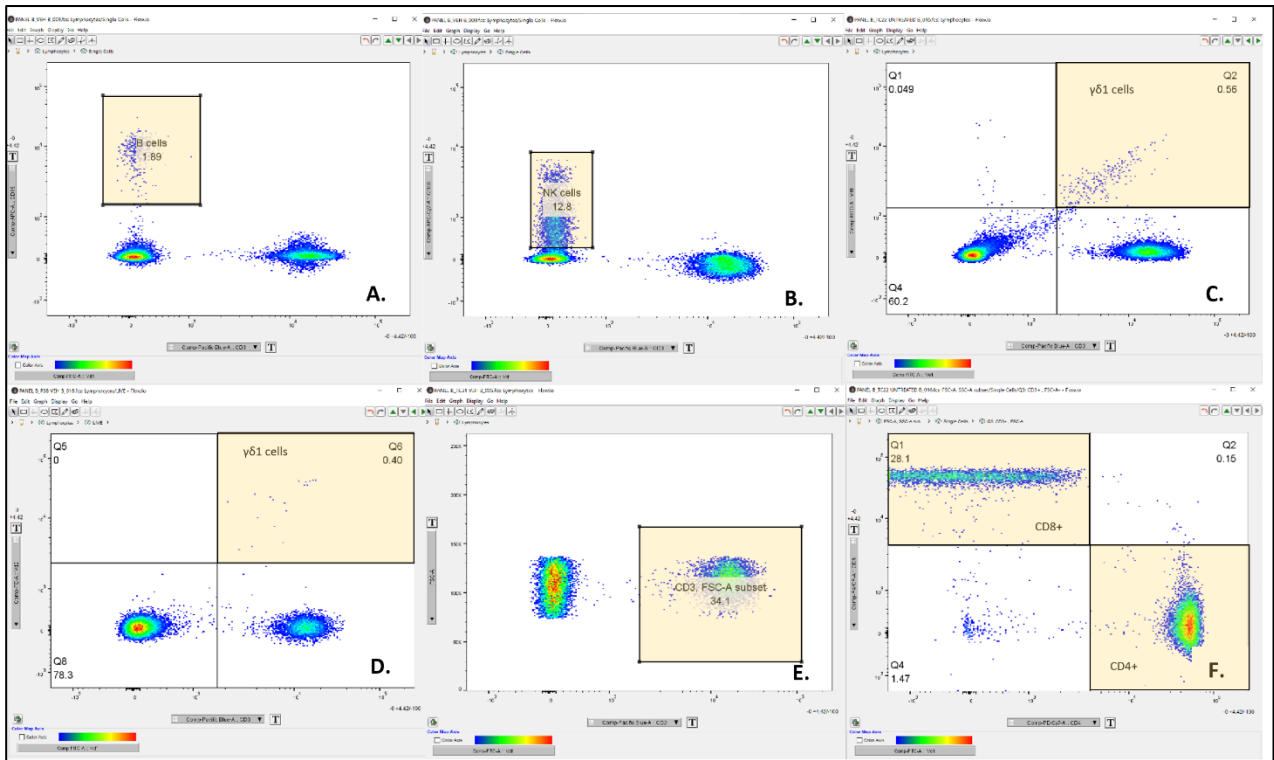


Figure 2.3: Flow cytometry gating strategy for the identifying lymphocyte populations.

A.) B cells (CD3-/CD19+), B.) NK cells (CD3-/CD56+), C.) $\gamma\delta 1$ (CD3+/ $\gamma\delta 1$ +), D.) $\gamma\delta 2$ (CD3+/ $\gamma\delta 2$ +), E.) CD3+ (FSC/CD3+), F.) CD8+ (CD8+/CD4-) and CD4+ (CD8-/CD4+).

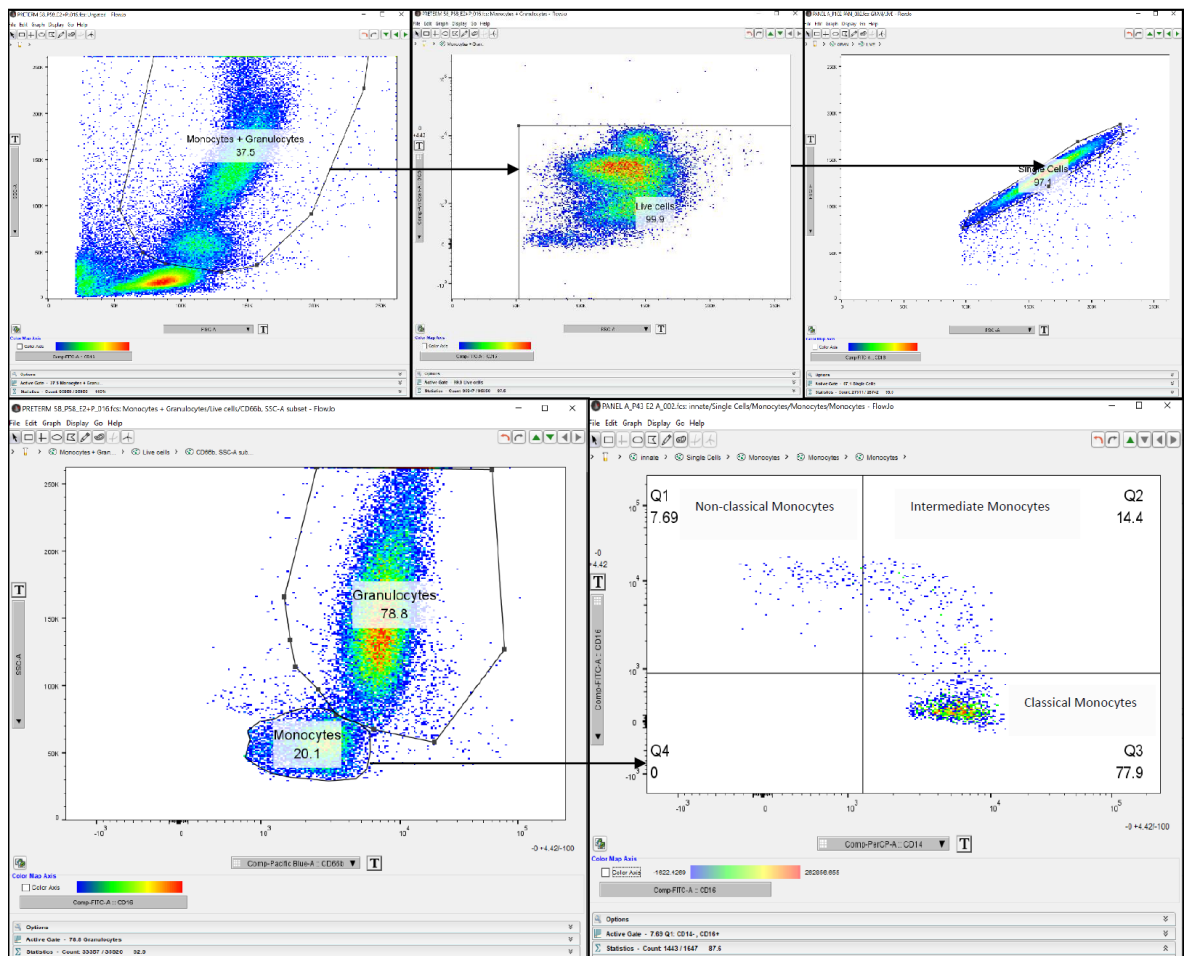


Figure 2.4: Flow cytometry gating strategy for identifying neutrophil and monocyte populations.

Neutrophils were identified based on light scattering properties and CD66b+.

Monocytes were identified based on light scattering properties and CD66b-. Monocyte sub-populations were identified based on CD14 and CD 16 expression: classical monocytes (CD14+/CD16-), intermediate monocytes (CD14+/CD16+), non-classical monocytes (CD14dim/CD16+).

2.8. RNA extraction, cDNA, RT-PCR

RT-PCR is a laboratory technique in which RNA is reverse-transcribed into complementary DNA and the target genes of interest are then amplified by polymerase chain reaction. Our samples constituted untreated whole blood in RNeasy[®] solution stored at -80°C until analysis was performed. At the time of analysis samples were removed from storage and defrosted to room temperature.

2.8.1. RNA extraction

RNA extraction was performed as per the manufacturer's instructions (life technologies, California, USA) using the RiboPure™ Blood Kit. Samples were first centrifuged in a microcentrifuge for 1 minute at 13,000 RPM. The resultant supernatant was then removed by aspiration without disturbing the cell pellet. 800 ul of lysis solution and 50 ul of sodium acetate was then added to the cell pellet and this was vortexed thoroughly to resuspend the pellet and allow cell lysis. 500 ul of acid-phenol:chlorophorm was then added to the cell lysate and samples were vortexed thoroughly. This solution was then kept at room temperature for 5 minutes. Samples were centrifuged for 1 minute at 13,000 RPM to separate the (lower) organic and (upper) aqueous phases. The clear aqueous phase containing the RNA was then removed without disturbing the dark organic phase, resulting in approximately 1 ml of RNA solution. The organic phase which contains heme and proteins was then discarded. 500 ul of 100% ethanol was then added to the RNA-containing aqueous phase and samples were vortexed briefly. The solution was then applied to a filter cartridge assembly and centrifuged at 13,000 RPM for 10 seconds to allow the liquid to pass through the filter. The flow-through was then discarded leaving the RNA trapped in the filter. 700 ul of Wash Solution 1 was applied to the filter and this was again centrifuged at 13,000 RPM for 10 seconds to allow the wash solution to pass through the filter. The flow-through was again discarded. This process was repeated twice more using Wash Solution 2/3 and the flow-through was discarded each time. Following the final wash, the filter cartridge was replaced in the same collection tube and this was centrifuged at 13,000 RPM for 1 minute to remove any residual fluid. Following this, the filter cartridge was placed in a clean collection tube and 50 ul of elution solution (preheated to 75°C) was pipetted onto the centre of the filter. The

cap was closed, and this was left to stand for 20 seconds before being centrifuged at 13,000 RPM for 30 seconds to recover the RNA. This process was repeated using another 50 ul of elution solution and then the filter cartridge and collection tube were centrifuged for 1 minute to recover all elution solution in the collection tube. The filter was then discarded leaving only the eluted RNA.

RNA purity and concentration were determined by using the NanoDrop ND-100 Spectrophotometer and analysed using ND-1000 Ver.3.1.2 software. This produced an absorbance curve for each sample, and RNA purity was calculated using the ratio of absorbance at 260 and 280 nm. A ratio of ≥ 1.6 for RNA suspended in water was considered the acceptable purity level. Samples with a ratio of < 1.6 were to be considered suboptimal and indicated possible contamination with phenol or protein. None of the samples processed fell outside this range. RNA samples were then stored at -80°C .

2.8.2. *cDNA synthesis*

Following purity and concentration determination, RNA was diluted to a working concentration of 1 ug/20 ul using RNase-free water. cDNA was synthesised from template RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). A 2X mastermix solution was prepared as per Table 2.3 for each sample. 20 ul of mastermix was added to 20ul of 1 ug/20 ul RNA solution (final volume 40 ul) for each sample. A separate no-amplification control (NAC) mastermix was prepared in which enzyme was replaced with equivalent volume of RNase-free water and this was added to a control RNA sample. A no-template control (NTC) in which the RNA solution was replaced by RNase-free water was also prepared and standard mastermix was added. The settings for the thermal cycling process were as follows: 10 minutes at 25°C , 120 minutes at 37°C , 5 minutes at 85°C and hold at 4°C . The final concentration of cDNA was 1ug/20 ul (50 ng/1 ul) and this solution was stored at -80°C until further analysis was planned.

Component	μl
10X buffer	4
25X dNTPs	1.6
10X RT Random Primers	4
Multiscribe Reverse transcriptase	2.0
Rnase-free water	8.4
Total per reaction(μl)	20

Table 2.3: Volumes of each component required for RT mastermix for each sample.

2.8.3. *TaqMan Real Time PCR*®

Quantification of gene expression was undertaken using the TaqMan Real-Time PCR® method. Real-time PCR is a technique in which a gene of interest is amplified and quantified in real time as PCR is in progress through use of a gene-specific fluorescent reporter. In TaqMan real-time-PCR® the TaqMan probe is an oligonucleotide specific to the gene of interest and which has both a 5' fluorophore and a 3' quencher which, while connected, inhibits fluorescence. During real time PCR this oligonucleotide attaches to the gene sequence of interest upstream of the primer. As Taq polymerase extends the primer on the 3' end it will eventually meet and degrade the oligonucleotide probe separating the fluorophore and quencher. The fluorophore is therefore no longer inhibited and hence the fluorescence detectable at any point in the reaction corresponds to the amount of gene template present.

TaqMan® universal PCR mastermix was used in combination with probes specific to the genes of interest. The following genes were investigated: Bruton's tyrosine kinase (BTK), AR, TLR7; IRAK-1; IKK- γ ; apoptosis-associated speck-like protein containing a CARD (ASC); AIM2; NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) and interleukin 1 beta (IL-1 β).

Samples were prepared for real time PCR in a laminar flow cabinet and both reagents and samples were prepared on ice. The volume of each reagent/sample added to each well was as per Table 2.4. Prior to plating, cDNA, which had previously been stored at a concentration at 50 ng/ul, was diluted to 5 ng/ul using RNase-free water. All samples were analysed in triplicate on a 384-well plate using the Quant Studio 5 real-time PCR system®. Settings for thermal cycling were as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute.

The PCR cycle at which the fluorescence becomes detectable above background noise is termed the C_T value. An increase in C_T value of 1 represents a doubling of the amount of the target gene present. ΔC_T values were calculated by subtracting the average C_T value of a control gene (GAPDH) measured in a sample from the average C_T value for a target gene in the same sample. The average ΔC_T value of the control group for a gene of interest was then subtracted from the ΔC_T of an individual sample and the resulting value is termed the $\Delta\Delta C_T$. Relative quantification

was calculated for each gene of interest and was expressed as fold change relative to a control group using the $2^{-\Delta\Delta C_T}$ method according to the following formula:

$$\text{Fold Change} = 2^{-\Delta\Delta C_T}$$

Component	1x volume (μl)	Manufacturer
TaqMan® Universal PCR buffer mix	5.0	Applied Biosystems
TaqMan® gene expression assay	0.5	Applied Biosystems
RNase free water	2.5	Qiagen
cDNA (5ng/ μ l)	2	
Total	10.0	

Table 2.4: Per-reaction volume of each component needed for TaqMan Real-Time PCR.

These volumes represent the amount of each component added to a single well on the 384-well plate.

2.9. Multiplex Enzyme Linked Immunosorbent Assay

Multiplex ELISA was utilised to quantify a selection of pro- and anti-inflammatory cytokines in the patients enrolled in this study. The Meso scale Discovery U-PLEX assay® was used to create a custom set-up for this experiment. The U-PLEX assay allows up to 10 cytokines to be evaluated on a single plate. To do this, biotinylated antibodies which are specific to the cytokines of interest are attached to U-PLEX linkers which self-assemble into designated positions within wells, where they remain adherent. Biological samples are then added, and cytokines within these samples are bound by the biotinylated antibodies which are now adherent to the surface of the well in specific positions unique to individual antibody-linker complexes. Detection antibodies conjugated with electrochemiluminescent labels are then added and these then bind to the relevant cytokines which are attached to unique locations within the well. This creates a sandwich immunoassay in which antibodies tether the cytokine of interest to a particular geographical location and the cytokine is then bound in turn by detection antibodies which will fluoresce when a current is applied to the plate under the correct experimental conditions (Figure 2.6). The light intensity produced is representative of the volume of cytokine present.

The following cytokines were analysed on a custom-made MULTI-SPOT assay plate from Meso Scale Diagnostics (USA): EPO, interferon gamma (IFN- γ), interleukin 10 (IL-10), interleukin 18 (IL-18), interleukin 1 receptor antagonist (IL-1RA), interleukin 1 alpha (IL-1 α), interleukin 1 beta (IL-1 β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), tumour necrosis factor alpha (TNF- α), tumour necrosis factor beta (TNF- β), vascular endothelial growth factor (VEGF), interleukin 18 (IL-18) and interleukin 33 (IL-33). For analysis, serum of participants was transferred to 96-well multi-array plates and processed as per the manufacturer's instructions (Meso Scale Diagnostics, USA). The limits of detection were within expected ranges for each individual assay.

To create the individual U-PLEX-coupled antibody solutions, 200 μ l of each biotinylated antibody was added to 300 μ l of the chosen U-PLEX linker. A different linker was used for each antibody. This solution was vortexed and incubated at room temperature for 30 minutes. 200 μ l of Stop solution was then added and the solution was again vortexed and incubated at room temperature for 30 minutes. This resulted

in a 10X U-PLEX-coupled antibody solution in each case. For multi-array plates with 10 antibodies, 600 ul of each U-PLEX-coupled solution were combined to give a total volume of 6 ml. For plates with fewer than 10 antibodies, 600 ul of each U-PLEX-coupled antibody solution were combined and the solution was then made up to 6 ml using Stop solution. In both of these cases the result was a 1X multiplex coating solution. 50 ul of 1X multiplex coating solution was added to each well in the multi-array plate. Plates were sealed and then incubated at room temperature with shaking for 1 hour. Plates were then washed 3 times with 1X Meso Scale Diagnostics Wash Buffer (150 ul/well). Calibrator standard and detection antibody solution were prepared as per the manufacturer's instructions. 25 ul of Diluent 43 was added to each well and all sides of the plate were tapped gently. 25 ul of the prepared calibrator standard solution was added to the calibrator wells and 25 ul of serum was added to the other wells. The wells were then sealed and incubated at room temperature for 1 hour. The plates were then washed 3 times with Meso Scale Diagnostics Wash Buffer (150 ul/well). 50 ul of detection antibody solution was then added to each well and plates were sealed and incubated at room temperature for 1 hour. 150 ul of Read Buffer T was added to each well and the plates were analysed on a Meso Scale Diagnostics instruments. Results are presented in picograms/ml.

2.10. MicroRNA analysis

miRNAs are small, endogenous, non-coding RNAs of approximately 22 nucleotides in length which regulate gene expression within the cell. The two major methods by which miRNAs regulate gene expression are by cleaving complementary messenger RNAs (mRNAs) or by binding to and inhibiting the translation of mRNAs ³⁵⁵. In addition to their primary function within cells, miRNAs are also found in the extracellular space and circulate in many bodily fluids including blood, saliva, cerebrospinal fluid, urine and breast milk. Whether these extracellular miRNAs are predominantly by-products of cellular metabolism or whether their excretion is a regulated process is not yet understood, though notably extracellular miRNAs are highly stable compared to cellular RNA and have shown both cellular interaction and uptake suggesting some role in cell-to-cell signalling ³⁵⁶. Regardless of their potential biological actions, the presence or absence of particular extracellular miRNAs have been associated with a variety of disease states in both adults and children. Extracellular miRNAs have therefore been extensively investigated as potential biomarkers of disease activity and severity, and show great promise in diagnostic and prognostic studies. The TaqMan® OpenArray Human Advanced MicroRNA Panel, QuantStudio 12K Flex (Figure 2.7) is a fixed panel of 754 well-characterised human miRNA sequences from the Sanger miRBase v21. This panel allows a comprehensive miRNA profile to be constructed through a single high-throughput experiment and is designed to work with small sample volumes similar to those collected during this study.



Figure 2.6: The TaqMan® OpenArray Human Advanced MicroRNA Panel, QuantStudio 12K Flex used for microRNA quantification.

2.10.1. RNA extraction and purification

In our patient group, serum was collected from whole blood by centrifugation within 1 hour of collection. This serum was stored at -80°C until RNA extraction was performed. RNA extraction and purification was performed using Qiagen® miRNeasy® Serum/Plasma kit (Qiagen, Venlo, Netherlands). Serum was thawed from frozen and 200 µl of serum from each patient was aliquoted into RNase-free eppendorfs. 1ml of QIAzol Lysis Reagent was added to each 200 µl sample (total volume 1.2 ml) and this mixture was incubated at room temperature for 5 minutes. 200 µl of chloroform was added to each sample. Samples were then vortexed for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were then centrifuged for 15 minutes at 12,000 x g at 4°C. The RNA-containing aqueous phase was then transferred to a new collection tube without disturbing the interphase or organic phase. For each 300 µl of aqueous phase recovered, 450 µl of 100% ethanol was added (1.5 volume) and this mixture was pipetted to mix thoroughly. 700 µl of the resulting mixture was then transferred to an RNeasy MiniElute spin column within a 2 ml collecting tube. This was then sealed and centrifuged at 8000 x g for 15 seconds at room temperature to allow the solution to pass through the filter. The flow-through was then discarded and the previous step was repeated for any remaining aqueous phase/ethanol solution in volumes of maximum 700 µl. The flow-through was discarded. 700 µl of Buffer RWT was then added to the RNeasy MiniElute spin column and this was sealed and centrifuged at 8000 x g for 15 seconds. The flow-through was discarded. 500 µl of Buffer RPE was then added to the RNeasy MiniElute spin column and this was sealed and centrifuged at 8000 x g for 15 seconds. The flow-through was discarded. 500 µl of 80% ethanol was then added to the RNeasy MiniElute spin column and this was sealed and centrifuged at 8000 x g for 15 seconds. The flow-through and the collection tube were then discarded. The RNeasy MiniElute spin column was placed in a fresh collection tube. The lid of the spin column was then opened, and the spin column/collection tube was centrifuged at full speed for 5 minutes to dry the RNA-containing filter membrane. The flow-through and the collection tube were discarded, and the RNeasy MiniElute spin column was placed in a fresh 1.5 ml collection tube. 14 µl of RNase-free water was pipetted directly onto the centre of the filter membrane

and this was sealed and centrifuged for 1 minute at full speed to elute the purified RNA.

2.10.2. *cDNA synthesis*

TaqMan® Advanced miRNA cDNA Synthesis Kit was used to synthesise cDNA from the purified RNA isolated in the previous step. All samples and reagents were thawed on ice apart from 50% PEG 8000 which was kept at room temperature. Poly(A) Reaction mix was prepared in a 1.5 ml microcentrifuge tube as per Table 2.5. The poly(A) reaction mix was then vortexed and centrifuged briefly. 2 ul of the purified RNA solution from each patient were placed in individual reaction tubes and 3 ul of the poly(A) reaction mix was added to each (total volume 5 ul). The reaction tubes were then sealed and vortexed briefly. The reaction tubes were then centrifuged briefly to eliminate air bubbles and placed in the thermal cycler. The following settings were used for thermal cycling: 45 minutes at 37°C, 10 minutes at 65°C and then hold at 4°C. Ligation reaction mix was then prepared in a 1.5 ml microcentrifuge tube as per Table 2.6. This was then vortexed and centrifuged briefly. 10 ul of the ligation reaction mix was then transferred to each reaction tube containing the poly(A) tailing reaction product (total volume 15 ul). The reaction tubes were then sealed and vortexed briefly. The reaction tubes were then centrifuged briefly to spin down contents and placed into the thermal cycler. The following settings were used for thermal cycling: 60 minutes at 16°C and hold at 4°C. RT reaction mix was prepared in a 1.5 ml microcentrifuge tube as per Table 2.7. The RT reaction mix was then vortexed and centrifuged briefly. 15 ul of the RT reaction mix was added to each reaction tube (total volume 30ul). The reaction tubes were then sealed and vortexed briefly. The reaction tubes were then centrifuged briefly and placed in the thermal cycler. The thermal cycler settings were as follows: 15 minutes at 42°C, 5 minutes at 85°C and hold at 4°C. The miR-Amp reaction mix was then prepared in a 1.5 ml microcentrifuge tube as per Table 2.8. This was vortexed and centrifuged briefly. For each sample, 45 ul of the miR-Amp reaction mix was added to a fresh reaction tube and 5 ul of the RT reaction product was then added this (total volume 50 ul). The reaction tubes were then sealed and vortexed briefly. The reaction tubes were then centrifuged briefly to spin down contents and placed in the thermal cycler. The thermal cycler was initially set at 95°C

for 5 minutes, followed by 14 cycles of denaturation/extension (95°C for 3 seconds followed by 60°C for 30 seconds), 99°C for 10 minutes and hold at 4°C. This undiluted miR-Amp product was then stored at -20°C until real-time PCR was undertaken.

Component	1x volume (ul)
10X Poly(A) Buffer	0.5 ul
ATP	0.5 ul
Poly(A) enzyme	0.3 ul
RNase-free water	1.7 ul
Total	3 ul

Table 2.5: Per-reaction volume of the Poly(A) Reaction Mix for cDNA synthesis step in microRNA PCR.

Component	1x volume (ul)
5X DNA Ligase Buffer	3 ul
50% PEG 8000 (room temperature)	4.5 ul
25X Ligation Adaptor	0.6 ul
RNA Ligase	1.5 ul
RNase-free water	0.4 ul
Total	10 ul

Table 2.6: Per-reaction volume of the Ligation Reaction Mix for the adaptor ligation in cDNA synthesis step of miRNA PCR.

Component	1x volume (ul)
5X RT Buffer	6 ul
dNTP Mix	1.2 ul
20X Universal RT Primer	1.5 ul
10X RT Enzyme Mix	3 ul
RNase-free water	3.3 ul
Total	15 ul

Table 2.7: Per-reaction volume of the RT Reaction Mix for reverse transcription in the cDNA synthesis step of miRNA PCR.

Component	1x volume (ul)
2X miR-Amp Master Mix	25 ul
20X miR-Amp Primer Mix	2.5 ul
RNase-free water	17.5 ul
Total	45 ul

Table 2.8: Per-reaction volume of the miR-Amp Reaction Mix for the amplification of cDNA synthesised during miRNA PCR.

2.10.3. Real-time PCR

The TaqMan® OpenArray Human Advanced MicroRNA Panel, QuantStudio® 12K Flex, TaqMan® OpenArray® Real-Time PCR Master Mix and QuantStudio® 12K Flex OpenArray® Accessories Kit were used for the real time-PCR on the miR-Amp product (cDNA template) synthesised in the previous step. The cDNA template was diluted 1:20 with 0.1X TE buffer. TaqMan® OpenArray® Real-Time PCR Master Mix and the diluted cDNA template were combined in equal volumes in reaction tubes. 5 ul of this mixture was then added to individual wells of a 384-well sample plate according to a template designed in the QuantStudio® 12K Flex Software. The plate was then sealed, and the edges of the filled wells were marked with pen. The plate was then centrifuged at 1000 RPM for 1 minute and the area previously marked with pen was then scored with a sterile blade. The sealed area of the plate containing the reaction mixture was then exposed by removing the cover along the previously scored edges and the reaction mixture was transferred from the 384-well plate to the OpenArray® plates using the automated AccuFill instrument. The OpenArray® plates were then filled with immersion fluid, sealed and cleaned with ethanol. The OpenArray® plates were then run on the QuantStudio 12K Flex instrument (Figure 2.8) and results were later exported for further analysis.

For the purposes of analysis, all samples with C_T values of 40 across all miRNAs were excluded. Geometric mean normalisation was then undertaken whereby the geometric mean was calculated for all of the C_T values for each sample, and each sample's set of C_T values was scaled according to the ratio of mean C_T values across the samples ($\Delta C_T = C_{TmiRNA} - GMC_T$). $\Delta\Delta C_T$ was then calculated by subtracting the ΔC_T of a control sample from the ΔC_T of a given patient. Fold change was then calculated according to the following formula:

$$\text{Fold change} = 2^{-\Delta\Delta C_T}$$

Validation of target miRNAs from OpenArray results was performed using qRT-PCR based TaqMan miRNA assays according to the manufacturer's instructions. Expression was measured using QuantStudio real time PCR software. Fold change expression was calculated using the $2^{-\Delta\Delta C_T}$ method with miR-16-5p as a control gene.



Figure 2.7: The QuantStudio™ 12K Flex Real-Time PCR System.

Image source:

<https://www.thermofisher.com/order/catalog/product/4472380#/4472380>.

2.11. Statistical analysis

Data analysis was undertaken using IBM SPSS 24.0 and Microsoft Excel. Graphical comparisons were made using GraphPad Prism 8.0. Normality of data was assessed graphically by dot plots and histograms and where required the Shapiro-Wilk test of normality was also employed. Categorical variables were compared using χ^2 test. Unpaired, continuous data were compared using the independent sample t-test if parametric or the Mann-Whitney U test if non-parametric. Paired, continuous data were compared using paired t-tests if parametric or Wilcoxon matched pair analysis if non-parametric. Statistical significance was set at $p < 0.05$ for all analyses.

Chapter 3: Neonatal Sepsis

3.1. Current approaches to defining neonatal sepsis

3.1.1. *Introduction*

Despite the frequency with which premature neonates are affected by sepsis there is no consensus on the criteria which should be used for defining and screening for neonatal sepsis ¹⁴³. A variety of approaches exist, each with their individual merits, and the approach adopted by a NICU depends largely on local practice and clinician preference. The use of different definitions has obvious implications for clinical practice as decisions regarding management depend largely on whether a patient is felt to have sepsis on clinical grounds. The implications for research however are potentially further reaching as the lack of a standard definition for patients involved in sepsis research limits the ability of clinicians to interpret and generalise results from clinical and translational studies. The Sepsis-3 group in adult medicine have recently published guidance on defining and screening for sepsis in the adult population which address many of the issues which are also problematic within neonatology ¹⁴⁹. The Sepsis-3 definitions are intensely clinically focused and identify the presence of organ dysfunction as the cornerstone for identifying cases of sepsis, an idea that does not appear to be addressed in any existing definition of neonatal sepsis. Due to the unique nature of the neonatal population the definitions of Sepsis-3 cannot be directly translated into neonatal practice though they serve as a benchmark in how a consensus definition may be created for use in this population.

3.1.2. *Hypothesis*

Existing definitions of neonatal sepsis differ from each other in important ways. Organ dysfunction scoring and early identification of high-risk patients are not prominent among current definitions of neonatal sepsis. None of the widely used definitions of neonatal sepsis account for the unique clinical and physiological characteristics of the premature neonate and while an initiative like Sepsis-3 is needed within neonatology, several factors will make this work much more complex in the neonatal population.

3.1.3. *Aims*

To review the definitions of neonatal sepsis currently in use by international research and surveillance networks and compare these to the work undertaken by the Sepsis-3 group in adult medicine. In particular, to identify the shortcomings of the currently used definitions and highlight the unique challenges in creating consensus terminology posed by the premature neonatal population.

3.1.4. *Background*

Infection is one of the leading causes of morbidity and mortality in the neonatal population worldwide ^{120,357-360}. Approximately 22/1000 livebirths develop neonatal sepsis with mortality rate of between 11-19% in affected neonates ¹²⁰. While there is a recognised association between socioeconomic status and sepsis-related mortality ³⁶¹, neonatal sepsis remains an important health issue worldwide accounting for approximately 15.6% of neonatal mortality internationally ³⁶². The innate and adaptive immune responses of neonates are immature compared to those of adults and older children ³⁶³. There are also a variety of environmental exposures such as maternal GBS colonisation, prolonged rupture of membranes and maternal chorioamnionitis which are unique to the neonatal period and place the newborn at increased risk.

The initial recognition of sepsis is also more challenging in neonates as the presenting signs and symptoms are often non-specific such as alterations in feeding, lethargy and respiratory distress ³⁶⁰. The increased risk and subtle presentation mean that medical professionals must maintain a low threshold for investigating and treating neonates with suspected sepsis. Suspected infection and sepsis are therefore among the commonest causes of admission to hospital in the neonatal population ^{364,365}, and are responsible for considerable expenditure within healthcare ³⁶⁶. In addition to the short-term morbidity, sepsis can lead to a variety of long-term complications within the neonatal population ^{72,367}, meaning that expenditure and resource utilisation are not limited to the early neonatal period. The premature neonatal population have an even more pronounced immaturity of host immune defence mechanisms than term neonates and require increased medical intervention which may disrupt normal anatomical barriers ³⁶⁸, thereby further predisposing to infection. This is reflected by

the fact that there is a strong inverse relationship between the risk of acquiring sepsis and both gestational age and birth weight ³⁶⁹. Premature neonates are at high risk of multi-organ dysfunction during sepsis episodes ¹⁴¹ and have a mortality of up to 35% in cases of EOS ³⁷⁰ and between 18-36% in LOS ³⁷¹. EOS is thought to be acquired vertically or in the peripartum period whereas LOS is considered postnatally or nosocomially acquired and is most commonly observed in preterm neonates with complex medical backgrounds. While EOS and LOS differ in aetiology, both have been linked to adverse outcome with meta-analysis suggesting that septic term and premature neonates are at high risk of later neurodevelopmental impairment and CP ^{372,373}, though true estimation of sepsis-related morbidity is hampered by investigators using differing criteria for its diagnosis.

Although sepsis definitions differ between sources, there is a traditional view that infection, sepsis and septic shock represent distinct but related entities. Infection is generally used to describe any suspected or proven infective process, irrespective of aetiology or severity ³⁷⁴. Within the paediatric population, sepsis has classically been characterised as a syndrome of systemic inflammation resulting in clinical or laboratory abnormalities secondary to a proven infective process ³⁷⁴, most commonly a blood stream infection. In 2016 the Sepsis-3 group published consensus guidelines on the screening and definition of sepsis in adults and characterised sepsis as “life-threatening organ dysfunction caused by a dysregulated host response to infection” ¹⁰⁰. Within these guidelines Sepsis-3 has also published an updated adult definition of septic shock describing it as a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality ¹⁰⁰.

Due to the considerable mortality associated with sepsis in the neonatal period ³⁷⁵, it has been identified as high-priority areas for clinical and experimental research internationally ³⁷⁶. Thus, due largely to advances in medical care the incidence of neonatal infection and the resulting mortality have lessened substantially in recent decades ¹²⁸. A consensus definition of neonatal sepsis is required to continue progress in this area as at present there is no unified definition of neonatal sepsis and the definitions currently in use vary greatly ¹⁴³. Attempts have previously been made to

define sepsis within the larger paediatric population, such as those of the European Medicines Agency ¹⁴⁵ and the International Consensus Conference on Pediatric Sepsis ¹⁴⁴. However, these definitions are not specifically designed for NICU patients and do not address the unique challenges of defining sepsis in preterm neonates. While great strides have been made in sepsis management it is increasingly recognised that a validated consensus definition of neonatal sepsis is critical to improve identification and treatment of septic neonates and reduce sepsis-related morbidity and mortality ³⁷⁷. In addition, a unified definition of neonatal sepsis could improve antibiotic stewardship, assist in screening for neonates at risk of adverse outcome and facilitate comparison of sepsis-related research and audit between centres. Such a definition would have to facilitate early identification of septic neonates and would need to address the unique challenges of diagnosing sepsis and screening for organ dysfunction in preterm neonates.

The aim of this review is to discuss the definitions of neonatal sepsis currently employed by research and surveillance networks internationally highlighting common themes in current literature and the limitations of currently used definitions. Existing definitions will be discussed with reference to the recent progress made in the Sepsis-3 consensus work from adult medicine.

3.1.5. *Methods*

A Pubmed search was performed to identify experimental and observational studies from neonatal research and surveillance networks which specified criteria for the diagnosis of neonatal sepsis within their manuscripts. The following search string was used: ((neonat*[Title] OR preterm[Title] OR premature[Title])) AND (sepsis[Title] OR infection[Title]). Search results were limited to full text articles published in English, performed in humans and in the neonatal age range (<1 month). Studies were included only if they were published by international neonatal surveillance and research networks. Observational studies, interventional studies and review articles were included, and the references of relevant studies were also interrogated. The networks identified to have published relevant definitions in their work were: the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development

(NICHD) Neonatal Research Network; The Australian and New Zealand Neonatal Network (ANZNN); the Vermont-Oxford Network (VON); the Neonatal Infection surveillance Network (NeonIN); Canadian Neonatal Network; Australasian Study Group for Neonatal Infections (ASGNI); The Centre for Disease Control and Prevention (CDC) and NeoKISS. If a data dictionary, surveillance report or definitions manual of a network was published online this was also accessed.

Common themes across publications were identified for discussion and are listed in Table 3.1. These themes will be highlighted in the sections that follow and include: infective organism/microbiology; duration of therapy; contaminants and polymicrobial cultures; CONS sepsis; timing of sepsis; clinical signs and culture negative sepsis; laboratory data and subclassification of sepsis.

This literature review was undertaken as part of the work of the European Society for Paediatric Research section on neonatal Infection, Inflammation, Immunology and Immunisation (4I group). This group was formed in 2018 with and comprises of neonatologists from across the world with a special interest in issues relating to neonatal infection and immunology. One of the primary goals of this international group is to begin work on a consensus definition of neonatal sepsis which was identified as a priority shortly after the group formed ³⁷⁸. At present preliminary work is underway on reviewing literature in relevant areas including animal models of neonatal sepsis and sepsis screening criteria in randomized-controlled trials. The 4I group has met in person most recently in Maastricht, Netherlands in 2019 at the joint European Neonatal Societies meeting and following completion of the initial literature reviews it is hoped that work can begin in earnest on identifying criteria for defining and screening for neonatal sepsis. This review, authored by myself and reviewed by the 4I group, represents a piece of the formative work of this group in outlining the problems with existing neonatal sepsis definitions and the complexity of future work in this area.

3.1.6. *Adult sepsis definitions and their utility in childhood*

The Sepsis-3 group was established to create consensus definitions of sepsis and septic-shock within adult medicine. The resulting consensus work which was published in 2016 has been widely adopted into clinical practice and was successfully incorporated into the Surviving Sepsis campaign^{100,379}. The Sepsis-3 publications by Singer *et al.*¹⁰⁰ addressed many of the existing challenges in defining sepsis such as the use of heterogenous definitions, the lack of consensus on terminology, the need to relate diagnosis to clinical outcome and the need for screening tools to identify high-risk patients earlier in the disease course. Following the formation of an expert group relevant literature was systematically reviewed^{380,381}, face-to-face meetings were undertaken and a Delphi subsequently convened to decide on screening criteria and definitions. Resulting definitions and screening criteria were then validated in large health databases within the US and sent to relevant international societies in the field for peer review. The result is a comprehensive, evidence-based approach to the definition and diagnosis of sepsis and septic shock in the adult population. In keeping with the goal of producing clinically relevant and outcome-based diagnostic tools a robust screening tool, the sequential organ failure assessment score (SOFA) and the quick SOFA (qSOFA), were also created to allow earlier screening and treatment of at-risk patients (Table 3.2). The definitions created eschew much of the established sepsis lexicon which was vague or confusing, such as “SIRS” and “severe sepsis”, in favour of a simple, validated, clinically-focused approach to defining sepsis and septic shock with more emphasis on organ dysfunction and less on traditional microbiological results.

Adult sepsis patients who develop organ dysfunction have substantially increased mortality in the intensive care setting³⁸². The linear relationship between the number of organs impaired and sepsis-related mortality³⁸³ reflects the importance of organ dysfunction in predicting outcome. Sepsis-3 have therefore defined sepsis as “life-threatening organ dysfunction caused by a dysregulated host response to infection” and septic shock as a “subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality”¹⁰⁰. Sepsis-3 guidance states that organ dysfunction may initially be occult

and that all patients presenting with infection should be evaluated for organ dysfunction, and conversely any unexplained episodes of organ dysfunction should prompt screening for sepsis ¹⁰⁰. This new focus on organ dysfunction in updated definitions reflects the findings that organ dysfunction is not only a key criterion for the early diagnosis of sepsis, but the degree of organ dysfunction is of prognostic importance in sepsis patients both during and after episodes.

To screen high risk patients, the Sepsis-3 group devised the qSOFA which is an abridged version of the longer SOFA score used to identify organ dysfunction in sepsis ³⁸¹. The qSOFA can be performed quickly in a clinical setting and requires only 2 of the following criteria as a positive screening test for sepsis: increased respiratory rate $>22/\text{min}$, systolic BP $<100\text{ mmHg}$ and altered mental state. In the setting of infection, qSOFA ≥ 2 acts as a good screening test for sepsis and should prompt more thorough investigation for organ dysfunction using the longer SOFA score and potential escalation of therapy. In the setting of suspected sepsis, a positive qSOFA identifies patients who are at higher risk of death or prolonged ICU stay. Criticisms have been levelled against the new diagnostic and screening tools presented by Sepsis-3 and these include concerns around qSOFA sensitivity, practical problems of applying the tools in lower income countries and a fundamental disagreement with the idea that organ dysfunction is required for sepsis diagnosis ³⁸⁴. Systematic literature review does however largely support the use of the Sepsis-3 definitions and screening tools noting that a positive qSOFA is strongly associated with mortality in pneumonia ³⁸⁵, has improved sensitivity for mortality in emergency department patients with sepsis ³⁸⁶; and has improved specificity for ICU admission, organ dysfunction and mortality ³⁸⁷ when compared to other illness severity scores. However, these reviews all noted that there are concerns about the sensitivity of qSOFA when used alone and due to the limited pool of research available at present there is considerable heterogeneity between the studies included in each review. The Sepsis-3 group has acknowledged that while limitations do exist in the definitions and tools they have developed, no approach will be all-encompassing ¹⁰⁰ and the qSOFA and SOFA represent simple, outcome-based screening tools that have been clinically validated and can be performed at the bedside.

Researchers have attempted to apply principles from Sepsis-3 in a paediatric setting by adjusting the physiological parameters of the SOFA score to age-related cut-offs to produce a paediatric SOFA (pSOFA) ³⁸⁸. pSOFA was then used to test the Sepsis-3 definitions in patients aged <21 years admitted to ICU with confirmed or suspected sepsis and confirmed the utility of Sepsis-3 parameters in the paediatric population ³⁸⁸. Subsequent work in a mixed paediatric ICU cohort using similarly adjusted SOFA and qSOFA scores showed improved diagnostic performance compared to existing illness severity and organ dysfunction scores ³⁸⁹, again highlighting the potential utility in childhood. Most recently the Sepsis-3 definitions have been applied in a paediatric emergency department setting where qSOFA had moderate prognostic accuracy in predicting mortality and/or admission to paediatric ICU ³⁹⁰. Despite some initially promising results there remains some debate on the utility of Sepsis-3 work within the paediatric population ³⁹¹⁻³⁹³. Similar work is lacking in neonatology, though it is clear that the sepsis definitions currently in use among older paediatric patients are not accurate in term and preterm neonates ¹⁴³. Wynn and Polin have suggested that the work of Sepsis-3 could be adapted to a neonatal setting with considerable modification and have proposed a neonatal-specific SOFA (nSOFA) ³⁷⁷ with early work suggesting that this may predict mortality in VLBW neonates with LOS ¹²⁹ (Table 3.3). The National Institute for Health and Care Excellence (NICE) guidelines have adopted the adult definitions of Sepsis-3 though guidelines do not specify terminology for use in neonatal patients. While NICE have published algorithms for the management and risk stratification of sepsis in paediatrics based on similar principles to the Sepsis-3 group, the age-ranges are broad (0-5 years) ³⁹⁴ and neonatal guidance uses the terms infection and sepsis interchangeably without specifying how sepsis should be defined ³⁹⁵.

3.1.7. *Infective organisms and microbiology*

Identifying an infecting organism is among the most common criteria for diagnosing neonatal sepsis with growth from blood and/or cerebrospinal fluid (CSF) culture being the most common method of isolation. However given the high sensitivity and specificity of new PCR-based techniques in diagnosing infection ³⁶⁰

some researchers are now including this in their sepsis definitions. Urine culture is not included in most published definitions as the rate of neonatal urinary tract infection in EOS and in VLBW neonates is low ³⁹⁶, suprapubic aspiration is invasive, and it is not routinely recommended in evaluating suspected EOS by most expert bodies including the American Academy of Pediatrics ³⁹⁷. In contrast, urine microbiology is likely to be of greater utility among premature neonates with suspected LOS where approximately 11% may have evidence of urinary tract infection ³⁹⁸.

The NICHD considers only bacteria and fungi isolated from blood and CSF in their definitions of neonatal sepsis ^{14,370,399-410}. The CDC classifies sepsis as bacterial isolation from any normally sterile site ⁴¹¹. The ANZNN requires positive blood and/or CSF cultures or a positive PCR from CSF for diagnosis of sepsis ⁴¹². The ANZNN includes bacteria and fungi under the heading of sepsis but also lists specific criteria for the diagnosis of sepsis of viral aetiology. The VON criteria require the isolation of bacterial or fungal pathogens from a prespecified list in either blood or CSF culture ⁴¹³ for the diagnosis of EOS and LOS ⁴¹³. NeonIN uses positive bacterial culture from blood, CSF or suprapubic urinary aspirate in the diagnosis of bacterial sepsis ⁴¹⁴. NeonIN diagnoses invasive fungal infection separately from bacterial, requiring only a positive culture from blood or CSF without any additional criteria ⁴¹⁵. Positive bacterial, fungal and viral cultures of blood and/or CSF are required in the Canadian Neonatal Network for the diagnosis of sepsis ^{416,417}. Both bacteria and fungi cultured from either blood or CSF are included within definitions from most ASGNI publications ^{418,419}. NEO-KISS defines laboratory-confirmed bloodstream infection as isolation of bacteria from blood or CSF along with two other criteria from a list of laboratory and/or clinical abnormalities ⁴²⁰. In the case of bloodstream infection, the NEO-KISS guidance specifies that bloodstream infection must be primary and not be as a result of localised infection elsewhere such as an osteomyelitis or abscess ⁴²⁰.

The use of microbiological isolates in the diagnosis of neonatal sepsis is well-established within existing literature. While it is essential to correctly identify and treat causative pathogens, relying on microbiology testing alone to define sepsis has limitations. Blood cultures may have a prolonged incubation before becoming positive ^{421,422} and thus do not facilitate early diagnosis. In addition, reliance on microbiological

culture alone does not help in identifying organ dysfunction which has been linked to adverse outcome in affected neonates ¹³⁷ and has been a major focus of Sepsis-3 ¹⁰⁰. While there is clearly an important role for microbiological testing within sepsis management, the most commonly employed techniques do not facilitate early recognition or screening of high-risk patients. Newer “-omics” based technologies have potential to personalise the treatment of many diseases, including sepsis ⁴²³ though at present these remain experimental.

3.1.8. *Duration of therapy*

NICHD publications frequently feature the requirement for a minimum duration of ≥ 5 days of antibiotic therapy to diagnose sepsis within their publications ^{14,399,400,404-409}. The ANZNN requires antibiotic administration with therapeutic intent after the available clinical and laboratory evidence has been reviewed, though a minimum duration of therapy is not specified. VON requires a pre-specified duration of antibiotic therapy to diagnose CONS sepsis but not in any other form of sepsis ⁴¹³. The NeonIN surveillance network requires treatment with ≥ 5 days of antibiotic therapy for a diagnosis of bacterial sepsis to be made ⁴¹⁴. NEO-KISS specifies a minimum duration of antibiotics in cases of clinical sepsis only where ≥ 5 days of therapy are required ⁴²⁰. Similarly, the Canadian Neonatal Network requires ≥ 5 days of antimicrobial therapy in cases of clinical sepsis but also in cases of sepsis with organisms which might otherwise be considered contaminants ⁴¹⁷.

A specified duration of antimicrobial therapy is a common diagnostic criterion in existing literature though this is usually in cases where sepsis was suspected but could not be microbiologically confirmed (e.g. negative cultures or CONS). While not explicitly discussed in the literature reviewed, the use of a minimum duration of therapy does not appear evidence-based and the duration of therapy chosen (most often 5 days) is likely arbitrary. Most importantly, the duration of therapy can only be determined retrospectively and is influenced by local practice, and therefore does not reflect illness severity and cannot be used in the early identification of sepsis.

3.1.9. Contaminants and polymicrobial cultures

Contamination of microbial culture is the growth of a non-pathogenic organism which was introduced during sample collection or processing ⁴²⁴ and in a neonatal setting is most commonly caused by skin commensals. Polymicrobial cultures are those in which ≥ 2 organisms are identified on a single microbiological culture which may result from contamination or may represent concurrent infection with multiple organisms. Correctly identifying whether polymicrobial culture and culture of commensal organisms represent contamination is especially challenging in premature neonates where the immature immune system means that commensal organisms can potentially cause invasive infection and more than one organism may be causing clinical symptoms concurrently. As a result, many organisations provide lists of specific organisms which should be considered contaminants or specify extra criteria which may be needed to confirm sepsis in the case of a potential contaminant.

The NICHD specifies the organisms which would be considered contaminants if isolated from blood and/or CSF ^{14,370,399,401,402,404-410}, although one NICHD study classified cultures of these organisms as positive if ≥ 5 days of antibiotics were administered with therapeutic intent ⁴⁰⁵. The ANZNN ⁴¹² discuss CONS as a potential contaminant but do not specify other organisms which should be considered contaminants. The Canadian Neonatal network provide a list of common skin contaminants and a list of organisms which are considered pathogenic if isolated on culture ⁴¹⁷. NEO-KISS does not provide a list of contaminants but does provide a specific list of organisms which are considered pathogens if cultured ⁴²⁰. VON, NeonIN and ASGNI do not address potential microbial contamination.

The way in which polymicrobial cultures are addressed differs between NICHD publications. One NICHD study considered polymicrobial cultures positive in EOS if at least one organism was considered a true pathogen ⁴⁰⁶. Another NICHD study reported that if one or more bacteria were isolated then the culture was considered positive as long as the bacteria identified were not on a prespecified list of common contaminants ⁴⁰¹. Another NICHD publication used outcome to determine clinical significance with Wynn *et al.* considered cultures with 3 organisms positive only if the neonate died within 72 hours whereas cultures with 2 organisms were excluded if at

least one of the organisms was a potential contaminant ³⁹⁹. The ANZNN do not address polymicrobial culture in-depth, stating only that the growth of a mixed CONS or other skin flora was insufficient for sepsis diagnosis ⁴¹². Within VON and the CDC, multiple pathogens may be independently recorded but polymicrobial cultures are not otherwise discussed ^{413,425}. The Canadian Neonatal Network state that the culture of ≥ 2 organisms is normally considered contamination ⁴¹⁷, though neonates with multiple organisms on culture may still be diagnosed with sepsis ⁴¹⁶. ASGNI usually requires the pure growth of a single organism from either blood or CSF to diagnose neonatal sepsis ⁴²⁶⁻⁴²⁸, however one publication stated that a pure growth of “at least one” organism constituted a positive culture though further detail was not provided ⁴¹⁸. In cases of potential contaminants, ASGNI states that the diagnosis of sepsis may still be made if clinical signs are present though at least one of a specified list of laboratory abnormalities is also required ^{418,426,427}. Polymicrobial cultures are not discussed within NEO-KISS or NeonIN publications.

Premature neonates are at especially high risk of sepsis secondary to commensal organisms ⁴²⁹ and are disproportionately affected by polymicrobial sepsis ⁴³⁰. The ability to confidently differentiate these diagnoses from culture contaminants is therefore important in the interpretation of neonatal blood culture results but does not facilitate earlier diagnosis of sepsis.

3.1.10. *Coagulase-negative staphylococcal sepsis*

Although CONS represent the commonest contaminants of neonatal blood cultures, they are also the most frequently isolated pathogens in cases of LOS. The growth of CONS in the culture of a preterm neonate with suspected sepsis is therefore considered clinically significant. However, given the frequency with which cultures are contaminated with CONS many publications classify CONS separately and require additional clinical and laboratory abnormalities to help distinguish cases of contamination from episodes of genuine CONS sepsis.

The NICHD publications which discussed CONS sepsis required that additional criteria such as laboratory findings suggestive of infection ^{370,402,410} or clinical signs of

sepsis⁴⁰² be present in addition to positive blood cultures in order for the diagnosis to be made. NICHD publications generally classified CONS as a contaminant when isolated in cases of suspected EOS^{401,406}, though one NICHD publication permitted the diagnosis of CONS EOS if the treating clinician felt it represented genuine infection based on the clinical picture and available laboratory results⁴⁰². In order to diagnose CONS sepsis according to the VON definition, signs of generalised infection and the intention to treat with ≥ 5 days of antimicrobials were required in addition to isolation of CONS from either blood cultures or CSF⁴¹³. NeonIN required an intention to treat with ≥ 5 days of antimicrobials⁴¹⁴ in addition to isolation of CONS from blood culture in order to diagnose CONS sepsis. The NEO-KISS definition stipulated that additional laboratory criteria (high CRP, elevated IL-6 or 8, high immature-to-total neutrophil ratio, platelet count $<100 \times 10^9/L$ or total leucocytes $<5 \times 10^9/L$) were needed in circumstances where CONS was isolated on blood culture⁴²⁰. Additionally, patients needed to have two of the defined clinical and/or laboratory criteria provided in Table 3.4 and Table 3.5⁴²⁰. The only ASGNI publication providing diagnostic criteria for CONS sepsis defined it as episodes of LOS in which CONS was isolated⁴²⁷, a definition shared by the Canadian Neonatal Network⁴¹⁶. CONS sepsis is not discussed separately in ANZNN publications.

CONS sepsis is an important clinical entity within neonatology due its prominence in cases of LOS among the preterm population⁴³¹. While being able to confidently differentiate true CONS sepsis from culture contaminants is not relevant to early identification of high-risk patients it is an important consideration in future consensus definitions in neonatology due to the diagnostic difficulties that isolation of CONS represents.

3.1.11. *Timing of sepsis*

21% of preterm neonates will have ≥ 2 episodes of LOS during their inpatient stay¹³⁴. Since the number of sepsis episodes a neonate has is known to affect survival¹³⁹ it is important to clearly differentiate whether a clinical deterioration represents a new episode of infection or is merely the continuation of an established infection in which bacterial clearance has been inadequate. As a result, many neonatal networks have

specific criteria to help distinguish between sepsis episodes which may occur in close proximity but are clinically distinct.

NICHD definitions state that if the same organism is identified after ≥ 10 days of appropriate treatment or if a new pathogen is isolated at any time, they are considered as distinct sepsis episodes ^{404,407}. The ANZNN states that if the same organism is grown from blood or CSF ≤ 14 days from the last positive culture it is considered a repeat isolate rather than a new episode of sepsis ⁴¹². In cases where the same organism is isolated NeonIN requires ≥ 7 days between positive bacterial cultures, ≥ 10 days between positive CONS cultures and ≥ 14 days between positive fungal cultures to diagnose a new sepsis episode ^{414,415}. NEO-KISS states that 14 days since the beginning of the previous infection and a period during which the neonate displays no signs of clinical sepsis are required to make a new diagnosis of sepsis ⁴²⁰. ≥ 7 days between repeat cultures of the same organism or isolation of a new pathogen at any time are required in the Canadian Neonatal Network definition to mark new episodes of sepsis ⁴¹⁷. The CDC and ASGNI do not address the time interval required for positive cultures to be considered separate infective episodes. The time interval between episodes is not a universal consideration in existing neonatal sepsis literature. It is however pertinent to the discussion of neonatal sepsis as it impact the number of sepsis cases diagnosed within a NICU, an important metric of NICU performance, and is of prognostic value to in determining outcome ¹³⁹.

The time at which sepsis occurs is used to differentiate vertically transmitted infection, generally referred to as EOS from that acquired postnatally, generally termed LOS. In some instances, the time cut-off refers to the time of culture collection while others specify that it is the time of symptom onset. The CDC specifies EOS as sepsis which affects those < 7 days and LOS as sepsis aged ≥ 7 days ⁴¹¹. The NICHD specify that patients with positive cultures at ≤ 72 hours of life are classified as EOS, whereas those with positive cultures beyond this are diagnosed with LOS ^{14,370,399-410}. NEO-KISS does not use the standard terms “early” and “late” and instead designates any infection where symptoms occur at ≥ 72 hours of life as nosocomial sepsis ⁴³². VON uses a “day of life” cut-off ⁴¹³ with EOS classified as sepsis $\leq$ day 3 of life and LOS as sepsis after day 3 of life, a classification which has important implications depending

on the time of day at which a neonate is delivered. The ANZNN definition stipulates that neonates with onset of symptoms <48 hours have EOS and those with onset of symptoms $\geq$ 48 hours have LOS^{412,433}. Onset at 48 hours is also used in both the NeonIN surveillance network and the Canadian Neonatal Network to differentiate EOS from LOS, with positive culture at $\leq$ 48 hours representing EOS and positive cultures >48 hours representing LOS^{414,416}. The differentiation of EOS and LOS within the ASGNI was similarly based on timing of $\leq$ 48 hours (EOS) or >48 hours (LOS)^{418,426,428}. Though it is not specifically noted whether this represents the timing of symptoms or timing of initial septic work-up, there is mention within manuscripts that data on the timing of culture positivity was recorded so this is likely to be the reference point used^{426,427}.

The designation of sepsis as either EOS or LOS is well-established in neonatal literature and helps differentiate infections which are vertically transmitted from those acquired postnatally as both have distinct microbiological profiles⁴³⁴ and prognosis. Differentiating EOS and LOS is important from a treatment perspective and in collecting data on nosocomial infections, an important outcome measure in the care of premature neonates. Thus, while not aiding in earlier diagnosis of sepsis the designation of EOS or LOS is important for audit and prognosis and is an important consideration in future work on a consensus definition of neonatal sepsis.

3.1.12. *Clinical signs and culture negative sepsis*

The requirement for patients to have clinical signs suggestive of infection is common within published definitions of sepsis, though not universal. Most publications including clinical signs in their diagnostic criteria do not provide practical guidance on the specific signs that should be present and do not use continuous vital sign monitoring. NEO-KISS and the Canadian Neonatal Network are the only networks to provide a prescriptive list of the clinical features which should be considered as suggestive of sepsis if present^{417,420} (Table 3.5).

Few NICHD publications required clinical signs to be present in order to make a diagnosis of sepsis and none of those studies specified the signs they considered

pathological ^{370,402,403}. The ANZNN definition states that a clinical picture consistent with sepsis should be present but does not provide further detail on the specific clinical features of interest ⁴¹². VON uses clinical signs of infection in the diagnosis of CONS sepsis only and provides examples of some signs that may be present ⁴¹³, though these are not prescriptive. The Canadian Neonatal Network requires clinical signs of sepsis to be present in both laboratory-confirmed bloodstream infection and clinical sepsis ^{416,417}, and provides a specific list of the signs that should be used. Clinical signs are required for the diagnosis of sepsis in ASGNI publications. Some ASGNI publications provide no further detail ^{418,426}, and while others provide specific examples ^{427,428} they state that it remains at the discretion of the clinician as to what signs are used. NEO-KISS requires the inclusion of clinical criteria in the diagnosis of laboratory-confirmed, CONS and clinical sepsis, and provides a prescriptive list of the clinical criteria which should be used ⁴²⁰. The NeonIN surveillance network do not require clinical signs to be present for sepsis to be diagnosed ⁴¹⁴.

NEO-KISS is one of the few organisations which lists a separate diagnosis of “clinical sepsis” and it is defined as follows: ≥ 5 days of antimicrobial therapy, no infection at another site and blood cultures are either negative or were not obtained ⁴²⁰. Additionally, 2 criteria are required from a list of clinical and/or laboratory parameters listed for standard bloodstream infection (Table 3.4 and Table 3.5). One NICHD publication on neurological follow-up of former premature neonates included a diagnosis of clinical sepsis if ≥ 5 days of antibiotics were administered to a neonate with negative cultures in the setting of LOS ⁴⁰⁹. The Canadian Neonatal Network have specific criteria for the diagnosis of clinical sepsis requiring the presence of specific clinical signs, negative cultures, the absence of infection elsewhere and ≥ 5 days of antimicrobials ⁴¹⁷. Criteria for the diagnosis of clinical/culture-negative sepsis are not listed in the ANZNN, ASGNI, VON, CDC or NeonIN.

The use of clinical signs as a criterion in the diagnosis of sepsis in neonates seems logical as alterations in clinical parameters, are common early in the clinical course of those with sepsis-related mortality ⁴³⁵. However, the early signs and symptoms vary greatly between cases and may be anywhere on a spectrum between non-specific presentations to fulminant multiorgan dysfunction. Despite the incorporation of

clinical signs in diagnostic criteria of several organisations, none utilise organ dysfunction scores to grade severity or guide management, something which has been highlighted as key in adult literature ¹⁰⁰. Due to the difficulties in accurately detecting neonatal sepsis based on traditional clinical examination alone there is increasing interest in the use of complex physiological data and continuous vital sign monitoring to detect and predict neonatal pathophysiology ⁴³⁶. Big data analytics shows promise in assisting management of neonatal sepsis but requires further validation before it can be incorporated into practice ⁴³⁶.

3.1.13. *Laboratory data and biomarkers*

In addition to standard microbiological testing, several organisations require additional laboratory investigations to diagnose sepsis (Table 3.4). While infection biomarkers undoubtedly provide additional information to clinicians, care must be taken when they are used in defining sepsis. The cut-off values used for sepsis biomarkers are not universal, a fact that has potentially important implications for the sensitivity and specificity of the test ⁴³⁷. Several existing biomarkers such as CRP may be normal early in sepsis, meaning that the positive and negative predictive value of such tests may vary depending on the timing of collection and whether serial measurements are undertaken ⁴³⁸. Additionally, laboratory data are affected by the assay used within a particular institute and therefore changes in measurements over time may be more valuable than the absolute value obtained.

NICHD publications use additional laboratory data for the diagnosis of CONS sepsis only ^{370,402,407,410}. The tests used are commonly available haematological and biochemical investigations such as CRP and FBC ^{370,402,407,410}. Certain ASGNI publications required abnormal laboratory data in addition to positive blood/CSF cultures for sepsis diagnosis ^{418,428}. The abnormal laboratory data were clearly specified in one study and included CRP or abnormal FBC or blood film analysis ⁴¹⁸, though elsewhere specific testing was not listed ⁴²⁸. In other ASGNI studies, added laboratory data were used only to distinguish contaminants from true sepsis ^{426,427}. The NEO-KISS definition allows inclusion of laboratory data as part of the criteria used in cases of laboratory-confirmed, CONS and clinical sepsis ⁴²⁰. Notably NEO-KISS is the only

organisation to include IL-6 and 8 in their definitions, likely reflecting their favourable sensitivity and specificity early in sepsis ⁴³⁹. The Canadian Neonatal Network allow the use of antigen testing in the diagnosis of bloodstream infection, but do not provide any further detail ⁴¹⁷. The ANZNN, VON, NeonIN and CDC do not include additional laboratory data in their diagnostic criteria for sepsis.

In addition to aiding diagnosis, biomarkers have potential to help guide investigation and treatment in neonatal sepsis. NICE guidelines suggest that CRP levels may be used to plan further investigations such as lumbar puncture and that trends in CRP values may guide decisions around the duration of antibiotic therapy ³⁹⁵. Transcriptomic, proteomic and metabolomic investigations have potential to revolutionize the diagnosis and treatment of neonatal sepsis ⁴⁴⁰ though they remain largely unvalidated in neonatal patients and progress is limited, among other things, by the heterogeneous definitions of sepsis used by investigators ⁴⁴⁰. Most existing research on neonatal biomarkers focuses largely on detecting early inflammatory response to sepsis, rather than detecting organ dysfunction in response to infection. This contrasts with the work of Sepsis-3 in which the biomarkers used in screening (creatinine, bilirubin and platelet count) screen for the complications of sepsis, namely organ dysfunction, rather than trying to look for early rises in acute-phase reactants ¹⁰⁰. The Sepsis-3 group have also acknowledged that while newer biomarkers of organ dysfunction are becoming available, they would require extensive validation before potential inclusion in future versions of the SOFA score ¹⁰⁰.

3.1.14. *Subclassification of sepsis*

Few definitions subclassify sepsis though some networks provide different criteria for the diagnosis of meningitis and blood culture positive sepsis. NICHD publications rarely provided subclassification for sepsis however one study included early onset meningitis separately from EOS ⁴⁰⁸, and another provided a complex classification of neonatal infection as follows: clinical infection; EOS/LOS; sepsis with concurrent NEC; or meningitis with and without concurrent sepsis ⁴⁰⁹. Some NICHD publications designated CONS sepsis as either “definite infection, “possible infection” or “probable contaminant” depending on the microbiological, clinical and laboratory data available

^{370,407,410}. ASGNI publications do not subclassify sepsis though meningitis has specific diagnostic criteria, requiring either a positive CSF culture or a positive blood culture in combination with an elevated CSF white cell count and signs suggestive of the diagnosis ^{418,426-428}. In addition, one ASGNI paper had a further designation for fungal meningitis requiring either a CSF white count of $>10^9$ in the setting of a positive blood culture or fungal growth within CSF ⁴¹⁹. The Canadian Neonatal Network have separate diagnostic criteria for central line-associated bloodstream infection ⁴¹⁶, meningitis and ventriculitis ^{416,417}. NEO-KISS sub-categorises bloodstream infection if a central line was present at the time of infection or in the preceding 48 hours ⁴²⁰. The VON, CDC, NeonIN surveillance network and ANZNN do not subclassify sepsis and none of the organisations discussed provide separate definitions for premature and term neonates.

3.1.15. Discussion

At present there is no universally accepted definition of neonatal sepsis. While common themes are present in current literature, the existing definitions differ greatly from each other. Pathogen isolation in blood or CSF culture is one of the commonest criteria in current literature, reflecting the importance of accurately identifying the causative organism in guiding antimicrobial therapy ³⁶⁰. Much of the existing literature also highlights that while neonates, especially preterm neonates ⁴²⁹ and those with complex medical backgrounds ⁴⁴¹, are at high risk of polymicrobial sepsis and sepsis secondary to CONS, it may be prudent to apply additional diagnostic criteria to help differentiate these episodes from culture contaminants. Another important theme is the timing of sepsis with most organisations differentiating between EOS and LOS based on the timing of symptoms or culture collection. Differentiating sepsis in this way is used to separate vertical infections from those acquired postnatally, and helps to tailor antimicrobials based on likely pathogens ³⁶⁰. Accurately delineating the timing of infection relative to delivery is also important in identifying nosocomial infections in the premature population where this is an important metric of NICU performance. The requirement for a minimum duration of therapy, most commonly ≥ 5 days, is used as a criterion by some organisations when defining cases of neonatal sepsis. While not

evidence-based, treatment duration is likely used in identifying cases where there is persistent clinical concern from those in which antibiotics are started empirically. Clinical signs and laboratory data are included in the definitions of many organisations though they are not universal, are rarely an absolute requirement and in many cases the exact cut-offs or criteria are vague or left at the discretion of the treating clinician. The lack of standardised clinical and laboratory criteria in definitions reflects the fact that clinical presentation may be non-specific³⁶⁰ and laboratory data unhelpful⁴³⁸ early in neonatal sepsis. Lastly, many organisations acknowledge that some subclassification is needed within neonatal sepsis, either because these subtypes influence prognosis⁴⁴² or because subclassification dictates the duration and choice of treatment⁴⁴³.

Many existing neonatal sepsis definitions have either developed organically over time or have been developed for internal use by smaller organisations, and thus lack the extensive evidence-base and peer review which has resulted in recent developments within the adult field^{100,379}. For accuracy, the diagnosis of sepsis in neonatal literature is often retrospective, and primarily focuses on microbiological culture results. Giannoni *et al.* have found that mortality in septic neonates is greatest in those with concurrent hypotension requiring catecholamine treatment, and requiring mechanical ventilation¹³⁷. These findings suggest that, similar to the adult population, the presence of organ dysfunction is an important contributor to adverse outcome among neonates. This further highlights the need for both clearer guidance on the diagnosis of neonatal sepsis and the need for a robust screening tool to identify organ dysfunction in term and premature neonates at an early stage in the disease course. While the work of Sepsis-3 provides an excellent example of how a consensus definition could be created within neonatology, it is clear that the definitions and screening criteria created by the Sepsis-3 initiative are not suitable for direct translation into neonatal practice. The foremost reason for this is that neonates and adults are very distinct patient populations in whom sepsis presentation, disease progression, diagnosis, treatment and outcomes of interest differ greatly. In addition, many of the criteria which may potentially be used in a neonatal screening tool for sepsis, such as thrombocytopenia or renal failure, would themselves need evidence-

based definitions and adjustments for use at the extremes of viability where medical evidence is currently lacking. The clinical presentation in neonates also differs from that of adults with many of the initial signs and symptoms being non-specific ³⁶⁰. Standard laboratory investigations for sepsis in adults and older children also have poor sensitivity and specificity in neonates or may be influenced by gestational age ¹⁴². The use of simple physiological measurements in neonates is also much more challenging than in adults as the normal ranges of many clinical parameters such as blood pressure or heart rate differ based on gestational and postnatal age ⁴⁴⁴. There is also increasing recognition that traditional signs of perfusion such as blood pressure are not adequate markers of perfusion in the premature neonate ^{445,446} and while many modalities are available for circulatory monitoring in neonatology, no gold-standard exists ⁴⁴⁷. This has important implications for design of an organ scoring system or sepsis screening tool which might be analogous to the SOFA or qSOFA scores, both of which are reliant on well-defined normal ranges for vital signs ¹⁰⁰. In combination these factors make the development of a sepsis screening tool and creation of a consensus sepsis definition much more challenging in neonates, with particular difficulty arising is the identification of septic shock which is dependent on the accurate identification of circulatory abnormalities as per Sepsis-3 ¹⁰⁰.

Premature neonates are a unique cohort within neonatology as they are disproportionately affected by sepsis compared to term infants ⁴⁴⁸, and are at high risk of mortality following the onset of organ dysfunction ¹⁰¹. Identifying sepsis in premature neonates represents a particular challenge as clinical presentation is especially non-specific ⁴⁴⁹ and immaturity of organ systems at birth means that baseline organ dysfunction may be present as a result of prematurity. This has implications for screening as several of the parameters used in the SOFA score are affected by lower gestation or birth weight including: degree of respiratory support ⁴⁵⁰, hyperbilirubinaemia ⁴⁵¹, platelet count ⁴⁵², blood pressure ⁴⁵³ and creatinine ⁴⁵⁴. Even term neonates, in whom it should theoretically be easier to identify new-onset organ dysfunction, may have alterations of bilirubin ⁴⁵⁵ and creatinine levels for reasons other than sepsis ⁴⁵⁶. Few of the laboratory investigation and physiological parameters used in the SOFA have established normal ranges which account for the gestation or

day of life, and it is recognised that some investigations such as creatinine may be affected by maternal or placental factors and therefore change considerably in the early neonatal period ⁴⁵⁴. Changes in organ dysfunction from baseline or trends over time may therefore be more important in complex neonates, such as premature neonates or those with congenital abnormalities, who may have clinical or biochemical evidence of organ dysfunction at baseline related to their underlying diagnosis. The timing of clinical or laboratory assessment will also need careful consideration to account for the potential effect of transitioning on screening tool results recorded shortly after delivery. While not designed as a tool for the definition of sepsis, the “Neonatal Early-Onset Sepsis Calculator” provides an excellent example of how accounting for the transitioning period in sepsis screening can be successfully achieved ⁴⁵⁷. This tool allows a period of 2 hours after birth in which physiological abnormalities may be acceptable without necessarily affecting the results of the sepsis risk calculation. Such considerations are important not only for accuracy of diagnosis but also for antimicrobial stewardship and to avoid unnecessary escalation of care. Any neonatal-specific screening tool will therefore require either gestation-specific normal ranges to be established for the criteria used or will require screening criteria which account for the unique physiological and biochemical status of term and preterm neonates.

Long-term neurological outcome is among the most important metrics in neonatal medicine, and this is especially so in the premature population as survival without disability is heavily influenced by gestational age at birth ⁴⁵⁸. While adult neurological impairment is increasingly recognised among older sepsis survivors ⁴⁵⁹ it is not nearly as common or severe as in the neonatal population ¹³¹. Data on long-term neurological outcome is therefore rarely available in adult sepsis studies with most focusing on short-term outcomes, most commonly survival. This is relevant to note as much of the recent data on Sepsis-3 definitions and screening tools relates only to short term outcomes such as validation, diagnostic accuracy and mortality ^{460,461}. Within neonatology more emphasis is placed on long-term outcome data especially that relating to neurological outcome and organ dysfunction in ex-premature neonates surviving into childhood. The outcome data used to validate definitions in adults and

neonates are therefore likely to be very different and neonates involved in early studies of any future consensus definitions will require long-term follow-up.

A unified definition of neonatal sepsis is important to improve clinical care, facilitate audit of best-practice and improve the quality of future clinical and experimental research in the area. However, replicating the work of Sepsis-3 within neonatology is inherently more complex, and the definitions and screening tools which are produced from any such consensus definition are likely to differ considerably from those produced by Sepsis-3. Furthermore, the validation of a consensus definition of neonatal sepsis would require not only the short-term outcome data which has been the focus of much existing adult literature, but long-term follow-up to evaluate outcomes specific to neonatology. An evidence-based, validated consensus definition of neonatal sepsis is required but will be a challenging task. The formation of an expert committee with relevant experience, systematic literature review and initiation of a Delphi process to identify criteria for the definition of neonatal sepsis will all be required. In addition, a validated set of screening criteria are needed which account for the complex nature of sepsis presentation within the term and premature populations. Validation of any resulting definitions or screening criteria could first be performed retrospectively on existing datasets before being introduced into clinical practice on a prospective basis. Similar to the definitions and tools developed by Sepsis-3, neonatal screening tools should be designed for use at the bedside, should identify at-risk patients early in the disease course and should be validated against clinical outcomes. The resulting work could be incorporated into clinical initiatives similar to the Surviving Sepsis Campaign as well as providing clearer definitions for use in clinical and experimental work within the field.

3.1.16. *Conclusions*

Differing opinions on the true nature of neonatal sepsis have meant that multiple definitions of neonatal infection have evolved¹⁴³. Although certain criteria are common between definitions most differ greatly from each other and there is a lack of consensus on parameters and cut-offs. The Sepsis-3 group have successfully developed and validated new, simplified definitions and screening criteria for sepsis

which are based on the presence of organ dysfunction in adults with suspected infection. At present, definitions of neonatal sepsis in published literature are heterogenous with a heavy emphasis on microbiological results rather than organ dysfunction. The existing definitions are also problematic as they do not identify patients early, do not grade severity of disease and provides no prognostic information on affected patients. A consensus definition of neonatal sepsis has considerable scope to improve the care of at-risk neonates and the work of Sepsis-3 provides a clear roadmap on how such a definition may be developed.

Organisation	Early onset sepsis	Late onset sepsis	Pathogens	Culture/PCR	Duration of therapy	Contaminants	Polymicrobial culture	Interval between episodes	CONS Sepsis	Clinical signs	Culture negative	Laboratory data	Subclassification
National Institute of Child Health and Human Development (NICHD) (17, 51-63)	Positive culture ≤ 72 hours of life	Positive culture >72 hours of life	Bacteria and fungi	CSF culture. Blood culture	≥ 5 days of antibiotic therapy required	Specific list of contaminants provided	Approach varied between studies	>10 days between isolates of the same organism, or a new organism cultured at any time	Additional clinical or laboratory criteria required with positive culture	Required in some studies, not defined	Recognised in one publication, specific criteria provided	Required in CONS sepsis only	Meningitis classified separately. CONS: “definite”, “possible”, “contaminant”
Australian and New Zealand Neonatal Network (ANZNN) (65, 89)	<48 hours at time of symptom onset	≥ 48 hours at time of symptom onset	Bacterial, fungal and viral†	CSF culture. Blood culture. PCR on CSF	Not specified	Only CONS specifically discussed	Not discussed	>14 days between isolates of the same organism	Not discussed	Required but not defined	Not recognised	Not required	Not discussed
Vermont Oxford Network (VON) (66)	≤ 3 rd day of life	>3 rd day of life	Bacteria or fungal	CSF culture. Blood culture	≥ 5 days of antibiotic therapy specified for CONS sepsis only	Only CONS specifically discussed	Polymicrobial culture may be diagnosed	Not specified	Signs of generalised infection, ≥ 5 days antibiotic therapy and positive culture	Required for CONS only, examples provided	Not recognised	Not required	Meningitis included with sepsis
Neonatal infection network (NeonIN) (67,68)	≤ 48 hours at time of positive culture	>48 hours at time of positive culture	Bacteria	CSF culture Blood culture Suprapubic aspirate	≥ 5 days of antibiotic therapy required	Specific contaminants not specified	Not discussed	≥ 7 days between repeat bacterial isolates. ≥ 10 days between repeat CONS isolates. ≥ 14 days between repeat fungal isolates	≥ 5 days of antibiotic therapy with positive culture	Not discussed	Not recognised	Not required	Not discussed
Canadian Neonatal Network (69, 70)	≤ 48 hours at time of positive culture	>48 hours at time of positive culture	Bacteria, fungal and viral	CSF culture Blood culture	≥ 5 days of antibiotic therapy for clinical sepsis and in potential contaminants	List of common skin contaminants provided	Polymicrobial culture may be diagnosed	≥ 7 days between isolates of the same organism or a new organism cultured at any time	Positive culture in the setting of LOS	Required, specific list provided	Recognised, specific criteria provided	One of several possible criteria which may be used	CVC-related sepsis classified separately meningitis/ventriculitis listed

Australasian Study Group for Neonatal Infections (ASGNI) (71, 72, 80-82)	≤48 hours at time of onset*	>48 hours at time of onset *	Bacteria or fungal	CSF culture Blood culture	Not specified	Specific contaminants not specified	Approach varied between studies	Not specified	Positive culture in the setting of LOS	Required, examples provided	Not recognised	Occasionally**	Bacterial and fungal meningitis defined separately
Neo-KISS (73)	Excluded	>72 hours after birth or admission at time of symptom onset is "nosocomial"	Bacteria, fungal or viral	CSF culture Blood culture	≥5 days of antibiotic therapy specified for clinical sepsis only	Only CONS specifically discussed	Not discussed	An infection-free period of 14 days is required	Additional laboratory criteria with positive culture	Required, specific list provided	Recognised, specific criteria provided	One of several possible criteria which may be used	CVC-related sepsis classified separately; meningitis not discussed
Centre for disease control and prevention (CDC) (64, 79)	<7 day at time of positive culture	≥7 days at time of positive culture	Bacteria	Culture from a normally sterile site	Not specified	Specific list of contaminants provided	Not discussed	Not discussed	Not discussed	Not discussed	Not discussed	Not required	Not discussed

Table 3.1: summary of the criteria of each organisation discussed.

CSF: cerebrospinal fluid; CONS: coagulase negative staphylococcus; †Separate diagnostic criteria were required for viral sepsis in ANZNN publications. *(unclear whether symptoms or cultures) **Required in some publications to differentiate contaminants from true sepsis.

SOFA (Sepsis-related Organ Failure Assessment) Score criteria					
	Score				
System	1	2	3	4	5
Respiratory PaO ₂ /FIO ₂ , mmHg (kPa)	≥400 (53.3)	<400 (53.3)	<300 (40)	<200 (26.7) with respiratory support	<100 (13.3) with respiratory support
Coagulation Platelets, ×10 ³ /μL	≥150	<150	<100	<50	<20
Liver Bilirubin, mg/dL (μmol/L)	<1.2 (20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	>12.0 (204)
Cardiovascular	MAP ≥70mm Hg	MAP <70mm Hg	Dopamine <5ug/kg/min or dobutamine (any dose)	Dopamine 5.1-15 ug/kg/min or epinephrine ≤0.1 ug/kg/min or norepinephrine ≤0.1 ug/kg/min	Dopamine >15 ug/kg/min or epinephrine >0.1 ug/kg/min or norepinephrine >0.1 ug/kg/min
Central Nervous System Glasgow Coma Scale score	15	13-14	10-12	6-9	<6
Renal Creatinine, mg/dL (μmol/L) Urine output, mL/d	<1.2 (110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440)	>5.0 (440)
				<500	<200
qSOFA (Quick SOFA) score criteria					
Respiratory rate ≥22/min					
Altered mentation					
Systolic blood pressure ≤100mmHg					

Table 3.2: SOFA and qSOFA scores

Proposed nSOFA (neonatal SOFA) score					
	Score				
Respiratory	0	2	4	6	8
	Not intubated OR intubated, SpO ₂ /FiO ₂ ≥ 300	Intubated, SpO ₂ /FiO ₂ < 300	Intubated, SpO ₂ /FiO ₂ < 200	Intubated, SpO ₂ /FiO ₂ < 150	Intubated, SpO ₂ /FiO ₂ < 100
Cardiovascular	0	1	2	3	4
	No inotropes AND no systemic steroids	No inotropes AND systemic steroid treatment	One inotrope AND no systemic steroids	Two or more inotropes OR one inotrope AND systemic steroid treatment	Two or more inotropes AND systemic steroid treatment
Platelets (10 ³ /ul)	0	1	2	3	
	Platelet count ≥150 × 10 ³	Platelet count 100–149 × 10 ³	Platelet count <100 × 10 ³	Platelet count < 50 × 10 ³	

Table 3.3: nSOFA score

Organisation	Setting	CRP	White cell count	Full blood count	Platelet count	Immature: total white cell ratio	Base excess	Blood Glucose	Interleukin 6 + 8
National Institute of Child Health and Human Development (NICHD)	Diagnosis of CONS sepsis only (17, 54, 59, 63)	>1mg/dL	Not discussed	Mentioned, values not specified	Not discussed	Not discussed	Not discussed	Not discussed	Not discussed
Australasian Study Group for Neonatal Infections (ASGNI)	Required to diagnose sepsis (71, 82). To differentiate potential contaminants from true sepsis only (80, 81)	Mentioned, values not specified	<4x10 ⁹ /L >20x10 ⁹ /L	Not discussed	<150x10 ⁹ /L	Immature WBC ≥20% total WBC	Not discussed	Not discussed	Not discussed
Neo-KISS	Diagnosis of clinical, laboratory-confirmed and CONS sepsis (73) †	>2mg/dL	<5x10 ⁹ /L	Not discussed	<100x10 ⁹ /L	Immature neutrophils >20% total neutrophil count	BE < -10 mEq/l	>140mg/dl	Mentioned, values not specified

Table 3.4: Laboratory Test cut-offs used in diagnosis of neonatal sepsis

CONS: Coagulase Negative Staphylococcus; CRP: c-reactive protein; †I:T ratio, leucocyte count and platelet count are used in defining CONS sepsis only.

Organisation	Clinical criteria
National Institute of Child Health and Human Development (NICHD) (17, 57, 58)	Clinical signs required but not specified
Australian and New Zealand Neonatal Network (ANZNN) (68)	Clinical signs required but not specified
Canadian Neonatal Network (72)	Clinical signs required but not specified
Australasian Study Group for Neonatal Infections (ASGNI) (73, 82-84)	Diagnosis of sepsis requires clinical signs to be present Examples given: Fever, Hypothermia, temperature instability, apnoea, bradycardia, increased oxygen requirement, feed intolerance, lethargy, hypotonia
Vermont Oxford Network (VON) (69)	Diagnosis of CONS sepsis requires clinical signs to be present Examples given: Apnoea, temperature instability, feeding intolerance, worsening respiratory distress, hemodynamic instability
NEO-KISS (75)	One of several possible criteria which may be used Specific list provided: Fever, hypothermia or temperature instability, tachycardia, bradycardia or heart rate instability, new or more frequent apnoea, capillary refill time >2 seconds, skin colour, increased respiratory support, unstable clinical condition, apathy

Table 3.5: Clinical criteria used in the diagnosis of sepsis

3.2. Does late-onset sepsis with coagulase negative *Staphylococci* cause neurodevelopmental delay in premature neonates?

3.2.1. Introduction

LOS is the most commonly encountered form of sepsis in the premature neonatal population ⁴⁶² and CONS are the commonest causative organisms. In healthy children and adults, CONS represent normal skin commensals and rarely cause infection unless the host becomes profoundly immunocompromised or has an indwelling medical device ⁴⁶³. In premature neonates however, CONS represent a clinically important cause of increased mortality and prolonged hospital stay ⁴⁶³. Mortality rates with CONS sepsis are lower than in EOS and LOS secondary to gram-negative organisms ⁴⁶⁴ and thus, despite its prevalence within the premature population CONS sepsis is often thought of as a more indolent, less harmful form of infection ⁴²⁷. As a result, little is understood regarding the long-term neurological effects of CONS sepsis in the neonatal population.

To investigate the effect of CONS sepsis on neurodevelopmental outcome a literature search and synthesis of evidence was undertaken according to the “Best BETs” format ⁴⁶⁵. This process involves the use of a patient-focused clinical scenario to inform a critical appraisal of relevant medical literature. The result, a succinct and evidence-based answer to the proposed clinical scenario, is presented below.

3.2.2. Scenario

A premature neonate born at 25+1 weeks gestation is now 3 weeks old, on full enteral feeds and nasal continuous positive airway pressure (CPAP) in 21% oxygen. Following multiple apnoeic episodes and an increase in the oxygen requirement a sepsis evaluation is performed and empiric antimicrobial therapy is commenced according to local guidance. A pure growth of CONS is later isolated on blood culture. Your registrar has enquired about any possible neurodevelopmental complications resulting from this episode of CONS sepsis.

3.2.3. *Structured clinical question*

Do premature neonates [patient] requiring therapy for CONS sepsis [intervention] have adverse neurodevelopmental outcomes [outcome] compared to non-septic neonates [comparison]?

3.2.4. *Search strategy*

Secondary sources

The Cochrane library and DARE were searched and revealed no relevant systematic reviews.

Primary sources

A PubMed search was undertaken using the following search strategy:

((neurodevelopment*[Title/Abstract] OR development*[Title/Abstract])) AND (vlbw[Title/abstract] OR Low-birth-weight[Title/Abstract] OR low birth weight[Title/Abstract] OR preterm[Title/Abstract] OR premature[Title/Abstract])) AND (CONS[Title/Abstract] OR sepsis[Title/Abstract]). Results were limited to English publications in humans, within the infant age range (birth-23 months). The search revealed 573 publications, of which 7 represented relevant clinical studies: 1 systematic review and meta-analysis³⁷², 4 prospective cohort studies^{409,442,466,467}, 1 retrospective cohort study⁴⁶⁸ and 1 case-control study⁴⁶⁹. All papers included studied preterm neonates <37 weeks with a confirmed diagnosis of CONS sepsis in whom the outcomes of interest included either neuroimaging or detailed developmental evaluation (Table 3.6).

Citation	Study group	Study type	Outcome	Key Result	Comments
Alshaikh B et al 2013 (1)	3 studies (n=4431) of infants born <1500 grams or <27 weeks with CONS sepsis and developmental follow-up.	Systematic review and meta-analysis (2a)	Neurodevelopmental index (NI)	Odds ratio 1.31 (95% CI 1.09 to 1.57)	Subgroup analysis was performed on infants affected by CONS sepsis. CONS sepsis was significantly associated with adverse NI and CP.
			Cerebral palsy (CP)	Odds Ratio 1.7 (95% CI 1.02 to 2.87)	
Hemels MA et al 2012 (2)	Preterm infants <32 weeks with CONS sepsis (n=28) were compared to preterm infants without sepsis (n=42).	Prospective cohort study (2b)	MRI abnormalities	No significant differences between groups p>0.05	No significant differences in neurodevelopmental outcomes or white matter damage.
			Neurodevelopment at 24 months	No significant differences noted between groups p>0.05	
Alshaikh B et al 2014 (6)	Preterm infants <29 weeks gestation with CONS sepsis (n=105) compared to preterm infants without sepsis (n=227).	Retrospective cohort study (2b)	Cognitive Delay	Adjusted Odds Ratio 2.23 (95% CI 1.01 to 4.9)	After adjusting for confounders CONS sepsis was associated with cognitive delay but not major disability.
			Major disability	Adjusted Odds Ratio 1.14 (95% CI: 0.55 to 2.34)	
Mittendorf R et al 2001 (7)	Infants with preterm labour <34 weeks. Infants with CP (n=5) compared to healthy controls (n=102).	Case-control study (3b)	Incidence of cerebral palsy	Adjusted Odds Ratio 3.0, p=0.42	No significant association between CP and preceding CONS sepsis but CP group included only 5 patients.

Table 3.6: list of included studies

3.2.5. *Commentary*

LOS is a commonly encountered problem in the NICU which is associated with increased mortality ⁴⁰⁷, and prolonged hospital stay in paediatric patients ^{407,470}. CONS are recognised as one the most important causes of LOS among premature neonates ⁴⁷¹⁻⁴⁷⁹. Many factors predispose premature neonates to CONS sepsis including immature mucosal barriers, and impaired innate immune responses ⁴²⁹. Historically CONS have been viewed as more indolent infecting organisms as they are less frequently associated with focal complications such as osteomyelitis or meningitis, however they are more likely to cause persistent infection in VLBW neonates ⁴⁸⁰. CONS are also the most commonly isolated organism in cases of polymicrobial bloodstream infection where they have increased mortality and complications compared to single-organism infections ^{481,482}. The prevention of CONS sepsis is therefore a high priority in neonatal care ⁴⁸³ and several interventions including improved hand hygiene, central line management and more rigorous diagnostic criteria for nosocomial infection are known to reduce the incidence of neonatal CONS sepsis ^{473,484,485}.

CONS infections have also been associated with an attenuated neonatal inflammatory response ⁴⁸⁶, a factor which is potentially problematic in the premature neonate where immune responses are already impaired. It is now also recognised that every episode of bacteraemia within the premature population can cause cerebral injury even in the absence of meningitis ⁴⁸⁷. This fact is important given that CONS is the commonest cause of LOS among premature neonates ⁴⁶² and evidence from animal models supports the role of CONS in contributing to inflammatory brain injury ⁴⁸⁸. Additionally, CONS sepsis is a disease that targets a particularly high-risk neonatal population who are generally more medically complex, of lower gestational age and of lower birthweight ⁴⁸⁹⁻⁴⁹¹. Thus, while CONS have historically been viewed as less invasive infective organisms, CONS sepsis occupies an important place in models of premature neonatal sepsis as it often affects those patients who are more vulnerable to long-term neurodevelopmental complications.

The only systematic review and meta-analysis identified ³⁷² included observational studies comparing neurodevelopmental outcomes in VLBW neonates

with culture-proven sepsis and non-septic VLBW controls. A subgroup analysis within this publication identified 3 prospective cohort studies on neonates with CONS sepsis^{409,442,467} which all had similar methodology and all used the same developmental assessment scale (BSID) to follow-up participants at 18-24 months corrected age. While similar in many regards, inclusion criteria differed between studies (based on either birth weight or gestational age) and it is not clear whether assessors at developmental follow-up were blinded to previous sepsis episodes. Pooled analysis found significant associations between the presence of CONS sepsis and adverse neurodevelopmental outcome as measured by neurodevelopmental index and CP diagnosis.

1 retrospective cohort study of premature neonates born at <29 weeks with confirmed CONS sepsis evaluated neurodevelopment as the primary outcome⁴⁶⁸. The Wechsler Preschool and Primary Scale of Intelligence-Revised, Bayley Scales of Infant Development II or Stanford-Binet IV scales were used during follow-up. This study used a clear case definition, included all cases of confirmed CONS sepsis in patients <29 weeks, had an appropriate control population and had relevant follow-up data for most patients enrolled. This study found that CONS sepsis in those born <29 weeks was associated with cognitive delay but not major disability after adjusting for potential confounders such as gestational age and IVH.

Of the 4 prospective cohort studies identified from our literature review 3 were included within the systematic review on developmental outcomes discussed earlier. The remaining prospective cohort study by Hemels *et al.* examined MRI results and neurodevelopmental outcomes in preterm infants born <32 weeks with CONS sepsis and compared these to non-septic premature neonates. The study had clear case definitions and follow-up data was available at 15 and 24 corrected months in more than 90% of those enrolled. The study found no difference in findings of white matter injury on a term-corrected MRI brain between groups and no differences in neurodevelopmental outcome at 2 years using the Griffiths Mental Development Scales, though notably results were not corrected for potential confounders⁴⁶⁶.

1 case-control study was identified which examined the microbiological antecedents of CP by studying the blood culture results of CP patients who were

previously enrolled in a study of MgSO₄ in women entering premature labour at <34 weeks ⁴⁶⁹. Case definition for CONS sepsis was based on microbiological results alone, with no data on clinical assessment or other laboratory parameters. This study found no association between CONS sepsis and subsequent CP at 18 months after controlling for potential confounders, though notably only 5 patients with CP were included and detailed data on developmental scoring was not provided. Interestingly the same study purported an association between CONS cultured from maternal swabs and subsequent diagnosis of CP ⁴⁶⁹. While on the surface this may seem like an interesting observation, the authors do not prove causation, do not present mechanistic data to support the association observed and most notably the findings have not since been replicated in similar studies ⁴⁹².

3.2.6. *Conclusion*

The only systematic review identified after literature search found that CONS sepsis was associated with both adverse neurodevelopmental outcome and CP. While the inclusion criteria differed somewhat between studies included, the methodology and endpoints were otherwise similar, and the pooled data included 4431 infants. The remaining observational studies identified on literature review were smaller and of poorer design. The findings of these studies were variable with regards to neurodevelopmental outcome and notably several did not adjust for potential confounders. Also notable is that none of the studies included investigated the potential effects of differing antibiotic regimens on the developmental outcomes of those enrolled. Based on the literature search performed, best evidence suggests that CONS sepsis is associated with adverse developmental outcome, though all studies identified from literature search were observational in nature and their causal inference is limited by the inability to show a dose response relationship (Grade B).

3.2.7. *Clinical bottom line and CEBM grade of evidence*

1. CONS is the most common organism in LOS among preterm infants and is associated with adverse outcome (Grade B).

2. Similar to other episodes of neonatal sepsis, CONS sepsis is associated with developmental impairment in the preterm neonate (Grade B).
3. Due to the potential association with developmental impairment, episodes of CONS sepsis are important considerations during subsequent developmental follow-up of affected preterm infants (Grade B).

Chapter 4: Organ dysfunction scoring in preterm neonates

4.1. Introduction

Organ dysfunction is a common and serious complication of preterm delivery ¹⁰⁴. Adults ICU patients are similarly affected and clinical scoring systems based on organ dysfunction and illness severity, which are in routine use in adult intensive care management, are of great prognostic value ⁹⁸. Prognostic scores based on clinical illness severity are not a new idea in neonatology, though several issues limit the use of existing scores in neonatal practice ¹¹⁴. Some illness severity scores, such as the Neonatal Therapeutic Intervention Scoring System (NTISS), are complex and lengthy to perform making them impractical for use at the bedside ¹⁰⁹. Others, like the Clinical Risk Index for Babies (CRIB score) which was developed to predict neonatal mortality ¹⁰⁷, while easy to use, are based largely on demographic data from birth, cannot be used outside the initial hours of life and do not individualise prognosis based on the clinical status of the neonate. In addition, few of the neonatal scores proposed have been adequately validated outside their original description ⁴⁹³. In contrast, the Neonatal Multiple Organ Dysfunction score (NEOMOD), which was originally developed to measure preterm neonatal organ dysfunction in VLBW neonates ^{101,104}, is simple to perform, clinically validated and requires only routinely available clinical and laboratory data ^{101,103,104}. The NEOMOD score provides an individualised measure of neonatal illness severity in real-time and uses this to predict neonatal mortality (Appendix v). Thus, while infrequently used in clinical practice, the NEOMOD is useful as both a measure of acute illness and a predictor of later outcome.

In addition to the prematurity-related organ dysfunction present at birth, preterm neonates are also at increased risk of sepsis-induced organ dysfunction compared to neonates born at term ⁷². The Sepsis-3 initiative in adult medicine recently produced consensus definitions for adults sepsis and highlighted the pivotal role of organ dysfunction in defining and screening for sepsis ¹⁰⁰. They produced the SOFA and qSOFA scores to screen for those at high risk of adverse outcome in suspected sepsis, and these scores have subsequently been validated and widely adopted into clinical practice ¹⁰⁰. Following the lead of Sepsis-3, Wynn *et al.* recently proposed the nSOFA

as a measure of organ dysfunction to help screen for and predict outcome in cases of neonatal sepsis ¹²⁹ (appendix v). The nSOFA, an organ dysfunction score based on respiratory, cardiovascular and haematological dysfunction, predicts mortality in preterm neonates with confirmed LOS though the initial validation study used repeated measurements and the prognostic value outside of blood culture-positive sepsis has not yet been studied ¹²⁹. Given that the initial validation study used only patients with microbiologically confirmed sepsis, the value of nSOFA needs to be established in an undifferentiated cohort of patients undergoing sepsis evaluation. Additionally, the performance of the nSOFA in comparison to existing scores such as the NEOMOD has not yet been assessed.

While many organ dysfunction scores have been shown to predict neonatal outcome ^{114,493}, existing validation studies often require the use of repeated measurements taken over a prolonged period, limiting their practical applications in a clinical setting. The utility of isolated measurements of organ dysfunction in the first week of life and during episodes of sepsis by scores such as the NEOMOD and nSOFA have not yet been established. If validated, such simple measurements could prove a practical yet effective tool in detecting clinical deterioration, identifying high risk patients, guiding clinical care and counselling parents.

Male preterm neonates have poorer outcomes than females across a variety of clinical areas ⁴⁹⁴. The aetiology of these differences remains poorly understood and it is unclear if early life organ dysfunction is an important contributor. While preterm males are generally thought to have higher early life morbidity than females, there are no studies to date which have objectively measured and compared organ dysfunction early in life between the sexes. Additionally, while males are more prone to sepsis ³⁶⁰ and are at higher risk of mortality following sepsis ^{250,252}, patterns of organ dysfunction during sepsis episodes have not yet been compared between preterm males and females. Understanding potential sex differences in organ dysfunction both early in life and during episodes of sepsis could help guide clinical care in addition to identifying areas for future research.

4.2. Hypothesis

Organ dysfunction after birth and sepsis-related organ dysfunction may be higher in male versus female preterm neonates and may predict clinical outcomes.

4.3. Aims

- To evaluate the prognostic value of early life organ dysfunction measurement in the premature population.
- To evaluate the prognostic value of sepsis-induced organ dysfunction measurement in the premature population.
- To compare patterns of organ dysfunction between premature males and females in the early neonatal period.
- To compare sepsis incidence between male and female preterm neonates and examine sex differences in sepsis-induced organ dysfunction during these episodes.

4.4. Results

4.4.1. Patient characteristics

108 preterm infants had serial organ dysfunction scores recorded (64 male, 44 female). Sepsis episodes were classified as suspected, clinical or microbiological. Microbiological was diagnosed according to the ANZNN guidance ^{412,433} requiring clinical signs of sepsis along with a positive blood culture, CSF culture or PCR. Clinical sepsis was classified based on NEOKISS criteria requiring a clinical picture suggestive of infection, laboratory abnormalities and an intention to treat with ≥ 5 days of antimicrobials in the setting of negative cultures ⁴²⁰. Suspected sepsis represents all other cases in which a sepsis evaluation was undertaken, a minimum of 48 hours of intravenous antibiotic therapy was given but which did not meet the specific criteria for clinical or microbiological sepsis. A total of 154 episodes of suspected (n=110), clinical (n=29) and microbiological (n=15) sepsis were recorded in these patients. The mean gestation of those enrolled was 28.7 (± 2.5) weeks and mean birth weight was 1060 (± 366) grams. Baseline demographics were similar between male and female

infants with the exception of pre-eclampsia which was more common in female neonates (Table 4.1). Septic risk factors were similar between males and females (Table 4.1). While mean gestation did not differ between males and females it is notable that the gestation of females enrolled in this study follows a bimodal distribution (Figure 4.1). This means that although the mean gestation did not differ between males and females, neonates of extreme prematurity are over-represented among the female cohort.

	Male (n=64)	Female (n=44)	p-value
Admission demographics			
Gestation (weeks)	28.9 (± 2.3)	28.4 (± 2.7)	0.28
Birth weight (grams)	1132 (± 250.7)	1013.9 (± 356.9)	0.12
Mode of delivery:			0.27
SVD	8 (12.5%)	8 (18.1%)	
EMCS	50 (78.1%)	35 (79.6%)	
ELCS	6 (9.4%)	1 (2.3%)	
Antenatal steroids complete	58 (90.6%)	36 (81.8%)	0.16
Pre-eclampsia	4 (6.3%)	8 (18.1%)	0.046*
AEDF/REDF	12 (18.75%)	9 (10.5%)	0.77
Arterial cord pH	7.3 (± 0.1)	7.3 (± 0.1)	0.45
Arterial cord base excess	-4.3 (± 4.6)	-5 (± 4)	0.53
Venous cord pH	7.3 (± 0.1)	7.3 (± 0.1)	0.31
Venous cord base excess	-3.6 (± 3.9)	-4.1 (± 3.1)	0.54
Admission pH	7.2 (± 0.1)	7.3 (± 0.1)	0.12
Admission base excess	-6.7 (± 5)	-5.4 (± 4)	0.14
Admission lactate	4.6 (± 3.8)	4.4 (± 3.5)	0.82
Resuscitation details			
1-minute Apgar	5.9 (± 2.4)	6.4 (± 2.2)	0.23
5-minute Apgar	7.7 (± 2.2)	8.3 (± 1.6)	0.11
CPR required	2 (3.1%)	0 (0%)	0.24
IPPV required	46 (71.9%)	18 (40.9%)	0.004*
IPPV duration (minutes)	3 (± 3.5)	1.4 (± 2.9)	0.013*
Time of IPPV commencement (minutes)	1.5 (± 1)	1.6 (± 1)	0.71
Intubated at delivery	26 (40.6%)	9 (20.5%)	0.037*
Time of intubation (minutes)	8.6 (± 5.3)	10 (± 5.3)	0.49
Surfactant administration	20 (31.3%)	7 (15.9%)	0.08
Maximum FiO2 at delivery	74.1 (± 31)	63.1 (± 26.9)	0.06
Septic risk factors			
Spontaneous labour	14 (21.9%)	14 (31.8%)	0.15
Suspected chorioamnionitis	10 (15.6%)	5 (11.4%)	0.53
Maternal antibiotics in labour	28 (43.8%)	19 (43.2%)	0.71
Maternal GBS	3 (4.7%)	2 (4.5%)	0.67
PROM >18 hrs	19 (29.7%)	7 (15.9%)	0.2
Foul smelling liquor	2 (3.15%)	1 (2.3%)	0.69
Neonatal temperature on admission (°C)	37.1 (± 0.6)	36.8 (± 1.6)	0.34
UVC inserted	37 (57.8%)	19 (43.2%)	0.13
UVC duration	37.9 (± 48.29)	29.8 (± 47.3)	0.39
UAC inserted	28 (43.8%)	24 (54.5%)	0.18
UAC duration	33.8 (± 49.9)	22.7 (± 49.7)	0.26

Table 4.1: Comparison of baseline demographics, delivery room resuscitation and septic risk factors between male and female preterm neonates.

Continuous variables are presented as mean ($\pm$ standard deviation) and categorical variables are presented as absolute numbers. AEDF: absent end-diastolic flow; CPR: cardiopulmonary resuscitation; ELCS: elective caesarean delivery; EMCS: emergency caesarean delivery; FiO₂: fraction of inspired oxygen; GBS: Group B Streptococci; IPPV: intermittent positive pressure ventilation; PROM: prolonged rupture of membranes; REDF: reversed end-diastolic flow; SVD: spontaneous vaginal delivery; UAC: umbilical arterial catheter; UVC: umbilical venous catheter.* =significant at $p < 0.05$.

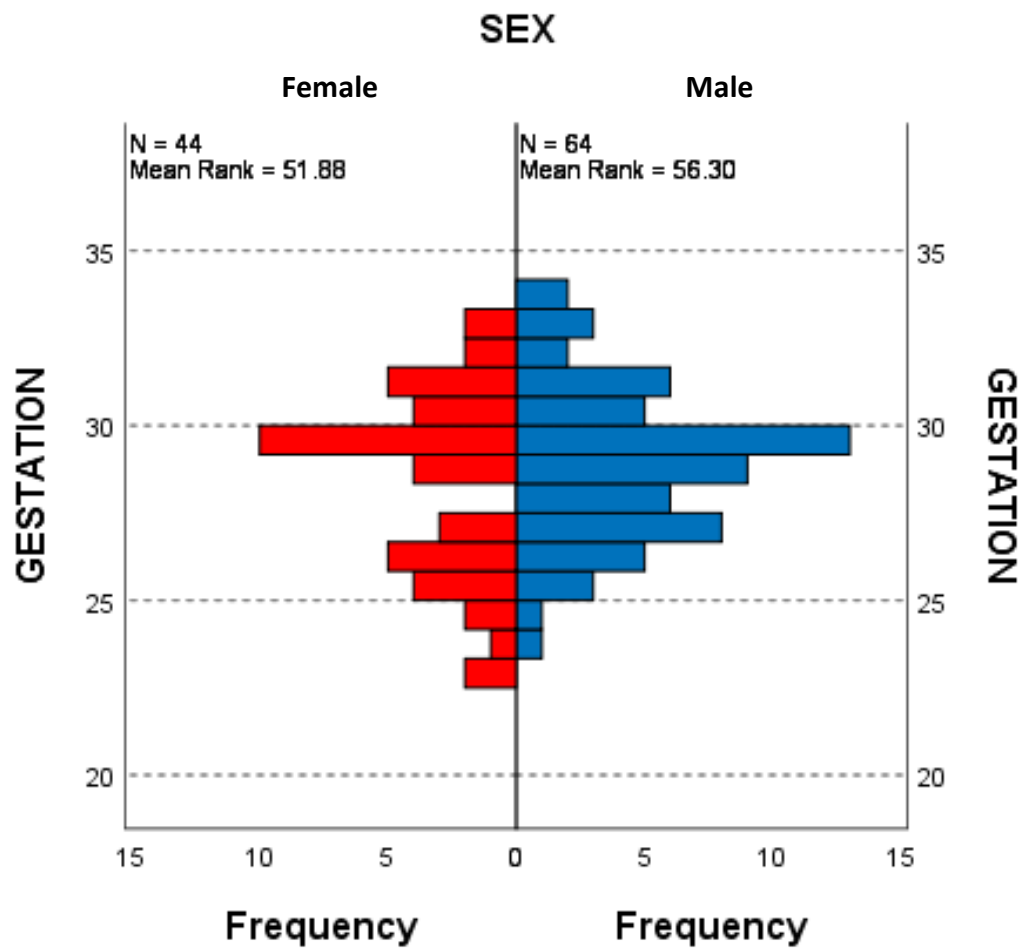


Figure 4.1: Histogram of gestation in male and female preterm neonates with organ dysfunction scores recorded.

Male gestation is normally distributed. Female gestation is bimodal with peaks in extreme prematurity and in moderate-late prematurity.

4.4.2. *Sex-differences in clinical outcomes*

Apgar scores were similar between males and females (Table 4.1). Males were more likely to require respiratory support in the form of IPPV ($p=0.004$) or intubation ($p=0.037$) and had a longer duration of IPPV ($p=0.013$) at delivery than females (Table 4.1). The only 2 neonates to require cardiopulmonary resuscitation were both male (Table 4.1). Male infants were more likely to develop PVL ($p=0.047$) and CLD compared to females ($p=0.034$) (Table 4.2). All other clinical outcomes were similar between male and female preterm neonates (Table 4.2).

	Male (n=64)	Female (n=44)	p-value
Death	2	4	0.18
Abnormal CRUS	29	14	0.17
IVH Y/N	7	7	0.56
Death/severe IVH	3	4	0.44
PVL	11	2	0.047*
ROP	12	10	0.63
Ventilated Y/N	31	16	0.3
Duration of mechanical ventilation	3.1 (3.1)	5.1 (11.2)	0.21
Surfactant administered	37	24	0.44
No. surfactant doses	0.8 (0.8)	0.7(0.7)	0.3
CPAP days	19.5 (17.9)	16.7 (21)	0.48
High flow days	4.3 (7.2)	2.7 (6.3)	0.26
NP Days	2.6 (5.6)	2.1 (9.4)	0.77
Maximum fractional inspired O ₂	45 (25.9)	42.8 (24.7)	0.66
Pulmonary haemorrhage (Y/N)	5	2	0.5
CLD	32	13	0.034*
PDA	24	16	0.7
Inotrope required (Y/N)	8	9	0.29
Parental nutrition duration	8.4 (5.6)	8.2 (6.4)	0.88
NEC	5	4	0.59
Days to full enteral feeds	8.3 (3.2)	8 (4.2)	0.69
Days to full bottle/breast feeds	50.7 (21.7)	50.4 (31.1)	0.96
Minimum haemoglobin	10.6 (2.8)	11.1 (3.7)	0.46
Maximum leukocyte count	18.4 (11.1)	20.8 (16.9)	0.37
No. antibiotic days	4.9 (5.7)	5.5 (8.12)	0.69
Length of stay	54.9 (22.3)	56.8 (32.9)	0.77

Table 4.2: Comparison of clinical outcomes between male and female preterm neonates.

Continuous variables are presented as mean ($\pm$ standard deviation) and categorical variables are presented as absolute numbers. IVH: intraventricular haemorrhage; PVL: periventricular leukomalacia; ROP: retinopathy of prematurity; CPAP: continuous positive airway pressure; NP: nasal prongs oxygen; CLD: chronic lung disease; PDA: patent ductus arteriosus; NEC: necrotising enterocolitis. *=significant at $p < 0.05$.

4.4.3. *Early life organ dysfunction and sepsis-related organ dysfunction*

Modified NEOMOD and nSOFA scores were calculated for all participants on the first and fifth day of life and values were collected as previously described¹⁰⁴. In cases of sepsis, NEOMOD and nSOFA scores were collected on the first day of sepsis evaluation only. NEOMOD and nSOFA scores showed significant positive correlations with each other on day of life 1 ($r=0.436$, $p<0.001$) and day of life 5 ($r=0.603$, $p<0.001$; Figure 4.2).

Baseline scores were examined in those who died before discharge and compared to those who survived. Comparisons of scores taken on day 1 of life showed that total scores were similar between survivors and those who died before discharge. However, cardiovascular dysfunction on NEOMOD was higher in those who subsequently died ($p=0.001$) (Figure 4.3). Comparison of scores taken on day of life 5 showed that neonates who died before discharge had significantly higher total NEOMOD ($p=0.019$) and nSOFA scores ($p=0.01$). Those who died before discharge also had higher levels of acid/base ($p<0.001$) and microvascular dysfunction ($p=0.04$) on NEOMOD scoring and higher levels of respiratory dysfunction on the nSOFA score ($p=0.005$; Figure 4.4).

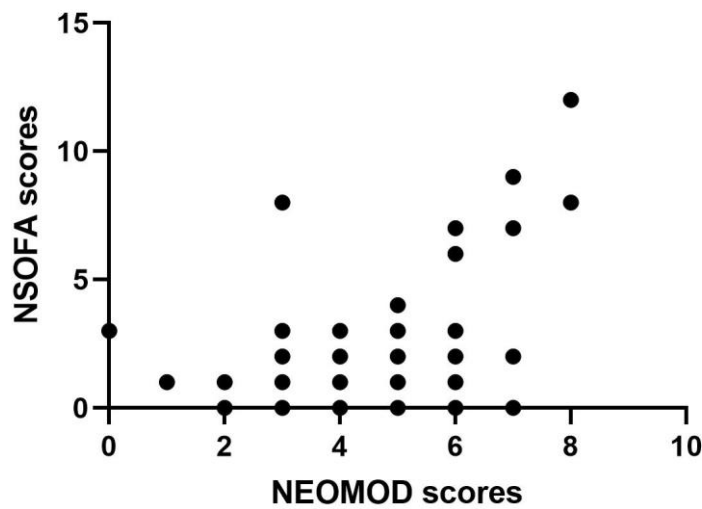
Baseline scores were then examined in those who has the composite outcome of death and/or IVH and compared to those who survived to discharge without IVH. On day of life 1 those in the death/IVH group had higher total nSOFA scores ($p=0.009$) and greater levels of cardiovascular dysfunction on both the NEOMOD ($p<0.001$) score and nSOFA score ($p<0.001$; Figure 4.5). On day 5 of life those in the death/IVH group had higher total NEOMOD ($p<0.001$) and nSOFA scores ($p<0.001$); higher neurological ($p<0.001$), haematological ($p=0.011$), respiratory ($p<0.001$), cardiovascular ($p=0.011$), acid/base ($p=0.02$) and microvascular ($p<0.001$) dysfunction on the NEOMOD score; and higher respiratory ($p=0.04$), cardiovascular ($p<0.001$) and platelet dysfunction ($p=0.011$) on the nSOFA score (Figure 4.6).

Those who died during a sepsis episode had higher total NEOMOD ($p<0.001$) and nSOFA scores ($p<0.001$) compared to those who survived the episode. Infants with sepsis-related mortality also had higher levels of haematological ($p<0.001$), gastrointestinal ($p=0.044$), cardiovascular ($p<0.001$), acid/base ($p=0.007$) and

microvascular dysfunction ($p=0.014$) on NEOMOD scoring and higher levels of respiratory ($p=0.011$) and cardiovascular dysfunction ($p<0.001$) on nSOFA scoring (Figure 4.7).

A.

Correlation of day 1 organ scores



B.

Correlation of day 5 organ scores

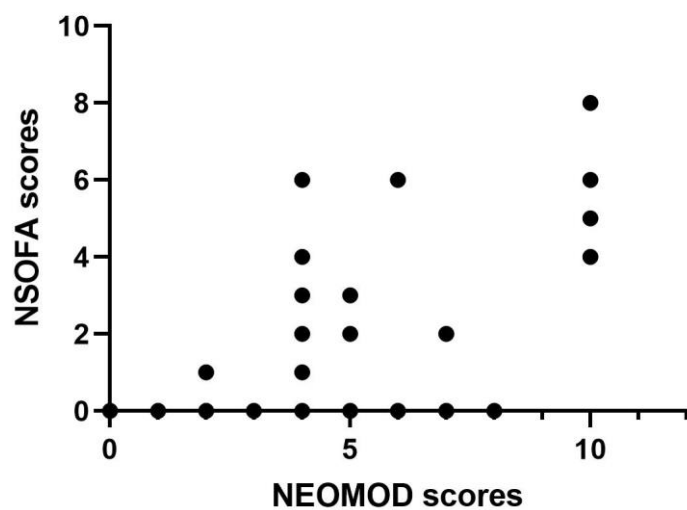
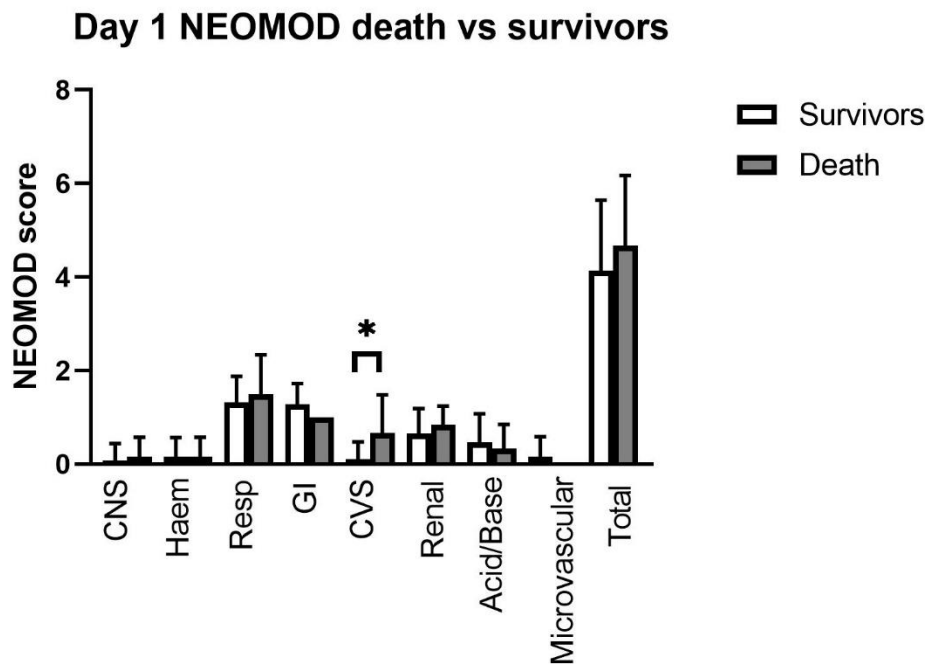


Figure 4.2: Comparison of NEOMOD and nSOFA scores recorded in early life

Comparison of NEOMOD and nSOFA scores recorded in early life in preterm patients (n=108). A.) NEOMOD and nSOFA scores on day 1 of life. B.) NEOMOD and nSOFA scores on day 5 of life.

A.



B.

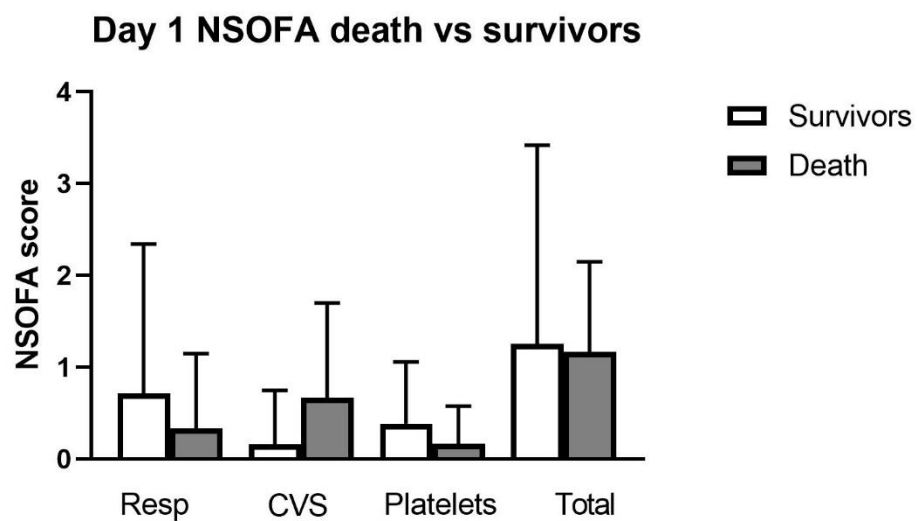
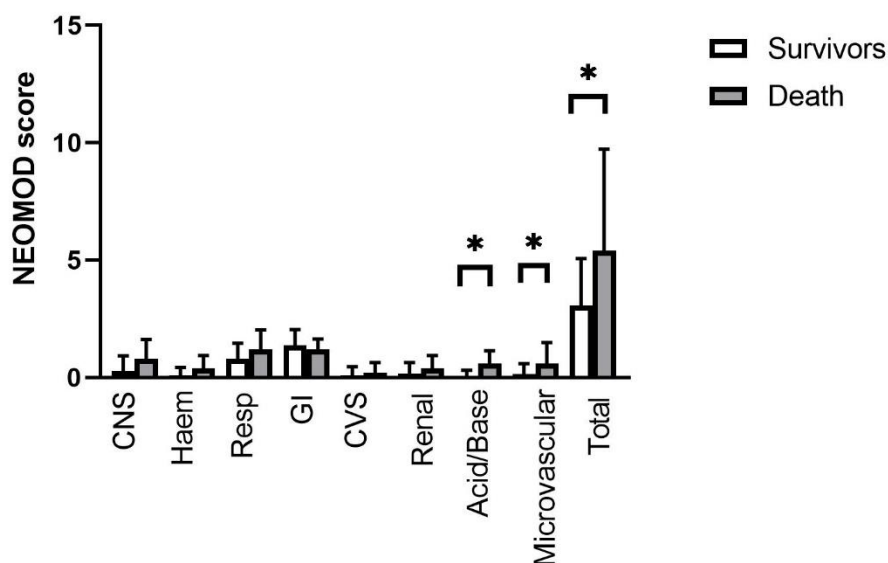


Figure 4.3: Comparison of organ dysfunction scores on day 1 of life in preterm neonates who died before discharge and those who survived.

Comparison of organ dysfunction scores on day 1 of life in preterm neonates who died before discharge (n=6) and those who survived (n=102). A) NEOMOD scores on day of life 1 in survivors and those who died before discharge. B) nSOFA scores on day of life 1 in survivors and those who died before discharge. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

A. Day 5 NEOMOD death vs survivors



B. Day 5 NSOFA death vs survivors

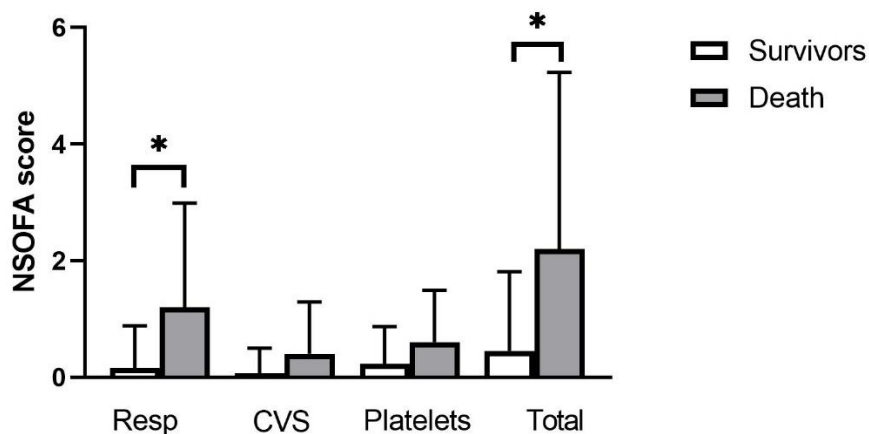


Figure 4.4: Comparison of organ dysfunction scores on day 5 of life in preterm neonates who died before discharge and those who survived.

Comparison of organ dysfunction scores on day 5 of life in preterm neonates who died before discharge (n=6) and those who survived (n=102). Values displayed represent mean and standard deviation. A) NEOMOD scores on day of life 5 in survivors and those who died before discharge. B) nSOFA scores on day of life 5 in survivors and those who died before discharge. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

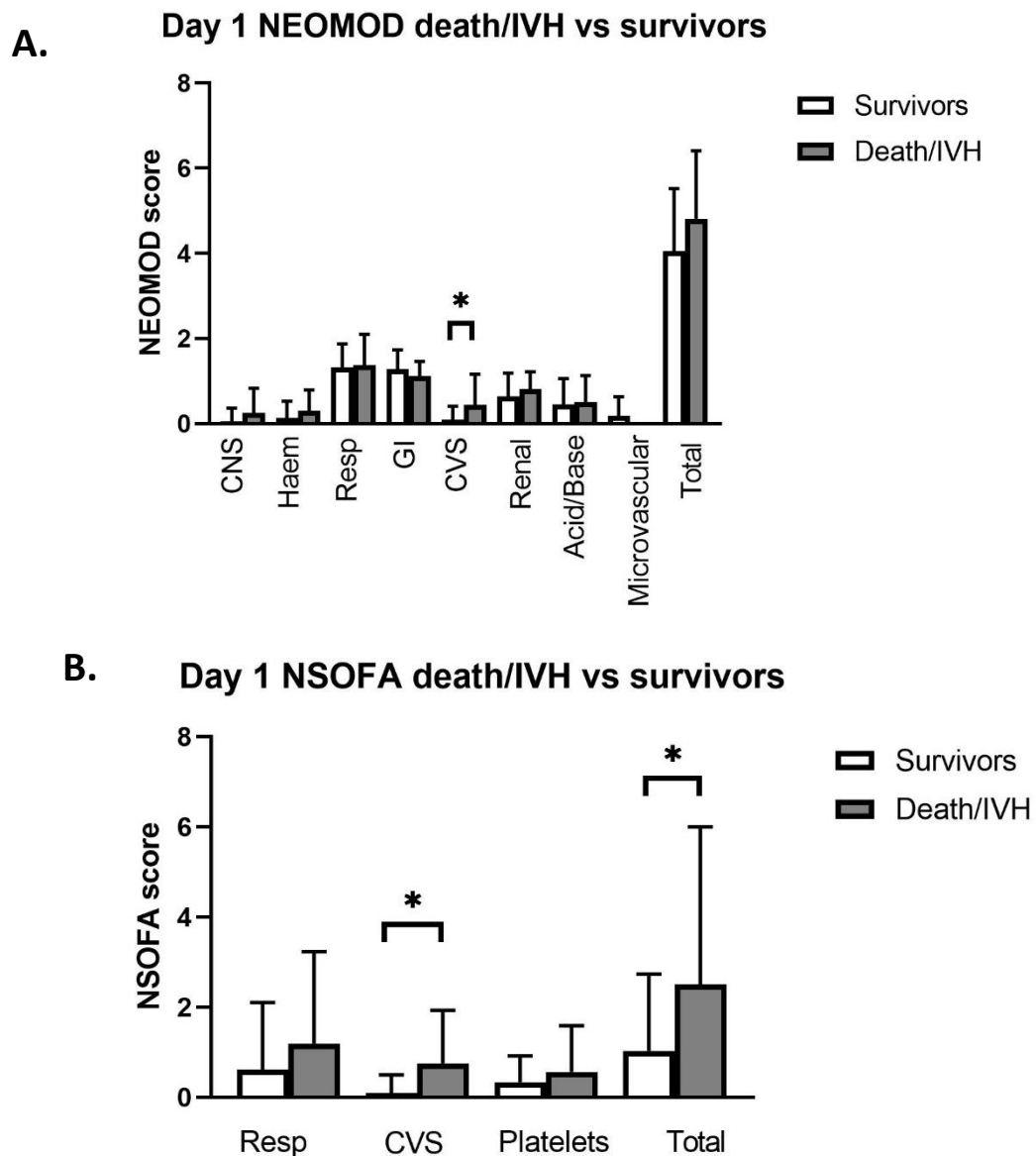


Figure 4.5: Comparison of organ dysfunction scores on day 1 of life in preterm neonates who had the composite outcome of death before discharge and/or IVH, and those who survived without IVH.

Comparison of organ dysfunction scores on day 1 of life in preterm neonates who had the composite outcome of death before discharge and/or IVH (n=17), and those who survived without IVH (n=91). Values displayed represent mean and standard deviation. A) NEOMOD scores on day of life 1 in those surviving without IVH and those in the death/IVH group. B) nSOFA scores on day of life 1 in those surviving without IVH and those in the death/IVH group. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

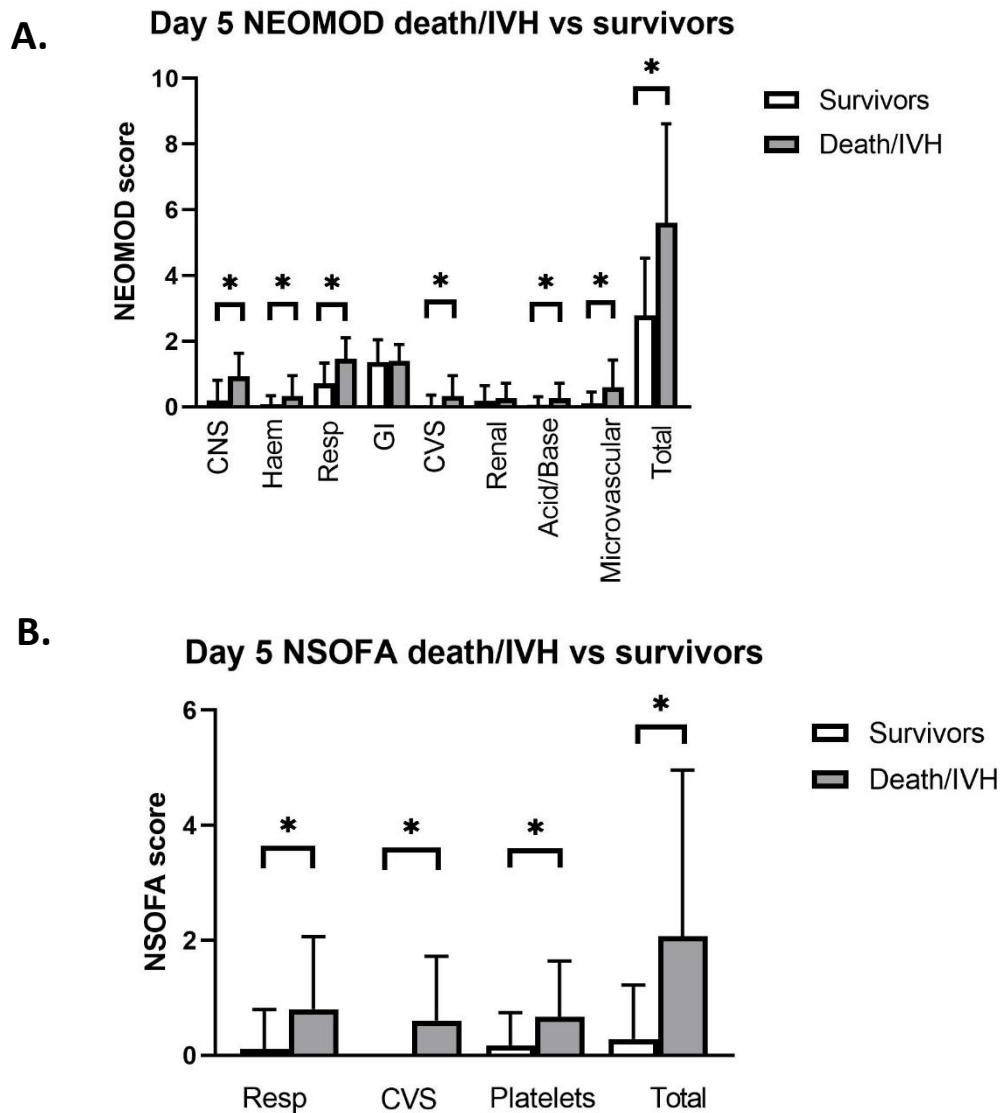


Figure 4.6: Comparison of organ dysfunction scores on day 5 of life in preterm neonates who had the composite outcome of death before discharge and/or IVH, and those who survived without IVH.

Comparison of organ dysfunction scores on day 5 of life in preterm neonates who had the composite outcome of death before discharge and/or IVH (n=17), and those who survived without IVH (n=91). Values displayed represent mean and standard deviation. A) NEOMOD scores on day of life 5 in those surviving without IVH and those in the death/IVH group. B) nSOFA scores on day of life 5 in those surviving without IVH and those in the death/IVH group. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

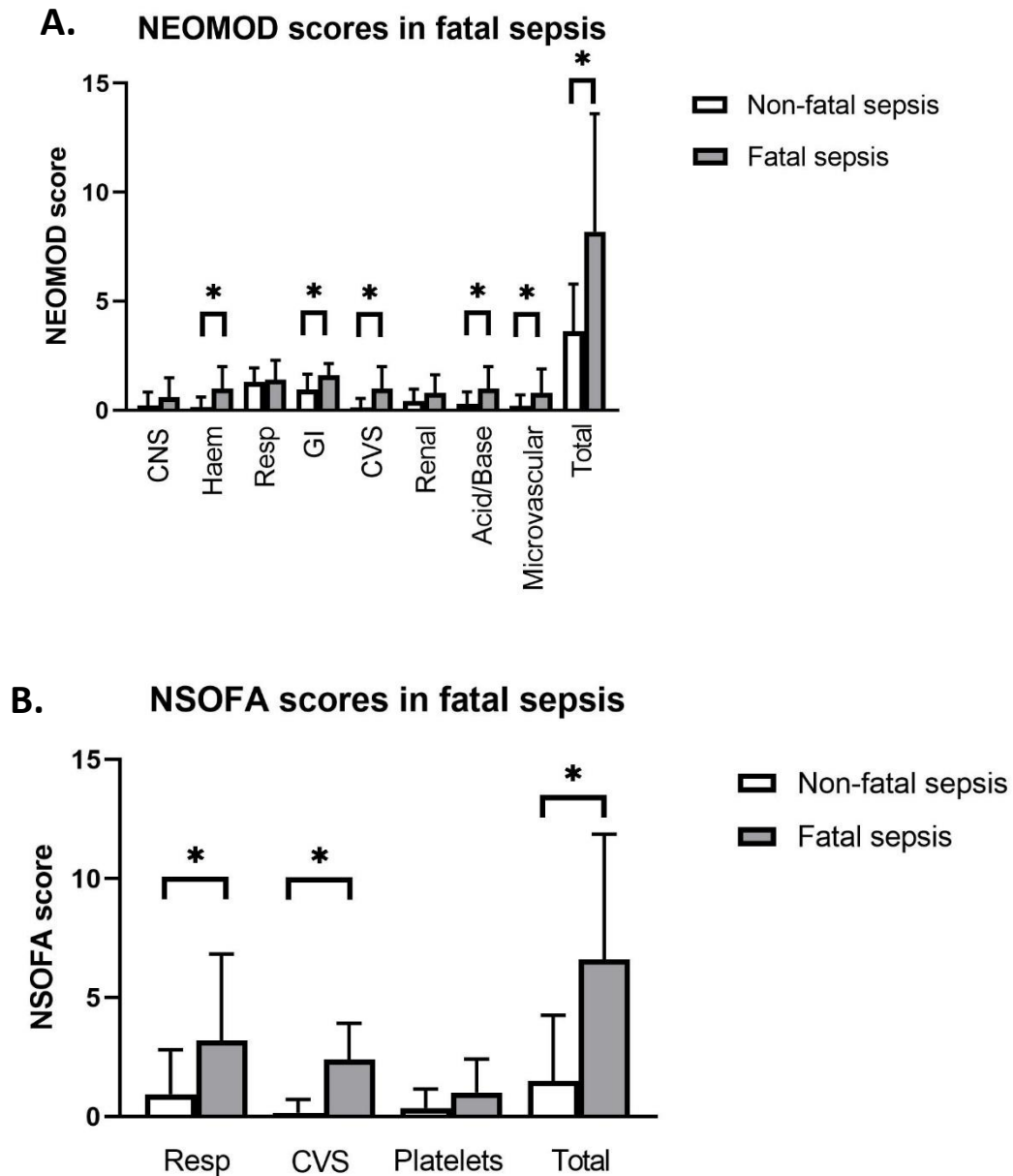


Figure 4.7: Comparison of organ dysfunction scores on the first day of sepsis in those who died of sepsis and those who survived.

Comparison of organ dysfunction scores on day 1 of infection during episodes of fatal sepsis (n=5) and non-fatal sepsis (n=149). Values displayed represent mean and standard deviation. A) NEOMOD scores in those who died of sepsis and those who survived. B) nSOFA scores those who died of sepsis and those who survived. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

4.4.4. Prognostic value of organ dysfunction in preterm neonates

Exploration of baseline organ dysfunction scores as a predictor of death using linear regression showed a significant relationship between total NEOMOD scores on day of life 5 and death before discharge ($p=0.031$) and between total nSOFA scores on day 5 of life and death before discharge ($p=0.031$). The predictive value of these scores were then examined further using receiver operator curves and no statistically significant association between the scores and death before discharge was demonstrated ($p>0.05$; Figure 4.8).

Exploration of baseline organ dysfunction scores as a predictor of the composite outcome of death/IVH using linear regression showed a significant relationship between total nSOFA on day of life 1 and death/IVH ($p=0.02$), total NEOMOD on day of life 5 and death/IVH ($p<0.001$) and total nSOFA on day of life 5 and death/IVH ($p=0.001$). The predictive value of these scores were then examined further using receiver operator curves (Figure 4.9). The area under the curve for day 1 nSOFA scores was 0.650 (95% CI: 0.514-0.804) and was significant at $p=0.032$. The area under the curve for day 5 NEOMOD was 0.759 (95% CI: 0.631-0.886) and was significant at $p<0.001$. Using the coordinates of the ROC day 1 nSOFA performed poorly as a predictor of death/IVH with the best cut-off value, that of ≥ 0.5 , having a sensitivity of 70% but a specificity of only 55%. Day 5 NEOMOD performed well as a predictor of death/IVH with a value of ≥ 3.5 having a sensitivity of 75% and a specificity of 77%.

Exploration of organ dysfunction scores during sepsis as predictors of death in cases of sepsis using linear regression showed a significant relationship between total NEOMOD scores and sepsis mortality ($p<0.001$), and a significant relationship between total nSOFA scores and sepsis mortality ($p<0.001$). The area under the curve for nSOFA was 0.815 (95% CI: 0.574-1.056) and was significant at $p=0.01$. nSOFA during sepsis performed well as a predictor of sepsis-related mortality with a value of >3.5 having a sensitivity of 80% and a specificity of 86% (Figure 4.10).

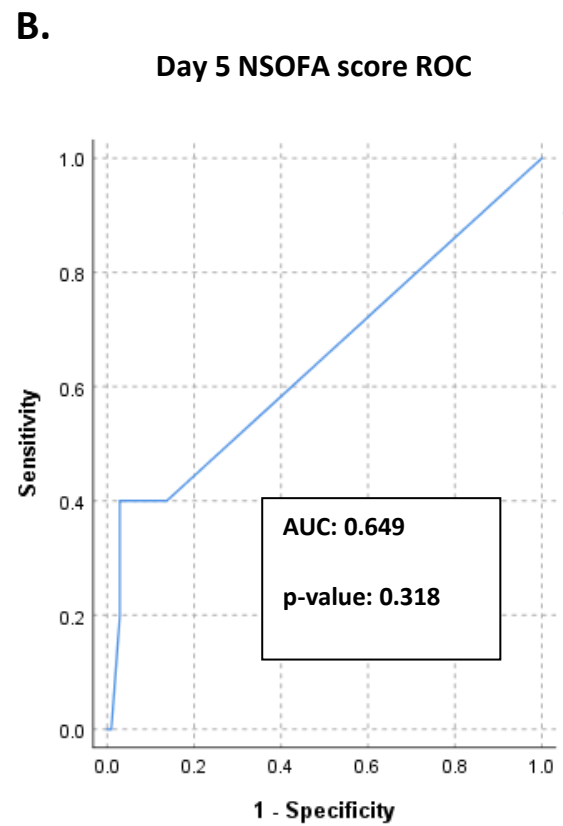
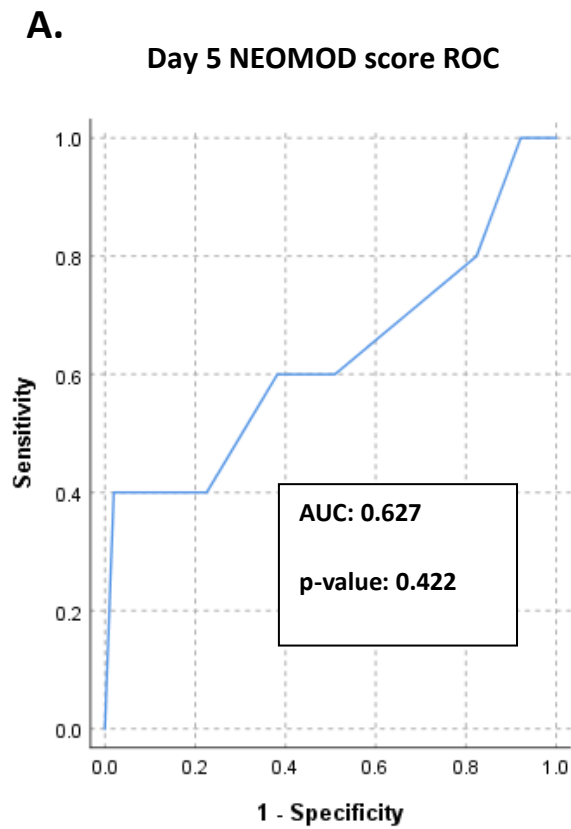
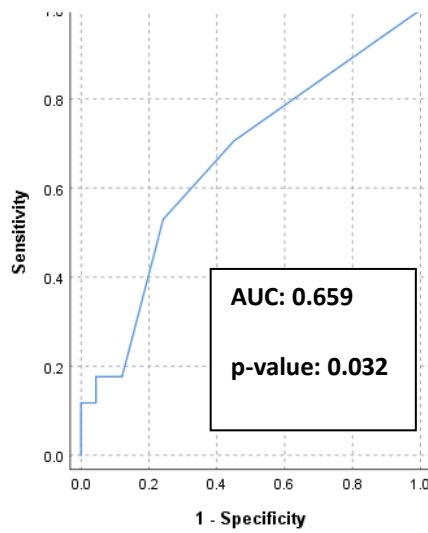


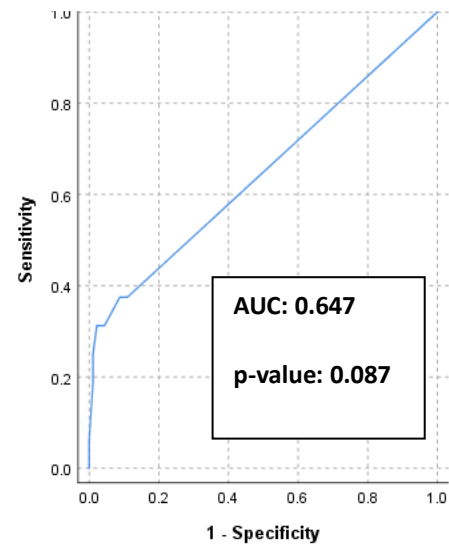
Figure 4.8: Receiver operator curves of organ scores on day 5 of life and death.

Receiver operator curves of organ scores on day 5 of life and death (n=108). A) Day 5 NEOMOD measurements. B) Day 5 nSOFA measurements.

A. Day 1 NSOFA score ROC



B. Day 5 NSOFA score ROC



C. Day 5 NEOMOD score ROC

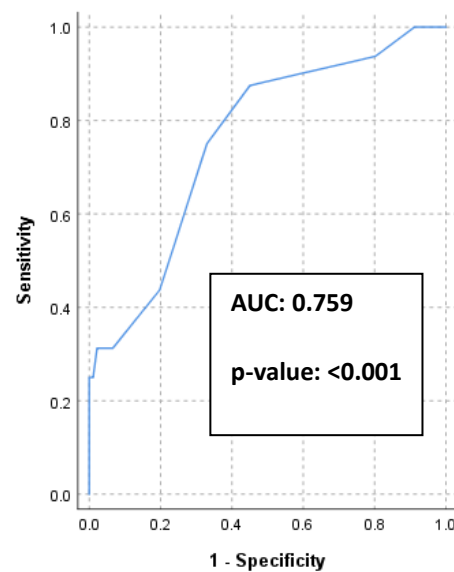


Figure 4.9: Receiver operator curves of early life organ scores and death/IVH.

Receiver operator curves of early life organ scores and death/IVH (n=108). A) Day 1 nSOFA measurements. B) Day 5 nSOFA measurements. C) Day 5 NEOMOD measurements.

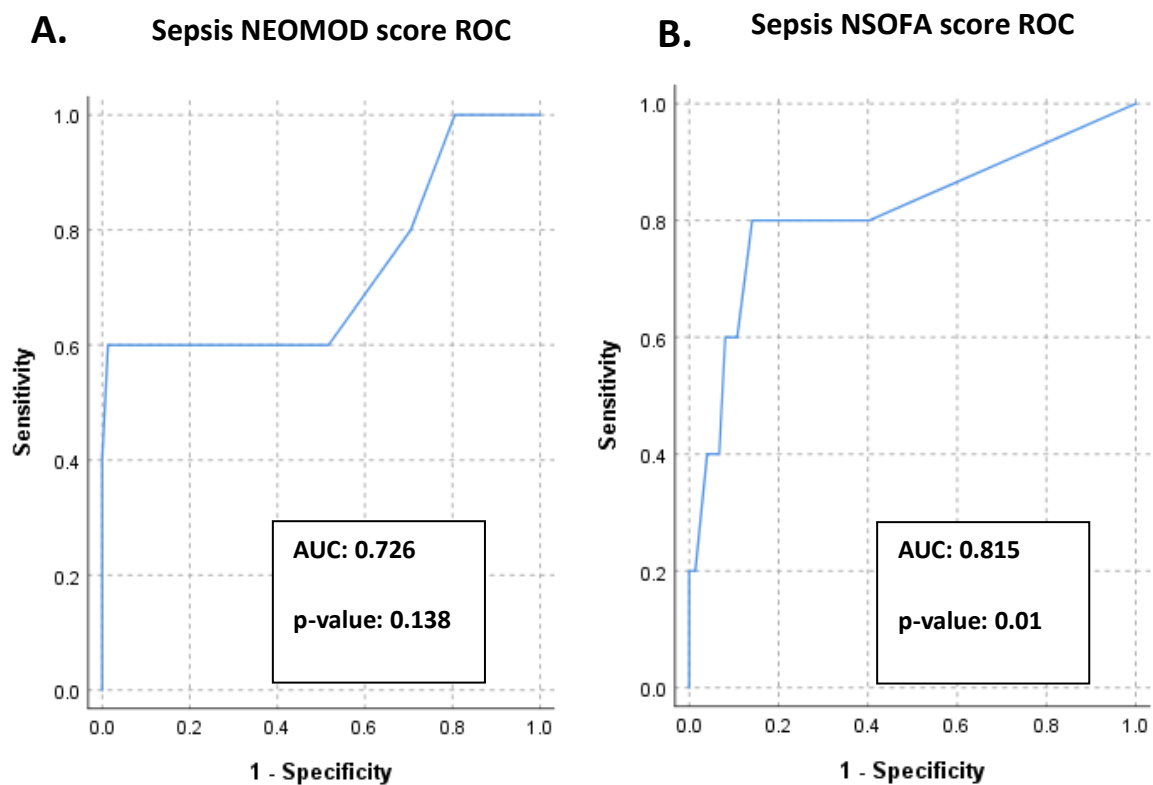


Figure 4.10: Receiver operator curves of organ dysfunction scores during sepsis episodes and sepsis-related mortality.

Receiver operator curves of organ dysfunction scores during sepsis episodes and sepsis-related mortality (n=108). A) NEOMOD measurements during sepsis. B) nSOFA scores during sepsis.

4.4.5. *Sex-differences in sepsis incidence and organ dysfunction*

Baseline scores on day 1 and day 5 were compared between male and female preterm infants. On day of life 1 male infants had significantly higher total NEOMOD scores compared to females ($p=0.016$). Males also had higher microvascular ($p=0.005$) and acid/base scores ($p=0.038$) compared to females. There was a trend towards higher total nSOFA scores in males on day of life 1, though this did not reach statistical significance ($p=0.078$). Males had significantly higher levels of respiratory dysfunction on nSOFA scoring on day 1 compared to females ($p=0.003$; Figure 4.11). On day of life 5 male infants had significantly higher total NEOMOD scores compared to females ($p=0.016$). Male infants also had significantly higher levels of gastrointestinal dysfunction on NEOMOD scoring on day of life 5 ($p=0.047$). nSOFA scores did not differ between males and females on day of life 5 (Figure 4.12).

The incidence of sepsis did not differ between males and females (Table 4.3). Comparison of organ dysfunction scores during episodes of suspected sepsis showed that male infants had higher total NEOMOD ($p=0.039$) and nSOFA scores ($p=0.024$). Males also had higher levels of gastrointestinal ($p=0.003$) and microvascular ($p=0.035$) dysfunction as measured on the NEOMOD score and greater levels of respiratory dysfunction as measured on the nSOFA score ($p=0.012$; Figure 4.13). Comparison during episodes of clinical sepsis showed similar total scores between males and females though males had greater gastrointestinal dysfunction as measured on the NEOMOD score ($p=0.027$; Figure 4.14). Comparison during episodes of microbiological sepsis showed similar total scores between males and females though females had higher levels of microvascular dysfunction ($p=0.013$) as measured on the NEOMOD score and higher levels of platelet dysfunction as measured by the nSOFA score ($p=0.008$; Figure 4.15).

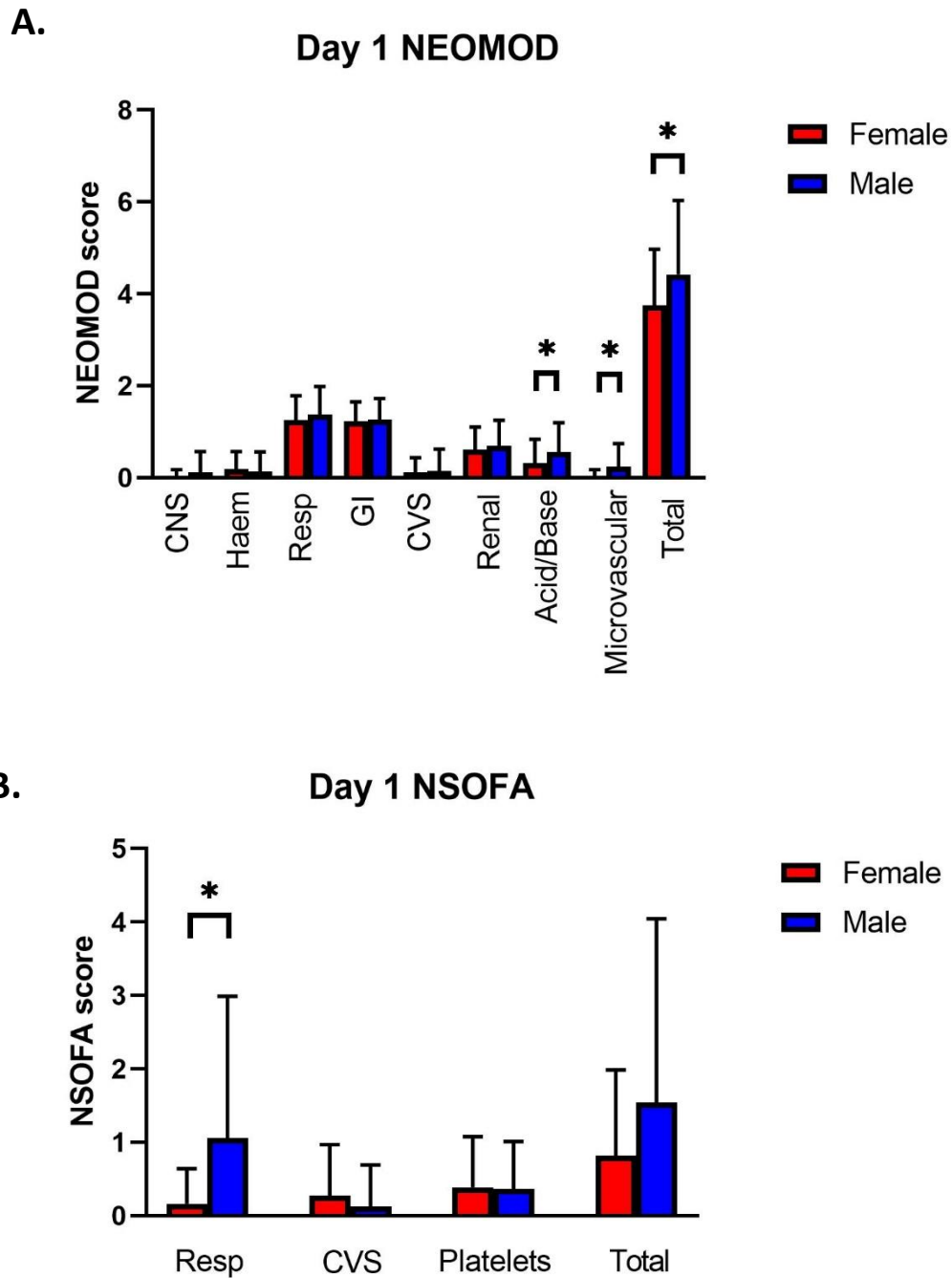


Figure 4.11: Comparison of organ dysfunction in preterm males and females on day of life 1.

Comparison of organ dysfunction in preterm males (n=64) and females (n=44) on day of life 1. Values displayed represent the mean and standard deviation. A) Day 1 NEOMOD scores. B) Day 1 nSOFA scores. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

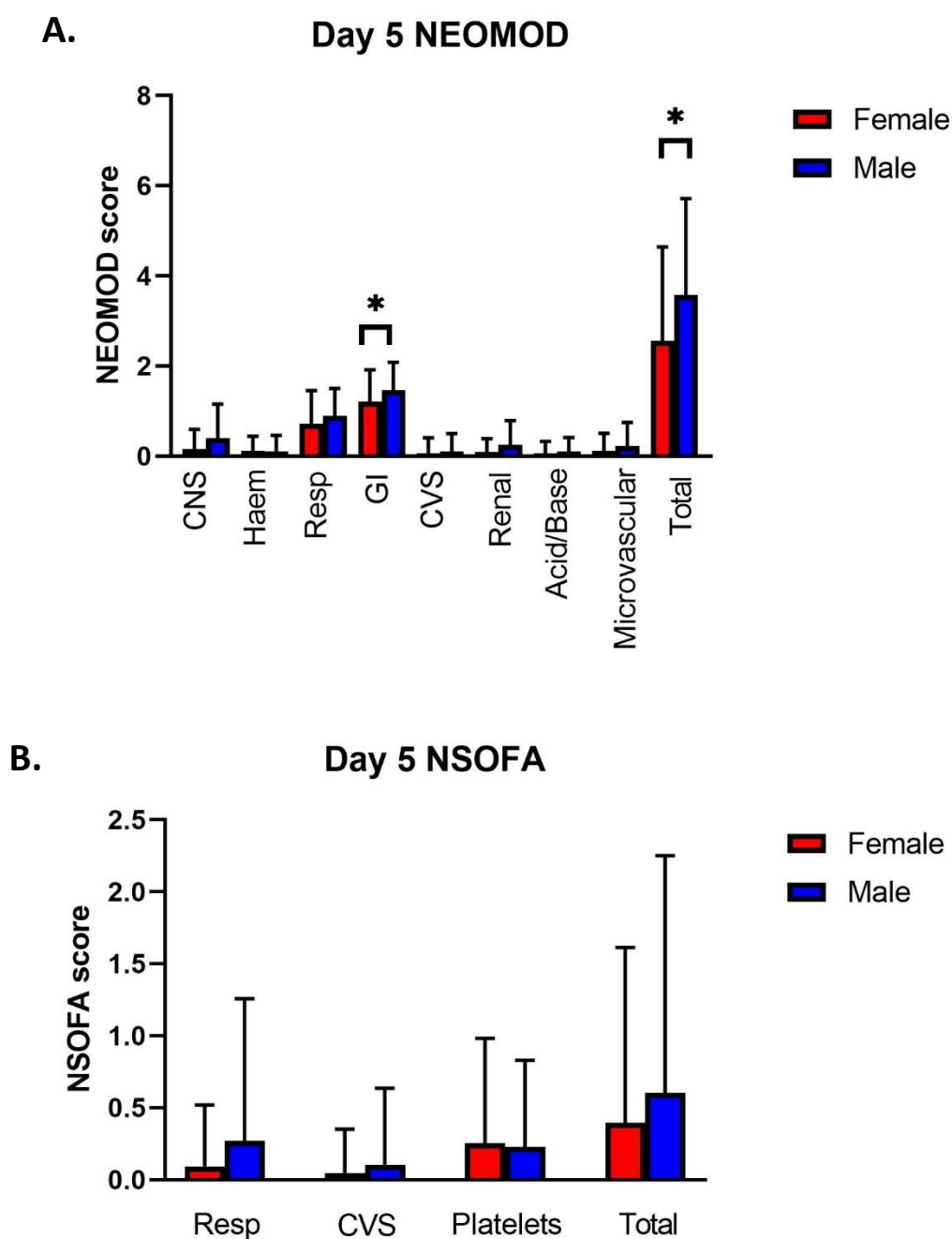


Figure 4.12: Comparison of organ dysfunction in preterm males and females on day of life 5.

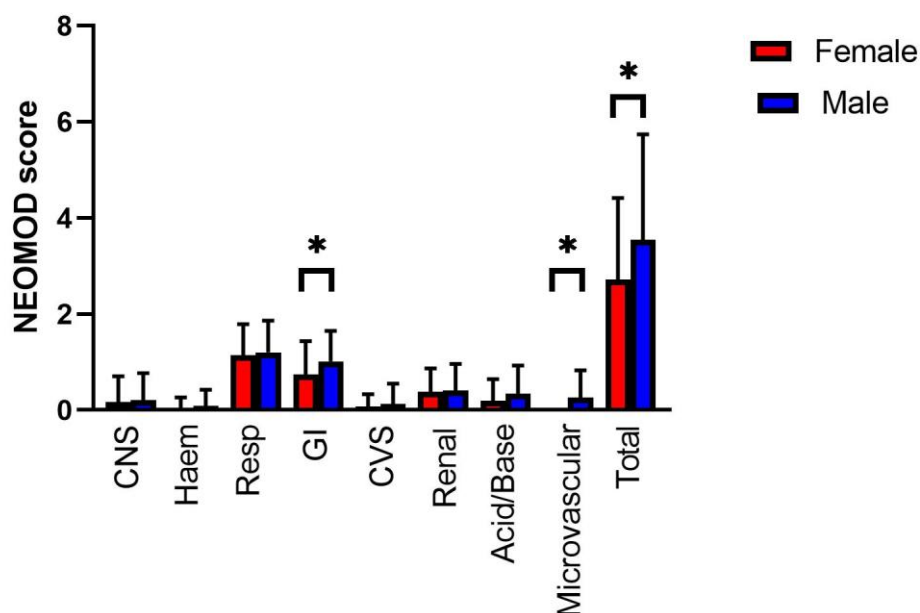
Comparison of organ dysfunction in preterm males (n=64) and females (n=44) on day of life 5. Values displayed represent the mean and standard deviation. A) Day 5 NEOMOD scores. B) Day 5 nSOFA scores. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

	Male (n=64)	Female (n=44)	p-value
Any sepsis episode	50 (78.1%)	32 (72.7%)	0.52
Microbiological	6 (9.4%)	9 (20.5%)	0.17
Clinical	18 (28.1%)	8 (18.2%)	0.14
Suspected	43 (67.2%)	30 (68.2%)	0.91
Meningitis	1 (1.6%)	0 (0%)	0.23
CONS	3 (4.7%)	3 (4.7%)	0.64
Pneumonia	4 (6.3%)	0 (0%)	0.09
EOS Microbiological	0 (0%)	0 (0%)	n/a
EOS clinical	9 (14.1%)	3 (6.8%)	0.24
EOS suspected	33 (51.6%)	21 (47.7%)	0.7
LOS Microbiological	6 (9.4%)	9 (20.5%)	0.1
LOS clinical	9 (14.1%)	5 (11.4%)	0.68
LOS suspected	26 (40.1%)	17 (38.6%)	0.87

Table 4.3: Comparison of sepsis episodes between male and female preterm neonates.

Comparison of sepsis episodes between male (n=64) and female (n=44) preterm neonates. Continuous variables are presented as mean ($\pm$ standard deviation) and categorical variables are presented as absolute numbers. Any sepsis episode=suspected/clinical/microbiological; CONS=coagulase negative staphylococci; EOS=early-onset sepsis; LOS=late-onset sepsis.

A. Suspected sepsis episodes NEOMOD



B. Suspected sepsis episodes NSOFA

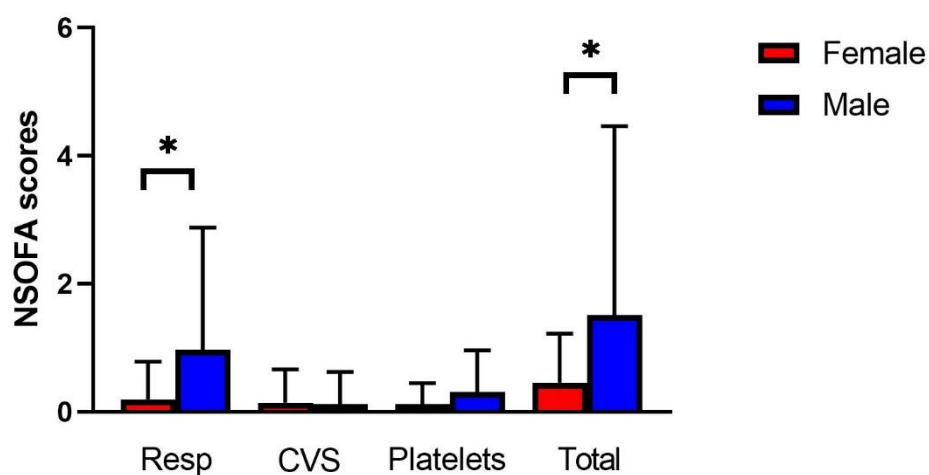
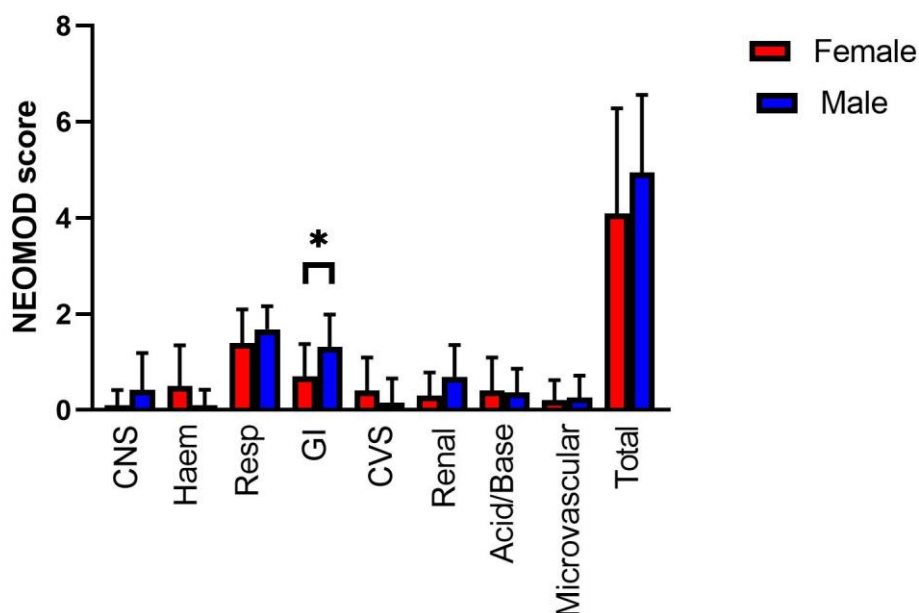


Figure 4.13: Comparison of organ dysfunction during all episodes of suspected sepsis in preterm males and females.

Comparison of organ dysfunction during all episodes of suspected sepsis in preterm males (n=68) and females (n=42). A) NEOMOD scores. B) nSOFA scores. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system.

*=significant at $p < 0.05$.

A. Clinical sepsis episodes NEOMOD



B. Clinical sepsis episodes NSOFA

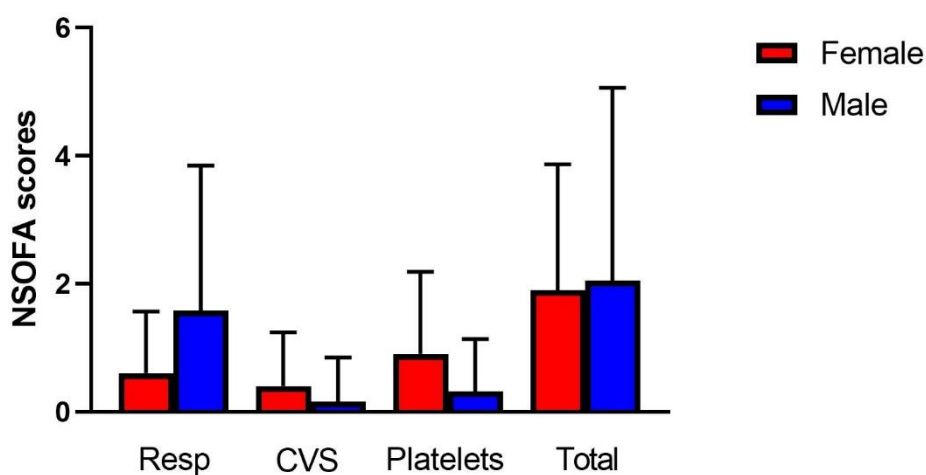
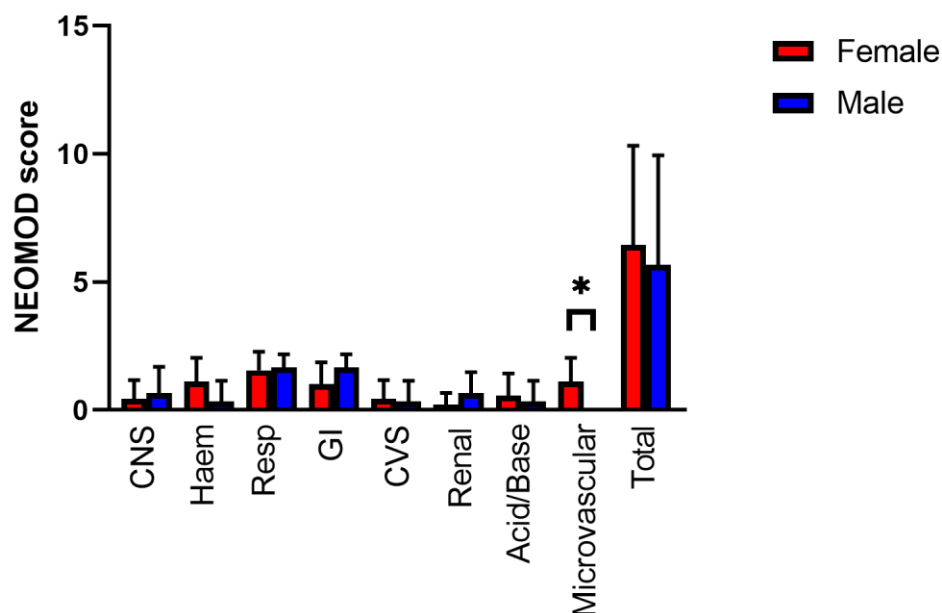


Figure 4.14: Comparison of organ dysfunction during all episodes of clinical sepsis in preterm males and females.

Comparison of organ dysfunction during all episodes of clinical sepsis in preterm males (n=19) and females (n=10). A) NEOMOD scores. B) nSOFA scores. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system.

*=significant at $p < 0.05$.

A. Microbiological sepsis episodes NEOMOD



B. Microbiological sepsis episodes NSOFA

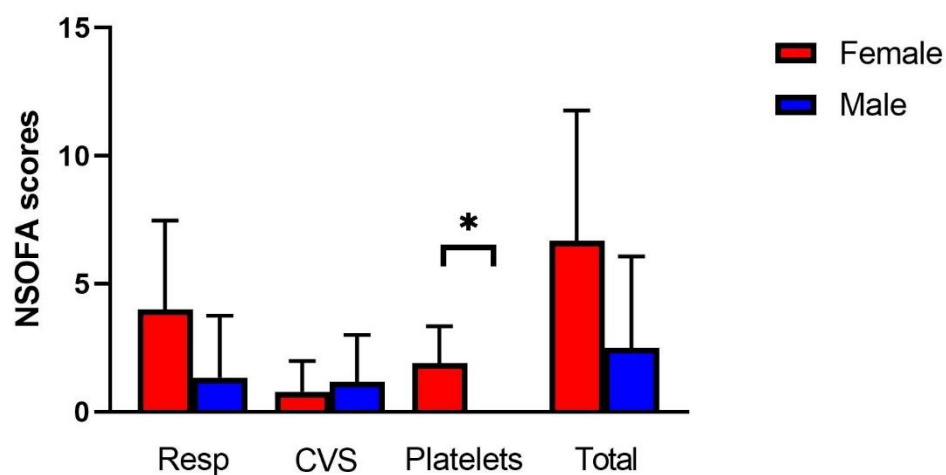


Figure 4.15: Comparison of organ dysfunction during all episodes of microbiological sepsis in preterm males and females.

Comparison of organ dysfunction during all episodes of microbiological sepsis in preterm males (n=6) and females (n=9). A) NEOMOD scores. B) nSOFA scores. CNS: central nervous system; Haem: haematological; Resp: respiratory; CVS: cardiovascular system. *=significant at $p < 0.05$.

4.5. Discussion

We evaluated 2 organ dysfunction scores in the premature population: the modified NEOMOD score, a tool to evaluate organ dysfunction in the preterm population, and the nSOFA, an organ dysfunction score which was recently developed as a predictor of mortality in VLBW neonates with sepsis ¹²⁹. While many organ dysfunction scores exist, the NEOMOD and nSOFA scores are quick, easy to perform at the bedside and are specifically designed for use in the premature population. In addition, the nSOFA represents the only organ dysfunction tool designed for use in preterm neonates with suspected sepsis and is the first attempt at applying the sepsis screening principles from Sepsis-3 in a neonatal population. Both scores had a significant and positive correlation with each other when performed within the first 24 hours and at 5 days of life.

Neonates who died before discharge had similar total scores on day 1 of life to those who survived, though they manifested greater cardiovascular dysfunction as measured on the NEOMOD score. On day 5 of life however, total NEOMOD and nSOFA scores were higher in those who died before discharge. In addition, microvascular and acid/base scores on NEOMOD, and respiratory scores on nSOFA were higher on day 5 in those who died before discharge. Similar trends were present when evaluating the composite outcome of death/IVH with greater day 1 cardiovascular dysfunction on both NEOMOD and nSOFA scores and greater total nSOFA scores compared to those who survived without IVH. Evaluations on day 5 however, showed that the death/IVH group had higher scores in almost all areas on both the NEOMOD and nSOFA scores. Similarly, at the time of sepsis screening, those neonates who subsequently died as a result of sepsis had significantly higher scores in almost all areas on both the NEOMOD and nSOFA score compared to those who survived.

NEOMOD and nSOFA scores were then further evaluated as predictive tests of death and death/IVH. Day 5 scores were the best predictors of death/IVH, with the NEOMOD performing best, likely reflecting its ability to assess dysfunction over 8 domains, giving a more global view of illness severity. During sepsis the nSOFA performed best as a predictor of mortality, in keeping with previous findings that nSOFA scores predict mortality in culture-positive LOS ¹²⁹.

We have shown that early life organ dysfunction strongly impacts short-term neonatal outcomes and it is those infants with persistent organ dysfunction, as measured on day 5 of life, that are at highest risk of later mortality. Regarding the NEOMOD specifically, our findings are consistent with previous work which showed that both the maximum NEOMOD ^{101,103} and mean NEOMOD scores ¹⁰⁴ during the first month of life are higher in neonates who died before discharge compared to survivors. The total number of organ systems showing dysfunction during the first month are also higher in those who died before discharge compared to survivors ¹⁰⁴, in keeping with our findings on day 5 of life. Notably, a syndrome of persistent inflammation and chronic organ dysfunction has been described in adults ³⁴⁹ and late-preterm neonates in surgical ICU ⁴⁹⁵ and has been linked to adverse outcome in both cases, supporting our findings of an association between persistent organ dysfunction on day of life 5 and death/IVH.

Many authors have used illness severity scoring to predict mortality or later neurological outcome in neonates ^{114,493}, though most studies either use lengthy, difficult to perform scores or require serial measurements and a prolonged period of assessment. Our findings suggest that simple, isolated measures of organ dysfunction may have a role in predicting preterm neonatal clinical outcome. In the case of the NEOMOD, previous validation studies used daily scores in the first month of life to predict outcome ^{101,103,104}, though our data suggests that repeat measurements within the first week of life may be of value. In the case of the nSOFA authors used repeated measurements several times per day to predict outcome in sepsis ¹²⁹, though we have shown that single measurements, while most useful in predicting sepsis-related mortality, may be of prognostic value even without suspected infection.

Differences in clinical outcome between males and females were evident from the very beginning of life with males requiring greater levels of resuscitation in the delivery room including increased use and duration of IPPV and intubation. Our findings of greater resuscitation requirements in males are in keeping with the findings of Stevenson *et al.* who noted that males are more likely to be intubated and require pharmacological resuscitation after delivery ⁴⁹⁶. It has been suggested that female infants exhibit a greater degree of gestational maturation which translates into

improved ability to transition to the postnatal environment ⁴⁹⁷. Genetic, immunological and hormonal differences between the sexes have been suggested to underlie this early female advantage ^{246,494} and may explain the differences we have observed.

Male infants had higher rates of both CLD and PVL. Our finding of a higher rate of CLD is in keeping with the findings of larger observational studies who found higher rates of CLD in males ^{498,499}, with authors again pointing to the improved respiratory development of premature females as a potential explanation ⁵⁰⁰. The higher rate of PVL among males in our study is in keeping with increased rates among males in published literature ^{499,501} and the identification of male sex as an independent risk factor for PVL ⁵⁰². The higher rates of PVL among males in our study and others potentially reflects the fact that male neonates are more sensitive to neurological insult than females ⁵⁰³.

The limited data on clinical outcomes which we present is in keeping with the large volume of published data suggesting poorer outcomes in premature males ⁴⁹⁴. However, many important clinical outcomes including mortality rates and IVH, both of which have been noted as more common among male preterm neonates ⁵⁰⁴⁻⁵⁰⁶, did not differ significantly between the sexes. This may be partially explained by the small sample size of this study which is likely under-powered to detect differences in all the clinical outcomes of interest. The other factor of note is the bimodal distribution of gestation within the female cohort compared to males. The gestation in males is normally distributed, whereas the female cohort has 2 peaks: one in the extreme preterm range and the other at moderate-late preterm gestation. The result is that despite similar mean gestation in both sexes, there is an over-representation of extreme preterm neonates in the female cohort. Given that extreme preterm neonates are disproportionately affected by morbidity and mortality in the preterm neonatal population, the female contingent therefore have a higher proportion of high-risk neonates. Despite this, the data on clinical outcomes presented still suggests that males have a greater need for medical intervention, poorer neurological outcomes and higher rates of respiratory morbidity after delivery, though the bimodal

spread of female gestation means that important differences may have been obfuscated.

Sepsis risk factors did not differ between the sexes, and the incidence of sepsis of any form did not differ between males and females in this study. Our findings conflict with much of the published literature in this area which suggests that male neonates are at overall increased risk of neonatal sepsis ²⁴⁷ along with community-acquired sepsis ¹³⁷, GBS sepsis ²⁴⁸ and sepsis secondary to drug-resistant or atypical bacteria ²⁴⁹. Our small sample size is likely the single largest reason that these differences were not present in our study though given that extreme preterm neonates are at especially high risk of sepsis ⁵⁰⁷ the skewed gestation of the female participants is also likely to have contributed.

While many manuscripts have acknowledged the higher morbidity in preterm males, and several organ scoring systems have been examined in the neonatal population ^{114,493}, studies using these scores to objectively compare illness severity between males and females are lacking. This is, to our knowledge, the first study which aims to directly compare organ dysfunction both at baseline and during periods of sepsis between males and females and thus the differences we have found are novel. Examining early life organ dysfunction shows a very clear pattern of increased illness severity among preterm males during the first week of life. All the differences detected on both day 1 and day 5 showed higher scores of organ dysfunction in males when comparing both total scores and, on a more granular level, when the individual contributors to these scores were examined. Males had higher levels of respiratory organ dysfunction on day 1 as measured by the nSOFA score, reflecting their increased early respiratory morbidity ²⁹⁴. Our findings are therefore reflective of both the increased severity of RDS in males and the higher need for respiratory support among preterm male newborns ²⁹⁴. Male infants had higher levels of microvascular and acid/base dysfunction on day 1, and gastrointestinal dysfunction on day 5 as measured by the NEOMOD score. Data examining sex differences in these areas is lacking and these findings are therefore novel. Though sex differences in microvascular dysfunction in neonates has not been previously described, hypoproteinaemia in the early neonatal period has been associated with severe adverse outcomes in the

preterm population ⁵⁰⁸ and hypoalbuminaemia has been suggested as predisposing both to sepsis and sepsis-related mortality ⁵⁰⁹. A publication by Ingemarsson *et al.* has suggested that acidaemia is more common among term male deliveries and is associated with adverse outcome ⁵¹⁰, though similar studies are lacking in the premature population. Gastrointestinal dysfunction was higher in males and our findings are supported by the limited available research which suggests that preterm males have higher bilirubin levels after delivery ⁵¹¹, though no evidence of sex-differences in liver enzymes in preterm neonates has been published. The persistence of higher NEOMOD scores in males at day 5 of life is of particular interest given that we have shown that persistent organ dysfunction predicts death/IVH in the VLBW cohort.

During episodes of suspected sepsis males had higher total nSOFA scores and NEOMOD scores compared to females, reflecting greater overall illness severity. Males also had higher levels of gastrointestinal and microvascular dysfunction as measured by NEOMOD scoring. As previously mentioned, data on sex differences in these areas are lacking though males do appear to have higher bilirubin levels after birth ⁵¹¹ and microvascular dysfunction has been linked to adverse sepsis outcome in preterm infants ⁵⁰⁹. Males had higher levels of respiratory dysfunction during episodes of suspected sepsis as measured by the nSOFA, a finding supported by the higher overall respiratory morbidity in preterm males ²⁹⁴. During episodes of clinical sepsis total scores were similar between males and females though males once again manifested more gastrointestinal dysfunction as measured by the NEOMOD score. Total scores did not differ between groups during microbiological sepsis though females had greater microvascular dysfunction as measured by the NEOMOD score and platelet dysfunction as measured by the NSOFSA score. No studies could be found explaining the microvascular differences and data on sex differences in platelet count was limited and somewhat contradictory. Some authors have suggested a male predominance in thrombocytopenia ^{512,513} and a slight female predominance in cases thrombocytosis ⁵¹⁴, while others have suggested there is no association between neonatal thrombocytopenia and sex ⁵¹⁵. No studies exist which specifically examine platelet count in males and females with neonatal sepsis therefore our findings are novel, though given the higher proportion of extreme preterms in the female

population the increased haematological dysfunction may be more reflective of gestational differences than true sex differences. It is also notable that our findings, while suggestive of a higher illness severity in males with suspected sepsis, may be limited by study design. Given that we recorded scores only during the first day of sepsis we may have detected more of the background organ dysfunction to which males appear to be predisposed and repeated measures during these episodes may have been more revealing. In addition, Rautonen *et al.* noted that males have higher mortality regardless of illness severity score ⁵¹⁶, thus while we suggest that the greater severity of organ dysfunction among males contributes to their adverse outcome it is unlikely to be the sole cause of the marked differences in outcome that we see clinically.

4.6. Conclusion

Persistent early life organ dysfunction is associated with adverse outcome in preterm neonates and the use of simple repeated measures of organ dysfunction within the first week of life has prognostic value in these patients. Single measurements of organ dysfunction on the first day of sepsis can predict death in preterm neonates undergoing septic work-up. These findings suggest that the use of organ dysfunction scores may help identify neonates at high risk of adverse outcome after delivery, detect clinical deterioration early in disease course and potentially guide management in cases of suspected sepsis. Organ dysfunction scoring may therefore be valuable in the early routine care of preterm neonates and, similar to the qSOFA score developed by Sepsis-3, may help identify high risk infants during cases of suspected sepsis.

Male preterm neonates manifested greater levels of early life organ dysfunction compared to females from day 1 of life persisting through the first week of life. Male neonates undergoing evaluation for suspected sepsis had greater total organ dysfunction scores compared to females. Given that persistent organ dysfunction predicts adverse outcome in the VLBW population, the novel finding of persistently greater levels of organ dysfunction in male infants, both early in life and during sepsis evaluation, are clinically significant findings.

While our findings highlight the potential utility of organ dysfunction scoring in clinical practice the ideal organ dysfunction score remains unknown. While we have evaluated two of the best available scores, the optimal approach to evaluating organ dysfunction among neonates remains unclear. One of the many problems in existing literature is that many scoring systems exist, and few are validated outside their initial description. Further investigation of the more robust among these scoring systems is therefore warranted to identify both the scores and individual parameters which perform best in screening for neonatal ill health and predicting neonatal outcome.

While we have identified organ dysfunction as an important marker of neonatal outcome and have highlighted the greater levels of organ dysfunction present among male neonates, the long-term implications of preterm organ dysfunction remain unknown. The role of the immune system in mediating neonatal sepsis-related organ dysfunction has been recognised⁵¹⁷, but is poorly understood. Similarly, the role of the immune system in preterm neonatal organ dysfunction outside of sepsis has not been well-established. Further study is therefore required to understand the influence of the immature immune system on neonatal organ dysfunction both during and outside of sepsis. Studies evaluating the persistence of organ dysfunction outside of the neonatal period and examining the relationship of both early-life and persistent organ dysfunction on later neurodevelopmental outcome are also required.

Chapter 5: The effect of gender and sex hormones on neonatal innate immune function

5.1. Introduction

Neonates are at increased risk of sepsis compared to older children and adults. Male neonates, particularly those born preterm are at especially high risk ²⁴⁷. In addition, males appear to be more susceptible to the adverse effects of infection-related inflammation with poorer clinical outcomes after sepsis episodes ²⁵⁰⁻²⁵². The reasons for the pronounced difference in sepsis susceptibility between the sexes is poorly understood though it has been hypothesised that a combination of immune, hormonal and genetic factors may contribute ²⁴⁶. In addition to the experimental evidence of differences in immune response between male and female neonates ²⁴⁶, the X-chromosome codes for several genes important in the innate immune response ³⁴⁴ and neonatal innate immune cells both express receptors for, and are inhibited by, maternal sex hormones ³³⁴.

Under normal circumstances every cell in the body will have one active copy of the X chromosome. In males the single copy of the X chromosome is always inherited from the mother and therefore all cells will express only maternally-inherited X chromosome genes. Females inherit one copy of the X chromosome from each parent and cells will randomly inactivate a single X chromosome meaning that female immune cells have potential to express X chromosome genes from either parent. This provides female immune cells with potential diversity in response to immune challenge ³⁴⁵. In addition, some X-linked genes may escape X chromosome inactivation meaning that expression of some X-linked genes may be higher than in males, again potentially allowing a more robust immune response in females. BTK is an enzyme encoded by the X chromosome with important function in a variety of innate immune processes including TLR-mediated pathogen recognition, cellular maturation, immune cell recruitment and NLRP3 inflammasome regulation ⁵¹⁸. IKK- γ is a protein encoded by the X chromosome which activates nuclear factor kappa-B in response to a variety of noxious stimuli ⁵¹⁹. IRAK-1 is an enzyme encoded by the X chromosome which has a key role in TLR signalling ⁵²⁰. TLR7 is a pattern recognition receptor encoded by the X

chromosome which recognises single-stranded RNA from viruses and GBS ¹⁹⁴. AR is also encoded by the X chromosome and is widely expressed on immune cells and is thought to affect immune response by regulating gene transcription ⁵²¹. Studies of X-linked genes are lacking in premature neonates but are important to fully understand the increased susceptibility of males to infection.

Given the increased susceptibility of premature male neonates to bacterial sepsis it is likely that their innate immune responses are not as robust as those of females. Neutrophils and monocytes are important in innate immune responses to infection, though their function in preterm infants is poorly characterised and it is unknown if their activity differs in male and female premature neonates. CD11b is a cell surface receptor which is crucial in effective neutrophil and monocyte adhesion and migration in response to infection ^{168,170}. CD11b is often used as a marker of immune system activation and has shown promise as a biomarker in neonatal infection and inflammation ¹⁸⁶⁻¹⁹⁰. Defects in CD11b-mediated immune cell migration have potentially important implications for innate immune response and have been shown to cause increased susceptibility to infection ¹⁷³⁻¹⁷⁵. TLR2 is a pattern recognition receptor involved in the recognition of pathogenic bacteria including CONS and GBS ⁵²². While TLR-2 is mainly involved in the recognition of gram-positive bacterial lipopeptides ⁵²², TLR-2 expression has been shown to increase in human leucocytes following LPS endotoxin exposure ^{523,524}. Defective TLR signalling causes increased severity of bacterial sepsis in mice ⁵²⁵ and TLR2 signalling has been linked to the adverse neurological effects of neonatal sepsis ⁵²⁶. At present, CD11b and TLR2 expression are poorly characterised in the premature population and much of the existing data on these receptors in peripheral immune cells is from cord blood which is known to be immunologically distinct from postnatally collected samples ³¹⁴. It is also unclear if differences exist in the activity of these receptors in premature males and females, and while female sex hormones are thought to have an important role in immune development it is unclear whether physiological concentrations of these hormones affect the expression of CD11b or TLR2 in monocytes and neutrophils of premature neonates.

5.2. Hypothesis

We hypothesised that sex differences in innate immune responses may be present in the neonatal population that contribute to the increased susceptibility of premature males to sepsis.

5.3. Aims

- To examine the in vitro effect of LPS, Pam3CSK4, E2 and Pg on the expression of CD11b and TLR2 in neutrophils and monocytes of male and female neonates.
- To examine the expression of X-linked genes involved in innate immunity in whole blood of male and female neonates.

5.4. Results

5.4.1. Patient Characteristics

The 21 preterm neonates (8 female, 13 male) who had whole blood collected for flow cytometry analysis had a mean gestation at birth (mean $\pm$ SD) of 28 ± 2 weeks and corrected gestation at the time of sampling was 30 ± 2.6 weeks. The mean birth weight of preterm neonates was 1084 ± 246 grams. Gestation at birth, corrected gestation at sampling and birth weight did not differ significantly between male and female preterm neonates ($p > 0.05$) (Table 5.1).

The 10 preterm neonates (5 female, 5 male) who had whole blood collected for qPCR had a mean gestation at birth (mean $\pm$ SD) of 29.4 ± 2.66 weeks and corrected gestation at the time of sampling was 31.3 ± 2.51 weeks. The mean birth weight of preterm neonates was 1084 ± 328 grams. Gestation at birth, corrected gestation at sampling and birth weight did not differ significantly between male and female preterm neonates ($p > 0.05$) (Table 5.2).

	Preterm males (n=13)	Preterm females (n=8)	p value (95% CI)
Gestation at delivery (weeks)	27.5 (± 2.2)	28.94 (± 1.4)	0.14 (-3.4 to 0.5)
Gestation at sampling (weeks)	30.6 (± 3.661)	31.2 (± 0.8)	0.67 (-3.6 to 2.3)
Birth weight (grams)	1025 (± 236.5)	1194 (± 242.3)	0.146 (-404.5 to 65.17)

Table 5.1: Demographics for preterm neonatal male and female flow cytometry samples.

Demographics for preterm neonatal male (n=13) and female (n=8) flow cytometry samples. Values presented represent mean $\pm$ standard deviation.

	Preterm males (n=5)	Preterm females (n=5)	p value (95% CI)
Gestation at delivery (weeks)	29.83 (± 2.3)	28.97 (± 3.2)	0.64 (-3.20 to 4.92)
Gestation at sampling (weeks)	32.3 (± 2.2)	30.3 (± 2.7)	0.23 (-1.56 to 5.51)
Birth weight (grams)	1162 (± 337.3)	1006 (± 336.9)	0.485 (-335.7 to 647.7)

Table 5.2: Demographics for preterm neonatal male and female qPCR samples.

Demographics for preterm neonatal male (n=5) and female (n=5) qPCR samples. Values presented represent mean $\pm$ standard deviation.

5.4.2. *The effect of sex on endotoxin-treated neutrophil and monocyte CD11b expression*

Neutrophils and monocytes were isolated based on size and granularity and delineated from each other based on CD66b positivity (granulocytes CD66b+, monocytes CD66b-). CD14 and CD16 expression was used to distinguish between classical (CD14+/CD16-), intermediate (CD14+/CD16+) and non-classical (CD14dim/CD16+) monocytes (Figure 2.4). Neutrophil CD11b expression was similar between preterm and term neonates at baseline ($p>0.05$). Neutrophil CD11b expression increased significantly in both term ($p<0.001$) and preterm ($p<0.001$) neonates following incubation with LPS. CD11b expression was higher in term neonates than preterm neonates following LPS treatment ($p=0.0057$) (Figure 5.1a). Total monocyte CD11b expression was similar between preterm and term neonates at baseline ($p>0.05$). Total monocyte CD11b expression increased significantly in both term ($p=0.0213$) and preterm ($p=0.007$) neonates following incubation with LPS (Figure 5.1b).

Classical monocyte CD11b increased significantly in both term ($p=0.0038$) and preterm ($p=0.0007$) neonates following LPS treatment. Classical monocyte CD11b expression was higher in term neonates than preterm neonates following LPS exposure ($p=0.0316$) (Figure 5.1c). CD11b expression did not change significantly in intermediate and non-classical monocytes of term or preterm neonates following LPS treatment (Figure 5.1d). CD11b expression was higher in preterm neonatal non-classical monocytes at baseline ($p=0.0445$) (Figure 5.1e).

The results of term and preterm neonates were then divided by sex and reanalysed (Figure 5.2). Neutrophil CD11b expression increased significantly in term males ($p=0.001$), term females ($p<0.0001$), preterm males ($p<0.0001$) and preterm females ($p=0.0091$) following LPS treatment. Neutrophil CD11b expression was higher in term females compared to preterm females following LPS treatment ($p=0.0451$), a difference which was not present in males (Figure 5.2a). Total monocyte CD11b increased significantly in term females ($p<0.001$) and preterm males ($p<0.001$) only following LPS treatment. Total monocyte CD11b was higher in term females compared to preterm females following LPS exposure ($p=0.0063$), a difference which was not

present in males (Figure 5.2b). Classical monocyte CD11b increased significantly in both term females ($p=0.0022$) and preterm males ($p<0.001$) following LPS treatment. Classical monocyte CD11b was significantly higher in preterm females compared to preterm males at baseline ($p=0.023$) (Figure 5.2c). Intermediate monocyte CD11b and non-classical monocyte CD11b did not increase significantly in any group following LPS treatment ($p>0.05$) (Figure 5.2d, Figure 5.2e).

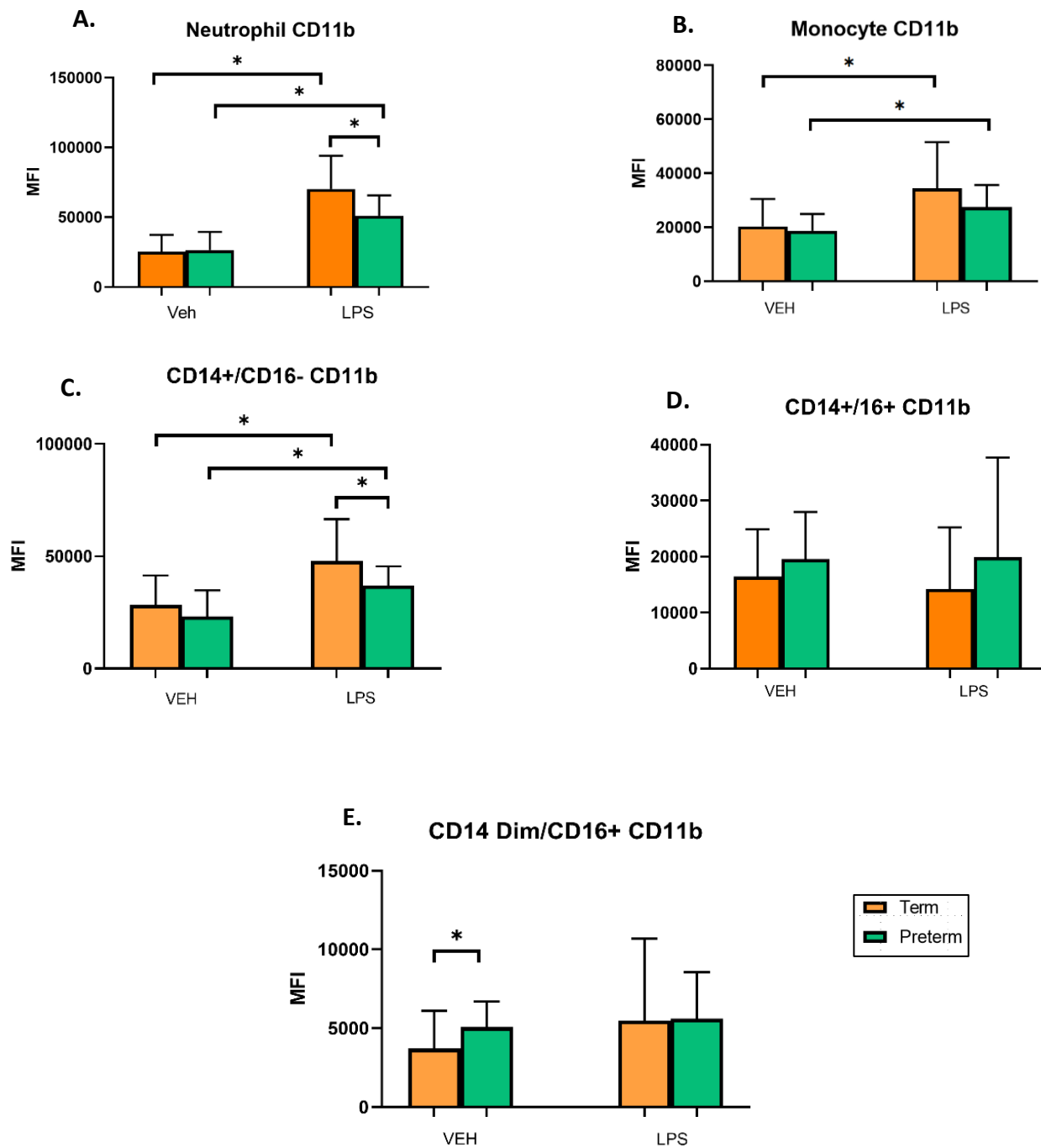


Figure 5.1: CD11b expression in neutrophils and monocytes in response to LPS (10 ng/ml) in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil CD11b; B) Total monocyte CD11b; C) Classical monocyte CD11b; D) Intermediate monocyte CD11b; E) Non-classical monocyte CD11b. *p<0.05; VEH=vehicle; LPS=endotoxin.

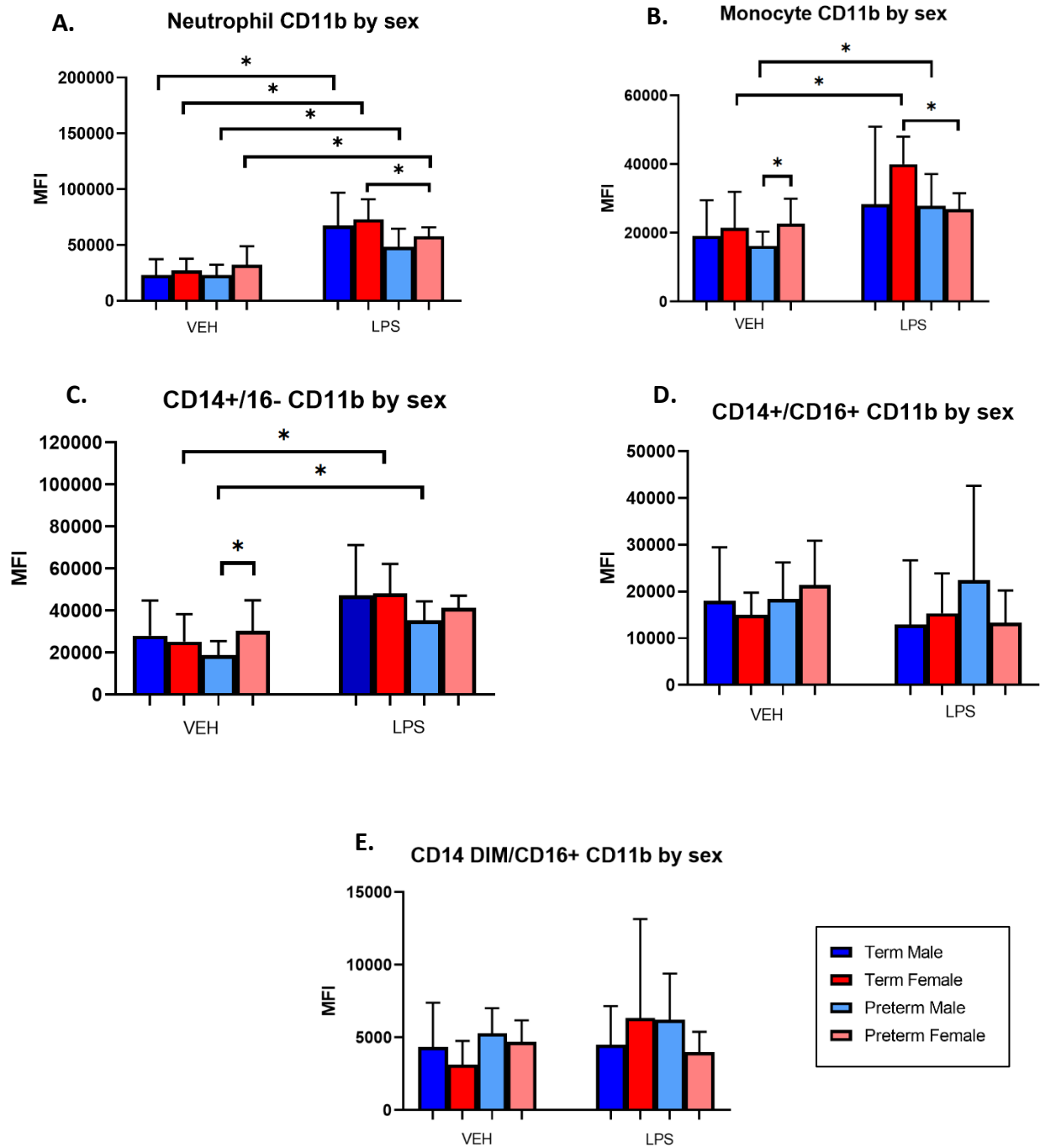


Figure 5.2: Sex differences in CD11b expression in neutrophils and monocytes in response to LPS (10 ng/ml) in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil CD11b; B) Total monocyte CD11b; C) Classical monocyte CD11b; D) Intermediate monocyte CD11b; E) Non-classical monocyte CD11b. *p<0.05; VEH=vehicle; LPS=endotoxin.

5.4.3. *The effect of sex hormones on endotoxin-treated neutrophil and monocyte*

CD11b expression

CD11b expression did not change significantly between untreated and E2-treated term or preterm neutrophils, monocytes, or monocyte subsets (Figure 5.3). CD11b expression did not change significantly between LPS-treated samples and samples treated with LPS and E2 in combination in term or preterm neutrophils, monocytes, or monocyte subsets (Figure 5.3).

The results of term and preterm neonates were then divided by sex and reanalysed (Figure 5.4). CD11b expression did not change significantly between untreated samples and E2-treated samples in term or preterm neutrophils, monocytes, or monocyte subsets of either sex (Figure 5.4). CD11b expression did not change significantly between LPS-treated samples and samples treated with LPS and E2 in combination in term or preterm neutrophils, monocytes, or monocyte subsets of either sex (Figure 5.4).

Treatment with E2 or Pg, either alone or in combination, did not significantly change CD11b expression from baseline in preterm male or female neutrophils, monocytes, or monocyte subsets (Figure 5.5). CD11b expression did not differ significantly between samples which were treated with LPS alone and samples which were treated with LPS along with either E2 or Pg in preterm neutrophils, monocytes, or monocyte subsets (Figure 5.5). CD11b expression increased significantly in both male ($p=0.001$) and female ($p=0.0091$) preterm neutrophils following LPS exposure (Figure 5.5a). CD11b expression increased significantly in male preterm total monocytes ($p=0.0004$) and classical monocytes ($p<0.0001$) following LPS treatment, but not in females (Figure 5.5b, Figure 5.5c). CD11b expression was higher at baseline in female preterm total monocytes ($p=0.0158$) and classical monocytes ($p=0.0230$) compared to males (Figure 5.5b, Figure 5.5c). CD11b expression was higher in preterm female classical monocytes when treated with E2 ($p=0.0456$), P+LPS ($p=0.0032$) and E2+P+LPS ($p=0.0292$) compared to males (Figure 5.5c).

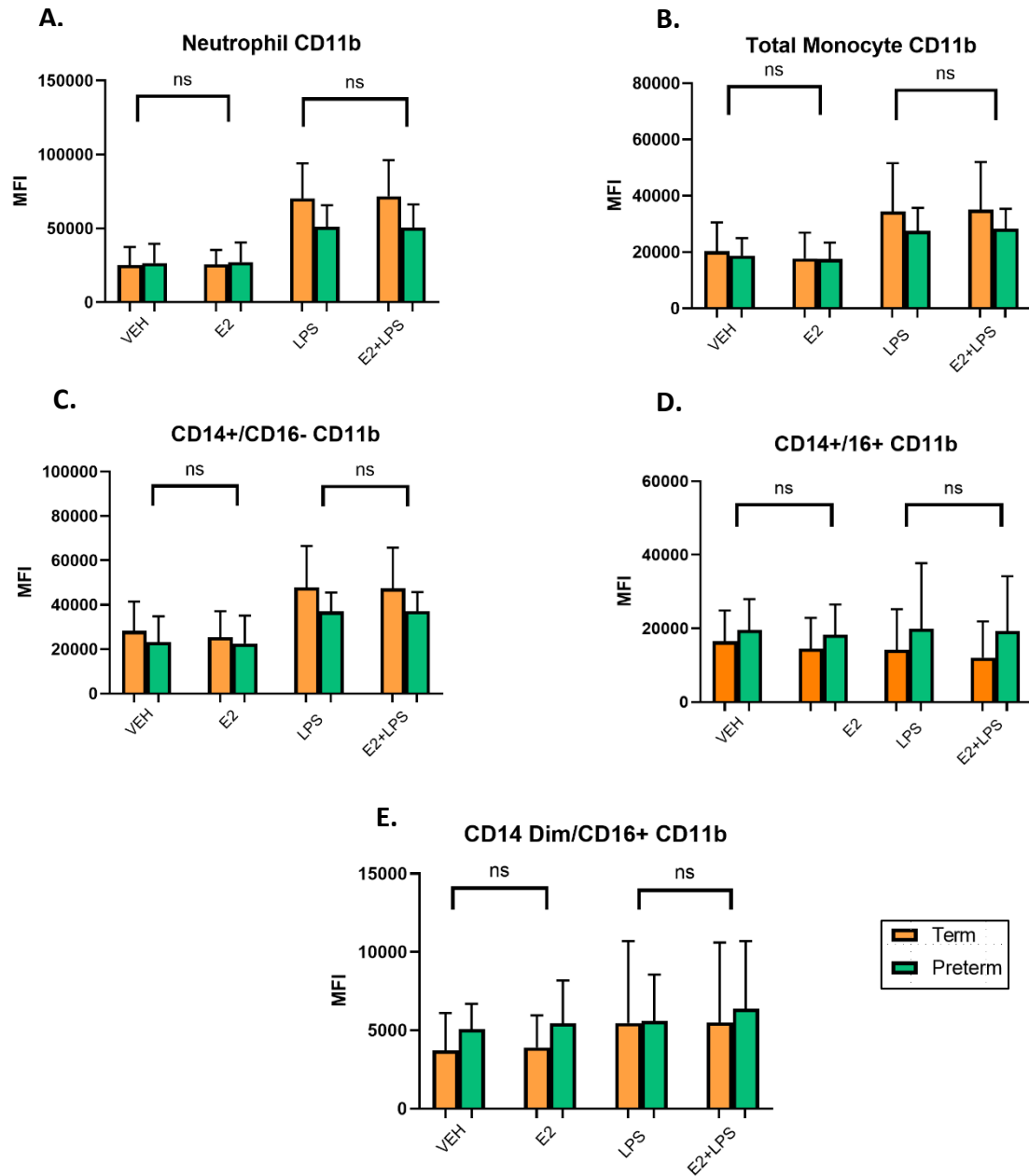


Figure 5.3: CD11b expression in neutrophils and monocytes in response to LPS (10 ng/ml) hormone treatments (10nM) in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil CD11b; B) Total monocyte CD11b; C) Classical monocyte CD11b; D) Intermediate monocyte CD11b; E) Non-classical monocyte CD11b. ns=non-significant; VEH=vehicle; E2=Estradiol; LPS=endotoxin.

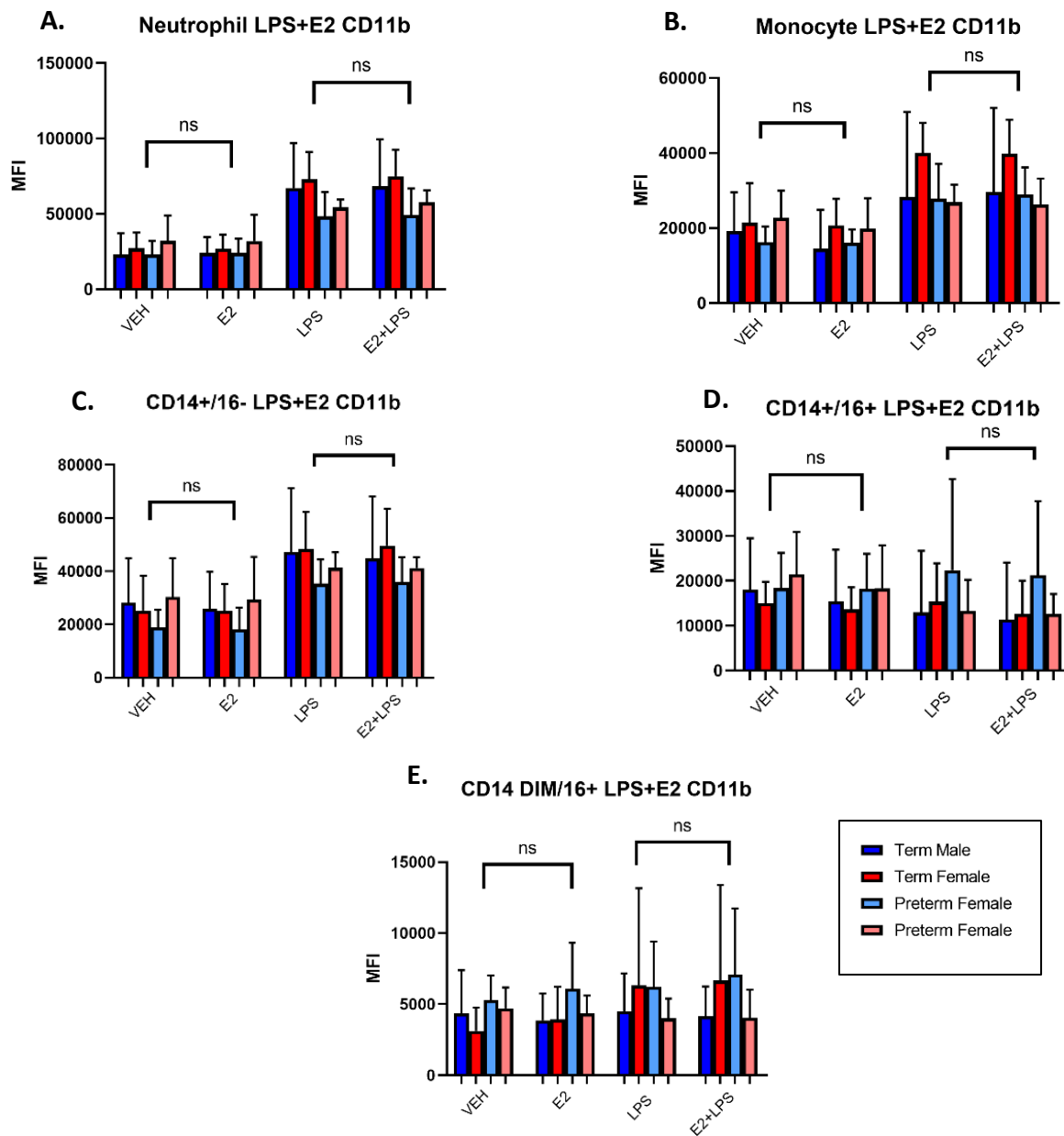


Figure 5.4: CD11b expression in neutrophils and monocytes of males and females in response to LPS (10 ng/ml) and hormone treatments (10nM) in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil CD11b; B) Total monocyte CD11b; C) Classical monocyte CD11b; D) Intermediate monocyte CD11b; E) Non-classical monocyte CD11b. ns=non-significant; VEH=vehicle; E2=Estradiol; LPS=endotoxin.

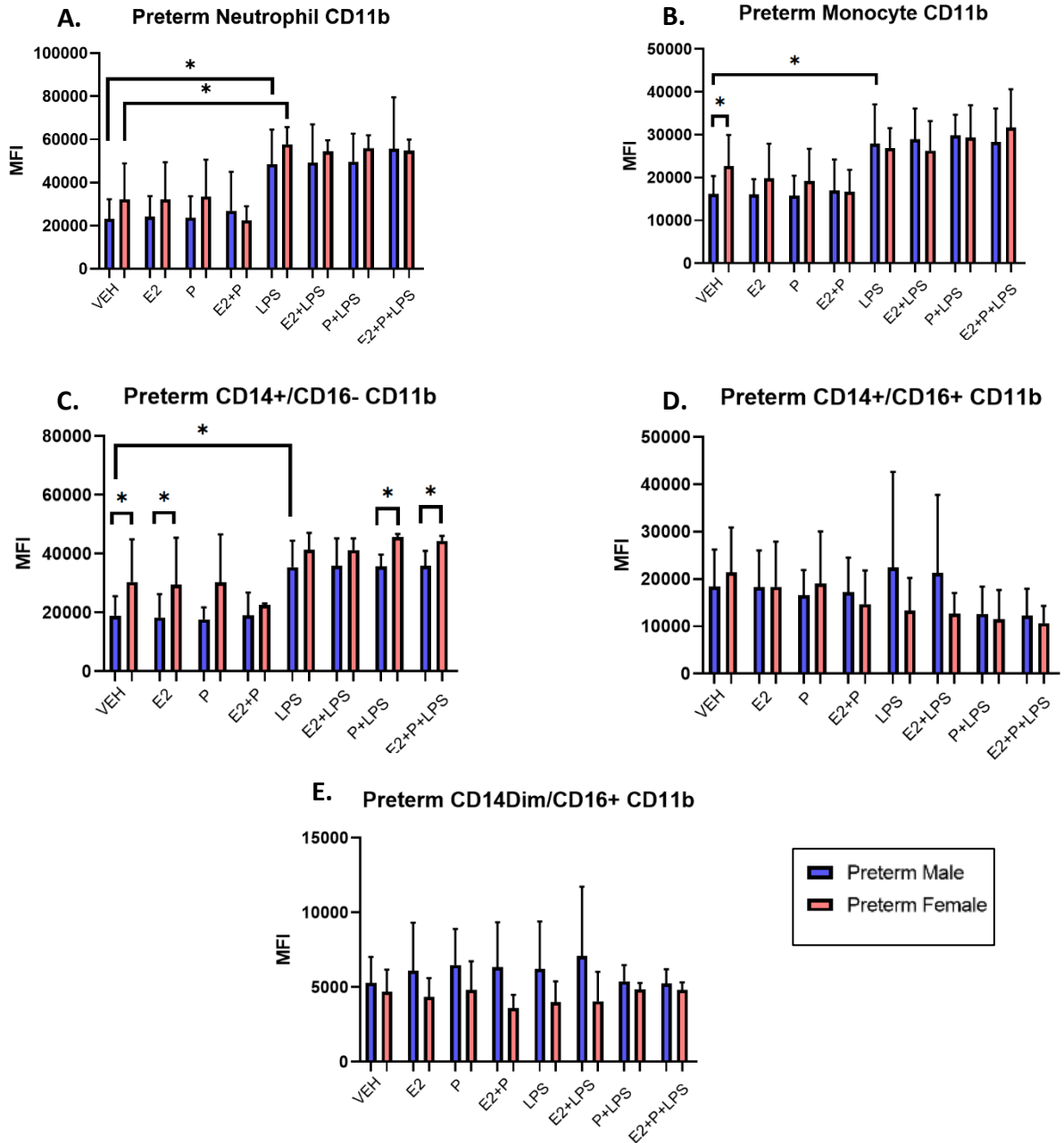


Figure 5.5: CD11b expression in neutrophils and monocytes of males and females in response to LPS (10ng/ml) and hormone treatments (10nM) in preterm males and females.

Values in preterm males (n=13) and females (n=8) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil CD11b; B) Total monocyte CD11b; C) Classical monocyte CD11b; D) Intermediate monocyte CD11b; E) Non-classical monocyte CD11b. *p<0.05; VEH=vehicle; E2=Estradiol; P=Progesterone; LPS=endotoxin.

5.4.4. *The effect of sex and sex hormones on Pam3CSK4-treated neutrophil and monocyte CD11b*

CD11b expression did not increase significantly in preterm male or female neutrophils, total monocytes or monocyte subsets following Pam3CSK4 treatment (Figure 5.6).

CD11b expression was higher in preterm females at baseline in total monocytes ($p=0.0158$) and classical monocytes ($p=0.0230$) compared to males. CD11b expression was higher in females when treated with E2 in classical monocytes compared to males ($p=0.0456$). CD11b expression was higher in male preterm non-classical monocytes for Pam ($p=0.0055$), E2+Pam ($p=0.0387$), P+Pam ($p=0.0233$) and E2+P+Pam samples when compared to females ($p=0.0071$).

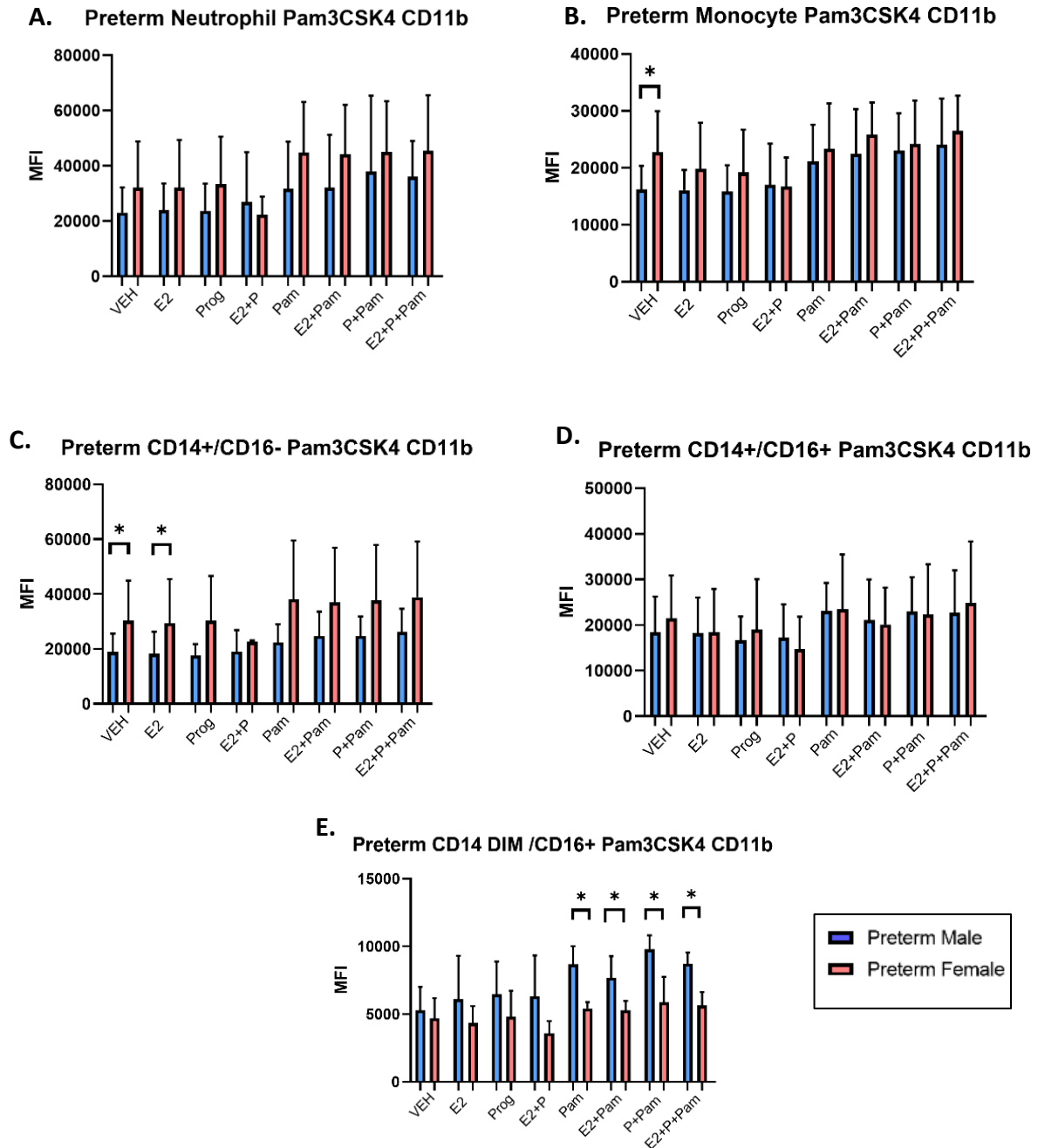


Figure 5.6: CD11b expression in neutrophils and monocytes of male and female preterm neonates in response to Pam3CSK (5 ng/ml) and hormone (10nM) treatments in preterm males and females.

Values in preterm males (n=13) and females (n=8) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil CD11b; B) Total monocyte CD11b; C) Classical monocyte CD11b; D) Intermediate monocyte CD11b; E) Non-classical monocyte CD11b. *p<0.05; VEH=vehicle; E2=Estradiol; P=Progesterone; Pam=Pam3CSK.

5.4.5. *The effect of sex on endotoxin-treated monocyte and neutrophil TLR2*

TLR2 expression did not increase significantly in term or preterm neutrophils following LPS treatment (Figure 5.7a). TLR2 expression increased significantly in both term ($p=0.0051$) and preterm ($p=0.0115$) total monocytes following LPS treatment (Figure 5.7b). TLR2 was significantly higher at baseline ($p=0.0135$) and following LPS treatment ($p=0.0346$) in preterm total monocytes compared to term neonates (Figure 5.7b).

TLR2 expression increased significantly in both term ($p=0.0002$) and preterm ($p=0.0039$) classical monocytes following LPS treatment (Figure 5.7c). TLR2 was significantly higher at baseline in preterm neonatal classical monocytes ($p=0.0339$) and intermediate monocytes ($p=0.0065$) compared to term neonates (Figure 5.7b, Figure 5.7c, Figure 5.7d). TLR2 expression was higher in preterm total and classical monocytes following LPS treatment ($p=0.0052$) compared to term neonates (Figure 5.7b, Figure 5.7c).

The results of term and preterm neonates were then divided by sex and reanalysed (Figure 5.8). Neutrophil TLR2 did not change following LPS treatment in any group and did not differ between groups at any treatment (Figure 5.8a). TLR2 expression increased significantly in total monocytes of both term females and preterm males following LPS treatment (Figure 5.8b). TLR2 expression was significantly higher in preterm female total monocytes both at baseline and following LPS treatment compared to term females (Figure 5.8b).

TLR2 expression increased significantly in term male, term female, preterm male and preterm female classical monocytes following LPS treatment (Figure 5.8c). TLR2 expression was higher in preterm female classical monocytes both at baseline and following LPS treatment compared to preterm males (Figure 5.8c). TLR2 expression was higher in preterm female classical monocytes both at baseline and following LPS treatment compared to term females (Figure 5.8c). TLR2 expression was significantly higher in preterm female intermediate monocytes at baseline compared to term females (Figure 5.8d). TLR2 expression did not change significantly in non-classical monocytes of any group following LPS treatment (Figure 5.8e).

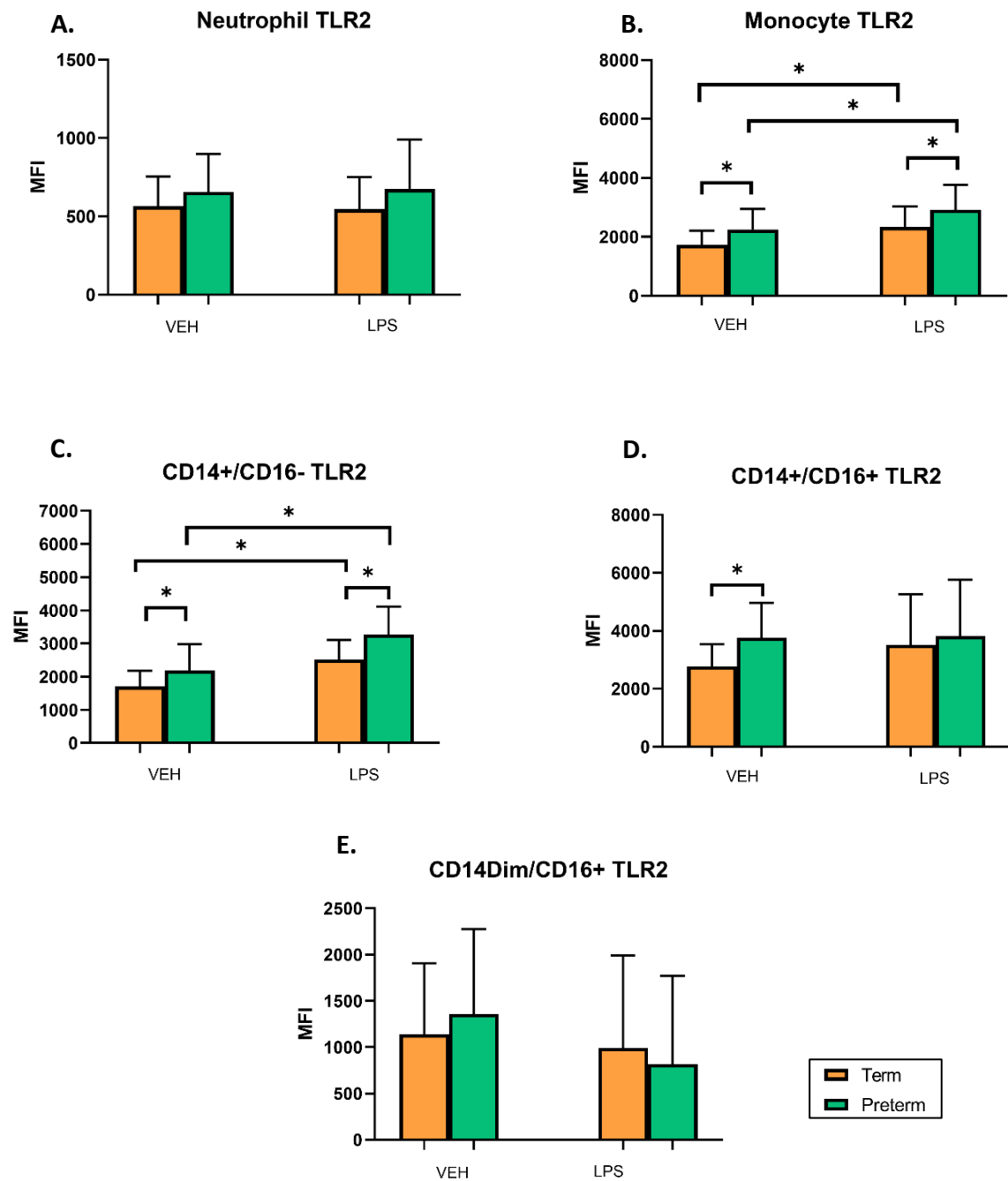


Figure 5.7: TRL2 expression in neutrophils and monocytes in response to LPS (10 ng/ml) in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil TLR2; B) Total monocyte TLR2; C) Classical monocyte TLR2; D) Intermediate monocyte TLR2; E) Non-classical monocyte TLR2. *p<0.05; VEH=vehicle; LPS=endotoxin.

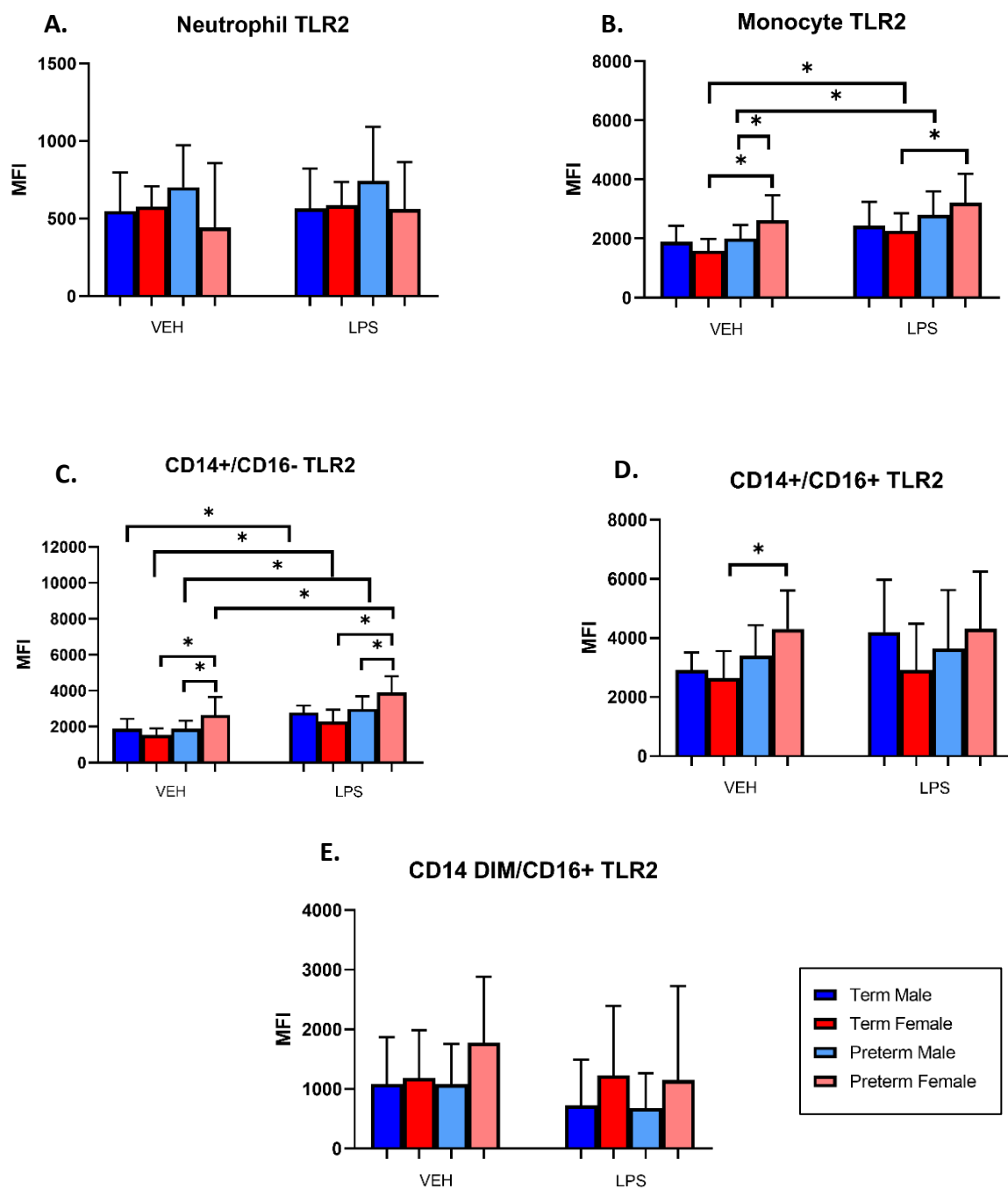


Figure 5.8: Sex differences in TLR2 expression in neutrophils and monocytes in response to LPS (10 ng/ml) in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil TLR2; B) Total monocyte TLR2; C) Classical monocyte TLR2; D) Intermediate monocyte TLR2; E) Non-classical monocyte TLR2. *p<0.05; VEH=vehicle; LPS=endotoxin.

5.4.6. *The effect of hormones on endotoxin-treated neutrophil and monocyte TLR2 expression*

TLR2 expression did not change significantly between untreated and E2-treated term or preterm neutrophils, monocytes, or monocyte subsets (Figure 5.9). TLR2 expression did not change significantly between LPS-treated samples and samples treated with LPS and E2 in combination in term or preterm neutrophils, monocytes, or monocyte subsets (Figure 5.9).

The results of term and preterm neonates were then divided by sex and reanalysed (Figure 5.10). TLR2 expression did not change significantly between untreated samples and E2-treated samples in term or preterm neutrophils, monocytes, or monocyte subsets of either sex (Figure 5.10). TLR2 expression did not change significantly between LPS-treated samples and samples treated with LPS and E2 in combination in term or preterm neutrophils, monocytes, or monocyte subsets of either sex (Figure 5.10).

Treatment with E2 or Pg, either alone or in combination, did not significantly change TLR2 expression from baseline in preterm male or female neutrophils, monocytes, or monocyte subsets (Figure 5.11). TLR2 expression did not differ significantly between samples which were treated with LPS alone and samples which were treated with LPS and either E2 or Pg in preterm neutrophils, monocytes, or monocyte subsets (Figure 5.11). TLR2 expression increased significantly in preterm male total monocytes following LPS treatment, but not females (Figure 5.11b). TLR2 expression increased significantly in both male and female classical monocytes following LPS treatment (Figure 5.11c). TLR2 expression was higher at baseline in preterm female total monocytes and classical monocytes compared to males. TLR2 expression was higher in preterm female classical monocytes following LPS treatment compared to males (Figure 5.11c).

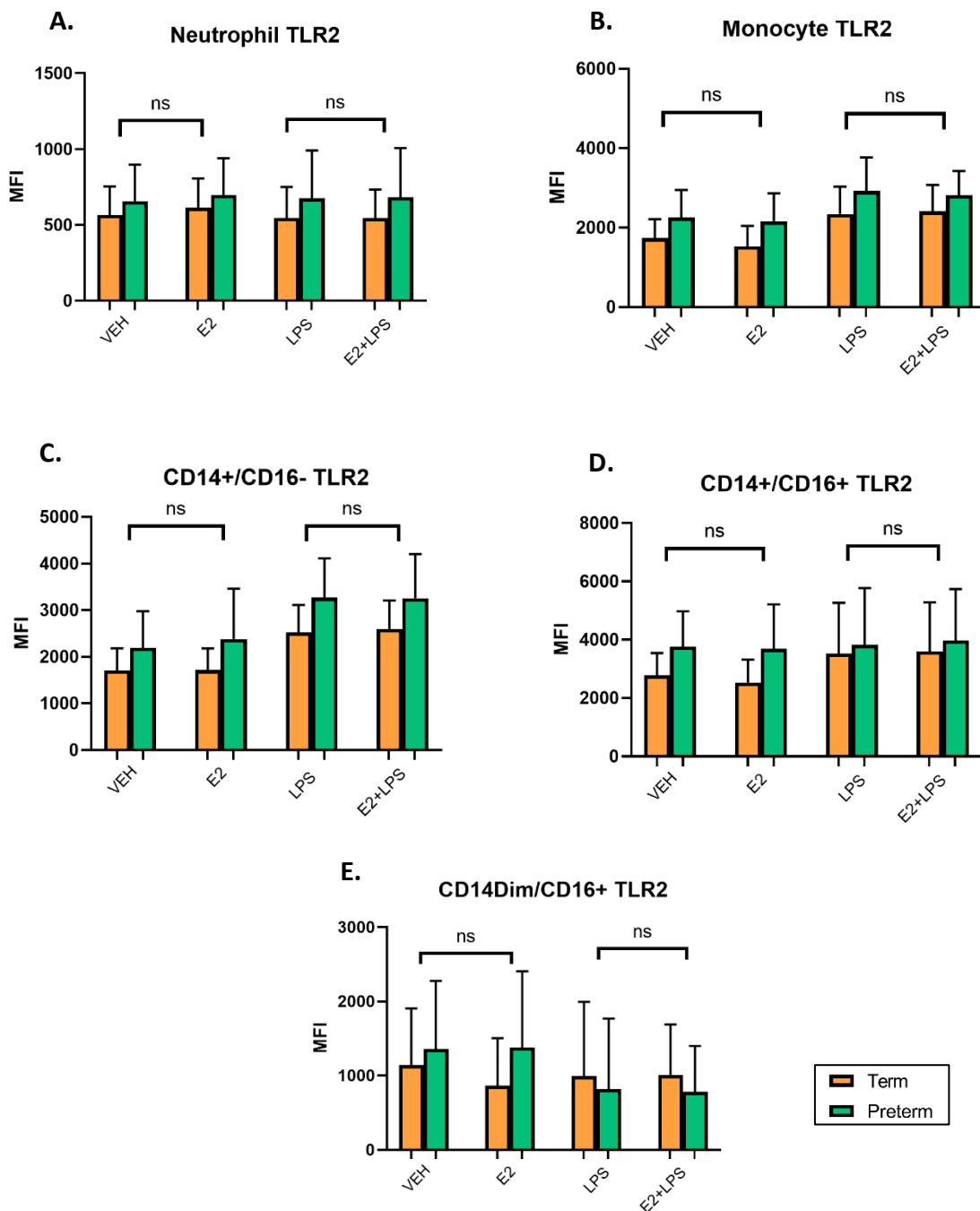


Figure 5.9: TLR2 expression in neutrophils and monocytes in response to LPS (10 ng/ml) and hormone (10nM) treatments in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil TLR2; B) Total monocyte TLR2; C) Classical monocyte TLR2; D) Intermediate monocyte TLR2; E) Non-classical monocyte TLR2. ns=non-significant; VEH=vehicle; E2=Estradiol; LPS=endotoxin.

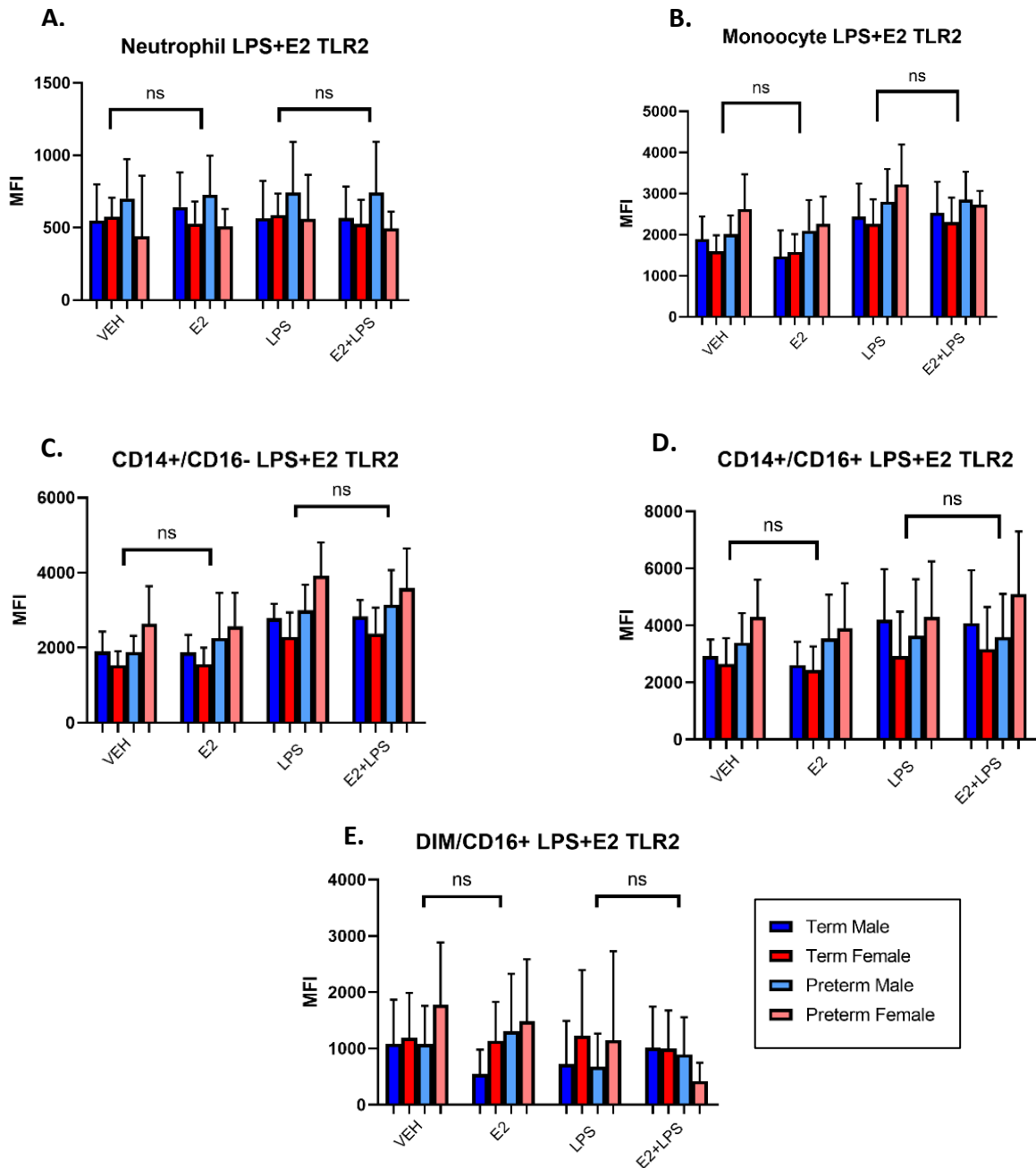


Figure 5.10: TLR2 expression in neutrophils and monocytes of males and females in response to LPS (10 ng/ml) and hormone (10nM) treatments in preterm and term neonates.

Values in preterm (n=21) and term neonates (n=19) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil TLR2; B) Total monocyte TLR2; C) Classical monocyte TLR2; D) Intermediate monocyte TLR2; E) Non-classical monocyte TLR2. ns=non-significant; VEH=vehicle; E2=Estradiol; LPS=endotoxin.

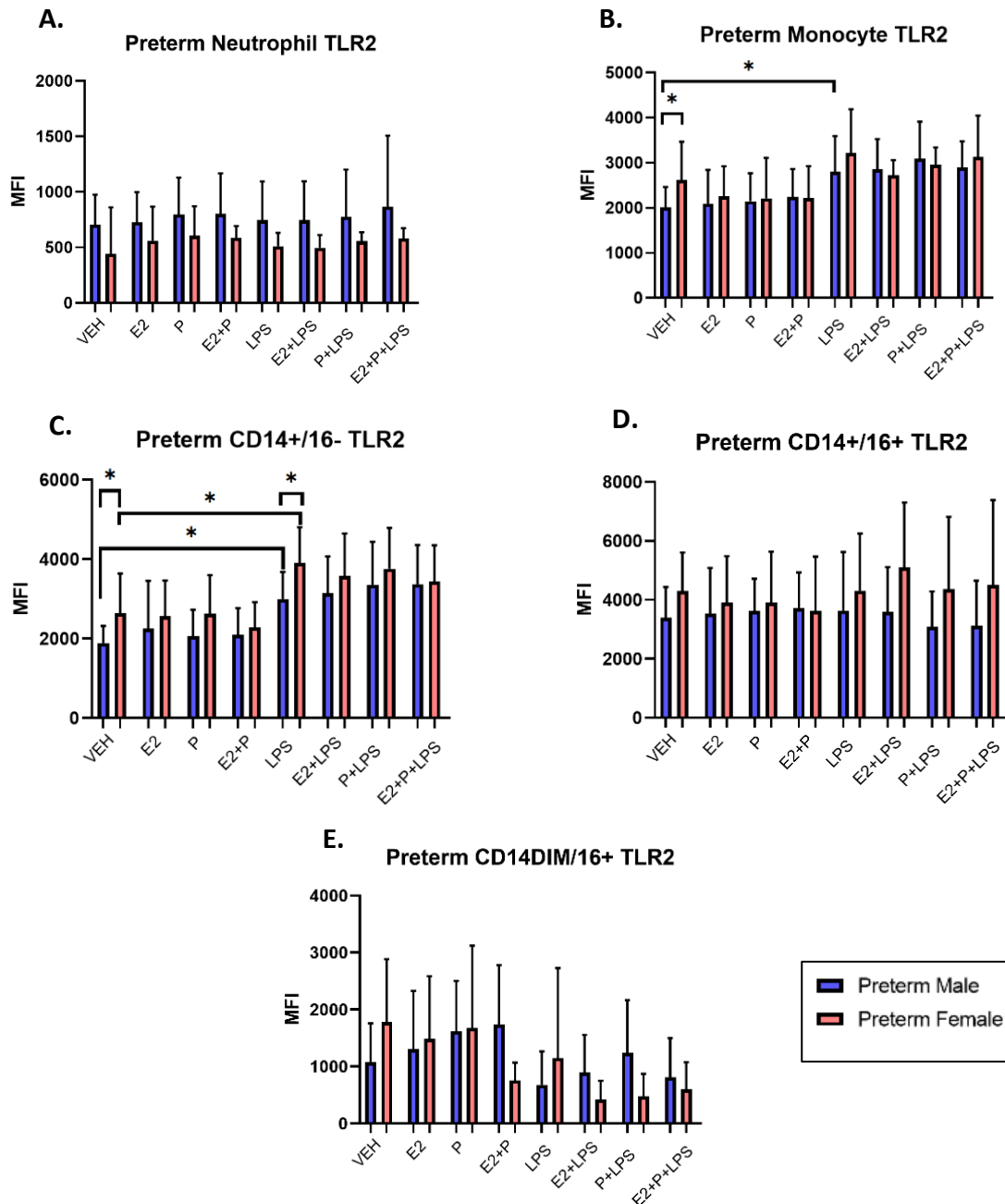


Figure 5.11: TLR2 expression in neutrophils and monocytes of males and females in response to LPS (10 ng/ml) and hormone (10nM) treatments in preterm males and females.

Values in preterm males (n=13) and females (n=8) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil TLR2; B) Total monocyte TLR2; C) Classical monocyte TLR2; D) Intermediate monocyte TLR2; E) Non-classical monocyte TLR2. *p<0.05; VEH=vehicle; E2=Estradiol; P=Progesterone; LPS=endotoxin.

5.4.7. *The effect of sex hormones on Pam3CSK4-treated neutrophil and monocyte TLR2 expression*

TLR2 expression did not increase significantly in preterm male or female neutrophils, total monocytes or monocyte subsets following Pam3CSK4 treatment (Figure 5.12). TLR2 expression was higher at baseline in preterm female total monocytes compared to males (Figure 5.12b). TLR2 expression was significantly higher in preterm female classical monocytes at baseline compared to males (Figure 5.12c).

5.4.8. *Lymphocyte percentages in male and female neonates*

Lymphocyte populations were isolated in 13 preterm neonates and 20 term neonates. Lymphocytes were isolated based on their size and granularity and the following lymphocyte populations were delineated based on cell surface antigen expression: NK cells (CD3-/CD56+), $\gamma\delta 1$ (CD3+/ $\gamma\delta 1$ +), $\gamma\delta 2$ (CD3+/ $\gamma\delta 2$ +), B cells (CD3-/CD19+), CD4+ T cells (CD8-/CD4+), CD8+ T cells (CD8+/CD4-). All preterm neonates received at least 1 dose of antenatal steroids before delivery and none received postnatal steroids before phlebotomy was undertaken. In each case B cells, NK cells, $\gamma\delta 1$ cells, $\gamma\delta 2$ cells, CD4+ T helper cells and CD8+ cytotoxic T cells were identified and their frequency, as a percentage of the total lymphocyte count, was compared between groups. Lymphocyte percentages did not differ between male and female preterm neonates (Figure 5.13a). Lymphocyte percentages did not differ between male and female term neonates (Figure 5.13b). Lymphocyte percentages did not differ between male term and preterm neonates (Figure 5.14a). Lymphocyte percentages did not differ between female term and preterm neonates (Figure 5.14b). The ratio of CD4:CD8 did not differ between male and female preterm neonates (Figure 5.15).

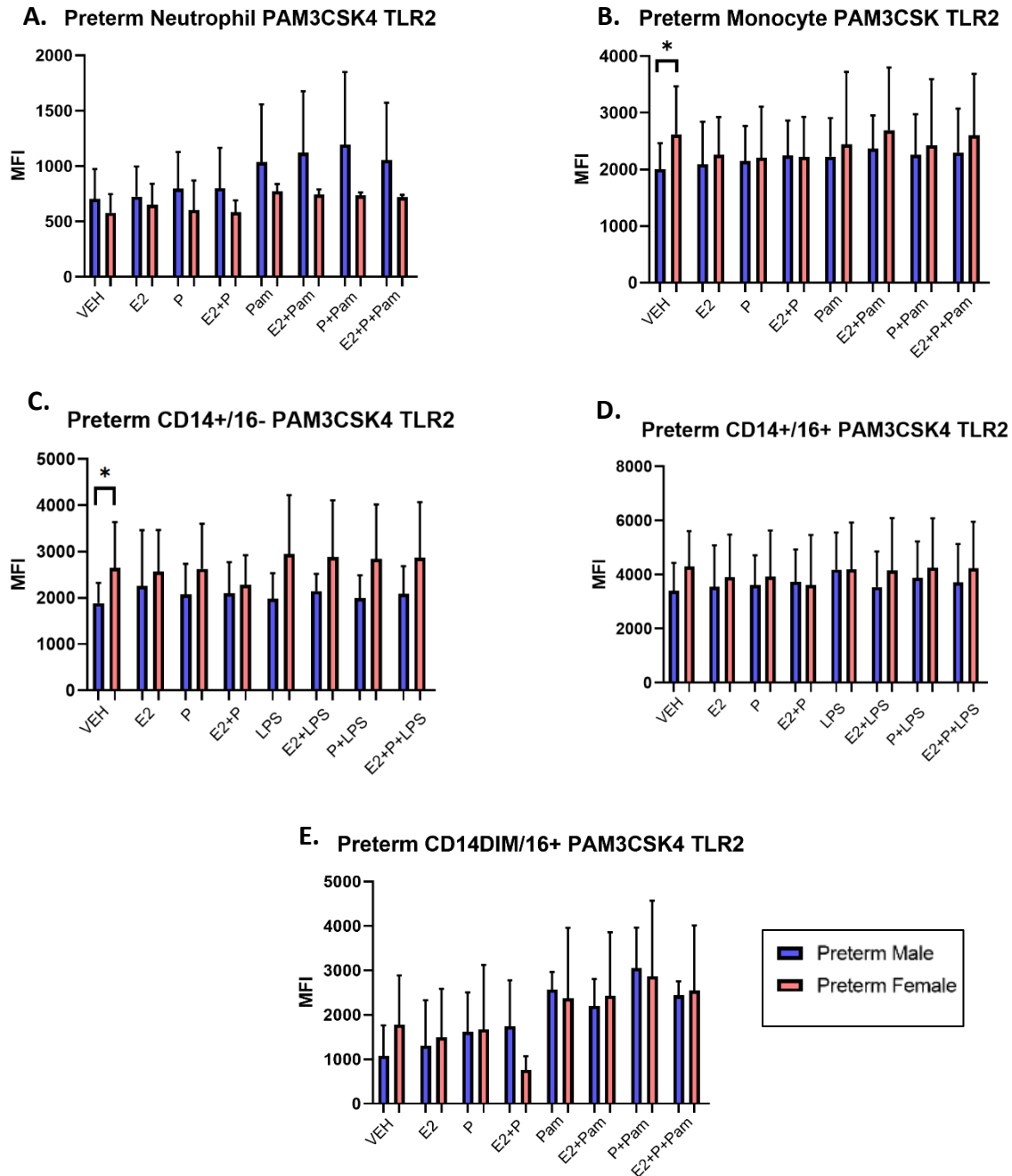
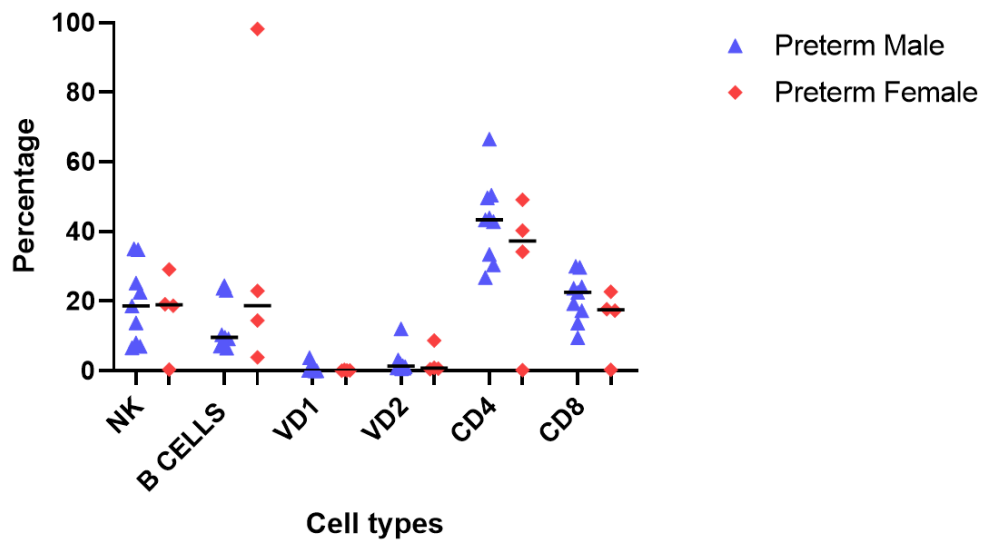


Figure 5.12: TLR2 expression in neutrophils and monocytes of male and female preterm neonates in response to Pam3CSK (5 ng/ml) and hormone (10nM) treatments in preterm males and females.

Values in preterm males (n=13) and females (n=8) are expressed as the mean $\pm$ standard deviation of the mean channel fluorescence (MFI). A) Neutrophil TLR2; B) Total monocyte TLR2; C) Classical monocyte TLR2; D) Intermediate monocyte TLR2; E) Non-classical monocyte TLR2. *p<0.05; VEH=vehicle; E2=Estradiol; P=Progesterone; LPS=endotoxin.

A. Lymphocyte percentages in preterm neonates



B. Lymphocyte percentages in term infants

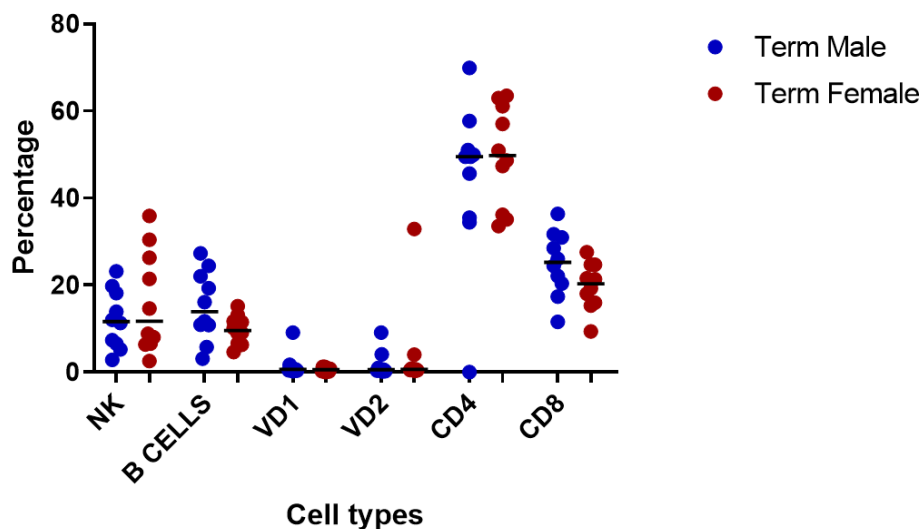


Figure 5.13: Lymphocyte percentages in peripheral blood of male and female term and preterm neonates.

Cell percentages are expressed as a percentage of the total lymphocyte count.

Percentages did not differ between groups. A) Lymphocyte percentages in male (n=9) and female (n=4) preterm neonates. B) Lymphocyte percentages in male (n=10) and female (n=10) term neonates. NK: natural killer; VD1: gamma delta 1 T cells; VD2: gamma delta 2 T cells; CD4: CD4+ T helper cells; CD8: CD8+ cytotoxic T cells.

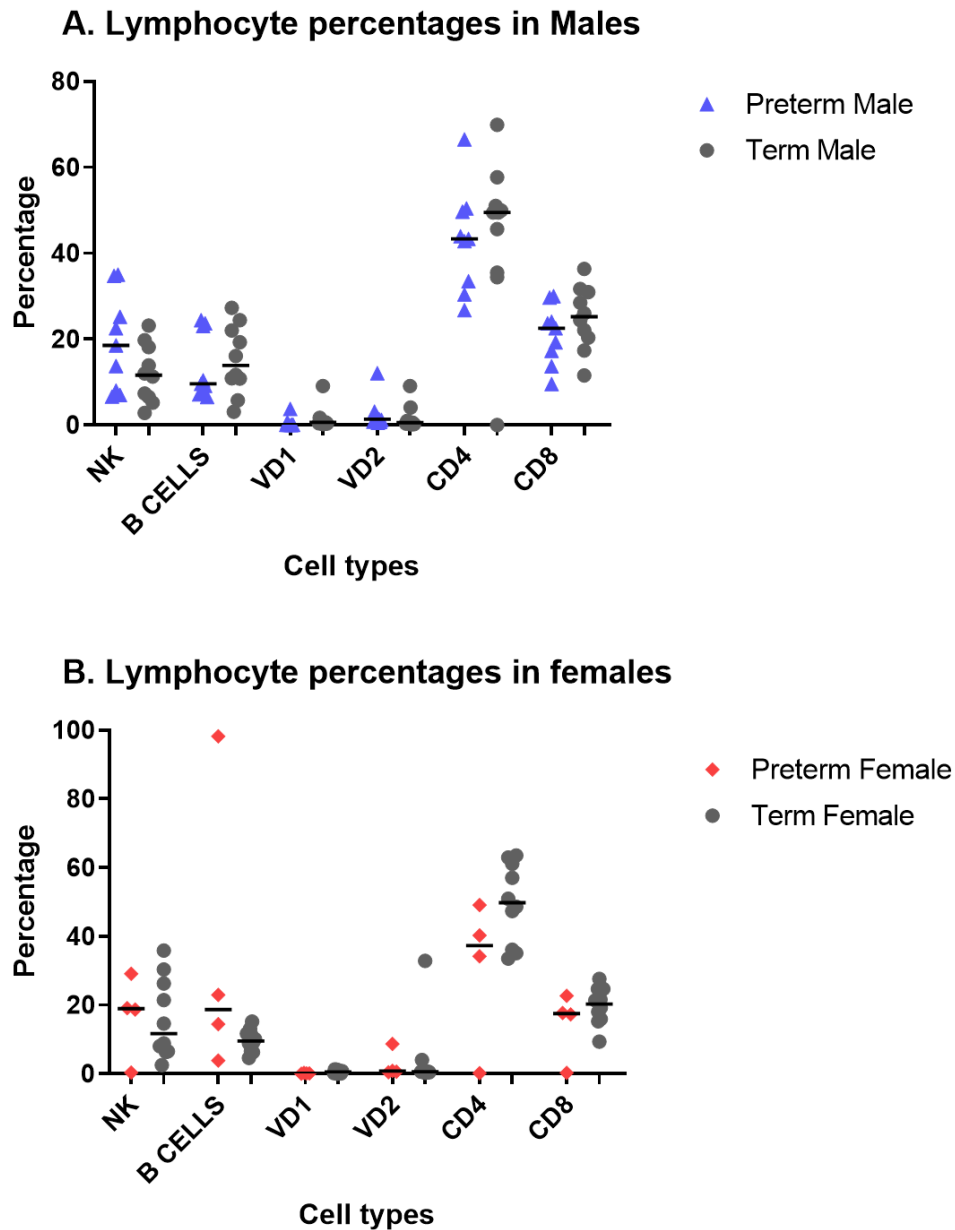


Figure 5.14: Lymphocyte percentages in term and preterm neonates divided by sex.

Cell percentages are expressed as a percentage of the total lymphocyte count.

Percentages did not differ between groups. A) Lymphocyte percentages in male term (n=10) and preterm (n=9) neonates. B) Lymphocyte percentages in female term (n=10) and preterm (n=4) neonates. NK: natural killer; VD1: gamma delta 1 T cells; VD2: gamma delta 2 T cells; CD4: CD4+ T helper cells; CD8: CD8+ cytotoxic T cells.

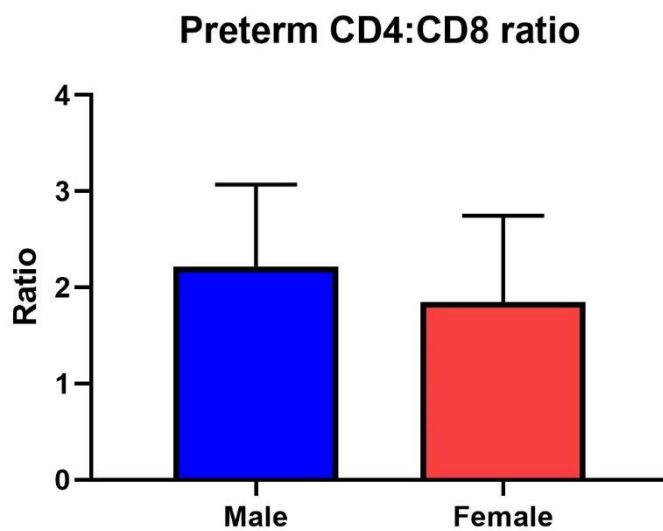


Figure 5.15: CD4:CD8 lymphocyte ratios in male and female preterm neonates.

Ration of CD4:CD8 lymphocytes in male (n=9) and female (n=4) preterm neonates.

Ratios did not differ between groups.

5.4.9. *The effect of sex on X-linked gene expression*

No significant differences were present in fold change expression in term or preterm neonates for any of the X-linked genes examined (Figure 5.15). Reanalysis of results divided by sex showed similar trends with no significant differences between groups for any of the genes examined (Figure 5.16).

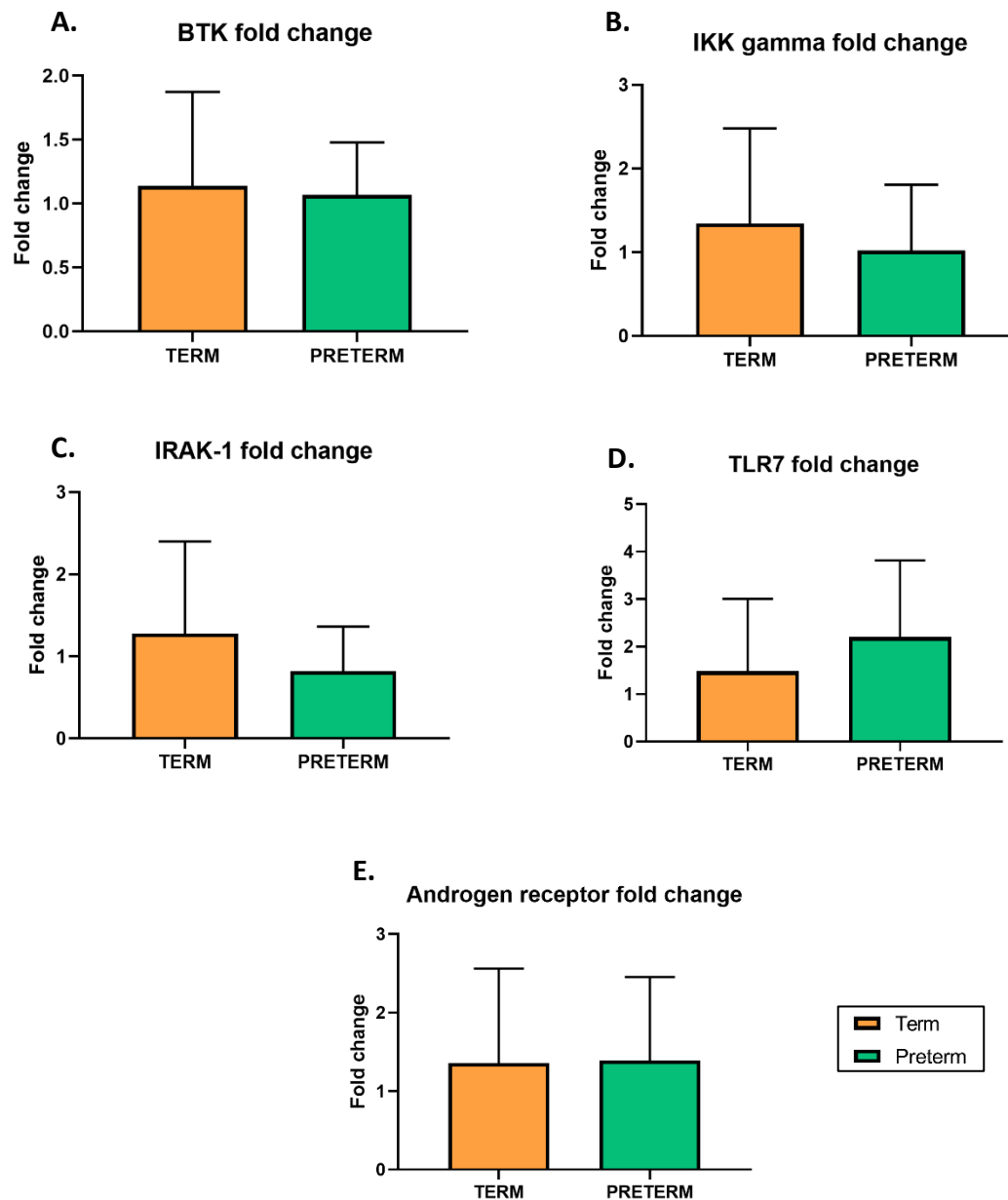


Figure 5.16: Expression of X-linked genes involved in innate immunity in term and preterm neonates.

Values in term (n=9) and preterm (n=10) neonates are expressed as the mean $\pm$ standard deviation of fold change. A) Bruton's tyrosine kinase (BTK); B) Inhibitor of nuclear factor kappa-B kinase subunit gamma (IKK- γ); C) Interleukin 1 receptor associated kinase 1 (IRAK-1) IRAK-1; D) Toll-like receptor 7 (TLR7); E) Androgen receptor.

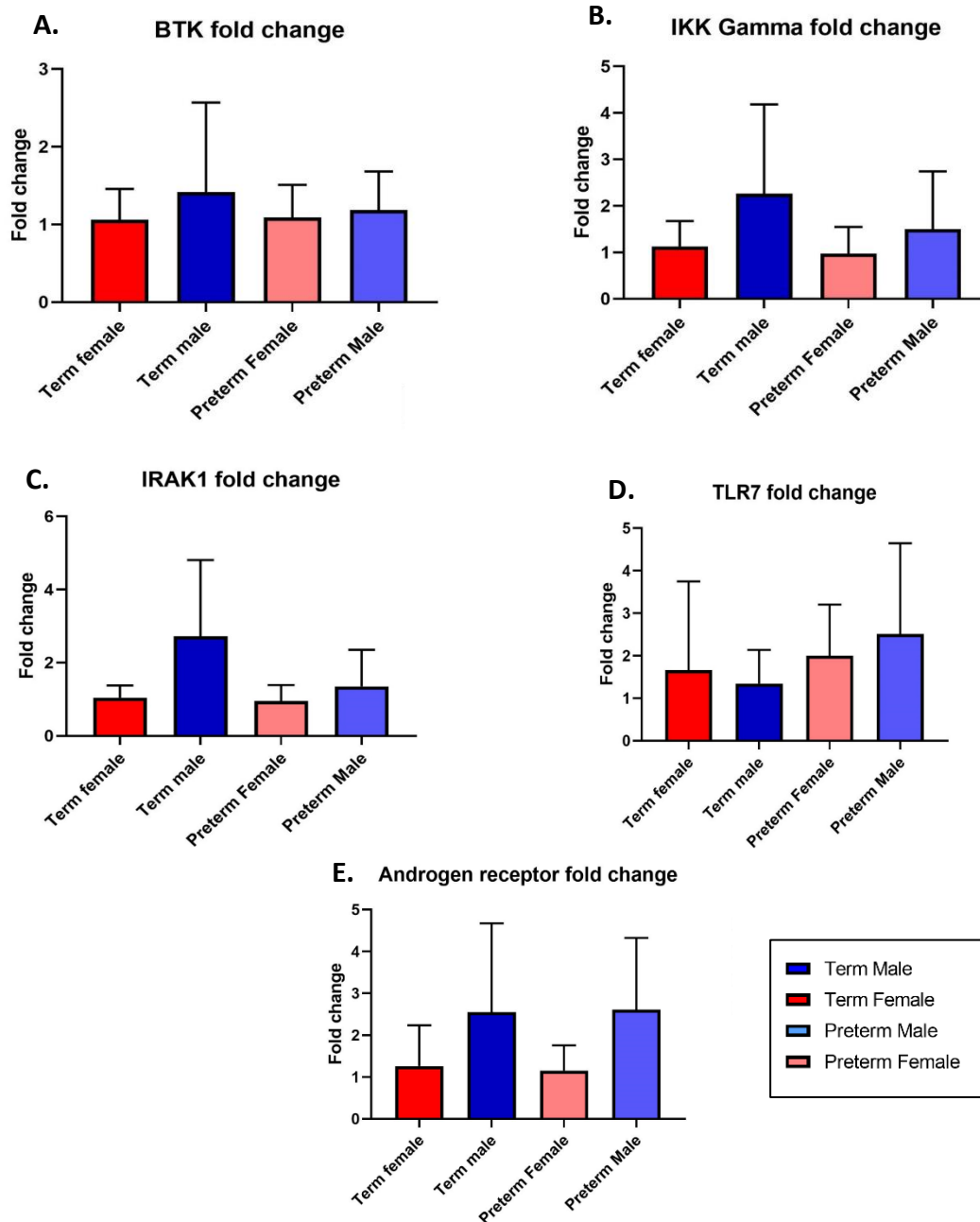


Figure 5.17: Expression of X-linked genes involved in innate immunity in male and female term and preterm neonates.

Values in term (n=9) and preterm (n=10) neonates are expressed as the mean $\pm$ standard deviation of fold change. A) Bruton's tyrosine kinase (BTK); B) Inhibitor of nuclear factor kappa-B kinase subunit gamma (IKK- γ); C) Interleukin 1 receptor associated kinase 1 (IRAK-1) IRAK-1; D) Toll-like receptor 7 (TLR7); E) Androgen receptor.

5.5. Discussion

Baseline neutrophil CD11b expression was similar in term and preterm neonates. Neutrophils of both term and preterm neonates had significantly increased CD11b expression following LPS exposure, though notably LPS-treated term samples had higher CD11b expression than preterms. Although it has been suggested that neutrophil CD11b/CD18 expression is similar in neonates and adults ⁵²⁷, it is more commonly accepted that neonatal neutrophils have diminished CD11b expression at baseline and in response to a variety of stimuli compared to healthy adults ⁵²⁸⁻⁵³⁰. The reduced neutrophil CD11b/CD18 expression in neonates is supported by the concurrent finding of impaired neonatal neutrophil chemotaxis ⁵³¹. This is thought to be due to a reduced cellular content of CD11b/CD18 ¹⁷⁶ which may reflect an increased proportion of immature cells in term neonatal blood compared to adults ¹⁷⁷. This cellular immaturity is likely to be even more marked in preterm neonates as total neutrophil CD11b/CD18 content has been found to be even lower at baseline in preterm neonates compared to infants born at term ¹⁸⁴.

Our findings therefore do not mirror published literature which suggests a diminished baseline CD11b expression in preterm neonates compared to term infants, though the timing of sample collection may explain our findings. Although total neutrophil CD11b/CD18 content is directly related to gestational age ¹⁸⁴, neutrophil CD11b expression increases in the postnatal period in both term and preterm neonates ⁵³². Given that our term samples were collected on day 1-2 of life and our preterm samples were taken at approximately 3 weeks after the date of delivery we are likely to have sampled term neonates at the absolute nadir of CD11b expression, while preterm neonatal CD11b is likely to have increased substantially from birth. This appears likely as previous studies have shown that preterm neonatal cord blood granulocytes ⁵³³ and antenatally collected fetal granulocytes ⁵³⁴ which have not experienced postnatal maturity, have diminished CD11b expression compared to term neonatal controls.

Our findings of a robust CD11b response among preterm neutrophils following LPS are supported by similar results in other studies ^{535,536} though authors once again found that preterm responses were diminished compared to healthy adult controls ⁵³⁶.

Given that term neonatal neutrophils have diminished CD11b expression in response to a variety of stimuli compared to adults ⁵²⁸, it seems logical that preterm responses would be even more impaired, as reflected by our finding of lower CD11b expression in preterms after LPS treatment compared to the more mature term neutrophils.

Dividing neutrophil results by sex showed no differences in baseline neutrophil CD11b between males and females among either term or preterm neonates. While term and preterm neonates of both sexes upregulated CD11b expression following LPS exposure, term female neutrophils appear to have the most robust endotoxin response, possibly reflecting the superior innate immune response of term females. Notably while male term and preterm neutrophil CD11b expression was similar following LPS exposure, term females had higher CD11b than preterm female, suggesting sex-specific CD11b ontogeny in these cells. These findings suggest that the response of females mature with advancing gestation, whereas male term and preterm neonates have very similar responses. Studies examining sex differences in neonatal CD11b expression are lacking and to our knowledge there are no other studies examining the effect of sex on preterm neonatal neutrophil CD11b on postnatally acquired blood samples. Studies in adults found no differences in neutrophil CD11b expression in males and females either at baseline or following inflammatory stimuli such as LPS ⁵³⁷ or phorbol myristate acetate ⁵³⁸. Following LPS exposure male and female mice have similar upregulation of CD11b, though following a dual insult of both ethanol and LPS male mice have significantly higher CD11b expression in neutrophils than female mice ⁵³⁹.

Total monocyte CD11b expression was similar at baseline in both term and preterm neonates with both groups showing significant elevations following LPS exposure. Term neonatal cord blood monocyte CD11b expression is lower at baseline than healthy adults ^{529,540} and has diminished LPS responsiveness compared to adults ⁵⁴⁰. Similar to granulocytes, diminished monocyte CD11b results from an increased proportion of immature white cells in neonates compared to adults ¹⁷⁷. Our findings of similar baseline CD11b in term and preterm monocytes conflict with existing reports which found that CD11b/CD18 expression on monocytes from preterm cord blood is lower than that of term infants ⁵⁴¹, though as detailed earlier the timing of sample

collection likely explains the similarities in baseline CD11b levels noted. Our findings of a robust CD11b response to LPS by preterm monocytes is supported by similar findings by other investigators ^{535,536}, and as with neutrophils these responses are diminished compared to those of adults ⁵³⁶.

To our knowledge no other studies comparing CD11b expression in monocyte subsets in the preterm population exist and data on term neonates is also lacking. Classical monocyte CD11b increased significantly in both term and preterm neonates following LPS exposure and while similar at baseline, these values were higher in term neonates following LPS exposure. Term neonates, who are at higher risk of bacterial infection than adults, have lower percentages of classical monocytes expressing CD11b in cord blood than adults ⁵²⁹. Our finding of a diminished LPS response in preterms therefore likely represents a further impairment in innate immune response as reflected clinically by their profound susceptibility to infection.

CD11b expression on intermediate monocytes in our study was not affected by LPS exposure and did not differ between term or preterm neonates. Data in preterms is lacking though intermediate monocytes of term neonatal cord blood have a similar CD11b expression profile to that of healthy adults ⁵²⁹. CD11b expression was higher at baseline in preterm non-classical monocytes though this difference was not present in LPS-treated cells. Again, data in preterm neonates is lacking and given that term neonatal cord blood has a similar expression profile of CD11b on non-classical monocytes compared to healthy adults ⁵²⁹ this represents a novel finding.

Examining monocyte CD11b by sex shows that at baseline preterm female total monocytes and classical monocytes have higher CD11b than preterm males. Female preterm classical monocytes also had higher CD11b expression than preterm males when treated with E2 or where samples were treated with LPS and Pg concurrently. This improved monocyte activation in preterm females likely reflects improved innate immune function as reflected clinically by their lower risk of sepsis. Additionally, only term females and preterm males have significant elevations in total monocyte and classical monocyte CD11b following LPS exposure. Similar to trends in the neutrophil population, LPS-treated term females had higher CD11b expression compared to preterm females, whereas the expression of CD11b was similar in LPS-treated term

and preterm males. Again, these findings suggest that the monocyte response of females matures with advancing gestation, whereas male term and preterm neonates have very similar responses. These sex differences in monocyte CD11b appear to be unique to the neonatal population as total monocyte CD11b expression on male and female peripheral blood monocytes in healthy adults are similar both at baseline and following stimulation ^{537,538}.

E2 did not affect the expression of CD11b on neutrophils or monocytes of term or preterm neonates of either sex. Pg either alone or in combination with E2 did not affect the expression of CD11b in preterm neonates. While a single study of rat monocytes treated with estriol at supra-physiological concentrations show reduced CD11b compared to baseline and LPS-treated samples ⁵⁴², our results are largely consistent with published literature in animals and adults which found no difference. Study of oophorectomized rats showed little difference in CD11b expression versus non-oophorectomized controls following experimentally induced injury ⁵⁴³. Murine eosinophils incubated with testosterone and Pg did not have any alterations in CD11b expression ⁵⁴⁴. Physiological concentrations of estrogen and Pg did not alter CD11b expression in neutrophils of healthy adults of either sex ⁵⁴⁵.

Though trends appear to be present in CD11b expression following Pam3CSK4 treatment in preterm neonates these did not reach statistical significance. CD11b expression was higher in preterm male non-classical monocytes following Pam3CSK treatment when compared to females, a finding which is unique to our study.

Neutrophil TLR2 did not differ between term and preterm neonates at baseline and did not increase in either group following LPS exposure. Preterm neonates had higher TLR2 expression at baseline in total monocytes, classical monocytes and non-classical monocytes. Both term and preterm total monocytes and classical monocytes had significant elevations in TLR2 following LPS treatment and in both cases preterm neonates had higher TLR2 expression following LPS exposure. Data on TLR2 expression in neonatal innate immune cells in comparison to adults is somewhat conflicting. The percentage of granulocytes expressing TLR2 has been suggested to be higher in term neonatal cord blood than in adults ⁵²⁹, whereas expression on cord blood monocytes has been identified as potentially similar to adults ⁵⁴⁶ or lower than in adult

populations ⁵²⁹. TLR2 expression may also differ between neonates and adults depending on the monocyte population being compared, with similar expression on non-classical monocytes and intermediate monocytes and lower expression in classical monocytes of term neonatal cord blood ⁵²⁹. Data comparing term and preterm neonatal TLR2 expression, which is largely focused on monocytes, is similarly conflicting with studies showing higher, lower and equivocal expression in preterm neonates compared to term infants ⁵⁴⁷. It has also been suggested that the expression of TLR2 increases rapidly after birth in neonatal monocytes, reaching term levels within 2 weeks of birth ⁵⁴⁸, though other authors have found little difference in TLR2 mRNA in peripheral blood of preterm neonates in the months after delivery ⁵⁴⁹. Our finding of higher TLR2 in VLBW preterm neonates may therefore represent rapid postnatal maturation of preterm TLR2 expression or simply reflect inherently higher TLR2 levels in the preterm population. Notably while preterm neonatal TLR2 expression may increase postnatally, cytokine responses do not mature as rapidly ⁵⁵⁰ and are impaired in comparison to both term and adult leukocytes ⁵⁵¹. In addition, while neonates are able to activate TLR2 signalling, it has been suggested that they are less well-equipped to terminate the TLR-mediated inflammatory response, potentially explaining their susceptibility to the adverse effects of gram positive infections like GBS ⁵⁵². Our findings of a robust TLR2 response in neonates following inflammatory stimuli is in keeping with findings that human neonates increase TLR2 mRNA in peripheral blood mononuclear cells and TLR2 protein in both monocytes and granulocytes in response to infection ⁵⁵³.

Dividing results by sex showed that total monocyte TLR2 increased significantly in preterm males and term females with LPS treatment. All classical monocyte groups had significant elevations in TLR2 following LPS exposure and expression in intermediate and non-classical monocytes did not change in either sex in term or preterm neonates. Notably the higher TLR2 expression in preterm neonates was largely attributable to preterm females. Preterm female total monocyte TLR2 expression was higher than both term females and preterm males at baseline, and higher than term females following LPS. Preterm female classical monocyte TLR2 was higher at baseline than term females and preterm males, and higher than term

females following LPS. Preterm female untreated intermediate monocytes TLR2 was also higher than term females. It is unclear why TLR2 is higher in preterm females compared to term females, given the increased susceptibility of preterm neonates to gram positive infections. The difference between preterm males and females is however reflected clinically by differences in infection susceptibility. The sex difference noted is novel and may be limited to the preterm or early neonatal population as TLR2 expression on monocytes of older children does not differ between males and females ⁵⁵⁴.

E2 treatment did not affect the TLR2 expression of term or preterm neonates of either sex. Pg, either alone or in combination with E2 did not affect the expression of TLR2 in preterm neutrophils or monocytes. While there is convincing evidence that sex hormones influence the immune system ⁵⁵⁵, studies examining the ability of sex hormones to modulate pattern recognition receptor expression in monocytes and neutrophils are limited, and mostly from adults and animal models. Pg has been shown to modify TLR4 mRNA levels in adult mononuclear cells in a dose-dependent fashion ⁵⁵⁶ and inhibits LPS-induced TLR4 upregulation in murine macrophages ⁵⁵⁷. Pg also decreases TLR7 expression in mononuclear cells of adults with hepatitis C infection ⁵⁵⁸. Estrogen and Pg in combination reduce the expression of TLR2 mRNA and protein on peripheral blood monocytes in adults ⁵⁵⁹ and at high doses increase cell surface expression of TLR4 in murine macrophages ⁵⁶⁰. A study by Calippe *et al.* however found that E2 did not affect the cell surface expression of TLR4 in murine macrophages ⁵⁶¹. The only study in neonatal patients found that while Pg has potential to increase TLR2 mRNA in cord blood mononuclear cells it does not affect TLR2 protein expression in these cells as assessed by western blot ⁵⁶². Notably the TLR2 agonist Pam3CSK4 did not significantly affect TLR2 expression in male or female preterm neonates.

Animals models of neonatal infection highlight the crucial role of TLR2 in clearing CONS infection ⁵⁶³, protecting against GBS sepsis ⁵⁶⁴, and regulating mucosal response to bowel inflammation ⁵⁶⁵. In addition, the beneficial effects of both probiotics and breast milk in protecting the neonatal bowel are thought to be partially mediated by their effects on TLR2. ^{566,567}. TLR2 activation secondary to sepsis has

however been implicated in sepsis-induced brain injury ⁵⁶³ and repeated early life systemic activation of TLR2 adversely affects brain development in mice ⁵⁶⁸. Thus, the decreased TLR2 expression in preterm males may explain their susceptibility to gram positive bacterial infection while the ability of preterm males to significantly increase expression in response to inflammatory stimuli may explain their susceptibility to adverse neurological outcome following sepsis.

Lymphocyte percentages did not differ between term and preterm neonates of either sex. The percentages of each cell type isolated were similar to those published by other sources in the term and preterm population ^{152,569}. CD4+ T cells were the most abundant cell type overall in keeping with the findings of other authors ^{152,569}. In our population $\gamma\delta$ T cells were the least frequently identified cell type of those examined in term and preterm neonates, in keeping with the findings of Prabhu *et al.* who identified lymphocyte populations in the cord blood of 76 term neonates ³⁵⁴. Preterm females had a high mean B cell percentage relative to other groups though notably this was due to a single outlying value and did not differ significantly from other groups. A study by Amatuni *et al.* on 562 neonates of various gestational ages showed that while preterm neonates are lymphopaenic relative to infants born at term, lymphocyte subset percentages showed little variation between extremely preterm and term neonates, in keeping with our findings ⁵⁶⁹. Berrington *et al.* performed a similar study of 67 neonates and again found that despite relative lymphopaenia among the preterm neonates enrolled, lymphocyte subset percentages were similar between term and preterm neonates ¹⁵². This same study noted that when preterm patients who had received postnatal steroids were included in the analysis NK cell percentages were higher and T helper cell percentages lower in preterm neonates ¹⁵², a finding we could not investigate as none of the neonates studied had postnatal steroids administered.

Sex differences in term and preterm neonatal lymphocyte counts are not well studied but our findings are supported by the limited available data. Amatuni *et al.* found that neonatal sex does not significantly affect lymphocyte populations in preterm neonates, in keeping with our results ⁵⁶⁹. The lack of sex differences in lymphocyte percentages is also supported by a study from Nikulshi *et al.* on 4128

children who found that although lymphocyte subsets differ between male and female children with advancing age these sex differences are not present in the first 6 month of life ⁵⁷⁰.

Studies on sex-differences in X-linked gene expression in peripheral blood in the neonatal population are lacking and very few examine preterm neonates. We found that BTK expression was similar in term and preterm neonates and dividing results by sex did not reveal any differences. O'Driscoll *et al.* found that BTK mRNA expression, while lower in neonates than adults, does not differ between cord blood monocytes in male or female term neonates ³⁰⁵, in keeping with our findings. We found no differences in IKK- γ between term and preterm neonates and no differences between males and females. O'Driscoll *et al.* found that IKK- γ mRNA is lower in mononuclear cells of term neonates compared to adults but did not differ between males and females ³⁰⁵, in keeping with our findings. We found no differences in IRAK-1 expression between term and preterm neonates. Zhang *et al.* made serial measurements of peripheral blood IRAK-1 in former preterm neonates and found that these did not change significantly between 28 weeks gestation and 34 weeks corrected gestation ⁵⁴⁹, in keeping with our findings. We found no differences in IRAK-1 expression between males and females in either term or preterm neonates. This conflicts with a study which detailed higher IRAK-1 mRNA and protein expression in female umbilical cord mononuclear cells compared to males ³⁰⁵. We found no differences in TLR-7 expression between term and preterm neonates of either sex. Zhang *et al.* found that TLR7 mRNA in peripheral blood of preterm neonates increase in the months after delivery ⁵⁴⁹ with authors stating that levels had increased significantly by 34 weeks gestation. We may therefore have expected term neonates to have higher expression than preterm neonates, though studies in term infants suggest that peripheral blood expression is weak even in cases of clinical inflammation such as meconium aspiration syndrome ⁵⁷¹. AR expression did not differ between term and preterm neonates and while expression appears to be higher in males this did not reach statistical significance. No other studies examining AR expression in term and preterm neonates could be found for comparison.

5.6. Conclusion

Our findings highlight the important differences in innate immune response between preterm and term neonates and suggest important differences between preterm males and females. Much of the existing literature on neonatal peripheral blood immune cells is from analysis of cord blood, which is thought to be immunologically distinct from postnatal samples³¹⁴, and our study is therefore one of the few which evaluates neonatal innate immunity in postnatally acquired peripheral blood.

CD11b was generally higher after endotoxin exposure in term neonates, reflecting the compromised innate immune response of preterm neonates. Our findings of higher TLR2 expression in preterm neonates are surprising given the profound susceptibility of preterm neonates to gram positive infection, though much of the existing literature comparing TLR2 expression in term and preterm neonates is conflicting and thus our findings may represent a real difference.

We have shown that female preterm neonates have higher CD11b and TLR2 in many cells compared to preterm males, likely reflecting their superior innate immune defences. We have also shown that CD11b and TLR2 expression is different in female term and preterm neonates but similar in males of differing gestation, potentially reflecting sex differences in immune cell maturation.

E2 and Pg did not affect CD11b or TLR2 expression in term or preterm neonates of either sex indicating that if sex hormones modulate immune response in these patients it is not through these receptors. The X-linked genes examined did not differ between term or preterm males and females suggesting that at this stage in life X chromosome inactivation may not be an important source of immunological advantage in females.

Chapter 6: miRNA expression in male and female neonates

6.1. Introduction

miRNAs are small non-coding sequences of RNA of approximately 22 nucleotides in length that function as post-transcriptional regulators of gene expression ⁵⁷². An individual miRNA may have numerous potential target mRNAs and conversely, an individual mRNA may be the target of many miRNAs. miRNAs are also among the most abundant gene regulators and given that an individual miRNA may have a potentially diverse range of biological actions, collectively miRNAs are among the most important regulators of gene expression in humans ⁵⁷³.

In addition to their presence within cells, miRNAs have also been identified within paediatric serum ⁵⁷⁴, plasma ⁵⁷⁵, saliva ⁵⁷⁶, respiratory secretions ⁵⁷⁷ and maternal breast milk ⁵⁷⁸. Whether these extracellular miRNAs are predominantly by-products of cellular metabolism or whether their excretion is a regulated process is not yet understood, though notably extracellular miRNAs are highly stable and have shown both cellular interaction and uptake, suggesting some role in cell-to-cell signalling ³⁵⁶. Due to the remarkable stability of circulating miRNAs ⁵⁷⁹, there is great interest in their use as potential biomarkers of disease ^{580,581}.

Preterm neonates are at increased risk of adverse clinical outcome compared to older infants. The risk of health complications in neonates is inversely related to gestation and while much has been published on the potential role of miRNAs in neonatal respiratory disease ⁵⁸²⁻⁵⁸⁷ the effect of miRNAs on mediating many of the other complications of prematurity is largely unknown. Female sex is thought to confer a biological advantage in many disease processes throughout life. Part of this advantage is thought to be mediated by genetic factors, more specifically by the X chromosome which contains many genes important to immune response ³⁴⁴. Female X chromosome mosaicism, X chromosome inactivation escape and X chromosome skewing have all been suggested as important factors providing women with a potentially more robust immune response than men ³⁴⁵. In addition to coding for many genes important in immune response, the X chromosome codes for approximately 10% of the miRNAs discovered in humans to date ⁵⁸⁸, which has been hypothesised as another important source of immunological advantage. Given that an

individual miRNA may have hundreds of targets it has been suggested that even modest differences in miRNA expression between males and females may have a cascade-like effect, with potentially significant effects on gene expression ³⁴⁸. Sex differences have previously been identified in the plasma miRNA expression profile of male and female children with cystic fibrosis ⁵⁷⁵, though the effect of sex on miRNA expression in paediatric disease is not well studied.

Although miRNAs are thought to play an important role in modulating neonatal immune response ⁵⁸⁹ and many have been suggested as biomarkers of preterm neonatal respiratory pathology ⁵⁹⁰⁻⁵⁹³, the role of miRNAs in neonatal disease is poorly understood. In particular, little is known about miRNA expression in VLBW preterm neonates and what effect sex has on miRNA expression in the premature neonatal population. Given the increasing interest in the use of miRNAs as biomarkers of disease, it is important to establish if differences in miRNA expression exist between male and female neonates. Study of such sex differences will both improve our understanding of the male disadvantage noted clinically and establish if sex is an important variable to consider in future studies of miRNAs as biomarkers of neonatal disease.

6.2. Hypothesis

Differences in miRNA profiles exist between male and female preterm neonates and may explain some of the differences in clinical outcome between the sexes.

6.3. Aims

To examine the serum miRNA profile of male and female VLBW neonates and compare this to healthy controls.

6.4. Results

6.4.1. *Patient characteristics*

Serum was collected on 11 VLBW preterm neonates (5 male, 6 female) and 11 healthy term neonates (5 male, 6 female). The mean (SD) gestation of preterm neonates was 28.4 (± 2) (range 25-32) weeks and mean birth weight was 1075 (± 273) (range: 560-1460) grams. Patient characteristics did not differ between male and female preterm infants (Table 6.1).

	Preterm male (n=5)	Preterm female (n=6)	p- value
Birth weight (g)	1148.3 ($\pm$ 190.1)	1001.7 (273.1)	0.306
Gestation at delivery (weeks)	29.2 ($\pm$ 1.6)	27.5 ($\pm$ 2.1)	0.137
Gestation at phlebotomy (weeks)	31 ($\pm$ 1.5)	29.7 ($\pm$ 1.8)	0.193

Table 6.1: Demographics for male and female preterm neonates

Demographics for miRNA samples of male and female preterm (n=11) neonates.

Values presented represent mean $\pm$ standard deviation. g=grams.

6.4.2. miRNA expression profile in male and female neonates

The expression of 754 miRNAs was studied in the serum of enrolled patients. miRNAs were considered as expressed if their Ct value was <35 (Figure 6.1). The 10 miRNAs with the lowest mean Ct values in the patients sampled are displayed in Table 6.2. The miRNA expression matrix compiled by Haider *et al.*⁵⁹⁴ was consulted to evaluate the potential cell types of origin for the 10 miRNAs identified as having the highest expression. miRNA-16-5p was ubiquitously expressed in all 18 cell types listed by Haider *et al.* whereas the remaining 9 miRNAs were either not listed in the expression matrix or had expression levels uniformly below the cut-offs used by the authors⁵⁹⁴.

Analysis of the raw Ct data showed that premature female neonates have the highest average number of expressed miRNAs (Ct <35) and premature male neonates had the least, though this did not reach statistical significance (Figure 6.2). Of the 754 miRNAs examined, 86 were identified as being from the X chromosome. The expression of these miRNAs was examined in participants (Figure 6.3). Analysis of the raw Ct data showed that premature female neonates have the highest average number of expressed X-linked miRNAs (Ct<35) and premature male neonates the least, though this difference did not reach statistical significance (Figure 6.4).

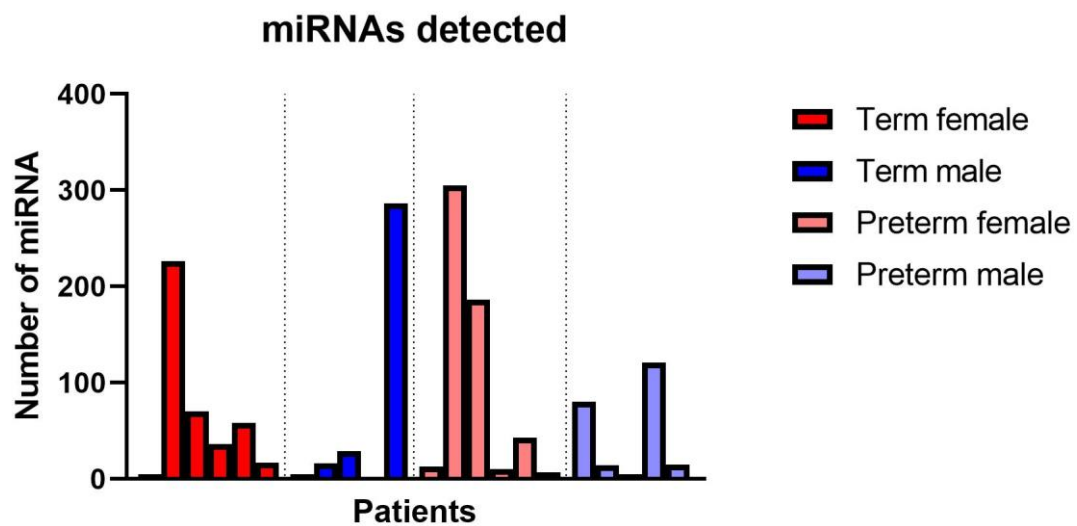


Figure 6.1: Number of miRNAs detected in participants.

This figure presents the number of expressed miRNAs (Ct <35) in preterm male (n=5), preterm female (n=6), term male (n=5) and term female (n=6) neonates.

miRNA name	Mean Ct values ($\pm$ Standard Dev)
miR-16-5p	25.82864 ($\pm$ 17.57)
miR-39-3p	27.43777 ($\pm$ 17.15)
miR-548p	29.08536 ($\pm$ 16.47)
miR-302b-5p	29.40473 ($\pm$ 16.13)
miR-552-3p	29.54718 ($\pm$ 16.06)
miR-548a-3p	29.60445 ($\pm$ 15.91)
miR-302d-5p	29.70991 ($\pm$ 15.68)
miR-518e-5p	29.77032 ($\pm$ 15.67)
miR-218-5p	30.22586 ($\pm$ 16.41)
miR-144-5p	30.24214 ($\pm$ 16.40)

Table 6.2: The 10 miRNAs with the highest mean expression.

The pooled Ct values of the miRNAs isolated from the OpenArray were analysed and the 10 miRNAs with the lowest mean Ct values across all samples are listed above.

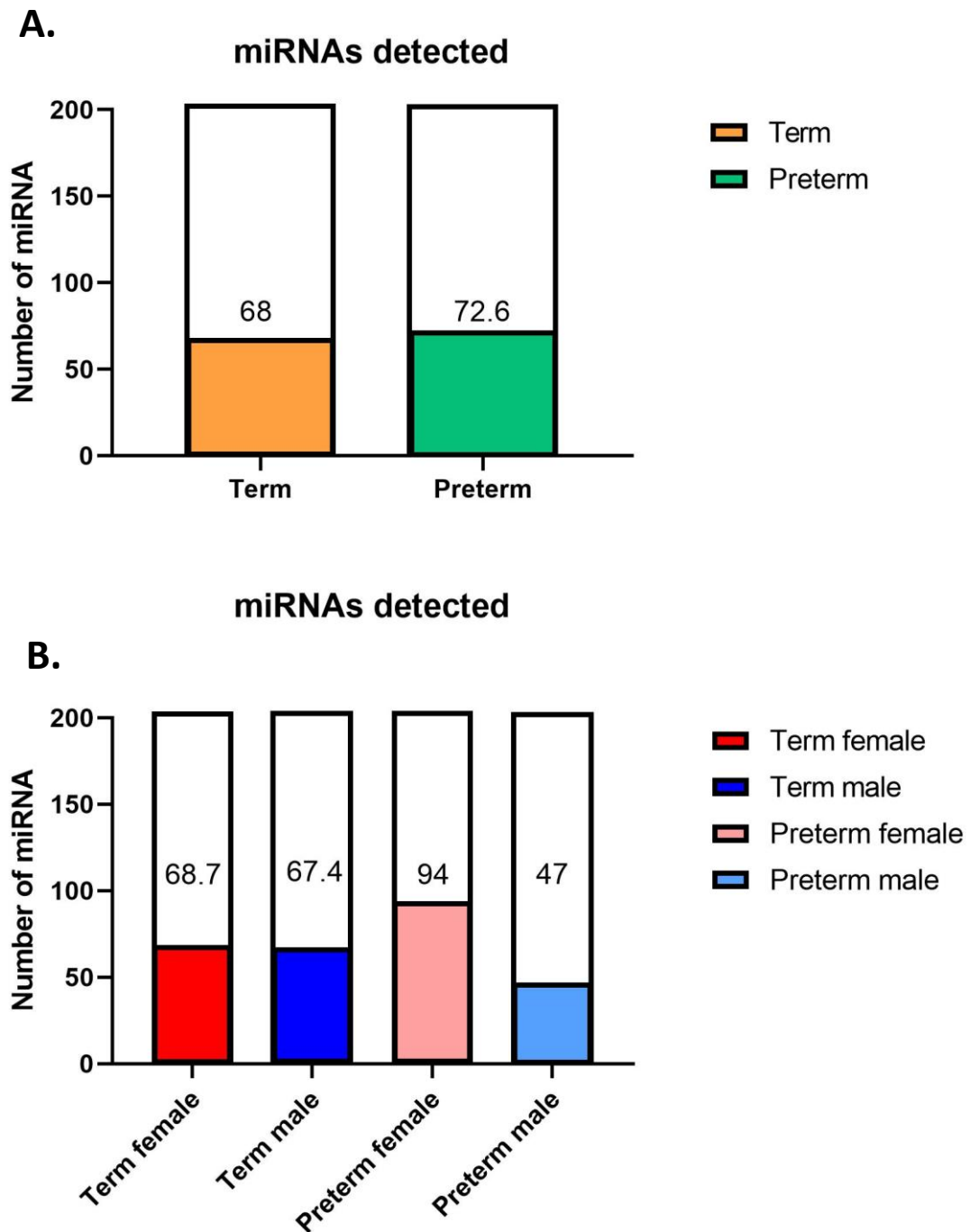


Figure 6.2: Mean number of expressed miRNAs (Ct <35) in each group.

Mean number of miRNAs detected (Ct<35) in term and preterm neonates. A) Mean number of miRNAs detected in term (n=11) and preterm (n=11) neonates. Means did not differ significantly between groups ($p>0.05$). B) Mean number of miRNAs detected in term female (n=6), term male (n=5), preterm female (n=6) and preterm male (n=5) neonates. Means did not differ significantly between groups ($p>0.05$).

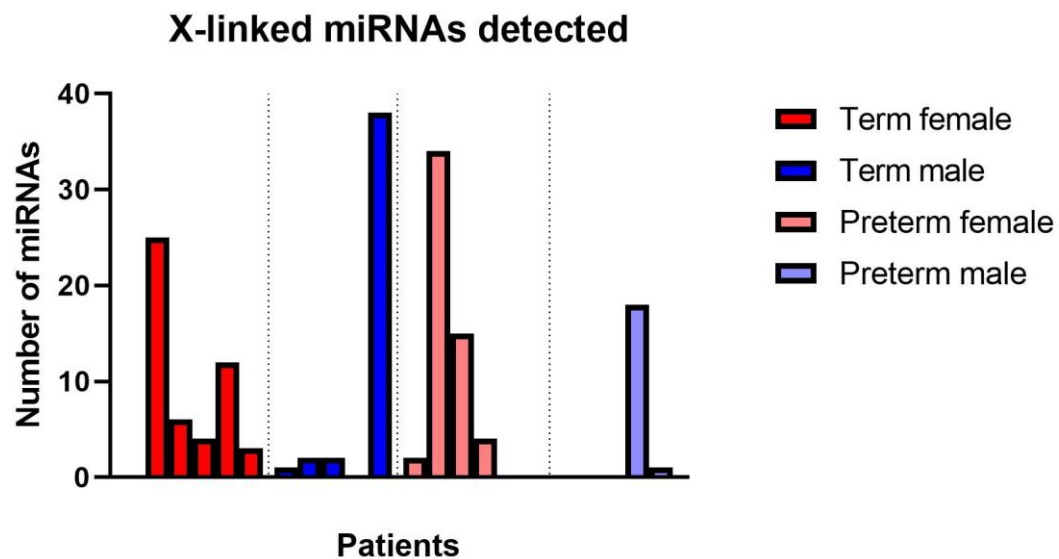


Figure 6.3: Number of X-linked miRNAs detected in participants.

This figure presents the number of expressed X-linked miRNAs (Ct <35) in preterm male (n=5), preterm female (n=6), term male (n=5) and term female (n=6) neonates.

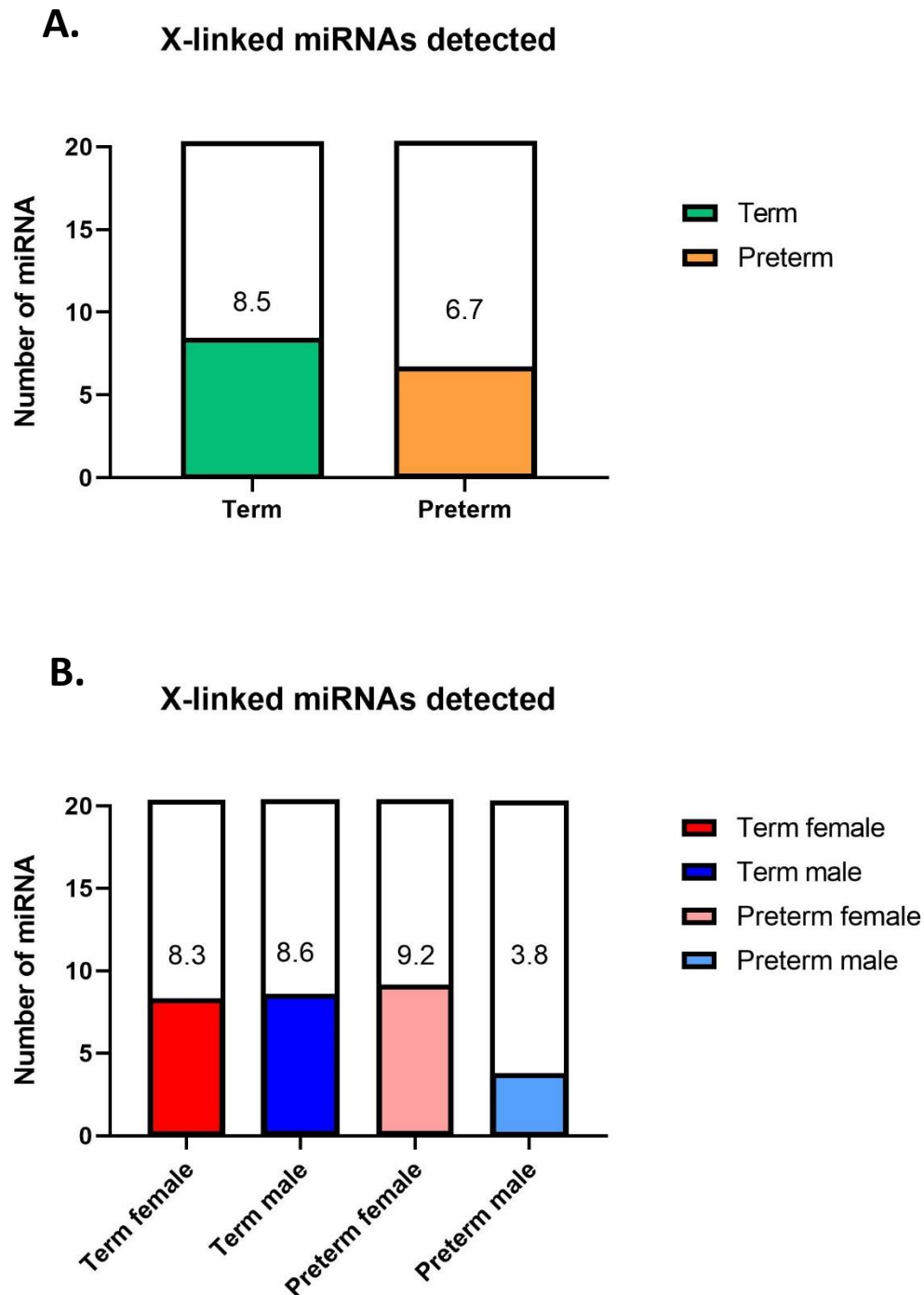


Figure 6.4: Mean number of expressed X-linked miRNAs (Ct <35) in each group.

Mean number of X-linked miRNAs detected (Ct<35) in term and preterm neonates. A)

Mean number of X-linked miRNAs in term (n=11) and preterm (n=11) neonates.

Means did not differ significantly between groups. B) Mean number of X-linked

miRNAs in term female (n=6), term male (n=5), preterm female (n=6) and preterm

male (n=5) neonates. Means did not differ significantly between groups (p>0.05).

6.4.3. *Differential expression analysis*

Geometric mean normalisation and fold change calculation was undertaken as detailed in chapter 2, section 2.10. Expression of each miRNA was compared between groups. miR-212-3p and miR-218-2-3p expression was significantly higher in preterm female than preterm male neonates ($p=0.045$ for both). miR-663b expression was significantly higher in term male neonates than both term female neonates ($p=0.039$) and preterm male neonates ($p=0.016$). No significant differences were detected in X-linked miRNAs between groups.

Validation of the OpenArray findings was then undertaken with qRT-PCR using TaqMan miRNA assays recommended by the manufacturer on the original serum samples from each neonate (Figure 6.5). While trends were present in the directions expected, neither miR-663b nor miR-212-3p differed between groups (Figure 6.5). miR-218-2-3p failed to validate on repeat testing (data not displayed).

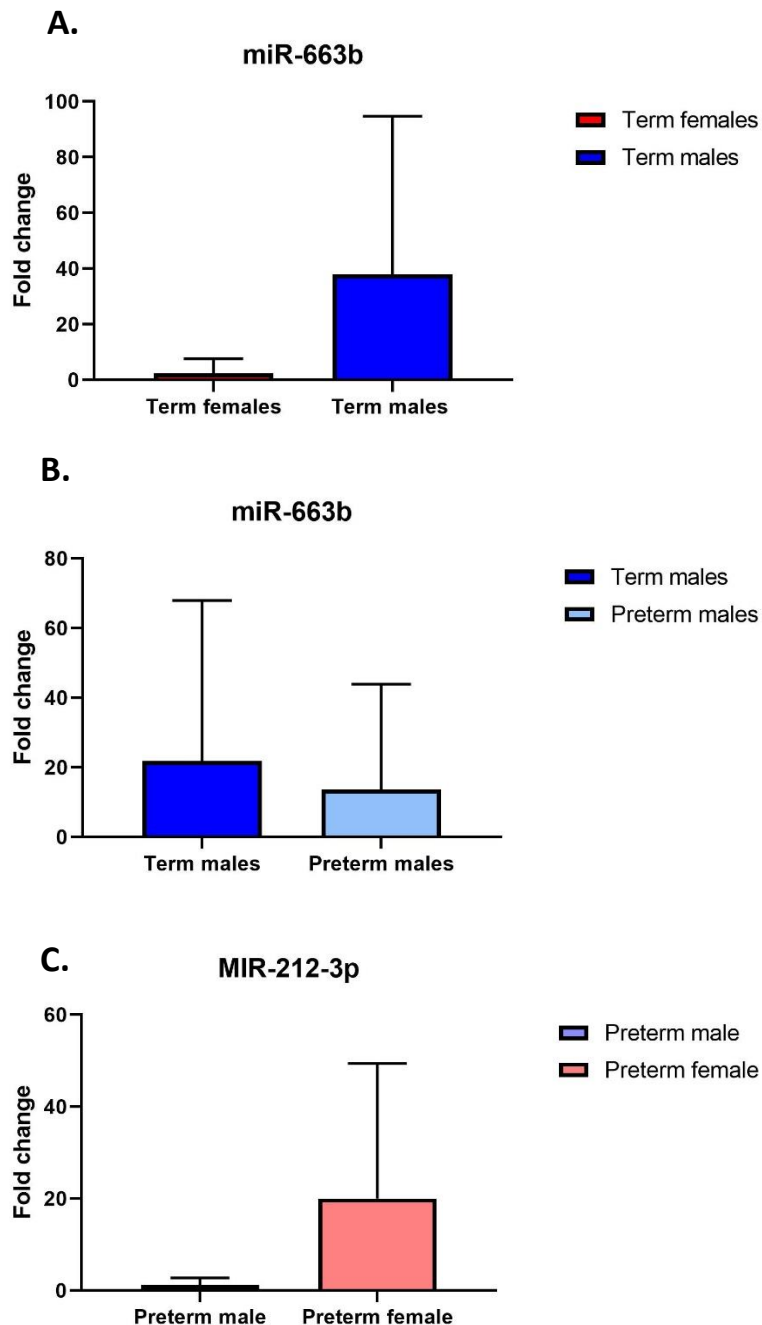


Figure 6.5: Validation of miRNAs of interest from OpenArray analysis.

Values displayed are mean $\pm$ standard deviation of fold change. A) Fold change comparison of miR-663b in term male (n=5) and term female (n=6) neonates. Fold change did not differ significantly between groups ($p>0.05$). B) Fold change comparison of miR-663b between term male (n=5) neonates and preterm male (n=5) neonates. Fold change did not differ significantly between groups ($p>0.05$). C) Fold change comparison of miR-212-3p in preterm female (n=6) neonates and preterm male (n=5) neonates. Fold change did not differ significantly between groups ($p>0.05$).

6.5. Discussion

miRNA profiles were examined in the serum of 11 VLBW preterm neonates and 11 healthy term control neonates. The mean number of miRNAs expressed in term and preterm neonates of either sex did not differ significantly between groups. Without separating groups based on sex, the mean number of expressed miRNAs is almost identical in the preterm and term populations, though after dividing groups by sex the profiles look very different. Term male and female neonates have similar mean numbers of expressed miRNAs though there is a trend towards preterm female infants having more miRNA detected and preterm male infants having the less. When the X-linked miRNAs were examined separately there were no significant differences between groups. Preterm neonates had slightly reduced overall expression of X-linked miRNAs compared to healthy term controls, though when these groups were examined by sex there was again a trend towards preterm male neonates having disproportionately lower expression than other groups, though this did not reach statistical significance. No individual X-linked miRNAs were found to differ between groups from OpenArray data. It has been suggested that miRNAs are a source of potentially important differences in male and female gene expression⁵⁹⁵, though findings on sex differences in circulating miRNA are largely based on data from older populations and are somewhat mixed. Mooney *et al.* found that sex did not significantly affect peripheral blood miRNA levels of healthy adults⁵⁹⁶ though other authors have found differences in one or more peripheral blood miRNAs^{580,597}, some of which may have important implications for fetal growth and neonatal well-being. miR-1323 for instance, which Duttgupta found to be upregulated in adult females⁵⁹⁷, is known to be reduced in the placental tissue of IUGR pregnancies⁵⁹⁸, suggesting a potential role in determining neonatal outcome. Mooney *et al.* have also previously identified miR-885-5p as differing in the plasma of boys and girls with cystic fibrosis, suggesting that it may be involved in the sex differences in respiratory outcome observed in the disease⁵⁷⁵. The only neonatal study evaluating the role of sex in circulating miRNA levels in humans was by Brennan *et al.* who performed RNA sequencing analysis of cord blood plasma in healthy term neonatal males (n=4) and females (n=4) and found no variation in overall miRNA expression between the sexes⁵⁹⁹. While clearly valuable, existing human studies on sex differences in miRNA

expression do not include preterm patients, and most frequently use either adult blood or cord blood. The use of cord blood in particular is potentially misleading as it is known to be immunologically distinct from that of postnatally acquired samples ³¹⁴ and up to 10% of cord blood samples may be contaminated by maternal blood ⁶⁰⁰, a fact which is clearly problematic for any genetic analysis. As a result, published literature in humans provides little indication as to whether sex differences are to be expected in preterm neonates and larger human studies of similar design to our own are required to provide clarification. Animal studies examining the role of miRNAs on sex differences in neonatal outcome are similarly rare, though Zhang *et al.* found that neonatal female mice had higher expression miR-30a after hypoxia-induced lung injury, and found decreased expression of miRNA-30a in post-mortem lung tissue of patients with CLD ⁶⁰¹. The effect of sex hormones on neonatal miRNA expression has not been established though androgen inhibition during pregnancy has been shown to affect the lung miRNA profile of male rats ⁶⁰².

The most abundant miRNAs within the neonatal groups appear to represent a profile unique to our population. Only mir-16-5p is a consistent finding among published literature detailing the most frequently identified miRNAs within study populations. This is likely due to the fact that much of the existing literature is from adult patients and neonates are likely to have different miRNA profiles due to their unique developmental stage and the relative abundance of immature cells. This is supported by a study from Lai *et al.* who investigated changes in miRNA profiles between preterm neonates (n=30) and adults, where they identified that peripheral blood miRNAs differ significantly between the populations with only one third of the miRNAs studied having similar expression in preterm neonates and adults ⁶⁰³. The miRNA profile may also differ significantly depending on the degree of prematurity as noted in the by Gródecka-Szwajkiewicz *et al.* who found that umbilical cord mononuclear cell miRNAs in term and preterm neonatal umbilical cord blood were distinct ⁶⁰⁴, which may explain why the most abundant miRNAs in our mixed population of term and preterm neonates were not common to other neonatal studies.

When referencing the work of Haider *et al.*⁵⁹⁴ the cells of origin for many of the most abundant miRNAs identified in this study could not be determined. miR-16-5p is ubiquitously expressed across many cell types and the other 9 most abundant miRNAs in our population were either uniformly poorly expressed in the cell types examined by Haider *et al.* or were not featured in their study. This again supports the idea that neonates have a unique miRNA profile representing their unique physiology where tissue and immune cells are immature and likely have a distinct miRNA environment to those of adults and older children. The circulating miRNAs we have isolated are likely reflective of this and supported by the findings of Yu *et al.* who noted that umbilical cord blood and adult peripheral blood had immune cell-specific differences in miRNA profiles⁶⁰⁵.

When fold change expression was compared across gestation and sex, 3 miRNAs were identified as potentially differing between groups. miRNA-663b, which has been identified as potentially inhibiting neuronal differentiation in humans⁶⁰⁶, was higher in term male neonates than both term female and preterm male neonates. mir-212-3p, which has been implicated in adult cardiac disease⁶⁰⁷ and epilepsy⁶⁰⁸, was higher in preterm female neonates compared to preterm male neonates. mir-218-2-3p, which has been implicated in human macrophage LPS responses⁶⁰⁹, was higher in preterm female than preterm male neonates. Validation experiments of these 3 miRNAs using qRT-PCR found no significant differences between any of the miRNAs studied. Trends in the directions expected were present for miR-663b and 212-3p though these did not reach statistical significance.

Recent publications on the use of miRNAs as biomarkers of disease in neonates are largely in umbilical cord blood of term neonates with neonatal encephalopathy where several miRNAs have been identified for further study⁶¹⁰⁻⁶¹³. Studies in term neonates have also identified several serum miRNAs as potential biomarkers of biliary atresia⁶¹⁴⁻⁶¹⁶. Exploratory analyses of circulating miRNAs in the preterm neonatal population are limited and we can identify no other studies which examine differences in miRNA profiles between male and female VLBW preterm neonates. None of the miRNAs identified in our study as potential targets have been investigated in previous neonatal biomarker studies. However, many authors have published findings which

suggest that miRNAs have a role in preterm neonatal disease and these are discussed briefly below.

Studies of miRNAs in preterm neonatal neurological disease are still in their infancy. Chapman *et al.* performed one of the few existing studies in the VLBW population and identified a number of miRNAs that may be differentially expressed in plasma of neonates with IVH or abnormal tone in the neonatal period ⁶¹⁷. Metin *et al.* examined miRNA expression in the plasma of 28 preterm neonates to identify potential biomarkers of ROP using qRT-PCR ⁶¹⁸. They found that the profiles of miRNAs linked to angiogenesis differed considerably between those with ROP and healthy preterm controls and identified that 2 miRNAs (miR-27b-3p and miR-214-3p) were significantly lower in those with late stage ROP.

miRNA profiles change considerably during lung development ⁶¹⁹ and animal models have implicated miRNAs in the pathogenesis of neonatal lung disease⁵⁸²⁻⁵⁸⁶. As a result, many authors have identified miRNAs as potential biomarkers of neonatal respiratory disease. Kan *et al.* found that the miRNA profiles in serum of preterm neonates with RDS vary considerably from those without RDS ⁵⁸⁷. Wu *et al.* found that miR-615-3p was higher in the whole blood collected from neonates with respiratory failure compared to healthy controls though the patient group in this study had heterogenous underlying conditions ⁵⁹⁰. A study by Go *et al.* in 73 preterm neonates identified that serum miR-21 is upregulated after birth in CLD patients and downregulated in controls ⁵⁹¹. In a case-control study of 30 preterm neonates, peripheral blood miRNAs have been used to identify 4 potential biomarkers (miR-152, miR-30a-3p, miR-133b and miR-7) of CLD in preterm neonates ⁵⁹². A study of 40 preterm neonates showed that miR-574-3p is significantly lower in the peripheral blood of those with CLD ⁶²⁰. A study by Lal *et al.* on 30 preterm neonates showed that miR-876-3p is a potentially important biomarker of CLD, with tracheal aspirate samples collected at birth showing significantly lower miR-876-3p in neonates who went on to develop CLD ⁵⁹³.

The potential utility of miRNAs as biomarkers of neonatal sepsis is another area of interest in preterm neonates. Cheng *et al.* found that miR-26a was reduced in the serum and mononuclear cells of neonates with sepsis compared to healthy controls ⁶²¹.

Liu *et al.* evaluated miR-181a in a cohort of 102 neonates with sepsis and found that reduced serum levels performed well as a biomarker of neonatal sepsis ⁶²², and that miR-181a inhibits monocyte LPS response by reducing TLR4 signalling. Similarly, miR-142-3p and let-7g in the cord blood of healthy term neonates have been shown to negatively regulate neutrophil IL-6 production ⁶²³, suggesting a role for miRNAs in innate immune responses to sepsis. Wang *et al.* found that in term and late preterm neonates (n=87) miR-15a and miR-16 were potentially useful biomarkers of neonatal sepsis ⁶²⁴ and that transfection of miR-15a and miR-16 into LPS-treated cells significantly reduced the expression of TLR4 and IRAK-1 ⁶²⁴. Cheung *et al.* performed a prospective cohort study in preterm neonates with suspected NEC and found that plasma miR-1290 may be useful diagnostically to differentiate NEC from suspected sepsis ⁶²⁵.

There are several important limitations to this study. Our sample size was small, with our study population containing only 11 term and 11 preterm neonates. This limits the power of our study, especially when further analysing results by neonatal sex, meaning that we may have missed important differences in the cohort studied. While the OpenArray used to generate the data presented provides a robust profile of over 750 well-described human miRNAs, performing RNA sequencing would likely yield a more accurate picture of the miRNA profile in term and preterm neonates. While providing more information, RNA sequencing requires a relatively large volume of blood meaning that while it is likely to provide more detail it is unfortunately not suitable for use in the population we have studied.

While miRNAs clearly have great potential as biomarkers of disease in preterm neonates there are several issues which complicate their use, apart from any potential sex differences in expression that may exist among preterm neonates. One of the many potential factors for consideration in identifying and validating miRNAs in neonatal disease is the effect of maternal and environmental factors which are unique to the neonatal population on miRNA expression. In a study of 24 pregnancies, Juracek *et al.* found that several miRNAs show significantly different expression in cord blood plasma depending on maternal characteristics including age, BMI and blood type ⁶²⁶. A study by Lee *et al.* in fetal tissues from 55 pregnancies showed that miR-223 was

significantly upregulated in several organ systems in cases of chorioamnionitis⁶²⁷, suggesting that perinatal inflammation affects tissue miRNA levels. The adoption of biomarkers of adult disease is also potentially complicated as evidenced by the findings of Lai *et al.* This study found that peripheral blood miRNAs differ considerably between preterm neonates (n=30) and adults with only one third of the miRNAs studied having similar expression in the two populations⁶⁰³. While the narrative of this paper was focused on development and aging these findings have clear implications for the use of miRNAs as biomarkers in neonates and highlights the need for further study in the preterm population. Lastly, in many cases it is unclear if miRNAs are involved in the pathogenesis of the conditions in which they are identified or whether they are merely representative of cellular injury⁶²⁸. Our limited understanding of preterm neonatal immune response and the place of miRNAs therein therefore limits progress in this area and an improved understanding of neonatal immunity may help in selecting biomarkers of preterm disease from the many potential miRNAs available to investigators.

6.6. Conclusion

This study found no significant differences in the miRNA profiles of term and preterm neonates of either sex. The 3 miRNAs of interest identified from the OpenArray profile were not validated in subsequent qRT-PCR investigations and have not been the target of previous biomarker work within neonatology. Term male and female miRNA profiles were similar in the number of expressed miRNAs in serum. While not reaching statistical significance, expression profiles of male and female preterm neonates do appear different with females having a greater number of average miRNAs expressed than males. This warrants further investigation in a larger patient group as, if present, such sex-differences would have significant implications on the use of biomarkers in the preterm population going forward. Collectively, our findings suggest that miRNA profiles are similar in neonates of opposite sex at term but require further investigation in the preterm population. This information adds to our overall understanding of miRNA profiles in term and preterm neonates and highlights important areas for further study.

Chapter 7: Sex differences in Inflammasome and cytokine responses in preterm infants

7.1. Introduction

Neonates are at increased risk of sepsis compared to adults and older children, this increased risk is especially marked in the preterm population. While there are many potential reasons for this increased risk, it is believed that the immaturity of the preterm immune system is a significant contributor ³⁶³. One of the key pieces in innate immune response are cytokines, which are small proteins secreted by cells which have important roles in cell signalling and have a diverse range of biological functions. While preterm neonates with sepsis are able to mount pro- and anti-inflammatory cytokine responses ⁶²⁹, preterm neonatal immune cells are known to have defects in cytokine response compared to term neonates and adults ^{630,631}. Data on circulating cytokine levels in preterm neonates is limited. Some have suggested that blood levels of most cytokines appear to increase with advancing gestation ²¹⁶, while others have found that trends are more complex and differ depending on the cytokine examined ²¹⁷. Additionally, it has been suggested that that immune response of preterm neonates may be more weighted towards a pro- rather than anti-inflammatory response as reflected by the endotoxin response of macrophages in preterm neonates which have diminished IL-10 secretion following stimulation ⁶³².

The inflammasome is a multi-protein complex which forms in the cytoplasm of cells in response to cellular damage and is another important component of the innate immune response to infection. Inflammasome formation results in both inflammatory cell death and the secretion of the markedly pro-inflammatory cytokines IL-1 β and IL-18 ⁶³³. The NLRP3 and AIM2 inflammasomes are among the best studied and are critical components of innate immune response to infection ^{634,635}. Both AIM2 and NLRP3 accumulate in the cytoplasm following activation and interact with ASC to form an inflammasome complex resulting in the accumulation of pro-caspase-1, which then auto-cleaves. The resulting activated caspase 1 then undertakes the proteolytic conversion of the inactive forms of both IL-18 and IL-1 β to their active forms.

Inflammasome activation has been implicated in many disease processes ²²⁸ though there is relatively little data in the preterm neonatal population.

Immune responses are thought to differ between male and female preterm neonates with female neonates generally thought to have a more robust immune response ²⁴⁶. Males neonate are thought to have a poorer ability to balance the pro- and anti-inflammatory response leading to excessive pro-inflammatory signalling. These differences manifest clinically as a greater susceptibility to sepsis ²⁴⁷ as well as a greater tendency towards the adverse effects of sepsis-related inflammation among male neonates ⁴⁹⁴. Though it has been suggested that cytokine responses to endotoxin may differ between the sexes in neonates ³⁰¹ and adults ^{636,637}, data is somewhat mixed and very little study has been undertaken in this area within the preterm neonatal population. Hormones have been shown to modulate neonatal immune response ⁵⁶² and despite markedly elevated levels of circulating female sex hormones *in-utero* little data is available on whether E2 and Pg affect the immune response of male and female neonates differently. A better understanding of the differences in innate immune response between male and female neonates and the role of sex hormones in modulating this response would help us understand the unique susceptibility of male neonates to sepsis and potentially identify therapeutic targets for future study.

7.2. Hypothesis

We hypothesised that male and female neonates have differing cytokine responses and inflammasome gene expression which may impact clinical outcome.

7.3. Aims

To examine genes involved in the inflammasome (NLRP3, AIM2, ASC and IL-1 β); a comprehensive array of pro- and anti-inflammatory cytokines (IL-1 α , IL-1 β , IL-1RA, IL-6, IL-8, IL-10, IL-18, IL-33, IFN- γ , TNF- α and TNF- β) and cytokines involved in hypoxia (EPO) and angiogenesis (VEGF) in response to inflammatory stimuli and sex hormones in male and female term and preterm neonates .

7.4. Results

7.4.1. Patient characteristics

The 10 preterm neonates (5 female, 5 male) who had whole blood collected for qPCR had a mean gestation at birth (mean $\pm$ SD) of 29.4 ± 2.66 weeks and corrected gestation at the time of sampling was 31.3 ± 2.51 weeks. The mean birth weight of preterm neonates was 1084 ± 328 grams. Gestation at birth, corrected gestation at sampling and birth weight did not differ significantly between male and female preterm neonates ($p > 0.05$) (Table 7.1).

The 40 preterm neonates (18 female, 22 male) who had whole blood collected for qPCR had a mean gestation at birth (mean $\pm$ SD) of 28.69 ± 2 weeks and corrected gestation at the time of sampling was 31.85 ± 2.5 weeks. The mean birth weight of preterm neonates was 1089 ± 304.9 grams. Gestation at birth, corrected gestation at sampling and birth weight did not differ significantly between male and female preterm neonates ($p > 0.05$) (Table 7.2).

	Preterm males (n=5)	Preterm females (n=5)	p value (95% CI)
Gestation at delivery (weeks)	29.83 ($\pm$ 2.3)	28.97 ($\pm$ 3.2)	0.64 (-3.20 to 4.92)
Gestation at sampling (weeks)	32.3 ($\pm$ 2.2)	30.3 ($\pm$ 2.7)	0.23 (-1.56 to 5.51)
Birth weight (grams)	1162 ($\pm$ 337.3)	1006 ($\pm$ 336.9)	0.485 (-335.7 to 647.7)

Table 7.1: Demographics for preterm neonatal male and female qPCR samples.

Demographics for preterm neonatal male (n=5) and female (n=5) qPCR samples.

Values presented represent mean $\pm$ standard deviation.

	Preterm males (n=22)	Preterm females (n=18)	p value (95% CI)
Gestation at delivery (weeks)	28.43 ($\pm$ 1.793)	29.01 ($\pm$ 2.241)	0.3693 (-1.87 to 0.71)
Gestation at sampling (weeks)	31.68 ($\pm$ 2.461)	32.06 ($\pm$ 2.636)	0.6403 (-2.015 to 1.254)
Birth weight (grams)	1058 ($\pm$ 315.1)	1126 ($\pm$ 296.6)	0.4908 (-265.4 to 129.6)

Table 7.2: Demographics for preterm neonatal male and female cytokine samples.

Demographics for preterm neonatal male (n=22) and female (n=18) cytokine samples.

Values presented represent mean $\pm$ standard deviation.

7.4.2. *The effect of sex on endotoxin-treated serum cytokine levels*

Whole blood from term and preterm neonates was incubated with endotoxin for 1 hour as described in chapter 2 and serum cytokine levels were measured using multiplex ELISA. Results were first compared between all term neonates and all preterm neonates (Figures 7.1-7.3). IL-1 β increased significantly in both term ($p < 0.0001$) and preterm ($p < 0.0001$) neonates following LPS treatment. IL-1 β was higher in term neonates both at baseline ($p = 0.0002$) and following LPS treatment ($p < 0.0001$) compared to preterm neonates. IL-18 increased significantly in preterm neonates following LPS treatment ($p = 0.0001$) but not in term neonates. TNF- α increased significantly in both term ($p < 0.0001$) and preterm ($p < 0.0001$) neonates following LPS treatment. TNF- α was significantly higher in term neonates following LPS treatment compared to preterm neonates ($p = 0.0107$). TNF- β was significantly higher at baseline in term neonates compared to preterm neonates ($p = 0.0095$). TNF- β increased significantly in both term neonates ($p = 0.0481$) and preterm neonates ($p < 0.0001$) following LPS treatment (Figure 7.1).

IL-1 α increased significantly in term ($p = 0.0022$) and preterm ($p < 0.0001$) neonates following LPS treatment. IL-1 α was higher in term neonates compared to preterm neonates following LPS treatment ($p = 0.0096$). IL-6 increased significantly in both term neonates ($p = 0.0141$) and preterm neonates ($p < 0.0001$) following LPS treatment. IL-6 was higher in term neonates both at baseline ($p < 0.0001$) and following LPS ($p = 0.0109$) treatment compared to preterm neonates. IL-8 increased significantly in both term neonates ($p < 0.0001$) and preterm neonates ($p < 0.0001$) following LPS treatment. IL-8 was higher in term neonates both at baseline ($p = 0.0312$) and following LPS ($p = 0.0001$) treatment compared to preterm neonates. IFN- γ was higher in term neonates than preterm neonates at baseline ($p = 0.0274$). IFN- γ increased significantly in preterm ($p = 0.0477$) but not term neonates ($p > 0.05$) following LPS stimulation (Figure 7.2).

IL-1RA was significantly higher in term neonates at baseline ($p = 0.0166$) and following LPS treatment ($p = 0.0167$). IL-1RA increased significantly in both term neonates ($p = 0.0172$) and preterm neonates ($p = 0.0017$) following LPS treatment. IL-10 increased significantly in term neonates following LPS treatment ($p = 0.0021$) but not in

preterm neonates ($p>0.05$). IL-10 was significantly higher in term neonates following LPS treatment compared to preterm neonates ($p=0.0155$). IL-33 increased significantly in both term neonates ($p=0.0203$) and preterm neonates ($p=0.0143$) following LPS treatment. EPO was higher in preterm neonates than term infants both at baseline ($p=0.0095$) and following LPS stimulation ($p=0.0366$). EPO levels did not increase in either group following LPS stimulation. VEGF increased significantly in both term ($p=0.0059$) and preterm ($p<0.0001$) neonates following LPS treatment. VEGF was significantly higher in term neonates both at baseline ($p=0.0052$) and following LPS treatment ($p<0.0001$) compared to preterm neonates (Figure 7.3).

Results were then divided by sex and reanalysed (Figures 7.4-7.6). IL-1 β increased significantly in term male ($p<0.0001$), term female ($p<0.0001$), preterm male ($p<0.0001$) and preterm female ($p=0.0160$) neonates following LPS treatment. IL-1 β was higher in term female neonates compared to both term male ($p=0.0016$) and preterm female ($p<0.0001$) neonates following LPS treatment. IL-18 increased significantly in both preterm male ($p=0.0064$) and preterm female ($p=0.0081$) neonates following LPS treatment. IL-18 was significantly higher in term females following LPS treatment compared to term males ($p=0.0237$). TNF- α increased significantly in term male ($p<0.0001$), term female ($p<0.0001$), preterm male ($p<0.0001$) and preterm female ($p=0.0002$) neonates following LPS treatment. TNF- α was significantly higher in term female neonates compared to preterm female neonates following LPS treatment ($p=0.0004$). TNF- β increased significantly in term male ($p=0.0031$), term female ($p=0.0384$), preterm male ($p<0.0001$) and preterm female ($p=0.0004$) following LPS treatment. TNF- β was significantly higher in preterm males following LPS treatment compared to preterm females ($p=0.0496$) (Figure 7.4).

IL-1 α increased significantly in term female ($p=0.0066$), term male ($p=0.0162$), preterm female ($p=0.0257$) and preterm male ($p<0.0001$) neonates following LPS treatment. Term females had higher IL-1 α than both term male ($p=0.014$) and preterm female ($p=0.0079$) neonates following LPS treatment. IL-6 increased significantly in term male ($p=0.0002$), term female ($p<0.0001$), preterm male ($p<0.0001$) and preterm female ($p=0.0055$) neonates following LPS treatment. IL-6 was significantly higher in term female neonates at baseline ($p=0.0019$) and following LPS

treatment ($p=0.0145$) compared to preterm female neonates. IL-6 was significantly higher in term male neonates at baseline ($p=0.0077$) and following LPS treatment ($p=0.0145$) compared to preterm male neonates. IL-6 was significantly higher in term female neonates compared to term male neonates following LPS treatment ($p=0.0373$). IL-8 increased significantly in term male ($p<0.0001$), term female ($p=0.0005$), preterm male ($p<0.0001$) and preterm female ($p=0.0180$) neonates following LPS treatment. IL-8 was significantly higher in term female neonates at baseline ($p=0.0073$) and following LPS ($p=0.0024$) treatment compared to preterm female neonates. IL-8 was significantly higher in term male neonates at baseline ($p=0.0257$) and following LPS treatment compared to preterm male neonates ($p=0.0170$). IFN- γ increased significantly in male preterm neonates only following LPS stimulation ($p=0.0471$). Term female neonates had higher IFN- γ following LPS stimulation compared to preterm females ($p=0.0403$) (Figure 7.5).

IL-1RA increased significantly in term female ($p=0.0046$), preterm male ($p=0.0007$) and preterm female ($p=0.0044$) neonates following LPS treatment. IL-1RA was significantly higher in term female neonates at baseline ($p=0.0247$) and following LPS treatment ($p=0.0039$) compared to preterm female neonates. IL-1RA was significantly higher in term male neonates at baseline compared to preterm male neonates ($p=0.0412$). IL-10 increased significantly in term female neonates following LPS treatment ($p=0.0023$) but not in other groups. IL-10 was higher in term female neonates compared to preterm female neonates ($p=0.0455$) following LPS treatment. IL-33 increased significantly in term male ($p=0.0005$), term female ($p=0.0048$), preterm male ($p=0.0442$) and preterm female neonates ($p<0.0001$) following LPS treatment. EPO was significantly higher in preterm females than term females both at baseline ($p=0.0011$) and following LPS stimulation ($p=0.0196$). EPO was significantly higher in preterm males than term males both at baseline ($p=0.0166$) and following LPS stimulation ($p=0.0007$). VEGF increased significantly in term male ($p=0.0011$), term female ($p=0.0106$), preterm male ($p=0.0001$) and preterm female ($p=0.0043$) neonates following treatment with LPS. VEGF was significantly higher in term female neonates compared to preterm females both at baseline ($p=0.0164$) and following LPS treatment ($p=0.0008$). VEGF was significantly higher in term male neonates compared to preterm

males at both baseline (0.0043) and following LPS treatment ($p < 0.0001$). VEGF was significantly higher in term female neonates compared to term males following LPS treatment ($p = 0.0464$) (Figure 7.6).

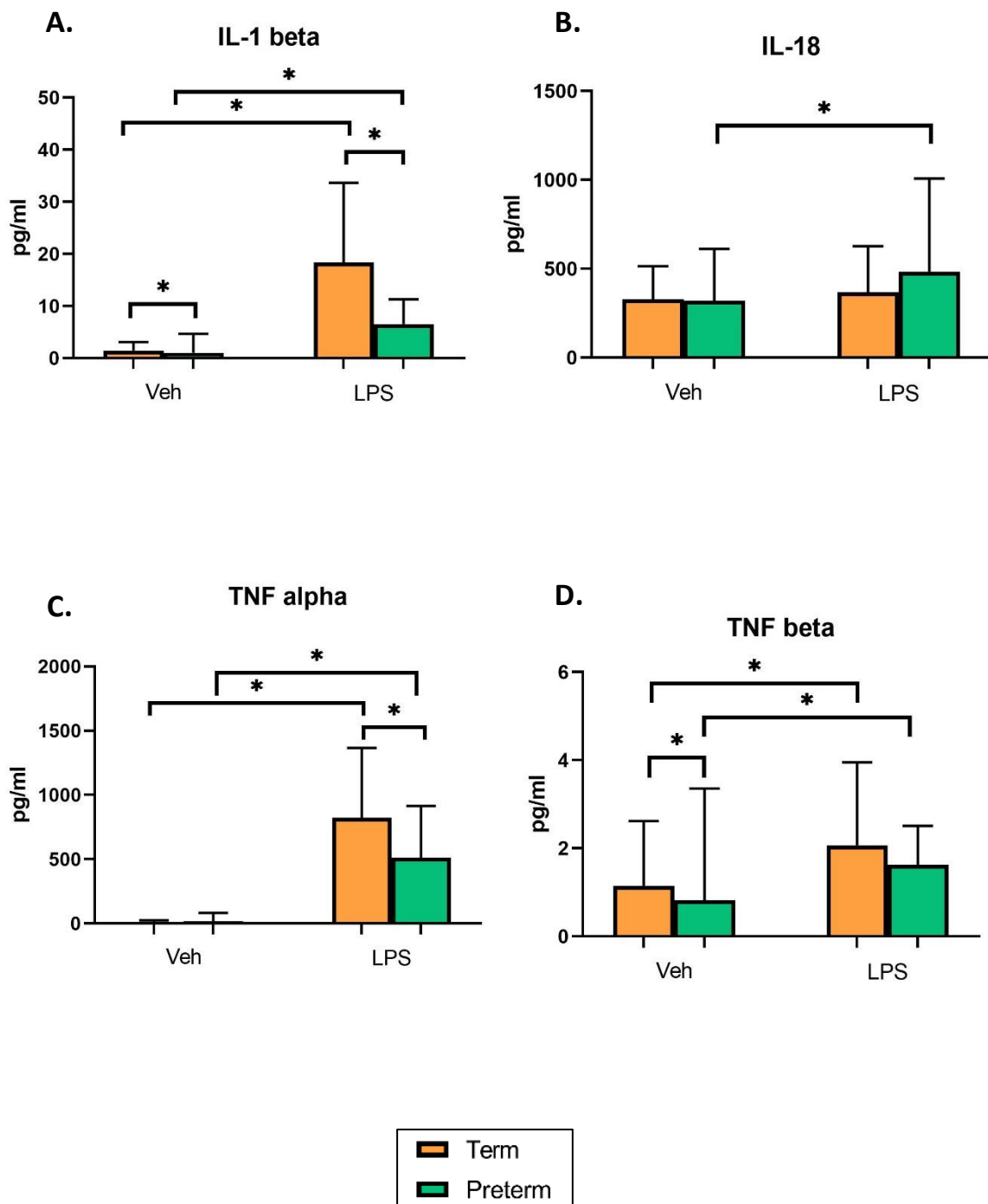


Figure 7.1: Serum IL-1 β , IL-18, TNF α and TNF β levels in term and preterm neonates following endotoxin treatment (10 ng/ml).

Serum IL-1 β , IL-18, TNF α and TNF β levels in term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A.) IL-1 β ; B) IL-18; C) TNF α ; D) TNF β . *=significant at p<0.05.

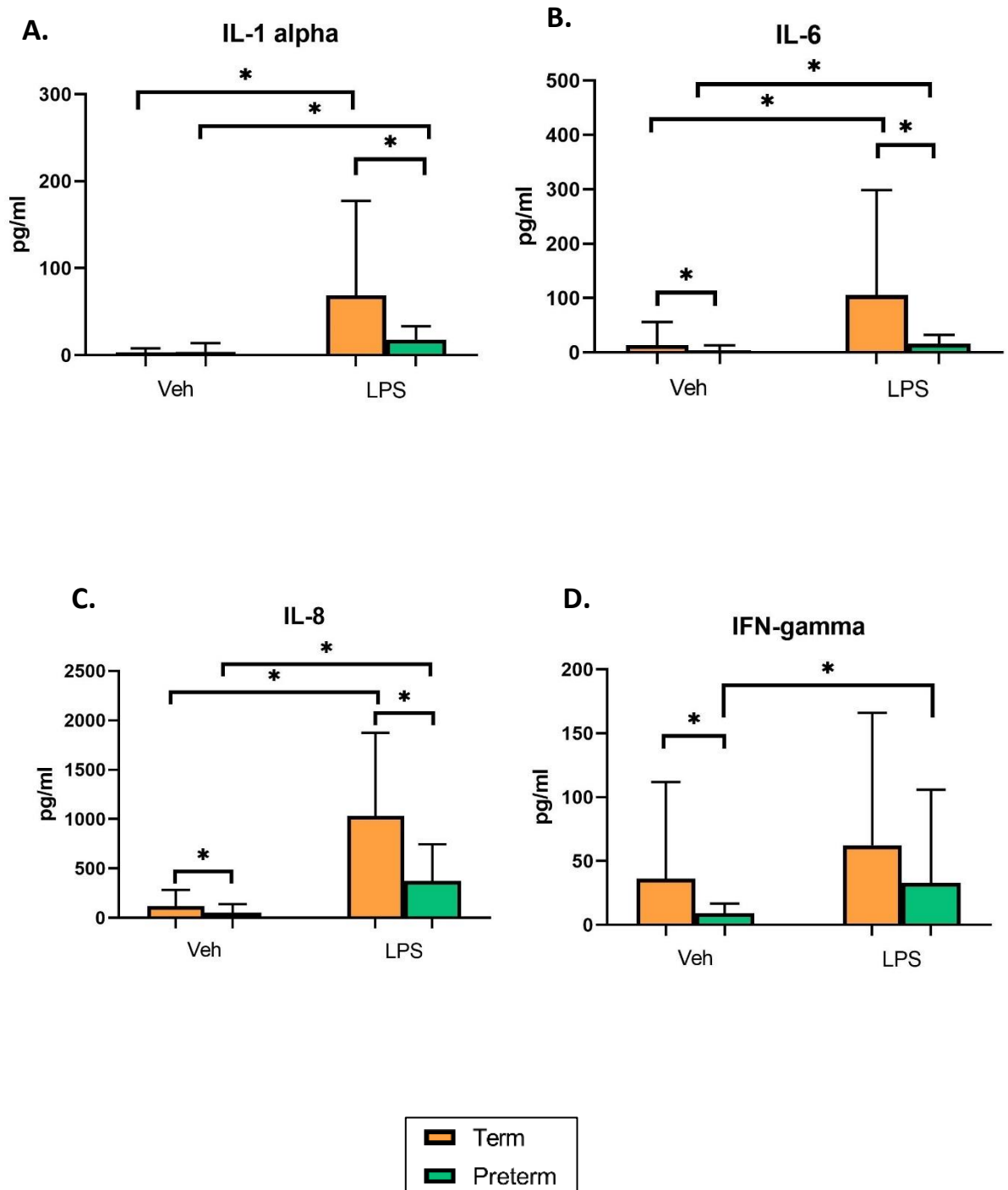


Figure 7.2: Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in term and preterm neonates following endotoxin treatment (10 ng/ml).

Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1 α ; B) IL-6; C) IL-8; D) IFN- γ . *=significant at p<0.05.

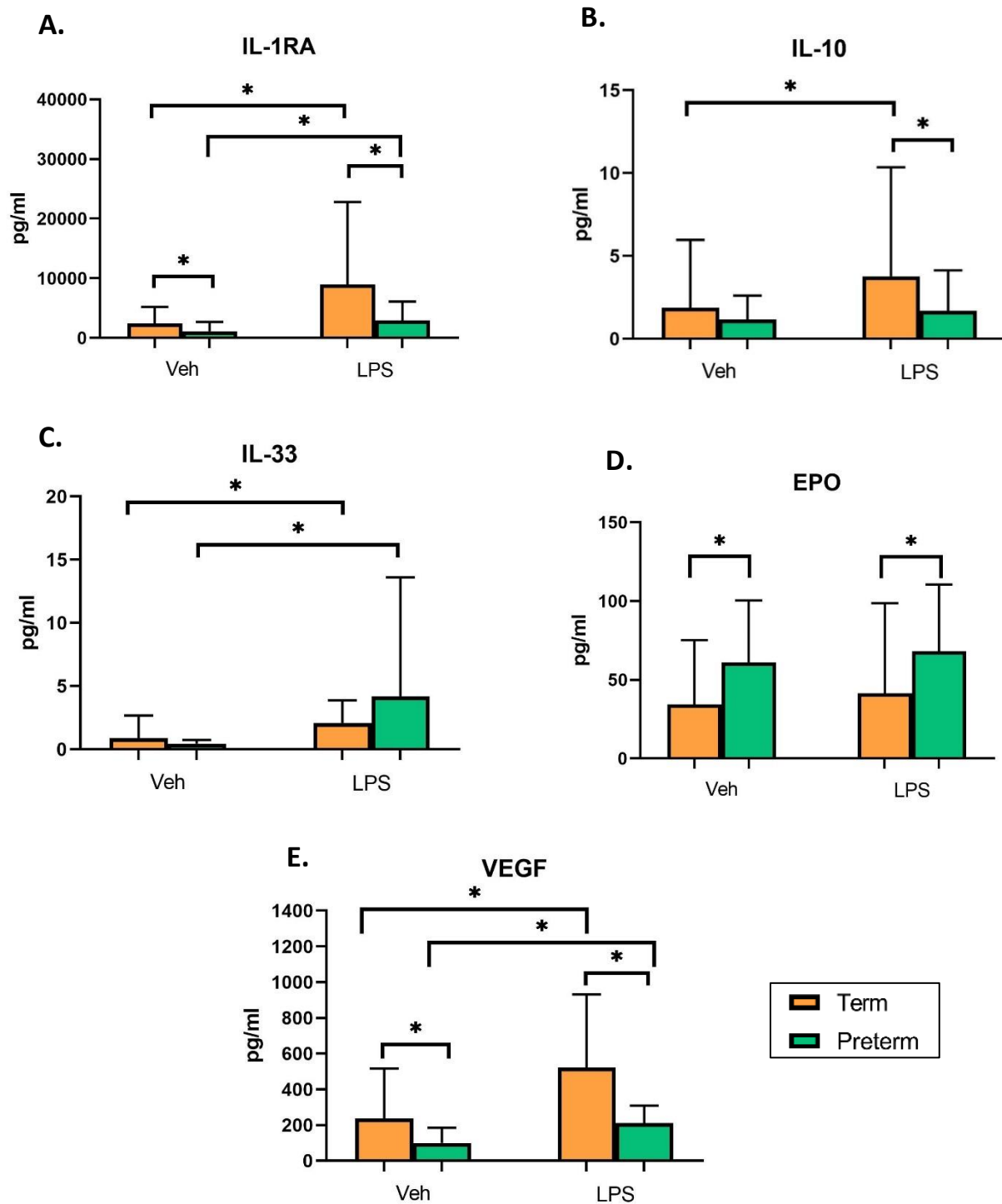


Figure 7.3: Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in term and preterm neonates following endotoxin treatment (10 ng/ml).

Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1RA; B) IL-10; C) IL-33; D) EPO; E) VEGF. *=significant at p<0.05.

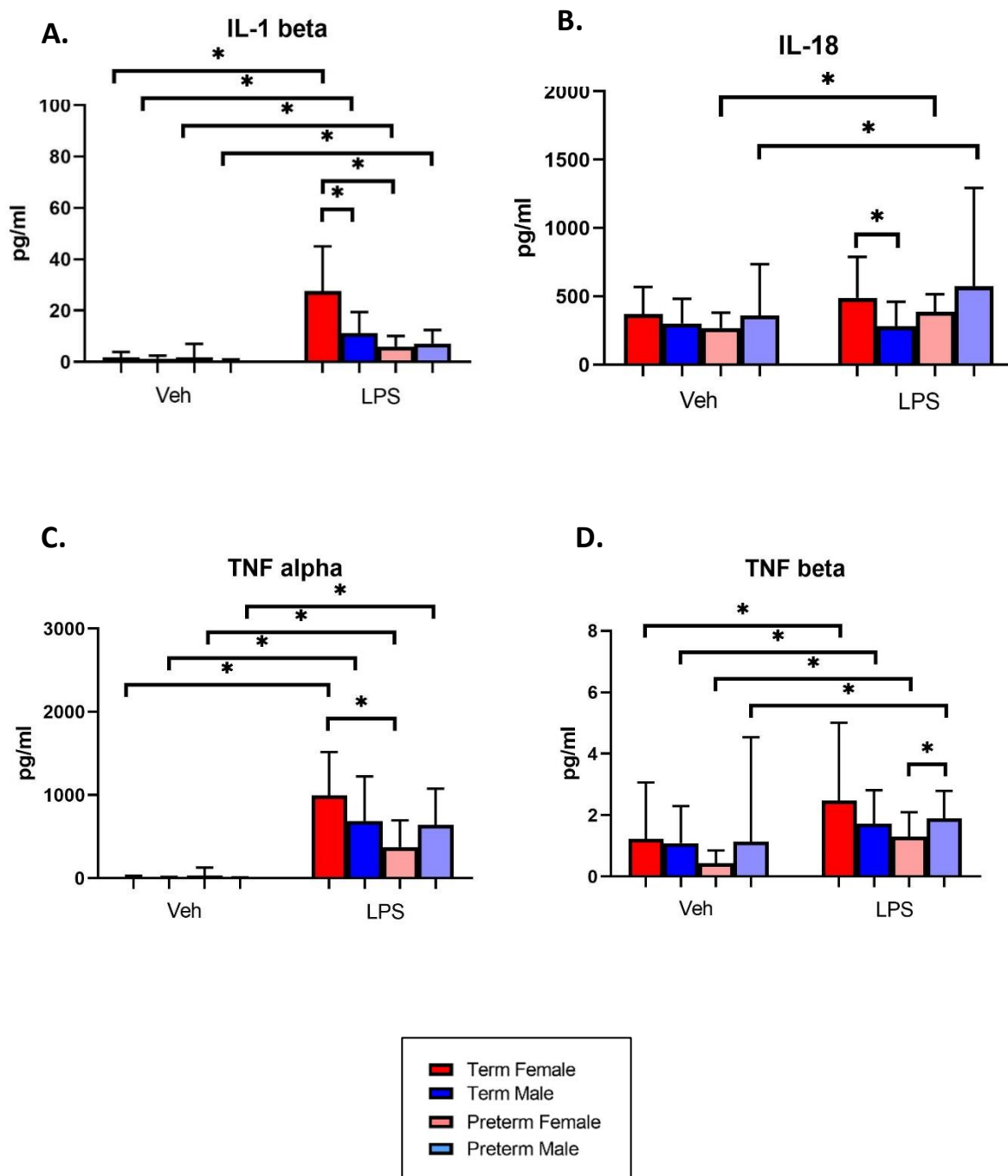


Figure 7.4: Serum IL-1 β , IL-18, TNF α and TNF β levels in male and female term and preterm neonates following endotoxin treatment (10 ng/ml).

Serum IL-1 β , IL-18, TNF α and TNF β levels in male and female term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A.) IL-1 β ; B) IL-18; C) TNF α ; D) TNF β . *=significant at p<0.05.

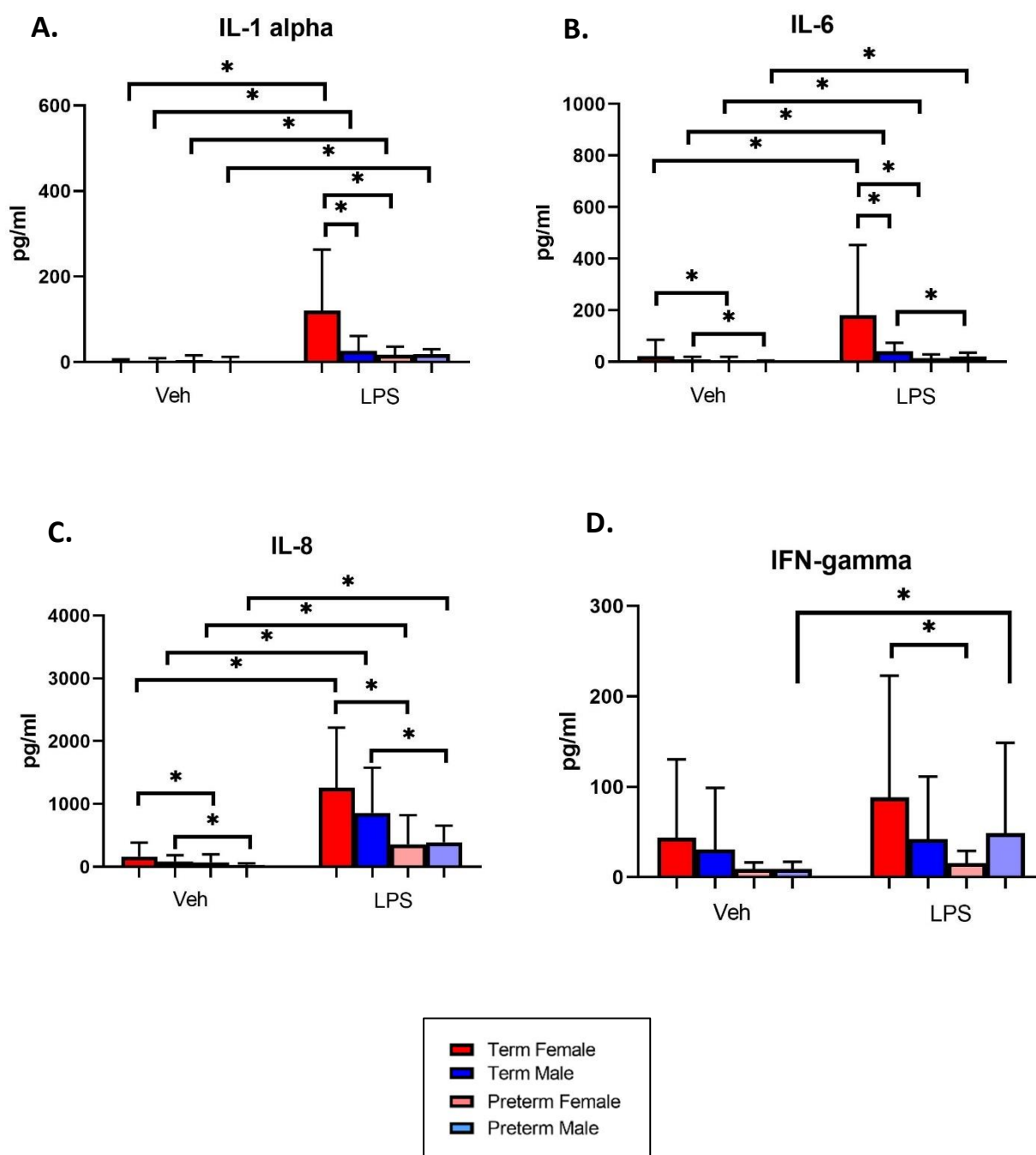


Figure 7.5: Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in male and female term and preterm neonates following treatment with endotoxin (10 ng/ml).

Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in male and female term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1 α ; B) IL-6; C) IL-8; D) IFN- γ . *=significant at p<0.05.

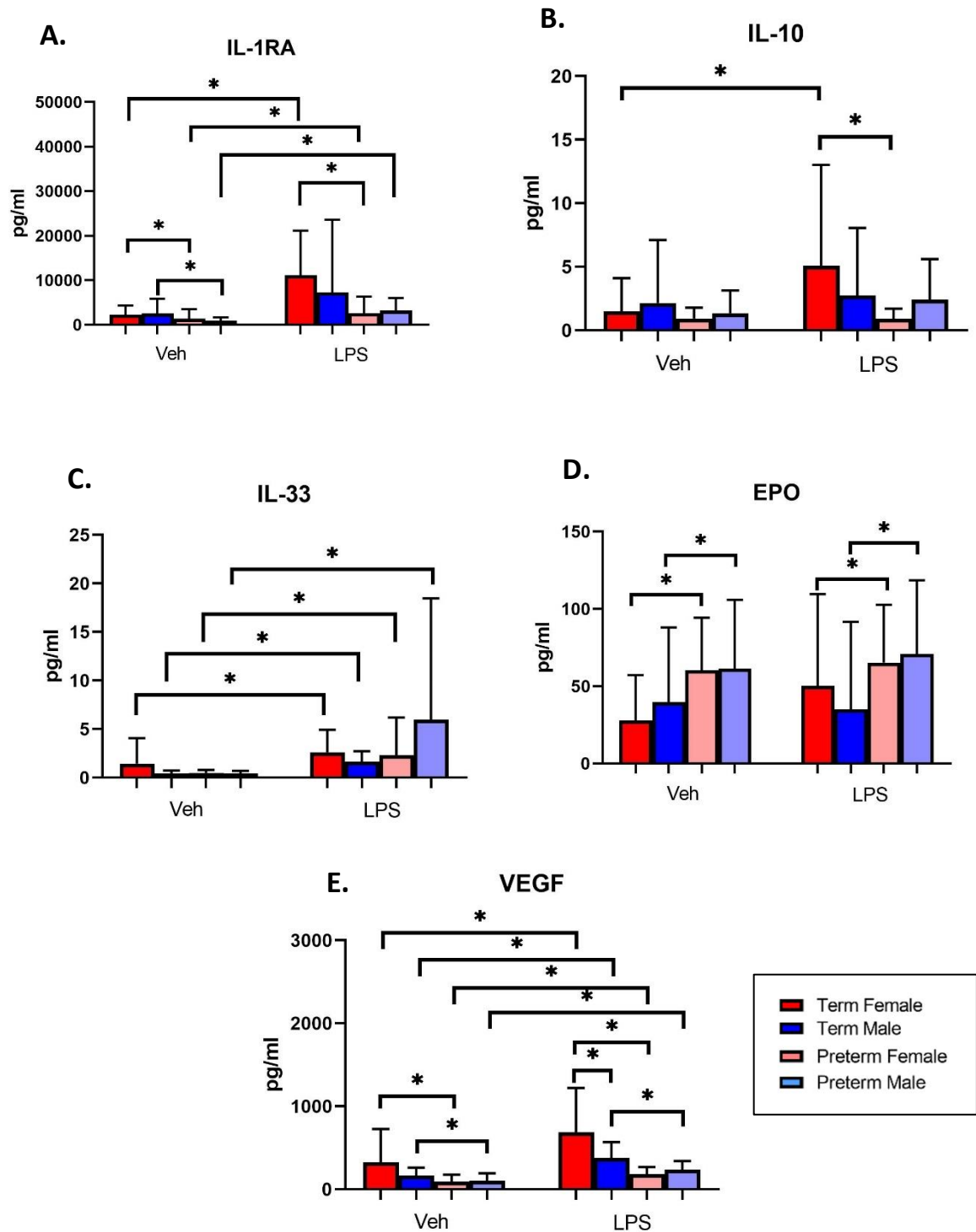


Figure 7.6: Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in male and female term and preterm neonates following treatment with endotoxin (10 ng/ml).

Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in male and female term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1RA; B) IL-10; C) IL-33; D) EPO; E) VEGF. *=significant at p<0.05.

7.4.3. *The effect of estrogen on endotoxin-treated cytokine levels*

To assess the potential role of sex hormones in immune modulation, cytokine levels in samples treated with E2 for 1 hour either alone or in combination with endotoxin were measured. For clarity the cytokine increases from baseline following LPS stimulation, which were discussed in section 7.4.3, are not displayed in the Figures 7.7-7.12. IL-1 β was higher in term neonates at baseline ($p=0.0002$), in samples treated with LPS ($p<0.0001$) and E2+LPS ($p=0.0037$) compared to preterm neonates. IL-1 β decreased significantly in preterm neonatal LPS-treated samples when incubated concurrently with E2 ($p=0.0497$). IL-18 increased significantly in preterm neonates following E2 treatment ($p=0.0156$). TNF- α was significantly higher in term neonates following treatment with LPS ($p=0.0107$) and E2+LPS ($p=0.0392$) compared to preterm neonates. Treatment with E2 did not affect TNF- α levels. TNF- β was higher in term neonates at baseline ($p=0.0095$) and following treatment with E2 ($p=0.0029$) and E2+LPS ($p=0.0006$). TNF- β decreased significantly in LPS-treated preterm samples when incubated with E2 ($p=0.0011$) (Figure 7.7).

IL-1 α was higher in term neonates treated with E2 ($p=0.0494$), LPS ($p=0.0096$) and E2+LPS ($p=0.0261$) compared to preterm neonates. IL-1 α levels were not affected by treatment with E2. IL-6 was significantly higher in term neonates at baseline ($p<0.0001$) and following treatment with E2 ($p=0.0024$), LPS ($p=0.0109$) and E2+LPS ($p<0.0001$). Treatment with E2 did not affect IL-6 levels. IL-8 was significantly higher in term neonates at baseline ($p=0.0312$) and following treatment with E2 ($p=0.0474$), LPS ($p=0.0001$) and E2+LPS ($p=0.0007$). Treatment with E2 did not affect IL-8 levels. IFN- γ was higher in term neonates at baseline ($p=0.0274$) and following treatment with E2+LPS ($p=0.008$). Treatment with E2 did not affect IFN- γ levels (Figure 7.8).

IL-1RA was higher in term neonates compared to preterm neonates at baseline ($p=0.0166$) and following treatment with E2 ($p=0.0372$), LPS ($p=0.0167$) and E2+LPS ($p=0.0280$). Treatment with E2 did not affect IL-1RA levels. IL-10 was significantly higher in term neonates following LPS treatment compared to preterm neonates ($p=0.0155$). Treatment with E2 did not affect IL-10 levels. Treatment with E2 did not affect IL-33 levels. Preterm neonates had higher EPO levels than term neonates at baseline ($p=0.0095$) and following treatment with E2 ($p=0.0008$), LPS ($p=0.0366$) and

E2+LPS (0.0005). E2 treatment did not affect EPO levels. VEGF was significantly higher in term neonates at baseline ($p=0.0052$) and following treatment with E2 ($p=0.034$), LPS ($p<0.0001$) and E2+LPS ($p=0.0002$) compared to preterm neonates. VEGF decreased significantly in LPS-treated samples of both term ($p=0.0439$) and preterm ($p=0.0032$) neonates when incubated with E2 (Figure 7.9).

Results were then divided by sex and reanalysed (Figures 7.10-7.12). Term females had higher IL-1 β than both term male ($p=0.0016$) and preterm female ($p<0.0001$) neonates following LPS treatment. Term females had higher IL-1 β than both term male ($p=0.0021$) and preterm female ($p=0.0021$) neonates following E2+LPS treatment. IL-1 β was significantly lower in LPS-treated samples when incubated with E2 in term male neonates only ($p=0.0427$). IL-18 was significantly higher in term females following LPS treatment compared to term males ($p=0.0237$). IL-18 was significantly higher in preterm males following E2+LPS treatment compared to term male neonates ($p=0.0402$). IL-18 levels were not significantly affected by treatment with E2. TNF- α was significantly higher in term female neonates compared to preterm female neonates following treatment with both LPS ($p=0.0004$) and E2+LPS ($p=0.0024$). TNF- α was significantly higher in term female neonates compared to term male neonates when treated with both E2 ($p=0.0258$) and E2+LPS ($p=0.0247$). TNF- β was significantly higher in term female neonates compared to preterm female neonates following treatment with E2 ($p=0.0044$) and E2+LPS ($p=0.0014$). TNF- β was significantly higher in preterm male neonates treated with LPS compared to preterm female neonates ($p=0.0496$). TNF- β decreased significantly in LPS-treated preterm male ($p=0.0417$) and preterm female ($p=0.0114$) samples when incubated with E2 (Figure 7.10).

Term females had higher IL-1 α than both term male ($p=0.014$) and preterm female ($p=0.0079$) neonates following LPS treatment. Term females had higher IL-1 α than both term male ($p=0.0185$) and preterm female ($p=0.0184$) neonates following E2+LPS treatment. IL-1 α levels were not affected by treatment with E2. IL-6 was significantly higher in term female neonates at baseline ($p=0.0019$) and following treatment with E2 ($p=0.0043$) and LPS ($p=0.0145$) compared to preterm female neonates. IL-6 was significantly higher in term male neonates at baseline ($p=0.0077$)

and following treatment with LPS ($p=0.0145$) and E2+LPS ($p=0.0362$) compared to preterm male neonates. IL-6 was significantly higher in term female neonates compared to term male neonates following treatment with LPS ($p=0.0373$) and E2+LPS ($p=0.0143$). IL-8 was significantly higher in term female neonates at baseline ($p=0.0073$) and following treatment with LPS ($p=0.0024$) and E2+LPS ($p=0.0014$) compared to preterm female neonates. IL-8 was significantly higher in term male neonates at baseline ($p=0.0257$) and following LPS treatment compared to preterm male neonates ($p=0.0170$). IL-8 was significantly higher in term female neonates compared to term males following treatment with E2 ($p=0.0481$) and E2+LPS ($p=0.0014$). IL-8 levels were not significantly affected by treatment with E2. IFN- γ increased significantly from baseline in preterm males ($p=0.0114$) following E2 treatment. Preterm male neonates had higher IFN- γ compared to female preterm neonates following E2 treatment ($p=0.0290$). Term females had higher IFN- γ than preterm females following treatment with LPS alone ($p=0.0403$) and E2+LPS ($p=0.0104$) (Figure 7.11).

Term female neonates had higher IL-1RA than preterm female neonates at baseline ($p=0.0247$) and following treatment with E2 ($p=0.0367$), LPS ($p=0.0039$) and E2+LPS ($p=0.0116$). Term male neonates had higher IL-1RA than preterm male neonates at baseline ($p=0.0412$). Term female neonates had higher IL-10 than term male neonates ($p=0.0035$) when treated with E2+LPS. Term female neonates had higher IL-10 than preterm female neonates when treated with LPS ($p=0.0455$) and E2+LPS ($p=0.0179$). IL-10 levels were not significantly affected by treatment with E2. IL-33 levels were not significantly affected by treatment with E2. Preterm male neonates had higher EPO levels than term male neonates at baseline ($p=0.0166$) and following treatment with E2 ($p=0.031$), LPS ($p=0.0007$) and E2+LPS ($p=0.0116$). Preterm female neonates had higher EPO levels than term female neonates at baseline ($p=0.0011$) and following treatment with E2 ($p=0.0257$), LPS ($p=0.0196$) and E2+LPS ($p=0.0388$). E2 treatment did not affect EPO levels in any group. VEGF was significantly higher in term female neonates compared to preterm females at baseline ($p=0.0164$) and following treatment with both LPS ($p=0.0008$) and E2+LPS ($p=0.0003$). VEGF was significantly higher in term male neonates compared to preterm males at both baseline ($p=0.0043$) and following LPS treatment ($p<0.0001$). VEGF was significantly

higher in term female neonates compared to term males following LPS treatment ($p=0.0464$) and E2+LPS treatment ($p=0.0237$). VEGF decreased significantly in LPS-treated samples from term male ($p=0.0455$), preterm male ($p=0.0432$) and preterm female ($p=0.0475$) neonates when incubated with E2 (Figure 7.12).

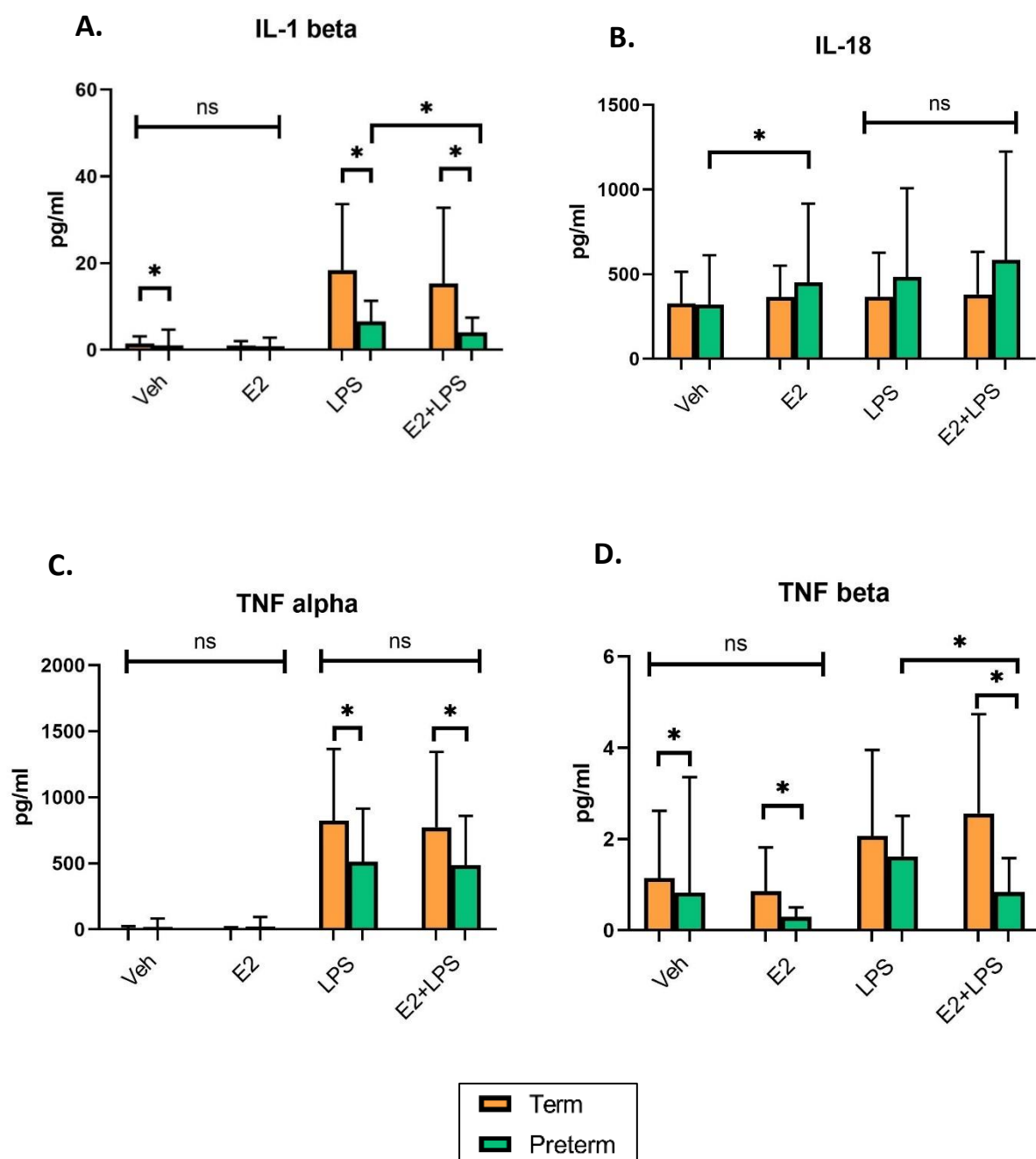


Figure 7.7: Serum IL-1 β , IL-18, TNF α and TNF β levels in term and preterm neonates following treatment with endotoxin (10 ng/ml) and estradiol (10nM).

Serum IL-1 β , IL-18, TNF α and TNF β levels in E2-treated male and female term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A.) IL-1 β ; B) IL-18; C) TNF α ; D) TNF β . *=significant at p<0.05; ns-non-significant.

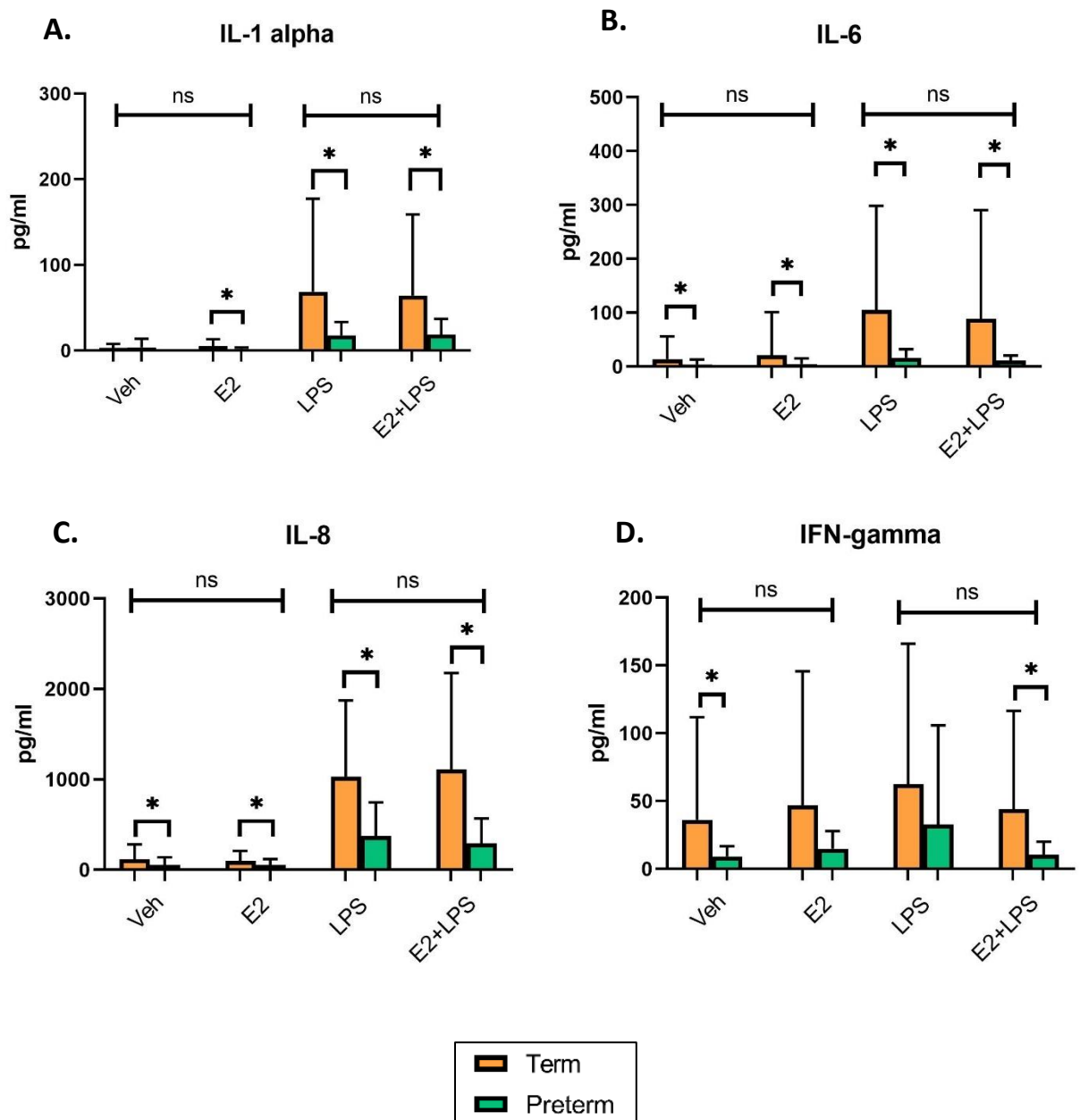


Figure 7.8: Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in term and preterm neonates following treatment with endotoxin (10 ng/ml) and estradiol (10nM).

Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in E2-treated term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1 α ; B) IL-6; C) IL-8; D) IFN- γ . *=significant at p<0.05; ns-non-significant.

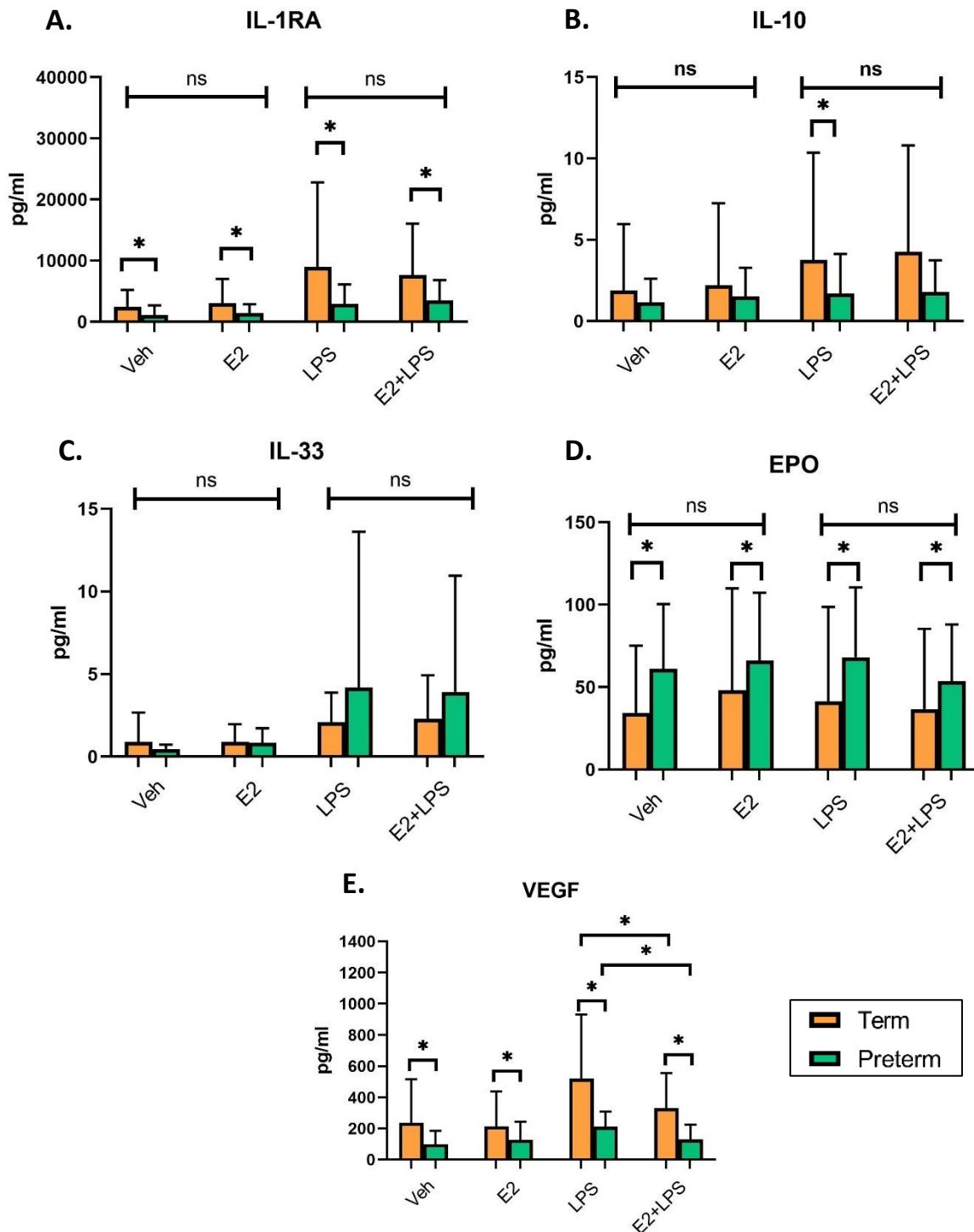


Figure 7.9: Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in term and preterm neonates following treatment with endotoxin (10 ng/ml) and estradiol (10nM).

Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in E2-treated term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1RA; B) IL-10; C) IL-33; D) EPO; E) VEGF. *=significant at p<0.05; ns-non-significant.

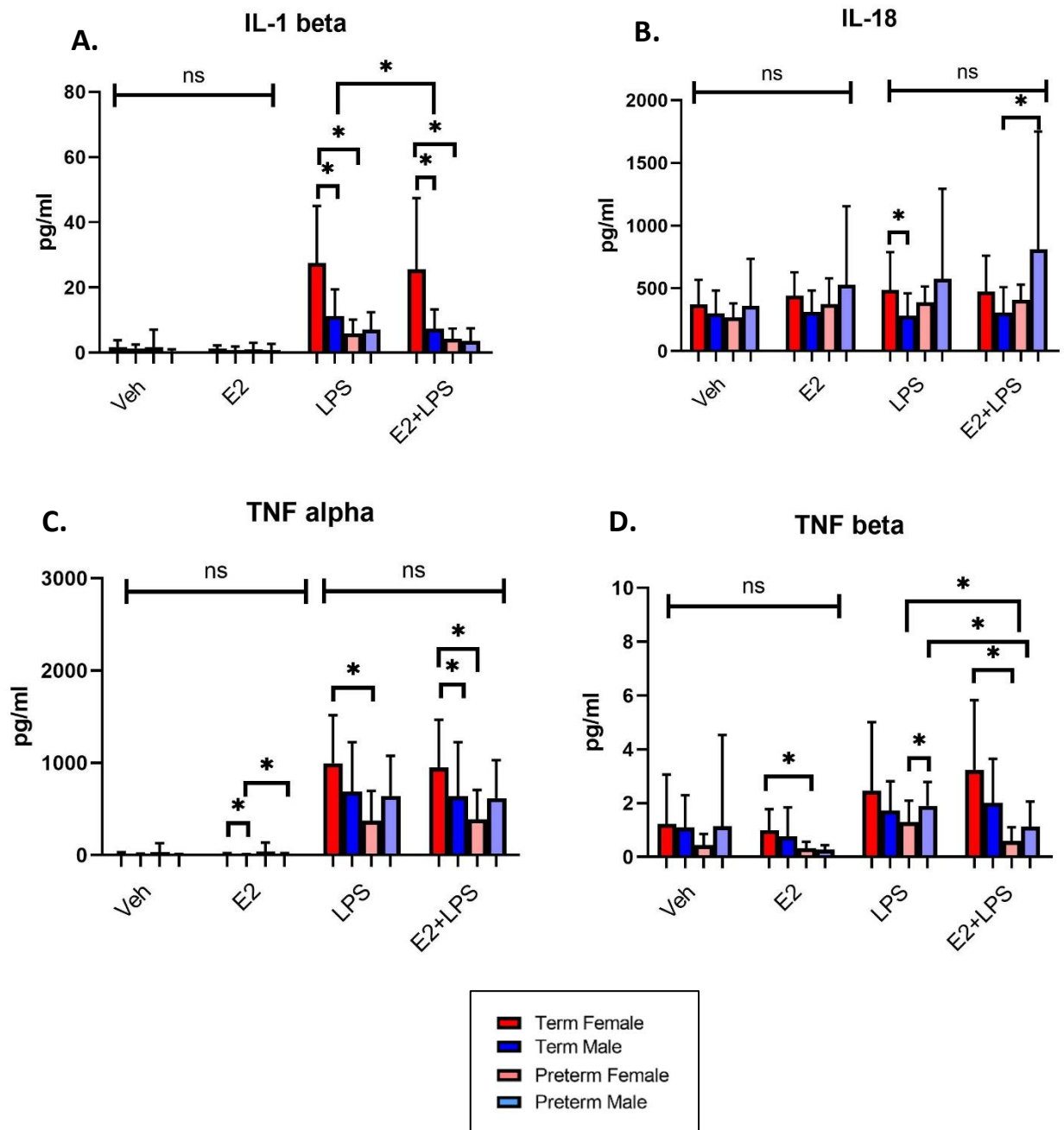


Figure 7.10: Serum IL-1 β , IL-18, TNF α and TNF β levels in male and female term and preterm neonates following treatment with endotoxin (10 ng/ml) and estradiol (10nM).

Serum IL-1 β , IL-18, TNF α and TNF β levels in E2-treated male and female term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A.) IL-1 β ; B) IL-18; C) TNF α ; D) TNF β . *=significant at p<0.05; ns-non-significant.

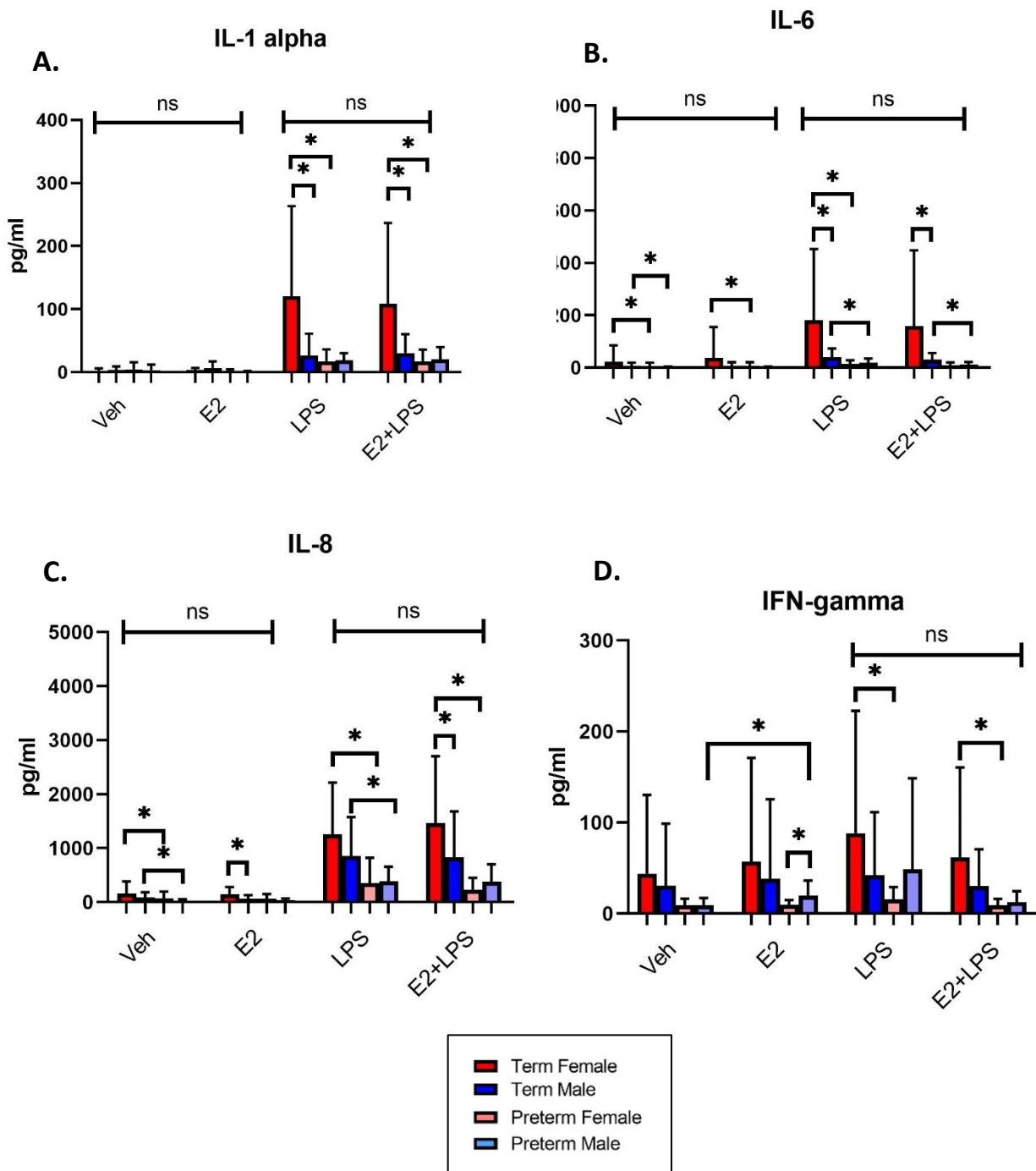


Figure 7.11: Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in male and female term and preterm neonates following treatment with endotoxin (10 ng/ml) and estradiol (10nM).

Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in E2-treated male and female term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1 α ; B) IL-6; C) IL-8; D) IFN- γ . *=significant at $p < 0.05$; ns-non-significant.

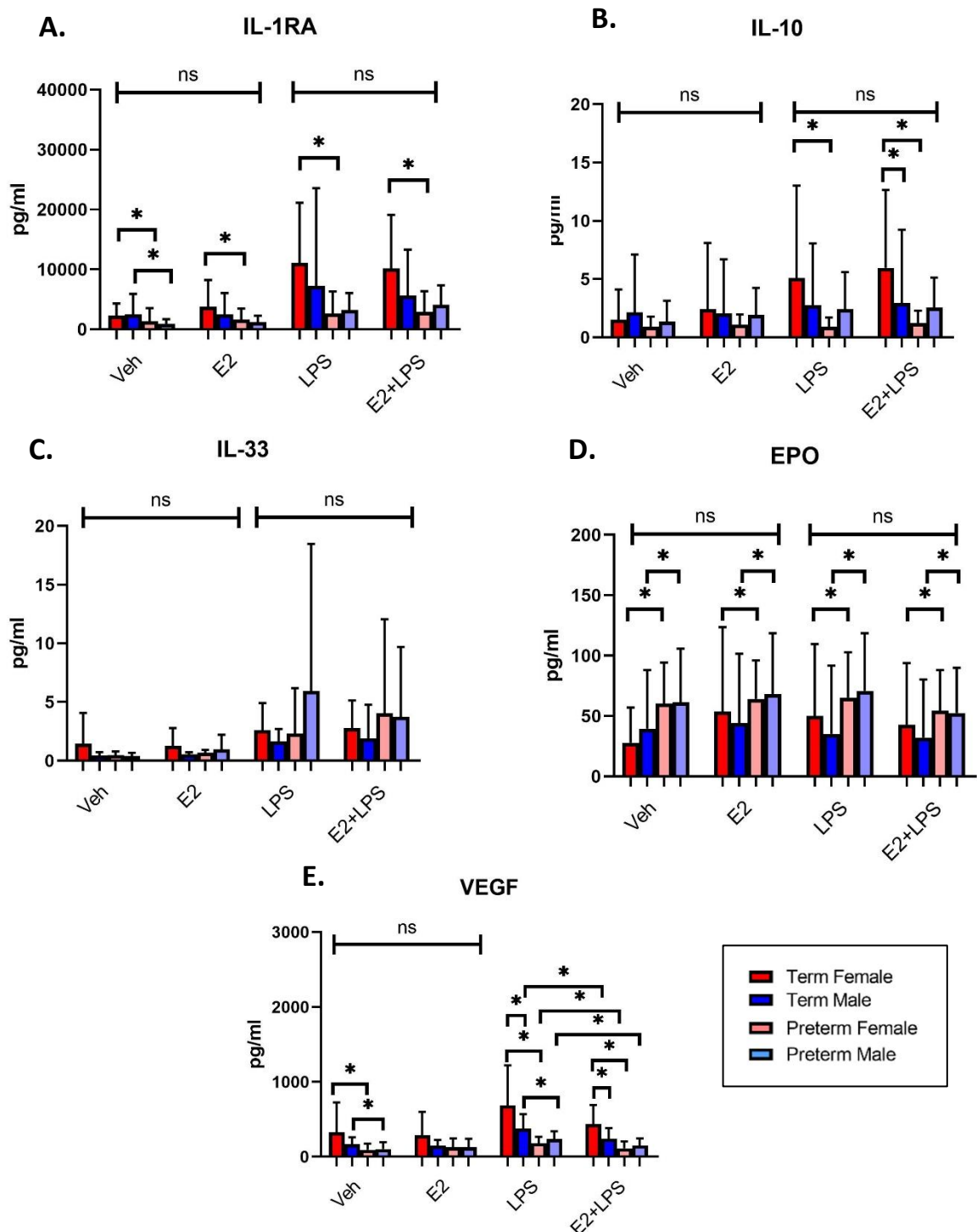


Figure 7.12: Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in E2-treated male and female term and preterm neonates following treatment with endotoxin (10 ng/ml) and estradiol (10nM).

Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in E2-treated male and female term (n=32) and preterm neonates (n=40). Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1RA; B) IL-10; C) IL-33; D) EPO; E) VEGF. *=significant at $p < 0.05$; ns-non-significant.

7.4.5 The effect of combined estrogen and progesterone on endotoxin-treated cytokine levels

To assess the potential role of sex hormones in immune modulation, cytokine levels in preterm neonatal samples treated with E2 and Pg for 1 hour either alone or in combination with endotoxin were measured. IL-1 β increased significantly in both male ($p<0.0001$) and female ($p=0.0160$) preterm neonates following LPS treatment. IL-1 β was significantly lower in LPS-treated samples when incubated with Pg ($p=0.0071$) or E2+Pg ($p=0.0474$) in female neonates only. IL-18 increased significantly in both preterm male ($p=0.0064$) and female ($p=0.0081$) neonates following LPS treatment. TNF- α increased significantly in both male ($p<0.0001$) and female ($p=0.0002$) preterm neonates. TNF- α was higher in preterm male neonates when treated with Pg+LPS compared to preterm female neonates ($p=0.0127$). TNF- α was significantly higher in preterm female neonates than preterm male neonates when treated with E2 ($p=0.0207$). TNF- β decreased significantly from baseline in male preterm samples treated with Pg ($P=0.001$) and E2+Pg ($P=0.0114$). TNF- β increased significantly in both preterm male ($p<0.0001$) and preterm female ($p=0.0004$) neonates following LPS treatment and was significantly higher in preterm male neonates following LPS treatment ($p=0.0496$). TNF- β decreased significantly in LPS-treated preterm female samples following the addition of E2 ($p=0.0114$), Pg ($p=0.0011$) and E2+Pg ($p=0.0038$). TNF- β decreased significantly in LPS-treated preterm male samples following the addition of E2 ($p=0.0417$), Pg ($p<0.0001$) and E2+Pg ($p=0.0008$) (Figure 7.13).

IL-1 α increased significantly in preterm male ($p<0.0001$) and female ($p=0.0257$) neonates following LPS treatment. IL-1 α was significantly lower in LPS-treated samples when incubated with Pg in female neonates only ($p=0.0211$). IL-6 increased significantly in both preterm male ($p<0.0001$) and female neonates ($p=0.0055$) following LPS treatment. IL-6 was significantly higher in male preterm neonates compared to females when treated with Pg+LPS ($p=0.0128$). IL-8 increased significantly in preterm male ($p<0.0001$) and preterm female ($p=0.0005$) neonates following treatment with LPS. IL-8 was higher in male neonates following treatment with Pg+LPS compared to preterm females ($p=0.0336$). IFN- γ increased significantly in male ($p=0.0471$) but not female ($p>0.05$) preterm neonates following LPS treatment.

IFN- γ was higher in preterm male neonates in samples treated with E2 ($p= 0.0290$) and LPS+Pg ($p=0.0467$). IFN- γ was significantly lower in LPS-treated samples when incubated with Pg in female neonates only ($p=0.012$) (Figure 7.14).

IL-1RA increased significantly in male ($p=0.0007$) and female ($p=0.0044$) preterm neonates following LPS treatment. IL-1RA was significantly higher in preterm male neonates when treated with Pg+LPS compared to preterm female neonates ($p= 0.0266$). IL-10 was significantly higher in preterm male neonates following treatment with Pg ($p=0.0291$), E2+Pg ($p=0.0293$), Pg+LPS (0.0062) and E2+Pg+LPS (0.0186). IL-33 increased significantly in both preterm male and preterm female neonates following LPS treatment. IL-33 was significantly lower in both male ($p=0.001$) and female preterm ($p=0.0054$) LPS-treated samples when incubated with Pg. IL-33 was significantly lower in both male ($p=0.0499$) and female ($p=0.003$) preterm LPS-treated samples when incubated with E2+Pg. EPO did not differ between preterm male and female neonates at any treatment. The addition of E2 and Pg alone or in combination did not affect the EPO concentration in either group. VEGF increased significantly in both preterm male ($p=0.0001$) and female ($p=0.0043$) neonates following treatment with LPS. VEGF decreased significantly in LPS-treated samples when incubated concurrently with E2 in preterm male ($p=0.0432$) and preterm ($p=0.0475$) female neonates (Figure 7.15).

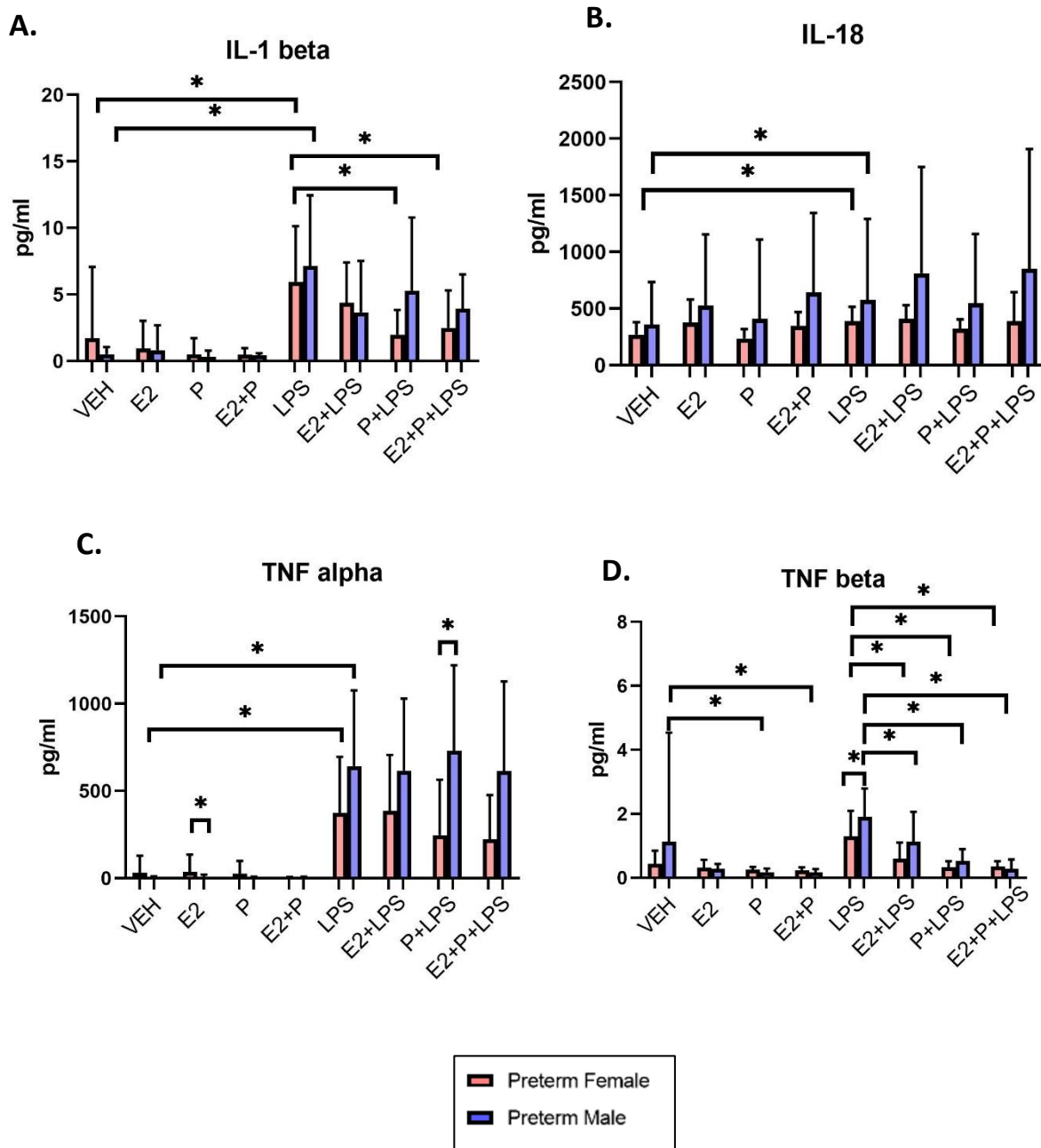


Figure 7.13: Serum IL-1 β , IL-18, TNF α and TNF β levels in hormone-treated male and female preterm neonates following treatment with endotoxin (10 ng/ml) and hormones (10nM).

Serum IL-1 β , IL-18, TNF α and TNF β levels in hormone-treated male (n=22) and female (n=18) preterm neonates. Values displayed represent mean $\pm$ standard deviation of pg/ml. A.) IL-1 β ; B) IL-18; C) TNF α ; D) TNF β . *=significant at p<0.05.

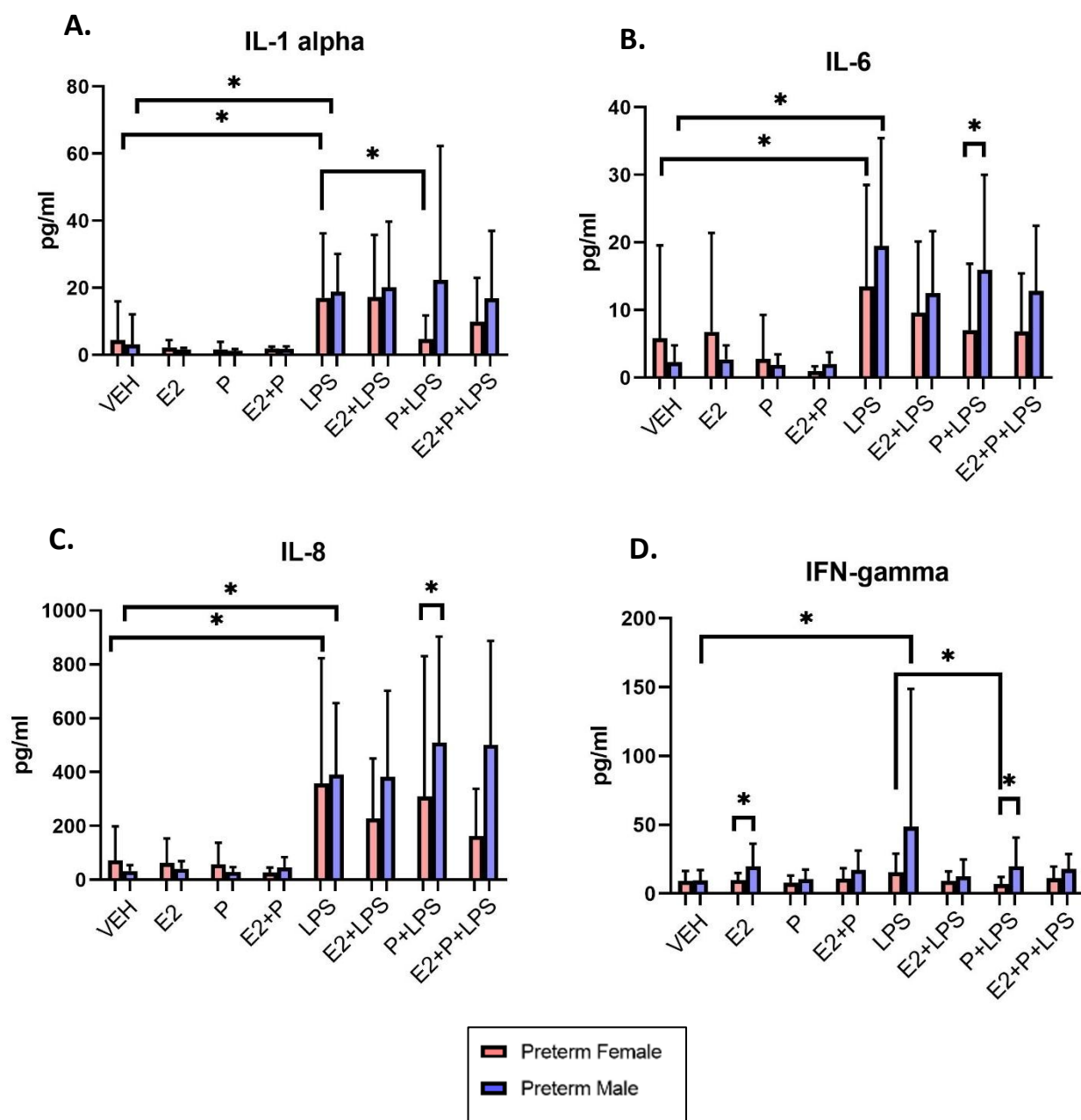


Figure 7.14: Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in hormone-treated male and female preterm neonates following treatment with endotoxin (10 ng/ml) and hormones (10nM).

Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in hormone-treated male (n=22) and female (n=18) preterm neonates. Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1 α ; B) IL-6; C) IL-8; D) IFN- γ . *=significant at p<0.05.

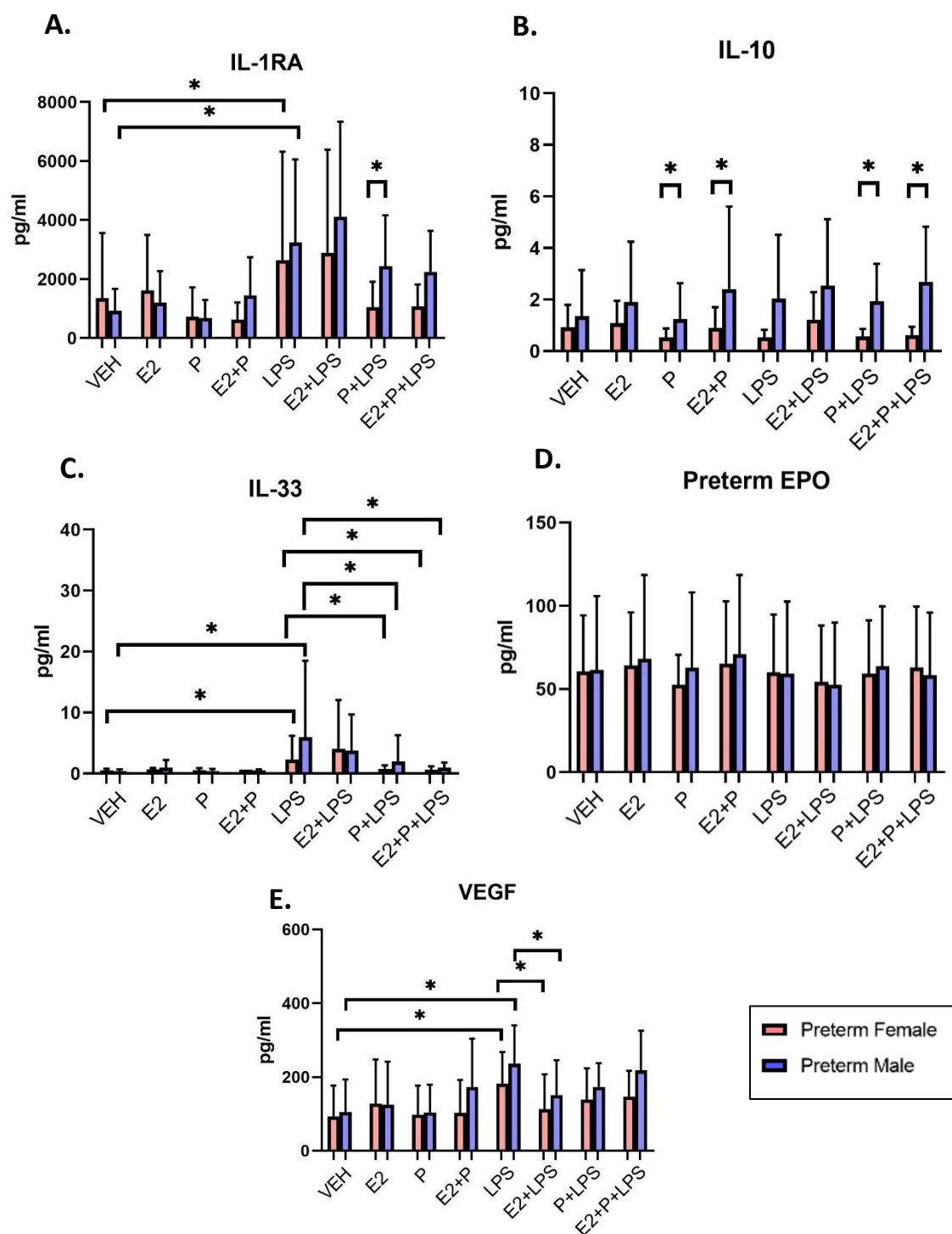


Figure 7.15: Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in hormone-treated male and female preterm neonates following treatment with endotoxin (10 ng/ml) and hormones (10nM).

Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in hormone-treated male (n=22) and female (n=18) preterm neonates. Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1RA; B) IL-10; C) IL-33; D) EPO; E) VEGF. *=significant at $p < 0.05$.

7.4.6 The effect of combined estrogen and progesterone on Pam3CSK4-treated cytokine levels

Gram positive organisms such as CONS are among the commonest and most important causes of sepsis in the preterm population⁶³⁸ and TLR2 is important in the recognition of such gram-positive bacteria. To assess the potential role of sex hormones in immune modulation, cytokine levels in samples treated with E2 and Pg for 1 hour either alone or in combination with the TLR2 agonist Pam3CSK4 were measured. IL-1 β increased significantly in preterm female neonates treated with pam3CSK4 ($p=0.0268$) but not preterm males. IL-1 β was higher in females treated with pam3CSK4 compared to males ($p=0.0064$). IL-18 decreased significantly in preterm males following pam3CSK4 treatment ($p=0.0178$). TNF- α did not differ significantly between groups with the addition of Pam3CSK4. TNF- β decreased significantly from baseline in male preterm samples treated with Pg ($P=0.001$) and E2+Pg ($P=0.0114$). TNF- β increased significantly in preterm female neonates following Pam3CSK4 treatment ($p=0.0004$). Preterm females had higher TNF- β following Pam3CSK4 treatment compared to preterm males ($p=0.0089$). TNF- β decreased significantly in both preterm female ($p=0.018$) and preterm male ($p=0.0435$) Pam3CSK4-treated samples when incubated with E2 (Figure 7.16).

IL-1 α increased significantly in preterm females when treated with Pam3CSK4 ($p=0.0335$), but not in males. IL-1 α was significantly higher in preterm females treated with Pam3CSK compared to males ($p=0.0003$). IL-6 did not differ between groups with the addition of Pam3CSK4. IL-8 did not differ between groups with the addition of Pam3CSK4. IFN- γ increased significantly in preterm females ($p=0.0074$) but not males ($p>0.05$) treated with Pam3CSK4. IFN- γ was higher in preterm males treated with E2 compared to preterm female neonates ($p=0.0290$) (Figure 7.17).

IL-1RA did not differ between groups with the addition of Pam3CSK4. IL-10 was significantly higher in preterm male neonates following treatment with Pg ($p=0.0291$) and E2+Pg ($p=0.0293$). IL-10 decreased significantly in preterm male neonates following treatment with Pam3CSK4. IL-33 did not differ significantly between groups with the addition of pam3CSK4. EPO did not differ between groups with the addition

of pam3CSK4. VEGF did not differ significantly between groups with the addition of pam3CSK4 (Figure 7.18).

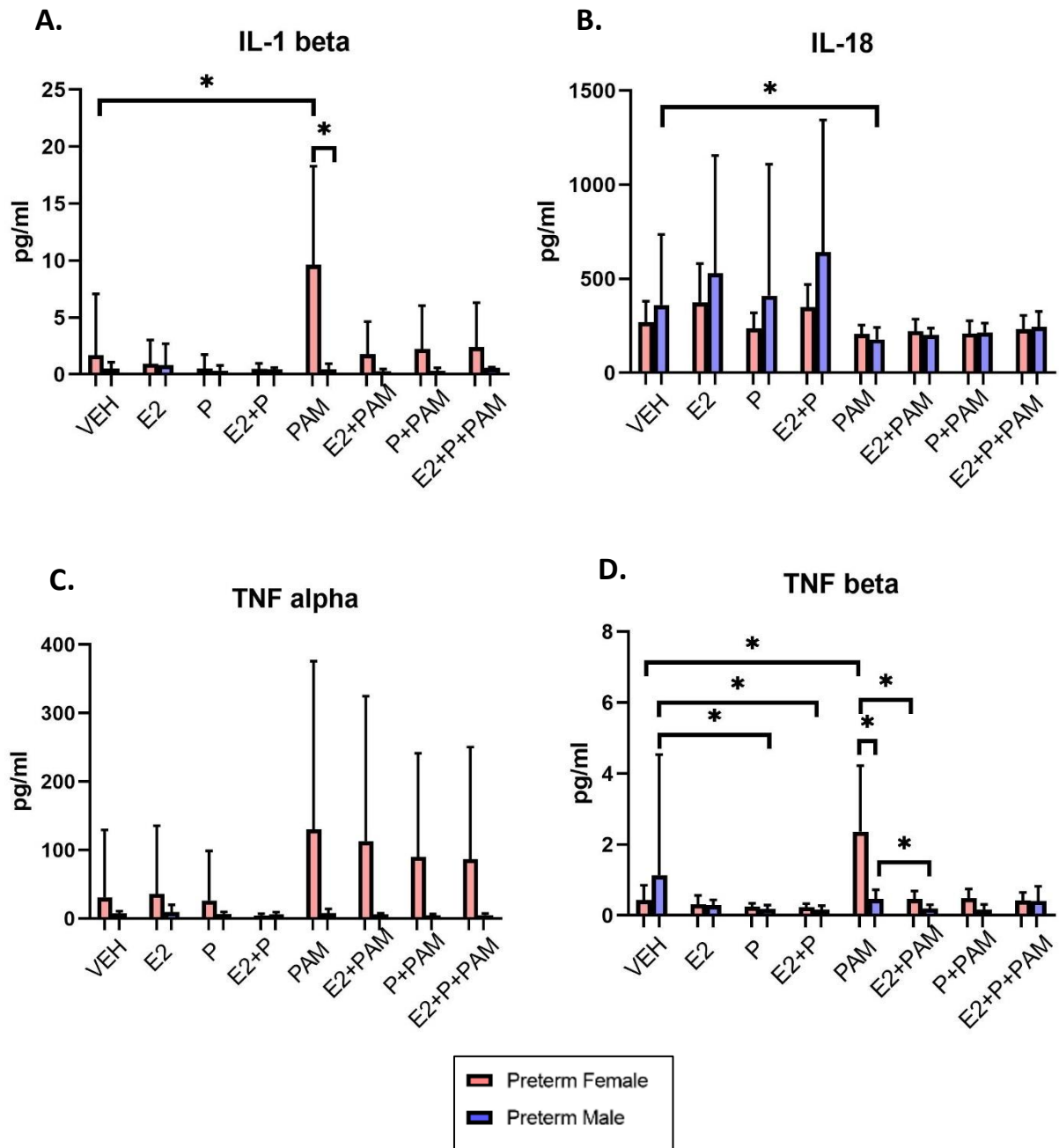


Figure 7.16: Serum IL-1 β , IL-18, TNF α and TNF β levels in male and female term and preterm neonates following treatment with Pam3CSK4 (5 ng/ml) and hormones (10nM).

Serum IL-1 β , IL-18, TNF α and TNF β levels in pam3CSK4-treated male (n=22) and female (n=18) preterm neonates. Values displayed represent mean $\pm$ standard deviation of pg/ml. A.) IL-1 β ; B) IL-18; C) TNF α ; D) TNF β . *=significant at p<0.05.

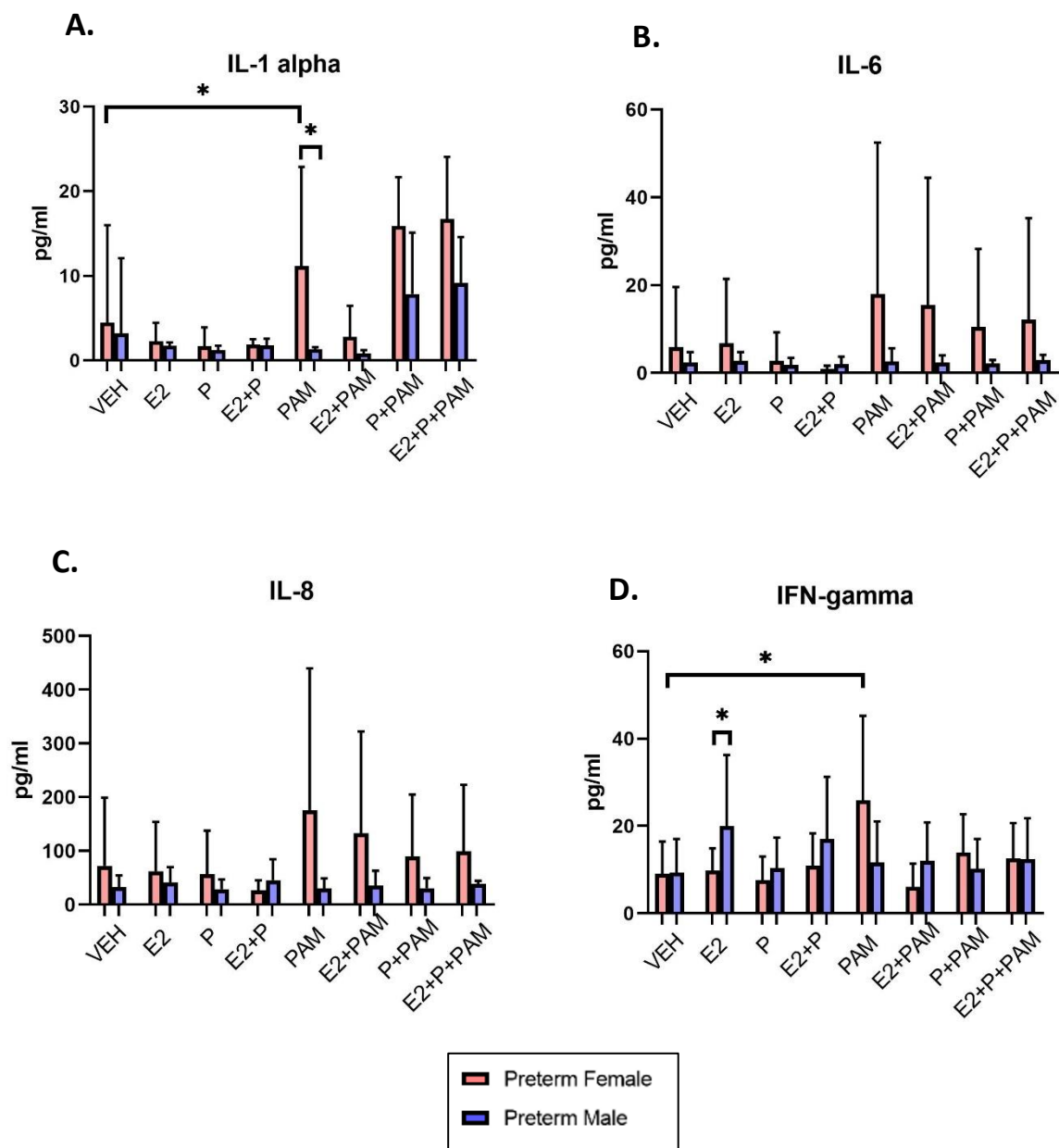


Figure 7.17: Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in male and female term and preterm neonates following treatment with Pam3CSK4 (5 ng/ml) and hormones (10nM).

Serum IL-1 α , IL-6, IL-8 and IFN- γ levels in pam3CSK4-treated male (n=22) and female (n=18) preterm neonates. Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1 α ; B) IL-6; C) IL-8; D) IFN- γ . *=significant at p<0.05.

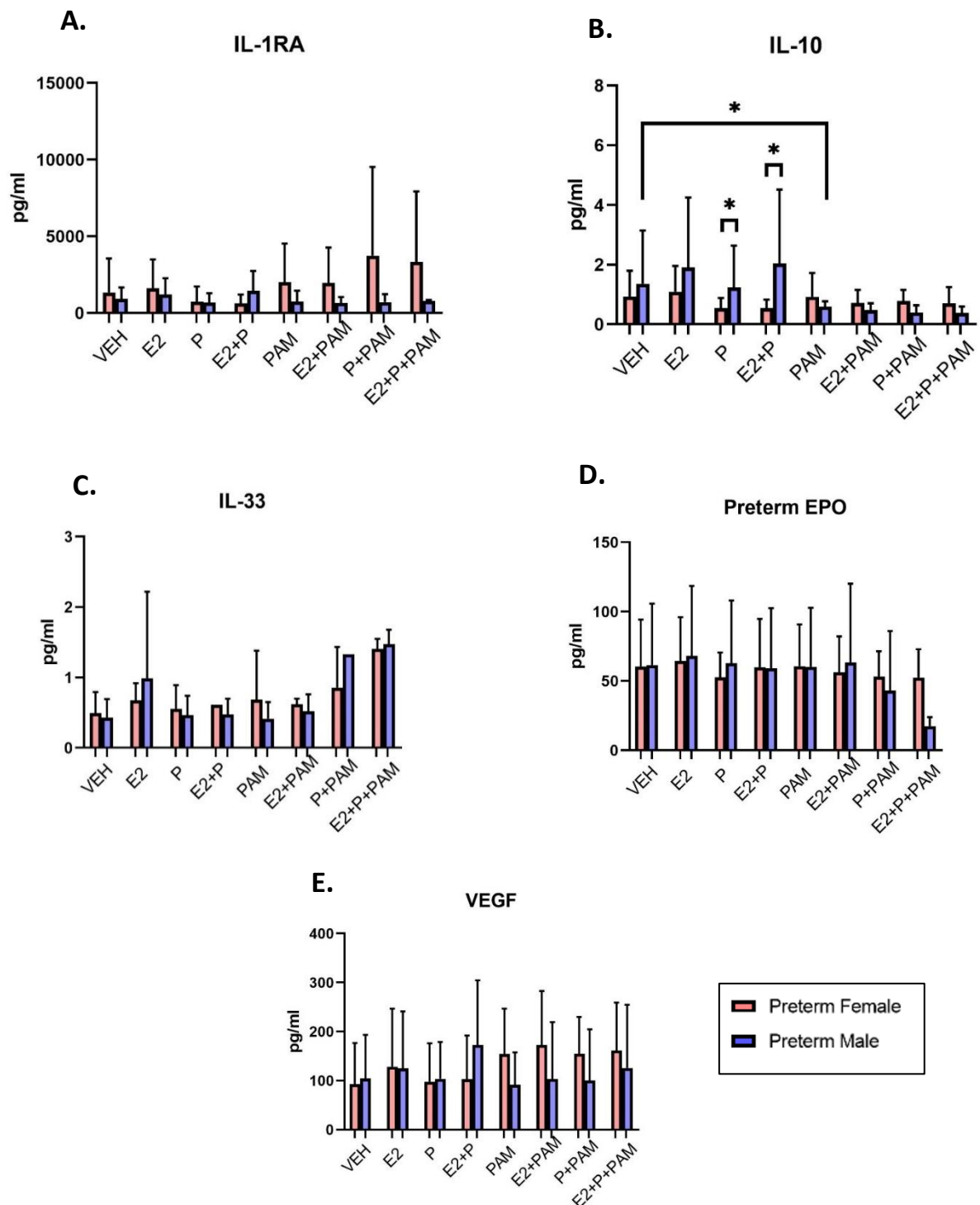


Figure 7.18: Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in male and female term and preterm neonates following treatment with Pam3CSK4 (5 ng/ml) and hormones (10nM).

Serum IL-1RA, IL-10, IL-33, EPO and VEGF levels in Pam3CSK4-treated male (n=22) and female (n=18) preterm neonates. Values displayed represent mean $\pm$ standard deviation of pg/ml. A) IL-1RA; B) IL-10; C) IL-33; D) EPO; E) VEGF. *=significant at $p < 0.05$.

7.4.4. *The effect of sex on inflammasome gene expression*

Given the sex differences in IL-1 β and IL-18 observed, inflammasome gene expression was then examined in term and preterm neonates. RNA was extracted from whole blood as described in chapter 2 and qRT-PCR was undertaken to examine the expression of NLRP3, AIM2, ASC and IL-1 β . Fold change was calculated relative to healthy term control neonates. Fold change expression did not differ between term and preterm neonates (Figure 7.19). When results were examined separately by sex, results did not differ between groups (Figure 7.20).

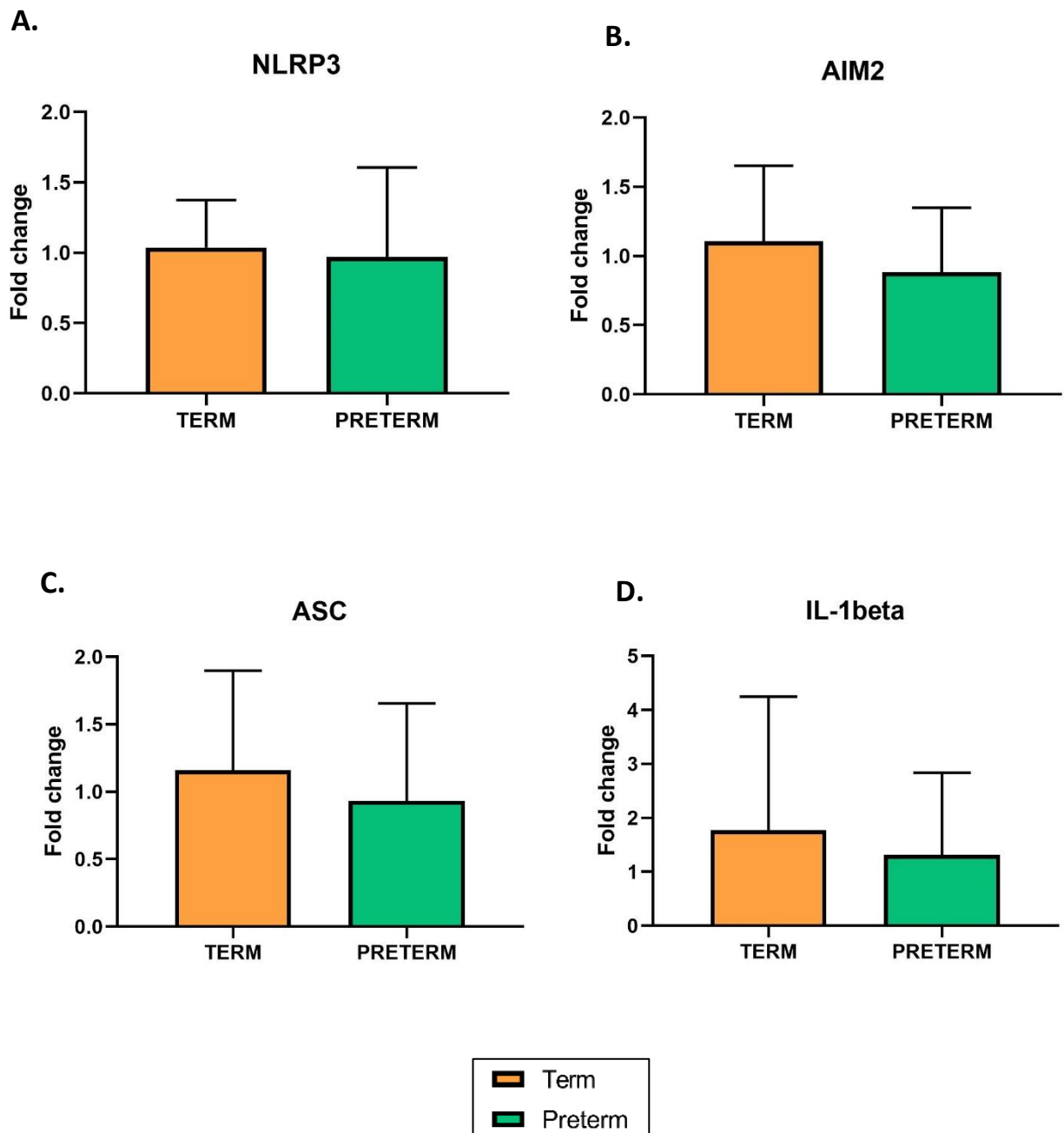


Figure 7.19: Inflammasome gene expression in term and preterm neonates.

Inflammasome gene expression in term (n=9) and preterm (n=10) neonates. Values displayed represent mean $\pm$ standard deviation fold change expression. Values did not differ between groups. A) NLRP3; B) AIM2; C) ASC; D) IL-1 β .

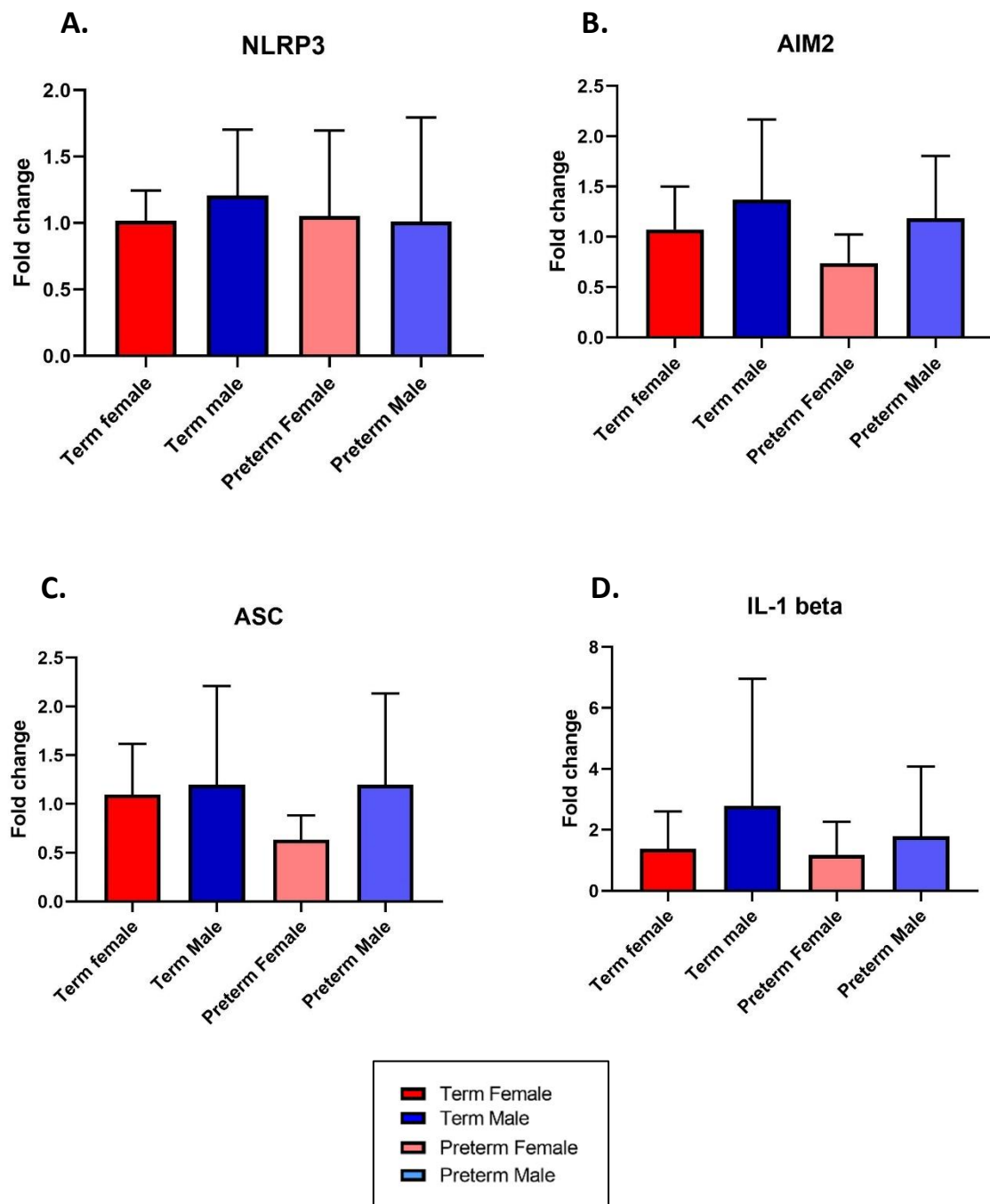


Figure 7.20: Inflammasome gene expression in male and female term and preterm neonates.

Inflammasome gene expression in male and female term (n=9) and preterm (n=10) neonates. Values displayed represent mean $\pm$ standard deviation fold change expression. Values did not differ between groups. A) NLRP3; B) AIM2; C) ASC; D) IL-1 β .

7.5. Discussion

The inflammasome genes NLRP3, AIM2, ASC and IL-1 β showed no difference in fold change gene expression between term and preterm neonates of either sex. No other studies could be identified which compared inflammasome gene expression between the sexes in neonates. Our findings of similar levels of gene expression in term and preterm neonates is surprising given the increased tendency towards infection in the premature population. Sharma et al. explored immaturity of the inflammasome in cord blood using western blot in premature neonates and found that ASC levels were similar to those of adults, though NLRP3 protein expression was reduced upon stimulation with endotoxin⁶³⁹. This may suggest that the inflammasome components studied may not vary between term and preterm neonates in the unstimulated state and that immaturity of the preterm inflammasome may only become evident following immune stimulation.

Though we did not observe differences in gene expression, when the inflammasome-related cytokines IL-18 and IL-1 β were examined by multiplex ELISA differences became evident. Both term and preterm neonates showed significant elevations in IL-1 β following treatment with LPS, though term neonates had a greater expression of IL-1 β both at baseline and in LPS-treated samples. The finding of higher IL-1 β in term neonates is supported by work from Strunk *et al.* who noted that IL-1 β expression by preterm mononuclear cells in response to infection increases in a gestationally-dependant manner⁶⁴⁰. This difference we noted in IL-1 β levels was due largely to term female neonates who had the most robust response in endotoxin-treated samples. The response of IL-1 β to endotoxin was similar in male and female preterm neonates though notably female preterm neonates had a significant elevation in IL-1 β following treatment with Pam3CSK4 and males did not. IL-1 β showed significant potential as a target for hormone therapy as preterm IL-1 β decreased significantly between LPS-treated samples and samples treated with endotoxin and E2 in combination, though dividing results by sex showed a significant reduction in term male neonates only. Preterm female neonates appear to be more sensitive to the immunomodulation effect of Pg with significant reductions in IL-1 β between LPS-treated samples and those treated with LPS and Pg in combination, a reduction that

was not present in preterm male neonates. IL-18 expression was similar in term and preterm neonates at baseline, though notably only preterm male and female neonates had significant increases in IL-18 following LPS treatment. This difference in LPS responsiveness may seem surprising given that circulating cytokines are often higher in neonates of increasing gestation but may reflect dysregulation of preterm neonatal immune response with a tendency towards a proinflammatory state. Zasada *et al.* also evaluated inflammasome responses in preterm neonates during infection and found that IL-18 was higher in those born extremely preterm compared to neonates of older gestation ²³³, in keeping with our findings. Notably IL-18 levels decreased significantly in preterm male neonates treated with Pam3CSK4 and not in females. Female sex hormones did not affect IL-18 levels in term or preterm neonates of either sex. Collectively this data suggests that a more robust inflammasome response in neonates of advancing gestation and neonates of female sex. Unstimulated samples showed similar inflammasome gene expression across gestation and sex, though circulating IL-1 β was higher in term neonates in the unstimulated state, in keeping with the findings of Bessler *et al.* who found lower IL-1 β in preterm neonatal cord blood in comparison to older children and adults ^{630,641}. This finding is also supported by another study evaluating LPS-stimulated cord blood cytokine levels in preterm infants (n=25) of various gestations and found that IL-1 β levels were significantly reduced at lower gestations ⁶³¹. When stimulated however sex differences become clear with term female neonates having the most robust IL-1 β response reflecting both their gestational and sex-related immune advantage. The reason for the greater increase in IL-18 in preterm neonates following endotoxin stimulation is unclear though this may simply reflect the immaturity of preterm innate immunity and an inappropriately pro-inflammatory response. In response to the TLR2 agonist pam3CSK4 we see that females had a significant response in IL-1 β and males had a significant reduction in IL-18, an attenuated response in this situation may underly the increased susceptibility to gram positive infections including CONS sepsis among male neonates. The only study showing sex-differences in inflammasome cytokine levels in neonates was by Kim-Fine *et al.* who found that male neonatal cord blood exhibits greater IL-1 β in response to LPS stimulation in comparison to female neonates ³⁰¹. Our findings directly contradict this paper though these authors used cord blood for their analyses, which is not a good

representation of postnatal immune response ³¹⁴. In addition to responding to the initial pathogen invasion, clinical studies have implicated IL-1 β in the development of CLD ⁶⁴² and data from rat models has implicated IL-1 β as an important mediator of sepsis-related brain injury in neonates ^{643,644}, highlighting a potentially important role in preterm clinical outcome. The aetiology of the sex differences in IL-1 β and IL-18 that we have noted are unclear. They may be the result of sex differences in a variety of cellular components including pro-cytokine production, caspase activity or TLR-mediated inflammasome priming, all of which require further study.

IL-1RA is an anti-inflammatory cytokine secreted by monocytes, neutrophils and macrophages that antagonises IL-1 action ⁶⁴⁵. IL-1RA has been shown to reduce the adverse cardiovascular effects of septic shock in pigs ⁶⁴⁶ and data from Lan *et al.* in rat models of brain injury suggests an important role for IL-1RA in attenuating LPS-induced brain injury ⁶⁴⁷. Several authors have suggested that neonates have robust IL-1RA responses compared to adults ^{648,649}, a finding that is supported by our results. Within our cohort, IL-1RA increased significantly in all groups following LPS treatment though notably this rise was greater at all treatments in term neonates, reflecting the relative maturity of their anti-inflammatory response to infection. The higher levels in term neonates were due largely to term female neonates who had the most robust IL-1RA response to inflammatory stimulation. While at baseline term male neonates had higher IL-1RA than preterm males this difference disappeared with LPS treatment inferring that the anti-inflammatory response of males does not mature effectively with increasing gestation. Notably the reverse is true in females where term female neonates had higher baseline IL-1RA than their preterm counterparts and the magnitude of this difference persisted and became more pronounced with stimulation, suggesting maturation of anti-inflammatory response in female neonates with increasing gestation. While sex differences in serum IL-1RA have not been examined in the neonatal population to date there is some limited evidence to support our findings. Bry *et al.* found that female neonates have higher levels of urinary and amniotic fluid IL-1RA than male neonates ⁶⁵⁰. Additionally, a study by Elsmen *et al.* evaluating the value of cord blood in predicting neonatal clinical outcome found that

IL-1RA has superior predictive value in female neonates ⁶⁵¹, potentially suggesting increased IL-1RA release in female neonates.

IL-10 is a potent anti-inflammatory cytokine secreted by a diverse range of immune cells. IL-10 has been identified as important in preterm neonatal outcomes with a study of 102 preterm neonates conducted by Koksai *et al.* finding that serum IL-10 is lower in preterm neonates who later develop CLD ⁶⁴². Within our study IL-10 was similar in all groups at baseline though increased significantly in term neonates only and was higher among term neonates compared to preterm neonates following LPS stimulation. Similar to IL-1RA, this increase among term neonates was due largely to term female neonates who were the only group to show significant responses to LPS. The levels of IL-10 were similar in term and preterm male neonates at baseline and following LPS stimulation though term females were greater than their preterm counterparts following LPS stimulation. This again suggests that females show improved anti-inflammatory cytokine responses with gestational maturation, a pattern not present in males. IL-10 decreased significantly in preterm males following Pam3CSK4 stimulation, potentially reflecting the increased susceptibility of preterm males to the deleterious inflammatory effects of gram-positive infection. No studies could be identified which examined neonatal sex differences in IL-10 though Dembinski *et al.* studied LPS-stimulated cord blood cytokine levels in preterm infants (n=25) of various gestations and found that IL-10 levels did not differ significantly with lower gestation ⁶³¹. This conflicts with our findings though notably the authors used cord blood which is known to be immunologically distinct from the postnatally acquired samples used in our study ³¹⁴.

The pro-inflammatory cytokine IL-1 α is raised early in sepsis and data from murine models has suggested that excessive IL-1 α production is an important factor in sepsis-related mortality ⁶⁵². IL-1 α increased significantly in both term and preterm neonates with greater levels in term neonates in samples treated with E2, LPS and E2+LPS. Following similar trends in other pro-inflammatory cytokines, this difference between term and preterm samples was largely due to term female neonates who had the most robust response in IL-1 α following stimulation. Preterm female neonates had a significant elevation in IL-1 α following the addition of Pam3CSK4 and had greater

levels following pam3CSK4 stimulation than their male counterparts, suggesting an improved immune response to gram positive infection in preterm female neonates.

IFN- γ , a cytokine produced by monocytes, NK cells and T cells is involved in a diverse range of pro-inflammatory actions was higher at baseline in term neonates in our study. Only preterm male neonates had a significant increase following LPS treatment. Term females had greater IFN- γ than preterm female neonates in samples treated with LPS and E2+LPS, while term and preterm males had similar levels at each treatment, reflecting a more robust immune response in females with increasing gestational maturity. Preterm males had greater IFN- γ in samples treated with E2 and Pg+LPS compared to preterm female neonates. Similar to trends with other cytokines, Pam3CSK4 treatment increased IFN- γ in preterm female neonates but not males. Dembinski *et al.* studied LPS-stimulated cord blood cytokine levels in preterm infants (n=25) of various gestations and found that INF- γ levels did not differ significantly with lower gestation ⁶³¹. No studies evaluating sex differences in neonatal IFN- γ could be identified.

IL-6, a pro-inflammatory cytokine involved in the acute phase response to infection and has been implicated in the development of IVH ⁶⁵³ and CLD ⁶⁴² in preterm neonates. Within our study IL-6 was higher in term neonates at both baseline and following LPS treatment when compared to preterm neonates, supporting the observation that mononuclear cell IL-6 secretion increases in a gestationally dependant manner ⁶⁴⁰. IL-6 increased significantly in both groups following LPS treatment, though the magnitude of response was much greater in term neonates. Our findings of higher levels in term neonates is supported by a study involving 22 preterm neonates conducted by Bessler *et al.* which found that LPS-induced IL-6 production by cord blood mononuclear cells is significantly lower in preterm neonates compared to term neonates and adults ⁶³⁰. Dembinski *et al.* also studied LPS-stimulated cord blood cytokine levels in preterm infants (n=25) of various gestations and found that IL-6 levels were significantly lower at lower gestation ⁶³¹. When examining by sex, all groups had a significant rise though, as with other cytokines, the magnitude of this response was much greater in term female neonates compared to other groups. Treatment with pam3CSK4 did not affect IL-6 levels in preterm

neonates. Kim-fine *et al.* found that male neonatal cord blood exhibits greater IL-6 in response to LPS stimulation in comparison to female neonates³⁰¹, a finding that directly conflicts with our findings of a more robust response among term female neonates.

IL-8, a chemokine involved in neutrophil chemotaxis was higher in term neonates with all treatments and increased in all groups following stimulation. Our findings of higher levels in term neonates conflicts with the findings of Dembinski *et al.* who studied LPS-stimulated cord blood cytokine levels in preterm infants (n=25) and found that IL-8 levels did not differ significantly with lower gestation⁶³¹, though notably this study examined cord blood. The findings of Strunk *et al.* were in keeping with our results, with these authors reporting lower levels of IL-8 gene expression and protein expression in preterm neonates compared to adults⁶⁴⁰. We found that term female neonates had the greatest rise and had levels that were significantly higher than the other groups at several treatments, though no other studies could be identified that compared IL-8 across the sexes in neonates.

IL-33 increased in all groups following LPS treatment and did not differ across gender or gestation. The addition of pam3CSK4 did not alter IL-33 levels. Studies on IL-33 are rare in neonates, though animal models have implicated it in the pathogenesis of CLD⁶⁵⁴. No studies could be identified that compared IL-33 between male and female neonates.

TNF- α , a potent pro-inflammatory cytokine which has a key role in pathogen defence, has also been implicated in the pathogenesis of septic shock and DIC and clinical studies have suggested a role in the development of CLD in preterm neonates⁶⁴². TNF- α increased in all groups following LPS stimulation and was higher in term neonates in LPS-treated samples, in keeping with findings of lower levels in preterm infants noted by other authors⁶⁴⁰. This difference was largely due to term female neonates who had higher levels than other groups at several treatments. TNF- α was not affected by treatment with pam3CSK4. Preterm male neonates had higher expression than preterm female neonates when treated with LPS and Pg in combination, though hormones had little effect on TNF- α levels otherwise. Published data on TNF- α in neonates of lower gestation is somewhat conflicting. In a study of 22

preterm neonates, Bessler *et al.* found that LPS-induced TNF α production by cord blood mononuclear cells is similar in preterm and term neonates ⁶³⁰, though a clinical study of 25 preterm neonates by Dembinski *et al.* found much lower levels at reduced gestation ⁶³¹.

TNF- β , a cytokine which activates innate immune responses was higher in term neonates at baseline and following treatment with E2 alone and in combination with LPS. All groups had a significant increase in TNF- β following LPS treatment and notably preterm male neonates had higher levels of TNF- β than preterm female infants when treated with LPS. TNF- β increased following pam3CSK4 treatment in preterm female neonates only. While no neonatal studies assessing sex-differences in neonatal TNF- β could be identified, the higher TNF- β in males is notable given that Zareen *et al.* found elevated TNF- β in school aged children with neonatal encephalopathy who had adverse developmental outcome ³⁵³.

VEGF is a protein that promotes vascular development and in neonates and has been most widely studied in patients with NE ^{655,656}. VEGF increased significantly in all groups and was higher both at baseline and following LPS in term neonates. Term females had the greatest levels in LPS-treated samples and Pam3CSK4 treatment did not alter VEGF levels. Much of the literature on VEGF in preterm neonates is in relation to ROP, where it has been suggested as a marker of disease activity ⁶⁵⁷, the differences we have noted are therefore of uncertain significance.

EPO is a hormone produced by the kidneys that promotes red cell production. A study of 50 preterm neonates Khosravi *et al.* showed that cord blood EPO correlated with later IVH ⁶⁵³. In our study EPO was significantly higher in preterm neonates compared to their term controls, likely reflecting the increased drive for haematopoiesis in the premature population. EPO did not differ between the sexes and was not affected by any treatment.

We found that E2 and Pg had potentially important effects on the expression of many of the cytokines studied, supporting the suggestion that sex hormones may have an important role in preterm male and female neonatal immune response ²⁴⁶. While neither IL-1RA nor IL-10 changed significantly with the addition of E2 it is notable that

IL-1RA and IL-10 were higher in preterm males in samples treated with Pg, suggesting some effect of sex hormones on serum concentrations of anti-inflammatory cytokines. IL-18, an inflammasome cytokine, was not affected by treatment with female sex hormones. IL-1 β however was affected by female sex hormones and notably the effect of E2 was greatest in the preterm population and female preterm neonates appeared more responsive to the anti-inflammatory effect of Pg. E2 treatment did not significantly affect IL-1 α levels though the addition of Pg to preterm female LPS-treated samples caused a significant reduction in IL-1 α levels, a difference which was not present in preterm male neonates. IFN- γ increased from baseline in preterm male serum when treated with E2 and the addition of Pg reduced IFN- γ in LPS-treated samples from preterm female neonates. While E2 did not significantly affect the expression of IL-33, preterm male and female LPS-treated samples had a significant reduction in IL-33 when concurrently incubated with Pg either alone or in combination with E2. Treatment with E2 and Pg reduced TNF- β levels from baseline in male preterm neonates but not females. Treatment with E2 and Pg alone and in combination significantly reduced TNF- β levels in preterm males and females across LPS-treated samples though notably term samples did not respond to hormone treatment. E2 also significantly reduced TNF- β in male and female samples treated with Pam3CSK4. E2 significantly reduced VEGF levels when added to LPS-treated samples of term male, preterm male and preterm female neonates, a fact which is surprising given that estrogen increases VEGF production during vascular injury in adults ⁶⁵⁸. Notably, male preterm neonates had higher IFN- γ , IL-8 and TNF- α levels compared to preterm females when treated with P+LPS. Studies of estrogen and Pg as immunomodulators in neonates are infrequent. The only study of similar design to ours was by Giannoni *et al.* who found that E2 and Pg strongly inhibit several important aspects of innate immune responses in newborns, including TNF and IL-6 ⁵⁶². Various agents including vitamin A ⁶⁵⁹, steroids ⁶⁶⁰ and NSAIDs ⁶⁶¹ have been suggested as potential modulators of neonatal cytokine response and we have identified a similar and potentially important role for female sex hormones in moderating neonatal cytokine responses. Both E2 and Pg showed potential to alter innate immune responses in term and preterm neonates and notably these responses often differed depending on neonatal gestation and sex. There is clearly a potential role for

immunomodulation in the treatment and prevention of neonatal sepsis and sepsis-related neuroinflammation in neonates and further work is needed to explore the effect of sex hormones on other aspects of the innate immune response. While we do not advocate administering hormones to preterm neonates, the effects we have noted are important in the context of antenatal steroids and maternal progesterone, two medications often administered to women with threatened preterm birth. Future clinical studies should focus on the effects of these medications on neonatal immune response as these antenatally administered medications may evoke similar effects to those we have seen, both are known to be safe and may present potential therapeutic avenues in the future.

In addition to their role in immune defence, cytokines are potentially useful in clinical management. A meta-analysis by Nist *et al.* found that elevated IL-1 β , IL-6, IL-8 and TNF- α in the serum of preterm neonates in the weeks after delivery are robust predictors of adverse neurological outcome ⁶⁶². Serum cytokines may also be good biomarkers of sepsis in high risk patients ⁶⁶³, may have a role in differentiating NEC from other forms of intestinal disease ⁶⁶⁴, are elevated in patients who develop complications of RDS ⁶⁶⁵ and may help to predict CLD ⁶⁴². In addition to diagnosis, it has been suggested that pro-inflammatory cytokine levels during sepsis may help guide therapy ⁶⁶⁶. The differences in responses that we have identified between the sexes have important implications in all these areas and highlights the need for future biomarker studies to include sex as a differential in both the planning and reporting of such studies.

Within our study it is notable that term female neonates had the most robust pro- and anti-inflammatory cytokine response. This balance of pro- and anti-inflammatory cytokine responses to sepsis has been suggested by many authors to be important in balancing the positive and adverse effects of inflammation in preterm neonatal sepsis ^{629,631,667}. Our data likely reflects the fact that female neonates, while mounting a significant pro-inflammatory response to promote pathogen clearance, are able to balance this with a simultaneous anti-inflammatory response, potentially mitigating the adverse effects of infection-related inflammation. Few studies have evaluated the impact of sex on neonatal blood cytokine responses and those that have

often use cord blood ³⁰¹ or amniotic fluid ⁶⁶⁸. This study is the first to evaluate a comprehensive array of pro- and anti-inflammatory cytokines in samples acquired postnatally in term and preterm neonates using sex as a key differentiator. While several studies of preterm cytokine responses exist, it is rare for authors to report results separately for males and females, making it challenging to discuss our findings in light of existing research. Nevertheless, we have identified important differences in male and female neonatal immune response and present data which adds considerably to our understanding of this complex area.

There are still considerable gaps in our knowledge on preterm neonatal cytokine responses. While cytokines have great potential as biomarkers in neonatal sepsis we have highlighted important sex differences in their circulating levels and others have shown that cytokine profiles of preterm neonates with EOS and LOS are distinct ²²⁶, as are those of ELBW neonates with different forms of microbiological sepsis ⁶⁶⁹, further complicating their use. Our findings support data suggesting a reduced capacity of preterm neonates to produce cytokines through associated defects in TLR signalling ⁶⁷⁰, though immaturity of cellular responses to cytokines ⁶⁷¹ may be equally important in preterm neonates and require further study. It has been suggested that levels of circulating cytokines may not increase in a linear fashion with increasing gestation ²¹⁷ and antenatal inflammation has the potential to alter cytokine levels in preterm neonates ²¹⁸. This may explain the apparent discordance in some of the published literature in this area and may be an important factor to control for in future studies.

Our results highlight the immaturity of preterm neonatal cytokine responses in addition to highlighting possible sex differences in cytokine expression and the potential biological role of E2 and Pg in modulating neonatal immune response early in life. Term female neonates had the highest levels of pro- and anti-inflammatory cytokines following stimulation. This reflects the improved ability of female neonates to fight infection while concurrently avoiding the potentially deleterious effects of excessive inflammation through a balance of pro- and anti-inflammatory signalling. The markedly elevated cytokine responses of term female neonates compared to preterm female neonates may reflect the advantage of increased gestation, whereas

their improved responses compared to male term neonates may represent the effect of sex.

7.6. Conclusion

Inflammasome gene expression is similar in male and female term and preterm neonates. Cytokine responses are distinct in term and preterm neonates and differ between males and females. Term female neonates have the most robust response with higher levels of both pro- and anti-inflammatory cytokines, reflecting the advantage conferred by their gestational maturity and female sex. This balance in pro- and anti-inflammatory responses is reflected clinically by better bacterial clearance and improved sepsis outcome. We have shown that female sex hormones have an important role in modulating neonatal immune response and our findings add to the existing literature that suggests an important role for hormones in the female immune advantage.

Chapter 8: Discussion

8.1. Introduction

Preterm birth is among the commonest causes of childhood mortality worldwide ¹¹⁹. Surviving preterm neonates are at high risk of adverse short- and long-term clinical outcomes compared to children born at term ^{19,672}. Due to the organ immaturity present, the short-term complications of prematurity can affect any organ system in the body and may have potentially devastating consequences ⁶⁷³. The neurological and developmental consequences of preterm birth have classically been the focus of most long-term follow-up studies though it is now recognised that ex-preterm children may have chronic, occult impairment of many other organ systems ¹⁹.

Multi-organ dysfunction is a common and serious short-term complication of preterm delivery ¹⁰⁴. It is recognised that those with greater medical requirements after delivery and during episodes of sepsis are at greater risk of adverse outcome and several authors have proposed illness severity scores incorporating organ dysfunction to provide prognostic information to parents and healthcare providers ¹¹⁴. Despite the abundance of organ scores available to neonatologists many are impractical, require serial recordings or are inadequately validated for clinical practice ^{114,493}.

Sepsis is the commonest cause of childhood mortality worldwide ¹¹⁹. The immaturity of preterm neonates extends to their immune system meaning that they are at even greater risk of sepsis ¹⁴², sepsis-related organ dysfunction ⁷² and sepsis-related mortality ³⁶⁰ compared to infants born at term. Despite the prominence of sepsis-related morbidity in term and preterm populations there is currently no unified definition of neonatal sepsis. The definitions currently in use are varied, based largely on microbiological results and are not designed to facilitate early identification of high-risk patients. The consensus definition of sepsis published by the Sepsis-3 group in adults highlights the central role of organ dysfunction in the definition of sepsis and advocates the use of organ dysfunction scores in screening for and defining sepsis ¹⁰⁰. This approach has not yet been adopted into neonatal practice though it is recognised that similar work is needed within neonatology ¹⁴³.

Male preterm neonates are at higher risk of adverse clinical outcome compared to females ⁴⁹⁴. The aetiology of these differences is poorly understood though a combination of genetic, immunological and hormonal differences are thought to contribute ²⁴⁶. Clinical studies evaluating patterns of organ dysfunction in male and female preterm neonates are lacking. Similarly, though an impaired male immune defence is thought to underly their increased sepsis susceptibility ²⁴⁶, there are few studies specifically evaluating this area.

Despite significant improvements in neonatal mortality in recent decades ^{8,14,128} there are still several important gaps in our knowledge: the optimal definition of neonatal sepsis is unclear, the role of organ dysfunction in predicting outcome has yet to be determined, the aetiology of the male neonatal disadvantage is poorly understood and the place of the impaired preterm immune response in all these areas remains unknown. We have chosen to review existing definitions of neonatal sepsis as a first-step in the important task of defining neonatal sepsis. We then investigated the role of organ dysfunction in screening for sepsis in keeping with the work of Sepsis-3 and evaluated organ dysfunction as a predictor of outcome in the preterm population. Lastly, we evaluated neonatal immune response in term and preterm neonates to further our understanding of the increased susceptibility to, and severity of, sepsis in preterm neonates. This immune work focused largely on innate immune responses by evaluating surface markers on peripheral blood immune cells; TLR expression; cytokine responses; miRNA, inflammasome and X-linked gene expression; and the role of hormones in modulating innate immune response. In the case of both organ dysfunction and immune studies we compared data between male and female infants in order to better understand the unique susceptibility of preterm male neonates to sepsis and adverse clinical outcome.

8.2. Existing sepsis definitions

Chapter 3 focused on reviewing the existing definitions of neonatal sepsis currently in use by international research and surveillance networks. This review was completed as part of a collaborative work with the ESPR section on Infection, Inflammation, Immunology and Immunisation (4I group) and was undertaken as a

preliminary step in the process of establishing a consensus definition of neonatal sepsis³⁷⁸. We discussed the definitions currently in use, identified common themes in neonatal sepsis literature and discussed these in light of the consensus work undertaken by the Sepsis-3 group in adult medicine.

We found that there were 8 organisations with definitions of neonatal sepsis within their publications. While common themes were present between the publications, these definitions differed greatly from each other, a sentiment echoed by other authors⁶⁷⁴. This is problematic for clinicians and researchers as there is potential to misclassify patients depending on the definition used. Apart from the obvious implications for clinical practice, the use of different definitions by different centres makes the results of research difficult to generalise.

Most existing definitions rely heavily on microbiological culture results and therefore do not facilitate early identification of patients, a concern also voiced by other authors¹²⁹. None of the definitions reviewed provided screening criteria for use in clinical practice and none incorporated the need for organ dysfunction despite recent work by the Sepsis-3 group suggesting that it is key to definition¹⁰⁰. Lastly, none of the definitions reviewed included a separate definition for premature neonates. This is problematic as preterm neonates are at especially high risk of sepsis while concurrently being more challenging to screen due to the non-specificity of existing clinical and laboratory tests in this population¹²³.

Our findings highlight the many problems with existing definitions and identify challenges for future consensus work in this area. There is therefore urgent need for a consensus definition of neonatal sepsis⁶⁷⁴, though the task of defining sepsis and creating screening criteria will be especially challenging in the premature neonatal population. The approach used by the Sepsis-3 group has been well-documented and provides a clear example of how this process may be replicated within neonatology¹⁰⁰. The process requires formation of an expert group, the completion of systematic literature reviews in the area, a formal Delphi process, consultation with expert groups within neonatology and then extensive clinical validation. The 4I group within ESPR represents the foundation of the expert group and the literature review process is currently underway with the follow-up of our manuscript, a systematic review of sepsis

definitions within randomised controlled trials, nearing completion. The Delphi process will require the incorporation of core outcomes methodology, similar to those used by other authors in the area ⁶⁷⁵, and the formation of a committee of international stakeholders before a formal review process can begin. Any definitions produced will need to be practical, clinically focused, provide screening criteria that identify patients early in disease and will require extensive clinical validation. Such consensus work, while challenging, is required to progress clinical care and research in neonatal sepsis as the continued use of current definitions is clearly not in service of our patients.

We also undertook a systematic review to evaluate the neurological effect of CONS sepsis on preterm neonatal neurological outcome. This was undertaken because despite the majority of preterm sepsis being LOS ⁴⁶² and CONS being the most common group of pathogens, it is classically thought that CONS represents a more indolent form of neonatal infection ⁴²⁷. We found that best evidence suggests that CONS sepsis adversely affects the neurological outcome of affected neonates, further highlighting the need for a definition of sepsis that relates diagnosis to later clinical outcome. Further studies are required to study the mechanism of CONS-related neurological injury which may be mediated by TLR signalling ^{568,676,677}.

8.3. Sex differences in organ dysfunction and clinical outcome in preterm neonates

We evaluated the role of organ dysfunction in the preterm VLBW population both early in life and as a predictor of outcome in episodes of suspected sepsis. Two scores were used: the NEOMOD, a score which was developed to measure early life organ dysfunction and use this to predict mortality ¹⁰¹ and the nSOFA, a score which was developed in accordance with the recommendations of Sepsis-3 to screen for high-risk neonates with suspected sepsis ¹²⁹. We found that organ dysfunction scores in the first week of life predicted death and the composite outcome of death/IVH and that scores on day 5 were the best predictors. We also found that organ dysfunction scores recorded at the time of sepsis evaluation predict outcome, highlighting both the

importance of organ dysfunction in neonatal sepsis and the potential utility of organ dysfunction in screening for neonatal sepsis. Our findings support the suggestion that assessment of organ dysfunction is useful in neonatal sepsis screening ³⁷⁷, in keeping with the idea that the principles of Sepsis-3 can be successfully applied in the neonatal population. While the potential utility of organ dysfunction in predicting clinical outcome and screening for sepsis that we have highlighted will require further validation, they are potentially important findings that will be useful in future consensus work on defining neonatal sepsis.

Male neonates in our study had greater levels of early life organ dysfunction than females. Male neonates also had greater levels of organ dysfunction when undergoing evaluation for suspected sepsis. While we can identify no other studies that directly compare organ dysfunction scores between male and female preterm neonates, our findings are supported by published literature suggesting that male neonates are at higher risk of early life morbidity ⁶⁷⁸ and sepsis mortality ^{250,252} than females.

When examining clinical outcomes, we found that preterm male neonates required greater intervention at delivery and had higher rates of both PVL and CLD compared to female infants. Many authors have highlighted the increased susceptibility of preterm male neonates to neurological injury ^{257,271}, adverse respiratory outcome ²⁹⁴ and instability at birth ⁶⁷⁸, though further study is required to better understand the aetiology of these differences.

Chronic organ dysfunction is likely under-recognised in former-preterm neonates ¹⁹ and it is unclear if the sex-differences in organ dysfunction that we have identified persist with increasing age. It has been suggested that chronic cardiovascular ^{282,283} and respiratory ^{290,293} dysfunction are greater in former-preterm male neonates though it is unknown if this is true of other organ systems and the impact on function and long-term survival is unknown. Future studies should examine whether the sex-differences we have observed in organ dysfunction persist into childhood and evaluate the relationship between early life organ dysfunction and long-term clinical outcome.

8.4. Sex differences in innate immune function

In chapter 5 we examined innate cell surface markers, the effect of sex hormones on these markers, X-linked gene expression and lymphocyte subsets in male and female neonates. We found that LPS responses in preterm neonates, while robust, were generally diminished compared to term infants in keeping with the diminished immune response of preterm neonates⁶⁷⁹ and their increased susceptibility to sepsis^{126,431}. Preterm female monocytes had higher CD11b and TLR2 expression compared to males in many instances, reflecting the superior innate immunity of preterm female neonates. The expression of TLR2, CD11b and several cytokines were similar in male neonates of different gestations but different in female term and preterm neonates, possibly reflecting differences in immune cell maturation between the sexes. Cytokine expression differed in term and preterm neonates with term neonates having higher circulating levels in most situations. In several instances clear sex-differences in cytokine responses were elicited in both term and preterm neonates and data from ELISA reflected the superior immune response of females with improved pro- and anti-inflammatory signalling in most cases.

TLR2 expression was higher in preterm monocytes than term monocytes, a difference mainly accounted for by preterm female neonates. Published literature on the expression of TLR2 in preterm neonates compared to term infants is mixed⁵⁴⁷ and the aetiology of the differences we have observed is unclear. Further study is required to evaluate TLR signalling in preterm neonates of both sexes, as despite the relative abundance of TLR2 expression in the cells examined, authors have suggested that TLR signalling in preterm neonates may be dysregulated^{548,551} and this requires further examination in VLBW male and female neonates both after delivery and later in childhood.

Sex hormones are known to modulate immune response^{246,324} and study of neonatal patients has shown that peripheral blood immune cells express estrogen receptors and certain forms of progesterone receptors³³⁴. Given the marked clinical⁴⁹⁴ and immunological differences²⁴⁶ in preterm neonates of differing sex it is likely that sex hormones have a significant role in modulating many aspects of innate immune response. Physiological concentrations of E2 and Pg did not affect the

expression of CD11b or TLR2 in term and preterm neonates of either sex. When serum cytokine levels were evaluated with multiplex ELISA we found that many cytokines were affected by the addition of sex hormones. Notably the effect of female sex hormones on cytokines, which was largely immunosuppressive, varied between term and preterm neonates and between neonates of opposite sex. This implies that the effect of sex hormones on immune response is likely complex and differs depending on the gestation and sex of the particular neonate. These findings are potentially clinically important as both progesterone and glucocorticoids are administered antenatally to mothers with threatened preterm labour and these medications may have a similar ability to modulate the neonatal immune response, representing potential therapeutic targets. While our findings highlight the importance of hormones in modulating term and preterm immune response there are many gaps in our current knowledge. The aetiology of these hormone-induced differences in cytokine levels are unknown. Neither TLR expression nor immune cell activation, as examined by CD11b expression, changed with the addition of E2 and Pg and the influence of these hormones was not examined in relation to inflammasome gene expression. Studies on neonatal cord blood have suggested that E2 and Pg may affect TLR signalling by inhibiting the NF- κ B pathway⁵⁶² though the effect on inflammasome signalling requires further study. Future studies investigating the action of sex hormones as immunomodulators should evaluate the effect of hormones on inflammasome signalling, investigate whether the effects of sex hormones on the NF- κ B pathway differ between male and female neonates and examine the effect of testosterone on these immune pathways. While attempts have been made to document postnatal hormone concentrations in preterm neonates⁶⁸⁰⁻⁶⁸², such studies are limited in number⁶⁸³, mostly use older techniques such as radioimmunoassay and often do not divide results based on the sex of the neonate³⁴⁰. A thorough evaluation of preterm neonatal hormone status using the current gold standard technique, mass spectrometry, is required to estimate how experimental findings may translate into an *in-vivo* environment. A better understanding of the interaction of neonatal gender, sex hormones and innate immune response would both progress our understanding of male infection susceptibility and identify potential targets for future therapeutic study

When examining X-linked genes involved in innate immune response we found no differences between the term and preterm patients studied. Following analysis of serum miRNAs we noted that term and preterm neonates appear to have a miRNA profile which is distinct from that of adults, as reflected by the most highly expressed miRNAs in samples analysed. Genetic factors have been suggested as contributing to the female immune advantage ³⁴² though when groups were separated by sex we did not identify differing expression of X-linked genes in the male and female infants studied. Additionally, when examining overall miRNA levels and X-linked miRNA levels in neonatal serum we did not note any significant differences between term and preterm neonates of either sex. While no significant differences were detected it is notable that in both cases preterm male neonates appeared to have disproportionately lower miRNA levels than all other groups, suggesting that similar analyses may yield positive results if repeated in a larger patient group. Sample size aside, the lack of discernible differences between groups may simply be because the effect of the X chromosome is not important early in life or it may be that the advantage conferred by 2 copies of the X chromosome comes from the diversity afforded by production of proteins from maternal and paternal copies, rather than differences in gene expression resulting from escape of X chromosome inactivation. Future studies should evaluate X chromosome skewing among preterm females neonates in response to septic challenge and early life organ dysfunction as this phenomenon has been observed in older women during illness ³⁴⁵ but has not been studied in neonatal disease to date.

Given the respiratory difficulties encountered by premature neonates, hypoxia is a frequent occurrence in clinical practice requiring the administration of supplementary oxygen. Hypoxia-inducible factors (HIFs) are important regulators of gene expression allowing adaptation to hypoxia with important roles in glycolysis, erythropoiesis, angiogenesis and cellular proliferation ⁶⁸⁴. HIF1 α is of particular note as it has potent pro-inflammatory actions and quickly accumulates in cells following periods of hypoxia ⁶⁸⁴. As with many areas of neonatal immunology, little research has been undertaken in the premature population though data from animal models suggests that HIF1 α is implicated in premature brain injury in infants with CLD ⁶⁸⁵ and

inhibition of HIF1 α is neuroprotective in hypoxic neonates ⁶⁸⁶. HIF1 α may therefore play an important role in promoting inflammation among preterm neonates and may be of particular interest with regard to sex-differences in inflammatory response given the poorer respiratory outcomes among premature males. Therefore, while not forming part of this study, characterisation of preterm neonatal HIF1 α expression is another important area for further research.

Lymphocyte subset percentages were similar between male and female term and preterm neonates. Sex differences in lymphocyte populations in preterm infants are not well-studied but our findings are in keeping with those of other authors ^{152,153,569}. Future studies should focus on examining lymphocyte function in neonates of different sex as preterm lymphocyte responses during sepsis are diminished compared to term neonates ⁶⁸⁷ but the effect of sex on this has not been examined. Innate lymphoid cells represent a particularly interesting avenue of investigation as animal models have suggested that these cells are hormone-responsive, have differing counts in males and females ⁶⁸⁸ and unlike most lymphocytes, these cells are more active in infants compared to adults ⁶⁸⁹.

8.5. Conclusions and future directions

Collectively this work demonstrates the profound susceptibility to sepsis, organ dysfunction and immune system dysregulation resulting from preterm birth and how neonatal sex interacts with these 3 areas. This thesis contributes to our current understanding of preterm neonatal sepsis both clinically and immunologically. It supports the considerable body of existing evidence which suggests adverse clinical outcomes in preterm males and progresses our understanding of the impaired immune function present among male preterm neonates. This thesis also highlights the role of sex hormone as important immunomodulators, both contributing to our understanding of the female immune advantage and suggesting that antenatal progesterone and glucocorticoids may represent potential areas of therapeutic study in preventing and improving outcome of neonatal sepsis.

Our findings, while novel, provide only very limited insights into sex differences in infection susceptibility in the preterm population and there are many areas that require further study. Future clinical studies should focus on defining and validating a consensus definition of neonatal sepsis which is suitable for use by clinicians and researchers. Future translational studies should examine innate immune responses during sepsis in male and female preterm neonates, examine the interaction between immune response and organ dysfunction and evaluate the influence of antenatal progesterone and steroid administration on preterm neonatal immune function. Further immunological studies should evaluate immune signalling upstream of the cytokines we have studied. In the TLR pathway this should examine whether differences in NF- κ B signalling exist between preterm male and female neonates. The effect of sex and sex hormones should also be evaluated in inflammasome signalling as little is known about the influence of these factors in neonates. Long-term follow-up studies are required to understand the interaction between neonatal immune function and long-term outcome. Such studies, while important, are lengthy and extend beyond the lifetime of this PhD. Two such studies are however currently underway and represent complementary work to that presented here. The first of these follow-up studies is an evaluation of sex hormone levels in preterm neonates enrolled in this study. We have biobanked urine, saliva and serum throughout this project to allow a comprehensive evaluation of preterm neonatal hormone status using mass spectrometry, the current gold-standard for hormone concentration measurement. In the future this will allow comparison between immune results recorded, organ dysfunction and hormone status at the time. The second such study is developmental follow-up of infants enrolled in this study to allow us to relate neonatal immune function, hormone status and organ dysfunction to long-term clinical outcomes.

In conclusion, this thesis progresses our understanding of neonatal sepsis, immune function and sex-differences in clinical outcome. These represent important areas for ongoing clinical and laboratory study and our findings represent an important contribution to existing literature.

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Appendix i: Ethical approval statements



'Excellence in the Care of Women and Babies'
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JM/MJ

10 October 2017

Dr Matthew McGovern,
PhD Student,
TCD
DUBLIN 2

Re.: Resubmitted Study No. 11 – 2017 – Gender and Neonatal inflammation in preterm outcomes: GENIE study

Dear Dr McGovern

Thanks a lot for attending the research ethics committee meeting on 20th September 2017 and clarifying points raised by the committee from the meeting in June. Your study is now approved in the Coombe Women & Infants University Hospital.

Yours sincerely

A handwritten signature in black ink, appearing to read 'Jan Miletin', written over a horizontal line.

Prof. Jan Miletin
Chairman

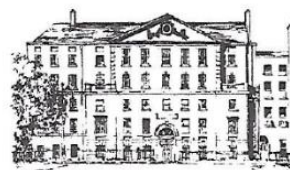
Copy: Prof. Eleanor Molloy, Prof. of Paediatrics, National Children's Research Centre, Prof. Martin White, Consultant Neonatologist, CW&IUH, Prof. John Murphy, Consultant Neonatologist, National Maternity Hospital, Prof. Afif El-Khuffash, Consultant Neonatologist, The Rotunda Hospital, Dr. John Kelleher, Consultant Neonatologist, CW&IUH, Dr Veronica Donoghue, Consultant Radiologist, NMH, Prof. Catherine Greene, Associate Prof, Microbiology, RCSI, Prof. Enda Roche, Consultant Endocrinologist, TCD, Prof. Denise McDonald, Consultant in Neurodisability, Paeds., Tallaght, Prof. Derek Doherty, Dept. of Immunology, TCD., Prof. Veronica O'Keane, Consultant Psychiatrist, TCD, Dr Mark Sherlock, Consultant Physician, Endocrinology, Tallaght., Prof. Arun Bokde, Dept. of Psychiatry, TCD, Prof. James Wynn, Neonatology, University of Florida, Prof. Wiebke Arlt, University of Birmingham.,



An tOspidéal Náisiúnta Máithreachais The National Maternity Hospital

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Máistir/Master: Dr. Rhona Mahony

PRIVATE AND CONFIDENTIAL

Professor Eleanor Molloy,
Consultant Neonatologist and Professor of Paediatrics,
Coombe Women's and Infants University Hospital,
Cork Street,
Dublin 8.

10th May 2017

Our ref: EC 11.2017

Re: Gender and Neonatal Inflammation in preterm outcomes: GENIE Study.

Dear Eleanor,

The above study was considered by the Ethics Committee at the National Maternity Hospital on the 08th May 2017. I am pleased to report that the study has received ethical approval.

There were a number of small points that perhaps might deserve attention.

1. In the consent form it might be worth mentioning that an MRI scan may also be undertaken.
2. In section F2.6 it mentions about taking samples of CSF and I presume that would only occur when the samples have been taken on medical grounds.
3. We also wondered whether a separate consent form for the controlled group would be required as these are normal babies who are simply having samples taken as a control.
4. The issue is how long the sample should be kept for. A compromise maybe for 7 years but at any point is that a time limit should be placed on how long samples are retained.
5. The issue of the 20 adult controls it wasn't clear from the research who where these and whether they are members of the Laboratory or are they other individuals in the community.
6. Finally in the consent form it should perhaps be explained to the families that samples are going to be sent to St. James for analysis and also being sent to Birmingham for analysis.



**THE
ROTUNDA
HOSPITAL**
DUBLIN

4th December, 2017.

Prof. Eleanor Molloy
Consultant Neonatologist and Professor of Paediatrics,
Coombe Women and Infants University Hospital,
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Dublin 8.

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Our ref: REC-2017-014 *(please quote this reference on all correspondence)*
**Re: Gender and Neonatal Inflammation in preterm outcomes:
GENIE study**

Dear Eleanor,

Many thanks for the amended documentation received in relation to the above research. I am pleased to advise that the requirements set out by the Committee in respect of your study have now been met. This being the case, ethical approval for the research is granted and it may now commence.

Please contact our Information Governance Manager, Sorchá Heaphy (01 817 6811, sheaphy@ROTUNDA.ie), in advance of the study start date to put together a confidentiality/data processing agreement.

You are requested to submit a progress report to the Committee in twelve months, and annually thereafter as applicable. We would also like to know when and where you publish or present your results. Please be aware of your responsibilities with respect to the Hospital's good research practice policies and guidelines.

Yours sincerely,

Kind regards.

Yours sincerely,

Professor Michael Geary,
Chairman,
Research Ethics Committee.

Appendix ii: Patient information leaflet

**GENIE Study: Gender and Neonatal
Inflammation in preterm outcomes**



Preterm Baby Information Leaflet

Study: Gender and Neonatal Inflammation in preterm outcomes: GENIE study

Investigators

PI: Professor Eleanor Molloy

Co-Investigators in Coombe Hospital: Prof Martin White, Dr. John Kelleher.

Your baby is being invited to participate in this study as she/he is a premature baby who has been admitted to the neonatal intensive care unit. The information below provides an explanation of the study which we are undertaking and it is important that you read and understand what we are doing in order to make an informed decision about participation. Read this information carefully and if there is any aspect of the study that is not clear please ask any questions that you wish. Please make sure that you are satisfied before agreeing to participate. Many thanks for your time and consideration of this invitation.

Purpose of the research

This study will be looking at the way in which hormone levels in boys and girls work on their immune system to protect against, and fight infection. To do this we would like to look at your baby's immune cells and hormone levels more closely during their stay in hospital, by collecting small samples of blood, urine and saliva before they go home. We would also collect information on your baby's general health and the function of their body organs while in hospital. We will not be changing your baby's care in any way during the study.

We are doing this because it will improve our understanding of the immune system in premature babies and help us to predict and more effectively treat infection. We believe that this will improve the care of other babies in the future.

Why as a Participant/Respondent have I been asked to take part in this study?

You have been asked to take part as your baby has been admitted to the neonatal intensive care unit because they were born prematurely. We are interested in learning more about your baby's immune system as premature babies are at higher risk of infection than older children and understanding why this is will help to care for other babies in the future. This study will be done in the intensive care unit at the Rotunda Hospital, the Coombe Women and Infants University Hospital and the National Maternity Hospital. This is a collaborative project across the maternity hospitals in Dublin as well as the medical laboratories in UCD, RCSI and Trinity College Dublin.

The voluntary nature of participation:

Taking part in this research is your decision. If you decide to take part you will be given an information sheet to take home with you and asked to sign a consent form. If you decide not to take

One copy of this form must be retained by the participant and one copy must be retained by the researcher

part or if you change your mind at a later date (before publication) you can withdraw your baby's information from the study.

During the Study

When babies are born premature they have a variety of tests and monitoring performed as part of their care in hospital. If you agree to take part we would like to look at your baby's immune system and hormone levels more closely during their time in hospital.

If you consent, we would like to examine blood taken around the time of birth and before your baby goes home (usually 3-4 weeks from birth). Blood would only be examined if your baby is having tests performed for other reasons and the amount collected would be between 0.5-1ml. If your baby has had blood taken already we may be able to use the samples which are remaining in the laboratory, pending your consent.

We would also collect a urine sample from your baby's nappy to check hormone levels and a saliva sample in the first week of life, and later if your baby has their vaccinations in hospital as this could be used to see their response to stress. Urine and saliva are not routinely collected in preterm babies but we do not believe that these tests would be uncomfortable or upset your baby. We would also collect information on your baby's general wellbeing and organ function, as well as information from examination of the placenta and details of the delivery.

Deciding to participate will not affect the level of care your baby receives. You may also decide to take part in some parts but not others.

Potential harms/risks

We do not foresee any potential harms/risks to your baby from these tests. Blood samples will only be taken with other bloods if needed as a routine part of your baby's day-to-day care and this will be decided by the consultant caring for your baby.

Potential Benefits

Taking part in this study will not change your baby's care but we believe that the information we collect will help other babies in the future. The results of our study will help doctors and nurses looking after premature babies to test for and treat infection. We also hope that we will be able to uncover information on new drugs which could protect and treat sick babies in the future.

Confidentiality

We will respect you and your infant's privacy. Personal data (such as name and date of birth) will be used by research staff to identify your baby while they are in hospital but will not be given to anyone else or be published. All those who access this data are trained healthcare professionals. The data produced from this study will be stored in a secure, locked location. Only members of the research team will have access to the data. Following completion of the research study the data will be kept for a period of 5 years and will then be destroyed. You have the right to withdraw from this research at any time without your child's care being affected, in such cases any personal data collected during the study will be destroyed.

One copy of this form must be retained by the participant and one copy must be retained by the researcher

At the End of the Study

Because this study is about collecting information there will be no changes to your baby's treatment. We will publish anonymised results and may use these results to plan future research. No individual baby will be identified as part of this process. After completion of the study data will be retained securely for comparison as per hospital policy and then destroyed.

Blood samples will be stored securely until we complete our tests in the laboratory. If any blood remains there is potential for samples to be kept anonymously and used for future study in Trinity College Dublin. Retaining these samples for future examination in our lab is entirely your choice and if you prefer we can safely dispose of the samples instead.

Other considerations

The study team may require access to your medical records and those of your baby. Again, any information collected will be completely anonymised.

Contact Details

If you have any questions about the study, please call:

Prof Eleanor Molloy, Consultant Neonatologist and Professor of Paediatrics, The National Children's Hospital, Tallaght Hospital on 01 896 3747.

Appendix iii: Patient consent form

GENIE Study: Gender and Neonatal
Inflammation in preterm outcomes



Preterm Consent Form – GENIE study: Gender and Neonatal Inflammation in preterm outcomes

Researcher: Dr. Matthew McGovern

Principle Investigator: Prof Eleanor Molloy

DECLARATION by participant: Please tick (✓) and provide your initials		
1.	I have read the information leaflet for this research study and I understand the contents.	Yes [] No [] initials []
2.	I have had the opportunity to ask questions and all my questions have been answered to my satisfaction.	Yes [] No [] initials []
3.	I fully understand that my/my babies' participation is completely voluntary and that I am free to withdraw from the study at any time (prior to anonymisation/publication) without giving a reason and that this will not affect my care/my babies care in any way.	Yes [] No [] initials []
4.	I agree that my/my babies' healthcare records will be accessed by the research team for the purpose of this research.	Yes [] No [] initials []
5.	I understand that information from this research will be published but that I will not be identified as a participant in this research in any publication.	Yes [] No [] initials []
6.	I understand that I/my baby will not be identified as a participant in this study (unless a legal requirement) and that the researchers may hold my personal information for 5 years after the study has been completed.	Yes [] No [] initials []
7.	I consent to having an extra blood sample saved if/when routine blood sampling is being performed, or for any remaining blood in the laboratory to be used for this research.	Yes [] No [] initials []
8.	I consent to having a urine sample collected from my baby.	Yes [] No [] initials []

One copy of this form must be retained by the participant and one copy must be retained by the researcher

9.	I consent to having saliva samples collected on my baby.	Yes [☐] No [☐] initials []
10.	I consent for my placenta/afterbirth to be examined.	Yes [☐] No [☐] initials []
11.	I consent for any blood remaining after completion of this study may be held in a secure biobank and may be used for further analysis in the future.	Yes [☐] No [☐] initials []
12.	I understand that I may freely withdraw from the study at any time without my care or the care of my baby being affected.	Yes [☐] No [☐] initials []
13.	I consent to have my clinical data collected, stored, retained and disclosed within the hospital I am attending for the duration of this study and destroyed when study is complete.	Yes [☐] No [☐] initials []
14.	I understand that the researchers undertaking this research will hold in confidence and securely all collected data and other relevant information.	Yes [☐] No [☐] initials []
15.	I freely and voluntarily consent to participating/allowing my baby participate in this research study.	Yes [☐] No [☐] initials []

PARTICIPANT'S NAME

Participant's signature: **Date:**

Name of person taking consent: **Signature:** **Date:**

Researcher: **Signature:** **Date:**

Appendix iv: Clinical proforma



Coláiste na Tríonóide, Baile Átha Cliath
Trinity College Dublin

Ollscoil Átha Cliath | The University of Dublin

GENIE study: Preterm infants

Maternal Diagnosis

Placenta Praevia	Y / N
IDDM	Y / N
Gestational DM	Y / N
HTN	Y / N
PET	Y / N
Epilepsy	Y / N
Thyroid disorder	Y / N
UTI	Y / N
Other diagnosis	

Antenatal medications

No. Doses steroids	
Timing steroids 1	
Timing steroids 2	
Magnesium	Y / N
Timing Magnesium	
Other medications	

Maternal Infection

Max temp in Labour	
Foul-smelling Liquor	Y / N
PROM >18 Hours	Y / N
ROM duration	
GBS status	Positive / Negative / Unknown
Maternal antibiotics in labour	Y / N
No. maternal doses antibiotics	
Baby temperature on NICU admission	

Delivery

Mode of delivery	Em CS / In labour CS / Pre-labour Elective CS / In Labour Elective CS / SVD cephalic / SVD cephalic
Instruments	None / Forceps / Suction

Presentation	Cephalic / Breech / Transverse
Complications	None / Head entrapment / Shoulder dystocia / cord prolapse / placental abruption / ruptured uterus / other:
Fetal distress	Non-reassuring CTG / Scalp pH
Other sentinel event	

Resuscitation

BMV/Neopuff	Start: Duration:
Intubated	Y / N Time:
CPR	Y / N Duration:
Adrenaline	Y / N ETT/IV Doses:
Fluid bolus	Y / N No. Of bolus:
Bicarb	Y / N
Curosurf in DS	Y / N Time:
Max FiO2 used	

Birth

Gestation	
Sex	M / F
Birth weight	grams
OFC	cm
Length	cm
APGAR	1min: 5min: 10min:
1 st gasp at	mins
Cord gas	pH: BE: Lactate:
1 st gas after delivery	CBG / VBG / ABG
1 st gas values	pH: CO2: O2: HCO3: BE: Lactate:

Respiratory

No. of total days intubated	
No. days traditional ventilation	
No. days HFOV	
No. days NO	
No. Days CPAP	
No. Days Hi flow	
No. Days low flow	
Max FiO2	
Pneumothorax	Y / N Chest Drain: T / N
Pulmonary haemorrhage	Y / N
Pneumonia	Y / N
Curosurf	Y / N
No. Curosurf doses	
CLD	Y / N
Home O2	Y / N

Cardiovascular

Inotropes	Y / N Adrenaline / Noradrenaline / Dopamine / Dobutamine / Milrinone / Hydrocortisone
Hypotension	Y / N
Lowest Mean BP	
Echo	Y / N Result:
PDA	Y / N
PDA treatment	None / Ibuprofen / Paracetamol / ligation / other:
UAC	Y / N Hrs in place:
UVC	Y / N Hrs in place:
PPHN	Y / N
ECMO	Y / N

Renal

Oliguria <1ml/hr	Y / N
Lowest urine output (ml/kg/hr)	
Abnormal renal us	Y / N result:
Max Urea	
Max creatinine	
Renal replacement:	Y / N

Haematology

Blood products	FFP / Fibrinogen / Cryoprecipitate / Platelets / factors
RCC	Y / N No. Of transfusions:
Max PT	
Max APTT	
Platelets <100	Y / N
Minimum platelet count	
Minimum Hb	
Minimum Fibrinogen	
Other haematological diagnosis:	

Gastrointestinal

NEC	Y / N Perforation: Y / N Surgery: Y / N
Days to full enteral feed	
Days to full enteral feeds	
Days to full bottle/breast feeds	
Feeds on discharge	Bottle / NG / Mixed
Total days of TPN	
Max ALT	
Max AST	

Max Ggt	
Min albumin	

Neurology

Seizures	Y / N
Anticonvulsants	Y / N Name:
Abnormal neurological exam at discharge	Y / N
Worst grade of IVH Right	
Worse grade of IVH Left	
PVL	Y / N
US 1	DOL: Results:
US 2	DOL: Results:
US 3	DOL: Results:
MRI	DOL: Results:
Morphine	Y / N
Ketamine	Y / N
Meningitis	Y / N Organism: PCR / Culture
CSF	Y / N WCC: RCC: Prot: Gluc:

Sepsis

EOS	Y / N Organism: Suspected / clinical / microbiological CNS involvement: Y / N
LOS	Y / N Organism: Suspected / clinical / microbiological CNS involvement: Y / N
Other infections	
No. Suspected sepsis episodes	
No. Confirmed sepsis episodes	
No. antibiotic days	
Max WCC	
Max Neuts	
Max CRP	

Outcomes

Discharged Home	Y / N DOL at discharge:
Transferred to another hospital	Y / N DOL: Where: Why:
Died	Y / N DOL: Cause:
Audiology	Pass / Fail
Ophthalmology	Max ROP: Treatment:

Appendix v: Neonatal sequential organ failure (nSOFA) components and scoring.

Scoring	0	1	2	3	4
	<i>If not intubated, respiratory score = 0; If intubated, respiratory score is doubled</i>				
Respiratory	SpO ₂ /FiO ₂ ≥ 300	SpO ₂ /FiO ₂ < 300	SpO ₂ /FiO ₂ < 200	SpO ₂ /FiO ₂ < 150	SpO ₂ /FiO ₂ < 100
Cardiovascular	No inotropes or systemic steroids	Systemic steroids alone	Single inotrope	2 or more inotropes	
Platelets (10 ³)	≥150	100-149	<100	<50	

Appendix v: Modified NEOMOD score proforma



Coláiste na Tríonóide, Baile Átha Cliath
Trinity College Dublin

Ollscoil Átha Cliath | The University of Dublin

GENIE study: baseline NEOMOD + suspected sepsis events

Study ID:		DOL:	
NEOMOD	2 points	1 point	0 points
CNS	Intraparenchymal blood / hydrocephalus / leukomalacia / cerebral atrophy	IVH	No haemorrhage / subependymal germinal matrix haemorrhage only
Haem	Plt <30 Wcc <3	Plt 30-100 WCC 3-5	Plt >100 WCC >5
Resp	Intubated	CPAP / FiO2 >21%	Spontaneous respirations
GI	Signs of NEC Total bili >102umol/dL ALT >twice normal	TPN Total bili 68-102umol/L ALT raised to <twice normal	Enteral feeds Total bili <68umol/L Normal ALT
CVS	Hypotension despite pharmacological support Persistent HR >180 or <100/min	Continuous pharmacological support needed to keep BP Adequate	Adequate BP HR 100 – 180/min
Renal	Urine <0.2mL/kg/Hr Creatinine >176umol/L	Urine 0.2-1mL /kg /Hr Creatinine 88-176umol/L	Urine >1mL/kg/Hr Creatinine <88umol/L
Acid – base	Base deficit >15mEq/L	Base deficit 7-15mEq/L	Base deficit <7mEq/L
Microvascular	Generalised edema Albumin <25g/L	Subcutaneous edema Albumin 25-33g/L	No edema Albumin >30g/L

Diagnosis: microbiological / clinical / suspected

WCC _____ NEUTS _____ MONOS _____ LYMPH _____ PLT _____ CRP _____ B/C _____ CSF _____