The Regulation of the Innate Immune System in Preterm Neonates

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ABSTRACT

Preterm delivery is the leading contributor to neonatal death globally, with half of these deaths being associated with inflammatory conditions. An immature ability to regulate the innate immune response may contribute to the higher rate of morbidity and mortality among preterm neonates. Neonates rely on their innate immune response to protect them from infection. Toll-like receptors (TLRs) are ubiquitously expressed innate immune receptors that signal for pro-inflammatory cytokine transcription. This process requires strict regulation at different levels of its intracellular signalling cascade to avoid pathological inflammation. MicroRNAs (miRs) are post-transcriptional regulators that have been identified as critical regulators of inflammatory gene expression. Currently, miR expression is sparsely characterised in the context of neonatal TLR signalling. This thesis therefore aimed to examine immune regulation by term and preterm neonates through characterising the expression of miRs that regulate TLR signalling in placenta and cord blood.

Cord blood and placenta were collected following term (≥ 37 weeks gestation), late preterm (32-36 weeks) and early preterm delivery (≤ 32 weeks), and assessed for baseline gene expression (Chapters 3 and 4). Cord blood was cultured and stimulated with TLR2, TLR3 and TLR4 agonists, and collected over a 24 hour period (Chapters 5 and 6). RNA was extracted and the relative expression of genes associated with TLR signalling (including select miRs) was quantified using qPCR. Bioinformatics analyses were used to determine network-level alterations in inflammatory signalling using publicly available microarray data on peripheral blood from neonates with infection (Chapter 7).

There was no difference in the expression of miRs or genes associated with TLR signalling in the placenta or cord blood between term and late preterm deliveries. Early preterm cord blood showed decreased *miR-155* and *let-7e* expression at birth, and demonstrated no change in miR expression following TLR stimulation despite increasing the expression of *IL6, IL10* and *NF-κB1*. Term and late preterm cord blood also increased *IL6* expression, but this occurred alongside increased anti-inflammatory *let-7e* expression. Further, term cord blood increased TNFα and IL6 protein production until 6 hours *in vitro*, while preterm cord blood continued to increase IL6 between 6 and

24 hours post-stimulation. Bioinformatics analysis confirmed an upregulation of innate immune pathways (including JAK-STAT and Nod-like Receptor signalling) in peripheral blood from preterm neonates with infection compared to term neonates with infection.

In summary, our findings demonstrate preterm cord blood is able to mount a robust response to TLR stimulation through increasing pro-inflammatory gene expression. As this response occurs in the absence of any change in the miRs we measured, we postulate that the inflammatory response is dysregulated in preterm neonates. These results are the first to suggest differential miR expression alters the neonatal innate immune response. As such, in addition to an increased risk of contracting infection, preterm neonates may be unable to regulate inflammation effectively. Inappropriate inflammatory signalling may therefore underlie preterm neonates' susceptibility to developing chronic and acute inflammatory morbidities.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Natalie Aboustate

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"If he can only perform good or only perform evil, then he is a clockwork orange—meaning that he has the appearance of an organism lovely with colour and juice but is in fact only a clockwork toy to be wound up by God or the Devil."

— Anthony Burgess, A Clockwork Orange

"Welcome to the Jungle."

— Guns n' Roses

For Anne.

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Abbreviation	Unabridged
APC	Antigen presenting cell
BLP	Bacterial lipopolypeptide
BPD	Bronchopulmonary dysplasia
CCL	CC chemokine (β-chemokine) ligand
CD	Cluster of differentiation
CLD	Chronic lung disease
CoNS	Coagulase negative *Staphylococcus*
CRP	C-reactive protein
ELBW	Extremely low birth weight
FIRS	Fetal Inflammatory Response Syndrome
FOXP3	Forkhead box 3, also known as scurfin
FOS	FBJ murine osteosarcoma viral oncogene homolog
GCSF	Granulocyte colony-stimulating factor
GMCSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome-wide association study
IFN	Interferon
Ig	Immunoglobulin
I-κB	Inhibitor of κB
IL	Interleukin
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
IUGR	Intrauterine growth restriction
IV	Intravenous
JNK	c-Jun N-terminal kinases
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBP	Lipopolysaccharide binding protein
LBW	Low birth weight
LSCS	Lower Segment Caesarean Section
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
LY	Lymphocyte antigen

Abbreviation	Unabridged
Mal	MyD88-like adaptor protein
MAPK	Mitogen-activated protein kinase
miR	microRNA
MCP	Macrophage chemotactic protein
MD2	Lymphocyte antigen 96
MyD88	Myeloid differentiation primary response gene 88
NEC	Necrotising enterocolitis
NET	Neutrophil extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NICU	Neonatal intensive care unit
NVB	Normal vaginal birth
PAMP	Pathogen associated molecular pattern
PE	Pre-eclampsia
PGN	Peptidoglycan
PIK	Phosphatidylinositol kinase
PMN	Polymorphonucleocyte
Poly I:C	Polyinosinic:polycytidylic acid
PPROM	Preterm pre-labour rupture of membranes
PROM	Prolonged rupture of membranes
PRR	Pattern recognition receptor
RA	Receptor antagonist
RAC1	Ras-related C3 botulinum toxin substrate 1
RDS	Respiratory distress syndrome
RIN	RNA integrity number
RIP	Ribosome inactivating protein
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
RPM	Rotations per minute
SARM	Sterile-α and Armadillo motif containing protein
SCN	Special care nursery
SGA	Small for gestational age
SOCS	Suppressor of cytokine synthesis
NVD	Normal vaginal delivery
TAB	TGFβ-activated kinase-1 binding protein

Abbreviation	Unabridged
TAK	TGFβ-activated kinase
TBK	TANK-binding kinase
Th cell	T-helper cell
TIR	Toll/IL-1 receptor domain
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNFR-associated factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T-cell
TRIF	TIR domain-containing adaptor protein-inducing IFNβ
VLBW	Very low birth weight
WT	Wildtype mouse strain

Please note that the gene nomenclature used in this thesis is in accordance with HUGO guidelines[1].

Chapter One:

Introduction & Literature Review

Preterm delivery is the leading precursor to adverse neonatal health outcomes and neonatal deaths[2]. As the rate of preterm delivery is increasing, prematurity has become a focus of WHO millennium development goals to reduce childhood mortality[2]. Approximately 75% of conditions leading to severe morbidity and mortality among preterm neonates are associated with inflammation. A better understanding of inflammatory processes by preterm neonates is therefore critical. Currently, innate immune responses and inflammatory regulation are poorly characterised in preterm neonates. This thesis includes a review that explores neonatal innate immune function, the potential impact of *in utero* perturbations on preterm immunity, Toll-like Receptor (TLR)-induced inflammation in cord blood and the regulation of these pathways by microRNAs (miRs). The reviewed literature explores different factors that may influence preterm neonates' increased susceptibility to inflammatory conditions.

Following a review of the literature, this thesis presents four experimental chapters that characterise the expression of genes associated with TLR signalling following preterm delivery. Specifically, the expression of these genes in the placenta (Chapter 3) and cord blood (Chapter 4) are characterised at birth, in addition to cord blood serum cytokines. Gene expression is also characterised in term and preterm cord blood following TLR stimulation *in vitro* (Chapter 5), including a sub-analysis of gene expression over 24 hours following stimulation (Chapter 6). Given the ubiquitous expression and influence of miRs, Chapter 7 explores theoretical networks involving TLR signalling in neonates using bioinformatics. This thesis concludes with a discussion of main findings in context of their broader clinical implications and future avenues for investigation: specifically, the idea that preterm neonates are unable to appropriately regulate TLR signalling through miR induction, which may place them at greater risk of inflammatory conditions.

1.1. PRETERM DELIVERY

15 million neonates are delivered prematurely (before 37 weeks of gestational age)[3] every year and this rate is increasing[3, 4]. Premature birth is classified into sub-groups according to gestational age at delivery, including: late (32-36 weeks), early (28-32 weeks) or extremely preterm (<28 weeks)[3]. Preterm delivery is the leading contributor to neonatal deaths, half of which are related to pathological inflammation[3]. Surviving preterm neonates have a significantly increased risk of morbidity, permanent disability or death during the neonatal period compared to their term-born counterparts[4, 5]. This places long-term social and financial strain on their families, communities and health resources[4, 6]. The prevention and management of preterm delivery is therefore prominently featured in the WHO's millennium development goal to reduce childhood mortality[3].

Preterm delivery is an obstetric syndrome encompassing conditions with varied aetiologies[7-9]. These can include maternal inflammation[10-13] and smoking during pregnancy[14], though, a majority of preterm births are idiopathic[7, 8]. In developed countries such as Australia, preterm delivery affects over 8% of live births[15, 16], with a greater prevalence amongst the Indigenous population (13.5% of live births)[16]. The increasing incidence of preterm delivery places more neonates at greater risk of developing severe inflammatory morbidities. A better understanding of these outcomes will facilitate improved clinical management and prevention strategies to reduce the burden of preterm delivery on society.

1.1.1. NEONATAL OUTCOMES FOLLOWING PRETERM DELIVERY

Despite representing only 1.7% of all deliveries, the management of preterm neonates accounts for 30% of all newborn healthcare costs[15]. Lower gestational age, birthweight, male sex, lack of antenatal steroid administration and multiple pregnancy are all associated with an increased risk of neonatal death[17, 18]. While preterm males have a higher average birth weight than preterm females[19], they have increased rates of infection and more severe complications[5, 19-21]. Males therefore represent 57% of admissions into the Neonatal Intensive Care Unit (NICU)[5, 15, 16] and are more prone to oxygen dependence and Respiratory Distress Syndrome (RDS) throughout the neonatal

period[10, 19, 20]. The adverse impact of preterm birth on long-term health outcomes is also well-established and includes higher rates of heart disease, stroke, type 2 diabetes, obesity, osteoporosis and neurodevelopmental disorders compared to adults born at term gestation[22-25]. Overall, it is well established that premature delivery predisposes the individual to life-long adverse health outcomes.

Infection is the leading cause of neonatal morbidity and mortality[26], with an increased risk of severe infection among preterm neonates compared to term[27]. The most common pathogens affecting neonates include Group B *Streptococci* (GBS), *Escherichia coli* and *Staphylococcus aureus*[28], which all underlie 70% of cases of neonatal sepsis [29]. Further, neonatal sepsis is associated with 42% of neonatal deaths within the first week of life[30]. Early-onset neonatal sepsis occurs within 72 hours of delivery and is associated with *in utero* insults such as chorioamnionitis[31] and funisitis[32], while late-onset neonatal sepsis is associated with nosocomial infection[33].

Preterm neonates are at higher risk of inflammatory morbidities than their term-born counterparts, including bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), sepsis, cerebral palsy and necrotising enterocolitis (NEC)[34]. Nosocomial infections commonly lead to morbidity due to the frequent exposure of preterm neonates to invasive clinical interventions[35-37]. Lung ventilation, respiratory surfactant administration and nasogastric feeding are all common within NICU[38] and these interventions have been associated with iatrogenic disease including infection and hypothermia[39]. In spite of system immaturity and clinical interventions, not all preterm neonates will develop inflammatory morbidities. Underlying mechanisms for this are yet to be identified.

90% of neonates who develop NEC are preterm, with the risk of disease being inversely associated with gestational age and birthweight[40]. The increased prevalence of NEC among preterm neonates demonstrates attenuated innate immune barriers and an immaturely regulated inflammatory response. NEC involves aberrant inflammation that affects the gastrointestinal tract, causing severe haemorrhaging, necrosis[41] and in 20% of cases, death[42, 43]. While the cause of NEC is unclear, dysbiosis and gastrointestinal immaturity appear to predispose preterm neonates to excessive inflammation in the gut (driven by both pathogenic and commensal bacteria)[44]. At birth, the gut is colonised by commensal bacteria, which are not harmful and do not normally elicit an immune

response. Typically, gut immune homeostasis develops through tolerance towards these commensal bacteria to prevent aberrant and unnecessary inflammation that can cause damage to the mucosa (which can lead to NEC). The transition from the *in utero* environment with low bacterial diversity to the high-diversity *ex utero* environment therefore needs to involve appropriate regulation of inflammatory pathways. The increased incidence of inflammatory morbidities in preterm neonates suggests that rather than having attenuated innate immunity, they exhibit an inappropriate and dysregulated inflammatory response. The specific immune events contributing to dysregulated neonatal inflammation and their impact on developing immunity remain poorly described[45-47].

1.2. THE IMMUNE SYSTEM

The immune system consists of a network of tissues, cells and mechanisms that protect the body from disease. An immune response involves recognising a foreign presence in the body, activating defence mechanisms and repairing collateral tissue damage. Each of these stages must be tightly regulated so that the response is sufficient to clear a pathogen without excessively damaging the host organism. Without regulation, immunity can become excessive, self-perpetuating or responsive to non-harmful cues.

Innate and adaptive immunity drive 'early' and 'late' response mechanisms, respectively. Innate immunity uses evolutionarily-conserved effector mechanisms to provide an immediate and generalised response to an antigen. Adaptive immunity develops subsequently and is more specific because it forms immunological 'memory' to an antigen, allowing for a faster response during later encounters with the same antigen. During the first two years of life, infants are reliant on their innate immune system for protection from pathogens[48] as they have yet to develop the immunological memory associated with adaptive immune responses.

1.2.1. INNATE IMMUNITY

Innate immunity is the first line of defence to a pathogenic presence in the body and comprises both passive and active components. Although it does not target specific pathogens, innate immunity provides a large-scale and fast-acting response. Its passive components can be anatomical or chemical, filtering a majority of immune threats by acting as a physical barrier, shedding pathogens or inhibiting pathogen growth. The first stage of an innate immune response occurs when an antigen evades these barriers and is distinguished from the 'self'. Antigens are recognised by generalised receptors on sentinel innate immune cells such as pattern recognition receptors (PRRs). PRRs are structured to recognise evolutionarily-conserved Pathogen Associated Molecular Patterns (PAMPs) on microbes or Damage Associated Molecular Patterns (DAMPs) expressed by host cells. PAMPs are essential for microbial survival and therefore, have minimal variability between different species of microbes. Toll-like Receptors (TLRs) are the best studied PRRs, with 11 classes characterised in humans[49]. When TLRs

recognise PAMPs, they initiate a cascade of intracellular signalling that leads to the expression of inflammatory mediators.

Inflammation is an innate immune response to sterile or infectious injury and can be localised or systemic. It begins when sentinel immunocytes recognise PAMPS and produce cytokines and chemokines to recruit other cells to the site of PRR ligation. The chemotaxis of cells to the affected area creates an influx of cytokines, complement proteins (that opsonise pathogenic organisms), antimicrobial proteins and chemotactic factors. While inflammation can be triggered by different molecular patterns, its ultimate goal is to clear antigens.

TLRs are ubiquitously expressed by innate immune cells including dendritic cells (DCs), macrophages and neutrophils. These immunocytes communicate with each other through cytokine signalling that occurs following TLR activation. Cytokines can act synergistically or antagonistically, and therefore link different stages of the inflammatory response. They regulate cellular growth and differentiation, pathogen-killing and the production of other immune mediators which influence inflammation. Different classes of cytokines can be grouped according to their pro- and anti-inflammatory roles. Interferons (IFNs) are typically expressed during responses to viral infection and lead to a cell-mediated or cytotoxic immune response, producing other pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNFα) and Interleukin-12 (IL12)[50]. Alternately, anti-inflammatory or tolerogenic cytokines including IL10 and Transforming Growth Factor-β (TGFβ) down-regulate inflammatory signalling. The overall balance of these different immune mediators leads to either perpetuation or resolution of inflammation.

Neonatal immunity continues to develop gradually following pathogenic exposures at birth, beginning with the introduction to maternal vaginal microflora during labour[51]. Exposure to an antigen-rich *ex utero* environment increases the importance of tolerogenic immune mechanisms for the neonate. As such, anti-inflammatory cytokines[52, 53] and an increased presence of regulatory T cells (Tregs) are observed in cord blood and peripheral neonatal blood compared to adult blood[54].

1.2.1.1. Toll-like Receptors

TLR activation is a central innate immune response mechanism that also establishes antigen presenting cell (APC) and lymphocyte maturation[55, 56]. Different classes of TLRs provide unique patterns of inflammatory protein expression specific to different ligands[57]. Specificity is conferred by TLR dimerisation and networks of signalling involving unique combinations of Mitogen Activated Protein kinases (MAPKs), adaptor molecules and transcription factors[58]. TLRs also have some upstream specificity, given their capacity to heterodimerise with other classes of receptors. Following TLR ligation, an intracellular cascade of protein activation occurs, which allows the development of a specific response based on the downstream signalling molecules recruited (Figure 1.1).

TLR signalling begins with the Toll/IL1-Receptor domain (TIR; common to all TLRs) binding one of five cytosolic adaptor proteins; MyD88, TRIF, TRAM, Mal or SARM[50, 59] (Fig. 1.1). All TLR signalling, except for TLR3, can occur via the MyD88-dependent pathway[55, 60]; where MyD88 forms a complex with the Mal adaptor that interacts with the TLR's TIR domain to recruit IRAK4 and IRAK1. TRIF is an adaptor for TLRs 3 and 4, and results in Interferon (IFN) expression independently of MyD88[59]. Both TRIF and TRAM are able to stimulate NF-κB through an alternate cascade of signalling, while also activating Interferon pathways through Interferon Regulatory Factors (IRFs) 3 and 7[59]. Alternately, the phosphorylation of IRAK1 following MyD88 induction leads to independent binding with TNF-Receptor Associated Factor-6 (TRAF6). IRAK1/TRAF6 binds the TAK1/TAB1/TAB2 complex and activate three pathways through TAK1, which phosphorylate transcription factors p38 and JNK, and the NF-κB inhibitor, IKK. In unstimulated immune cells, NF-κB exists in a quiescent state, bound to an Inhibitor of κB (IκB) unit[61]. The degradation of IKK results in the translocation of NF-κB into the nucleus, alongside p38 and JNK. Notably, p38 and ERK1/2 activation is decreased in term and preterm cord blood compared to adult monocytes following TLR2 and TLR4 stimulation[35, 62]. Overall, TLR signalling leads to the activation of MAPKs that translocate transcription factors NF-κB or IRFs into the nucleus that induce pro-inflammatory cytokine transcription including IFNs, IL1β, IL6, IL8, IL12 and TNFα.

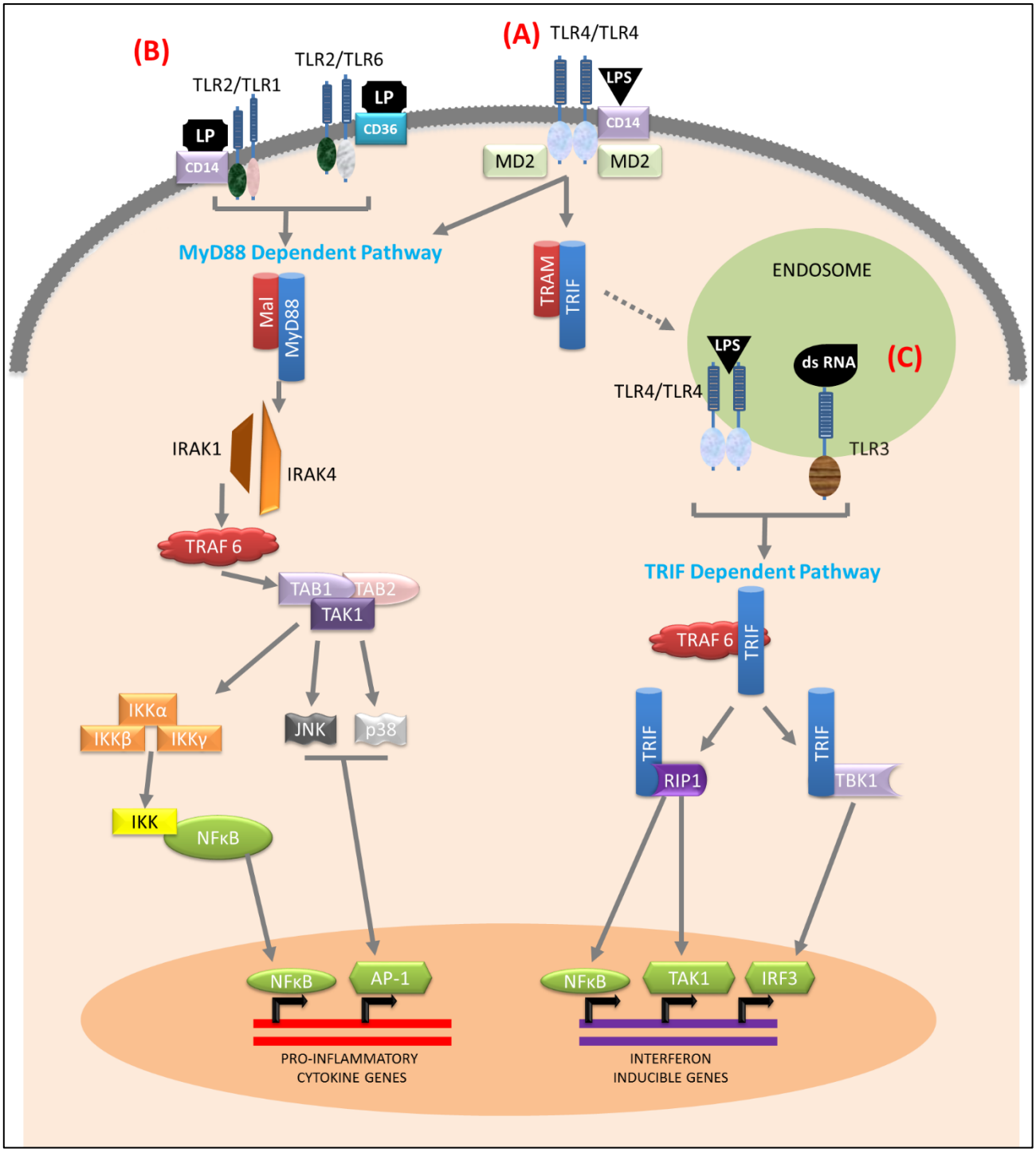

Figure 1.1. ***A diagrammatic representation of the TLR signalling pathway.*** **(A)** TLR4 forms a homodimer and proceeds to signal through both MyD88- and TRIF- dependent pathways when ligated by lipopolysaccharide (LPS). **(B)** TLR2 can heterodimerise with TLR1 or TLR6 and signals through the MyD88-dependent pathway following peptidoglycan (PGN) ligation. The MyD88-dependent pathway signals through a cascade involving IRAK and TRAF6, followed by the translocation of NF-κB or AP-1 into the nucleus to induce pro-inflammatory cytokine transcription. **(C)** An endosome containing the TLR4 homodimer or TLR3 dimer (bound to double stranded viral DNA), which can both signal via MyD88-independent or TRIF-dependent pathways. TRIF-dependent signalling occurs through the TRIF/TRAF6 cascade and ultimately results in the transcription of Interferon inducible genes.

1.1.1.1.1. TLR2

TLR2 can heterodimerise with either TLR1, TLR6 or TLR10 to allow for greater specificity in recognising peptidoglycan or lipoprotein components of gram-positive bacteria[56, 63]. In this manner, TLR2 recognises the largest range of ligands in the TLR family[63]. The ligation of either of its heterodimers will result in signalling through MyD88[60]. TLR1/2 is specific to the recognition of tri-acetylated lipopeptides[56, 60], while TLR2/6 recognises peptidoglycan and zymosan. Gram-positive bacteria maintain a cell wall containing peptidoglycan (PGN) and most species are commensal. However, Coagulase-negative *Staphylococcus* (CoNS) is a gram-positive bacterium, and is the most common cause of infection in preterm neonates[64]. Further, 32% of sepsis-related mortalities are associated with gram-negative infections, while 90% of neonatal septicaemia is caused by CoNS[39, 65].

Alterations in the human *tlr2* gene have been linked with susceptibility to *Staphylococcal* infection during sepsis[66]. This is also demonstrated by *TLR2*$^{-/-}$ mice, which express decreased pro-inflammatory cytokines compared to WT mice following exposure to *Staphylococci*[67]. Studies on murine neonates have also shown TLR2 expression by neutrophils is essential for clearing *Chlamydia pneumonia* infection, where *TLR2*$^{-/-}$ but not *TLR4*$^{-/-}$ murine neonates demonstrate more severe disease and prolonged infection compared to WT neonates[68]. Together, these studies demonstrate the importance of TLR2 in recognising and clearing gram-positive bacteria.

In humans, cord blood monocytes produce decreased TLR2 and TNFα and show decreased phosphorylation of MAPKp38 compared to adult monocytes. This may decrease the capacity for monocytic apoptosis during the resolution of inflammation in neonates[69]. Neonatal peripheral blood increases MyD88, TLR2 and TLR4 expression following gram-positive and gram-negative infections, but TLR4 and the co-signalling molecule MD2 only increase with gram-negative infections[70]. TLR2 is typically upregulated during diseases such as sepsis, which is often polymicrobial, confirming that the ability to heterodimerise increases its capacity to recognise a wider range of pathogens than any other TLR class[60, 71]. While the rate of phagocytosis and pathogen killing of gram-negative bacteria by cord blood granulocytes is equivalent to that observed in adult granulocytes, the same response to whole GBS is impaired in cord blood *in vitro*[72]. These findings are likely related to an increased incidence of contracting these infections and developing neonatal sepsis.

1.1.1.1.2. TLR3

TLR3 exists as a dimer in intracellular endosomal membranes and primarily recognises double-stranded viral RNA components. TLR3 heterodimer formation and the pleiotropic nature of TLR signalling allows for greater specificity in its downstream response. Notably, only TLR3 signals via the TRIF pathway, independently of MyD88. Interferon cytokines are characteristic of a Type 1 T-helper (Th1) cytokine response and are primarily induced via this signalling pathway. Notably, the TRIF pathway can also activate NF-κB through TRAF[73, 74]. The role of TLR3 is highlighted by *TLR3*$^{-/-}$ mice that cannot respond to viral stimulation with dsRNA or Polyinosinic-polycytidylic acid (Poly I:C)[75, 76] and therefore, cannot activate Th cells during viral infection[77]. Cord blood mononuclear cells (CBMCs) show neither constitutive *TLR3* expression, nor an inflammatory response to Poly I:C stimulation *in vitro*[78]. This inability to recognise viral stimuli may underlie the increased prevalence of viral infection among neonates compared to adults.

1.1.1.1.3. TLR4

TLR4 is best known for its LPS (or endotoxin) binding-specificity. LPS is a major component of gram-negative bacterial outer membranes. TLR4-deficient mice cannot respond appropriately to LPS, showing an increased bacterial load during infection and a higher rate of mortality compared to WT mice[55, 79, 80]. Similarly, humans with mutations in the *TLR4* gene are hypo-responsive to inhaled LPS under experimental conditions, demonstrating decreased lung volumes that are characteristic of respiratory restriction[81]. LPS recognition occurs on binding to LPS binding protein (LBP) in the serum, followed by CD14[55]. The co-receptors CD14 and LBP then form a complex with LPS at the cell membrane, prior to interacting with a TLR4 molecule homodimer [82]. The TLR4 homodimer exists as a complex with MD2[83] and following LPS/co-receptor ligation, it proceeds down the MyD88-dependent pathway or is translocated within an endosome and signals via the MyD88-independent, TRIF-dependent pathway[84]. TLR4 needs to be accompanied by appropriate co-receptors to be activated, as demonstrated by studies of TLR4 over-expression in a human kidney nephrocyte cell line, which are not responsive to LPS unless MD2 is present[63]. Blocking CD14 using antibodies decreases IL8 expression to a greater extent in term and preterm cord blood compared to adults and inhibits IL10 and TNFα expression following LPS stimulation[85]. This suggests IL10 and TNFα expression by neonates requires CD14/TLR4 signalling plus

CD14 and another TLR. Neonates may therefore require co-stimulation through multiple TLRs or co-receptors to achieve the same cytokine response as adults. Constitutive expression of CD14 is similar between preterm and term cord blood, but following LPS stimulation it is decreased in both preterm and term monocytes compared to adults[82, 86, 87]. In preterm cord blood, decreased CD14, TLR2, TLR4 and MD2 expression have all been reversed by incubation with IFNγ *in vitro*[82]. Overall, impaired viral cytokine pathways may contribute to attenuated TLR responses in neonates compared to adults.

Endotoxin tolerance

Endotoxin tolerance develops with the repeated ligation of TLR4 by LPS. Aberrant activation of the innate immune response or uncontrolled inflammation can lead to severe disease states, including septic shock. During sepsis, severe widespread tissue damage occurs due to inadequate immune regulation and the potent nature of the inflammatory response in the blood stream. Endotoxin tolerance or immune paralysis commonly develops during sepsis or severe infection, as the body reaches an immune threshold and cannot respond to further inflammatory stimulation[79]. This state of tolerance is characterised by decreased pro-inflammatory cytokine expression, and is observed in septic patients[88, 89]. This process is protective as it limits damage during chronic inflammation [79, 90].

Animals injected with TLR agonists during sepsis demonstrate increased rates of survival (compared to those not administered TLR agonists) [91, 92]. This phenomenon occurs as the body reaches a threshold in inflammatory signalling, which promotes tolerance rather than increased inflammation. Similarly, *IRAK1*$^{-/-}$ mice are unable to completely activate TLR signalling and therefore, cannot mount sufficient cytokine responses, making them resistant to endotoxic shock[90]. Studies in animals and human cell lines have linked endotoxin tolerance to NF-κB suppression[79, 93, 94]. This suppression occurs independently of TLR4, MyD88, Mal or TRAF6 expression, instead increasing IκB as a repressor of NF-κB induction[79]. The development of tolerance is dependent on the timing and dosage of endotoxin exposure, where chronic doses (e.g. sub-lethal doses of LPS administered over a longer period of time) result in more damage than acute exposures (e.g. one near-lethal dose of LPS)[95]. Though tolerance seems like a protective mechanism that limits excess inflammation, the nature of

immune suppression in the neonate can lead to secondary infections and death[96]. Therefore, appropriate pro-inflammatory stimulation is still required to clear the initial infection in order to decrease systemic inflammatory signalling.

In context of the neonate, general immune tolerance is critical in the transition to an antigen rich *ex utero* environment. At birth, the neonatal gut must develop enteric tolerance towards newly-established commensal bacteria. Intestinal explants show increased macrophage infiltration and decreased expression of the anti-inflammatory cytokine, TGFβ in human and murine preterm neonates compared to adults[97],[98]. Additionally, fetal intestinal epithelial cells (IECs) exposed to LPS show increased NF-κB and chemokine expression compared to neonatal and adult cells, although fetal macrophages are non-responsive to LPS challenge[99]. This study suggests that IECs remain in a state of LPS tolerance after birth for commensal bacterial colonisation. Seminal work by Nanthakumar et al. demonstrates increased NF-κB and MyD88 pathway gene expression in intestinal samples from aborted fetuses and sectioned intestine from preterm neonates with NEC[100]. This suggests the fetal and preterm neonatal intestine show less-mature tolerogenic mechanisms that may predispose them to developing conditions such as NEC.

1.2.2. PHYSIOLOGICAL EXPOSURES DURING PREGNANCY AND THEIR IMPACT ON NEONATAL IMMUNITY

Fetal immune development is influenced by maternal physiology and environmental exposures. Studies in primates and rodents have demonstrated that prenatal exposure to pathogens attenuates neonatal immune responses during the postnatal period[101-104]. When pregnant rhesus monkeys were exposed to perinatal stress (behavioural startling), their cord blood expressed decreased TNFα and IL6 compared to 'unstressed' controls[105]. LPS stimulation of these offspring's blood *ex vivo* showed decreased TNFα and IL6 expression and lower numbers of lymphocytes compared to offspring from unstressed mothers. This study suggests physiological insults to the primate mother perturb the immune development of her progeny. Rodents have also demonstrated alterations in neonatal immune function following maternal perturbations. Plasma from the offspring of dams exposed to LPS during gestation shows decreased expression of pro- and anti-inflammatory cytokines compared to unexposed pups[103]. These offspring also show decreased recruitment of monocytes, neutrophils and lymphocytes when re-

exposed to LPS during the neonatal period compared to unexposed pups[101, 106]. Overall, exposures to infection *in utero* appear to program the immune phenotype of offspring across their lifespan. While the concept of immune programming by *in utero* perturbations is important, it may have different short-term or long-term outcomes.

1.2.2.1. Intrauterine Inflammation

Inflammation is central to both term and preterm labour, where there is a shift from immune acquiescence to inflammatory activation of gestational tissues[9, 107, 108]. This influx of inflammatory mediators (i.e. the 'magnitude' of inflammation) is lower in term compared to preterm labour[107, 109]. The difference in inflammation between term and preterm labour is driven by distinct aetiologies, with preterm labour being more commonly associated with intrauterine infection[10-13]. As such, the increased expression of pro-inflammatory IL1β, IL6, IL8 and TNFα has been observed with preterm labour in cord blood and the maternal compartment[52, 110, 111]. Murine studies have also shown that blocking IL6 expression in amniotic epithelial cells *in vivo* prevents preterm delivery following IL1β and TNFα administration[112]. Additionally, anti-inflammatory placental IL10 expression is decreased in association with preterm parturition and chorioamnionitis (inflammation of the fetal membranes)[113].

Aberrant inflammation during pregnancy is frequently associated with adverse pregnancy outcomes including intrauterine growth restriction (IUGR) and neonatal sepsis[114]. A study of 701 Kenyan births found an increased incidence of low birth weight, still-birth and perinatal mortality in deliveries associated with acute placental inflammation relative to normal placentae[114]. Other prospective studies have supported these findings, observing an increased incidence of NEC and sepsis in neonates from pregnancies with chorioamnionitis[115]. While inflammation may be important for parturition, where excessive or poorly-timed, it can lead to adverse pregnancy outcomes.

Chorioamnionitis occurs in up to 65% of spontaneous preterm deliveries, with its prevalence inversely related to gestational age[116]. Clinical studies have associated chorioamnionitis with an increased incidence of the inflammatory morbidities including RDS[117], early-onset neonatal sepsis[46, 117, 118] and BPD[116, 119]. Increased amniotic TNFα expression and amniotic infection are also associated with increased neonatal mortality

and the development of RDS, intraventricular haemorrhage (IVH) and NEC[120, 121]. However, some have argued that intrauterine infection is protective against inflammatory disease, as chorioamnionitis is associated with the decreased prevalence and severity of RDS[122, 123]. This may be because RDS accelerates lung maturation via increasing surfactant production[124] through inflammatory signalling to lung tissues[125]. Further, when LPS or IL1β are infused directly into the fetal ovine trachea to cause local inflammation increased lung maturation is also observed[122, 126-128]. Alternately, antagonising IL1 expression in fetal sheep *in utero* and exposing them to LPS arrests systemic inflammation and decreases lung maturation (supporting a direct association with inflammation)[128] while inflammatory stimuli increase lung development, intrauterine infection impairs alveolarisation, increases severity of pulmonary hypertension in the newborn and decreases pulmonary blood flow in fetal sheep[129]. Together, these studies highlight that while inflammation can induce respiratory maturation, it is not necessarily beneficial for neonatal lungs.

60% of fetal deaths occur in the presence of chorioamnionitis[130, 131]. Ovine models of chorioamnionitis have demonstrated increased inflammation in the fetal ileum[132] and decreased anti-inflammatory $FOXP3^+$ expression[133], both of which are thought to precede inflammatory neonatal diseases such as NEC. Murine models of chorioamnionitis using intraperitoneal LPS administration show increased *IL1* and *IL6* expression by developing neurons and therefore, an increased incidence of fetal brain injury[134]. Chorioamnionitis and fetal inflammatory response syndrome (FIRS) are both associated with increased IL1β, TNFα, IL6 and IL8 in the human amnion and cord blood[135-138], and increased numbers of neutrophils in cord and peripheral blood collected from neonates[139]. Chorioamnionitis increases the risk of the FIRS, defined as fetal plasma (sampled by cordocentesis) containing concentrations of IL6 exceeding 11pg/mL[140, 141]. The diagnosis of FIRS is confirmed by placental histopathology which also shows inflammatory infiltration in the fetal compartment[138]. FIRS can be caused by microbes entering the amniotic cavity, resulting in excessive chemokine expression and neutrophil invasion[142]. The fetus responds to infection by increasing IL6 expression which can also result in funisitis and vasculitis[143]. IL6 is a systemic acting cytokine associated with chronic and acute inflammation, and its increased expression is consistent with a systemic fetal response that occurs during severe intrauterine infection[144]. FIRS affects 39-49% of preterm deliveries[140, 141] and may lead directly to

early activation of labour through inflammatory signalling[144, 145]. FIRS is also a risk factor for neonatal morbidity including early-onset sepsis[118], RDS, BPD, IVH, PVL and cerebral palsy[146]. Activation of the inflammatory response *in utero* is therefore a significant contributor to adverse pregnancy outcomes.

1.2.2.2. Mode of Delivery

Microbial exposure associated with vaginal delivery may influence fetal immune development by altering cord blood cytokine synthesis[147-149]. Cord blood from non-labouring Caesarean section deliveries has been associated with lower pro-inflammatory IL6, IL8, Granulocyte Macrophage Colony Stimulating Factor (GMCSF) and Granulocyte Colony Stimulating Factor (GCSF), but increased IL10 compared to vaginal deliveries[135, 148, 150]. Notably, studies demonstrating this have lacked appropriate control groups, i.e. cohorts comparing labouring to non-labouring Caesarean section, which are important for separating the effect of labour from exposure to vaginal microflora. Other authors have observed no difference in a number of cord blood cytokines (IL1α, IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL12p40, IL12p70, IL17, IL1RA, sIL2Rα, GMCSF, IFNα2, IFNγ, TNFα or TNF-β) between vaginal and Caesarean section deliveries[151].

Studies on cord blood immune stimulation present conflicting evidence concerning the impact of mode of delivery on neonatal immune function. *In vitro* stimulation of whole cord blood with TLR ligands has shown no difference in TNFα, IL6 and IL10 production between term infants born vaginally or via non-labouring Caesarean section[62]. Conversely, increased IFNγ and IL12 expression is observed in CBMCs from vaginal deliveries stimulated with the mitogens Conconavalin A (ConA) and Phytohaemagglutinin-A (PHA), or LPS, compared to non-labouring Caesarean sections[152]. A recent study in our laboratory has found no difference in cord blood cytokine expression according to mode of delivery for a range of cytokines (including GMCSF, IL1β, IL1RA, IL6, IL8, IL10, IL12p70, MCP1, sCD40L and TNFα protein expression), both at birth and in response to TLR2, 3 and 4 stimulation[153]. Therefore, differences in cytokine expression according to vaginal delivery may only be observed at birth and appear to have less of an impact on subsequent neonatal innate immune function (i.e. no difference following TLR stimulation).

1.2.2.3. Maternal Cigarette Smoking During Pregnancy

Maternal cigarette smoking during pregnancy creates a pro-inflammatory *in utero* environment[154] and is associated with decreased birthweight[155], restricted brain growth[156] and long-term impairments to child neurodevelopment[157]. 10-20% of women smoke throughout pregnancy, with higher rates observed in low socioeconomic and/ or poorly-educated populations[158]. Cigarette smoke is associated with increased reactive oxidative species (ROS) and oxidative stress marker production by cord[159] and neonatal peripheral blood[160]. CBMCs from neonates whose mothers who smoked during pregnancy express decreased constitutive IL6, TNFα and IL10[161] and following TLR2, TLR3 and TLR4 stimulation *in vitro* compared to non-smoking mothers[162, 163]. A study of a murine macrophage cell line showed that IL12 and NF-κB expression is decreased with LPS exposure when cells are co-cultured with benzene metabolites from cigarette smoke, compared to cells cultured with LPS alone[164]. Overall, it appears that TLRs, the TLR signalling cascade and cytokines are all decreased in cord blood associated with maternal smoking during pregnancy, which could contribute to altered neonatal innate immune function.

1.2.2.4. Antenatal Glucocorticoid Exposure

Natural increases in cortisol expression are observed across gestation and drive the final stages of fetal organ maturation[165, 166]. Perinatal practice guidelines therefore support the administration of synthetic glucocorticoids (betamethasone or dexamethasone) during the management of threatened preterm labour. Glucocorticoids aid fetal lung maturation through surfactant production and therefore, reduce the incidence of RDS[167, 168]. Antenatal steroid administration originates from Liggins' landmark work during 1969, where pregnant sheep were used to investigate the permeability of the placenta to glucocorticoids and preterm delivery[169]. The study demonstrated preterm parturition occurred when dexamethasone was infused directly into the fetal lamb, but not the pregnant ewe. Incidentally, Liggins discovered that preterm lambs exposed to dexamethasone showed increased lung aeration, which he postulated was a product of enzymatic activity induced by the glucocorticoid that lead to surfactant production. This was later confirmed by studies where prenatal cortisol exposure increased the synthesis of proteins and phospholipids that promote alveolar development, and induced surfactant production in preterm lamb and human lungs[170, 171].

In Liggins and Howie's follow-up, randomised controlled trial of antenatal betamethasone administration to mothers presenting in spontaneous preterm labour, a significant reduction in RDS and neonatal mortality was observed compared to mothers that were not administered betamethasone[172]. As a result, antenatal glucocorticoid administration has become routine practice for women presenting in threatened preterm labour[173]. Recent Cochrane reviews continue to support the use of antenatal glucocorticoid administration, indicating significant reductions in neonatal inflammatory diseases such as RDS, cerebral haemorrhage, IVH, NEC and systemic infection, as well as an overall reduction in neonatal death[168, 174-179]. Other than these associations, the mechanisms and potential effects of antenatal glucocorticoid exposure on subsequent neonatal immune function remain unclear. Notably, recent reviews and animal studies have argued that antenatal exposure to glucocorticoids may have unwanted side-effects including decreased growth[173, 180] and poor neurodevelopmental outcomes during infancy and childhood[181-184].

Glucocorticoids typically suppress inflammation through several mechanisms including reducing T-cell proliferation via IL2 suppression[165, 185], repressing endothelial adhesion molecule expression[186], inhibiting neutrophil chemotaxis[186], repressing transcription of pro-inflammatory cytokines[165, 187, 188] and chemokines[186, 187, 189], and decreasing the circulation of lymphocytes, monocytes and eosinophils[190]. These effects occur when cortisol binds to the glucocorticoid receptor in leukocytes, which then binds to glucocorticoid responsive elements on DNA or to transcription factors specific to TLR signalling genes[191-195]. The impact of glucocorticoids on neonatal immune regulation is highlighted by murine models of LPS exposure. Antenatal betamethasone exposure is associated with lower lymphocyte counts, reduced ROS production and decreased early-response cytokine production (TNFα and IL1β) in rodent offspring, indicating a state of immunosuppression[106, 196]. Rodent models of maternal stress during pregnancy (where endogenous glucocorticoids become elevated) also demonstrate suppressed lymphocyte proliferation, indicating glucocorticoids may exert long-term effects on adaptive immunity in offspring[197].

The impact of glucocorticoids on human neonatal immunity is unclear. Studies on cord blood have demonstrated altered immune responses following prenatal glucocorticoid exposure. Cord blood treated with dexamethasone *in vitro* increases CD14 expression and endocytosis, suggesting that it enhances monocyte differentiation compared to

untreated cord blood[198]. Evidence from this study was, however, obtained independently of antigenic stimulation and while dexamethasone-treated cord blood increased IL10 and decreased IL12 compared to untreated cord blood, its effect on inflammatory function may be different. Other studies have shown that glucocorticoids decrease pro-inflammatory cytokine expression in cord blood *in vitro* following viral stimulation[165] and inhibit neutrophil chemotaxis during LPS exposure[189]. Alternately, high doses of dexamethasone have been found to increase NF-κB-luciferase activity in mice with endotoxaemia[199], supporting increased inflammatory signalling. Overall, the impact of these typically immunosuppressive compounds on preterm neonatal immunity *in vivo* remains contentious[165, 200].

1.3. NEONATAL INNATE IMMUNITY

Neonates demonstrate an increased reliance on tolerogenic immune mechanisms compared to adults during their transition to the *ex utero* environment. A common misconception arising from a tolerogenic immune state at birth is that neonates are immunosuppressed. Current literature shows varied functional capacity in different aspects of immunity. For example, *IL6 mRNA* and protein expression appears to be increased in whole cord blood stimulated with LPS compared to adults[51, 201, 202]. Conversely, some have observed no difference between CBMCs and adult mononuclear cell IL6 production following stimulation with GBS, lipoteichoic acid (LTA) or LPS[203]. The same study also showed that the cord blood IL6 response to GBS was greater than single TLR stimulation (LTA or LPS). This may occur because whole bacteria are complex stimulants, expressing many unique ligands. However, others have used FACS to show cord blood monocytes express decreased IL6 following stimulation with whole, heat-killed GBS compared to adults[204]. This could highlight a decreased capacity by CBMCs to respond to multiple stimuli simultaneously compared to adult leukocytes. Overall, simultaneous activation of different TLRs is common during infection because microbes can express multiple ligands and therefore it is likely that neonatal immunity functions on a conditional basis according to the pathogenic species encountered.

Preterm delivery attenuates specific aspects of neonatal immunity. For example, preterm cord blood shows leukopenia and lymphopenia that persists into the postnatal period for up to seven months[205-208]. Additionally, neonates have a decreased proportion of circulating neutrophils[209, 210] and their storage pools are also depleted due to limited numbers of progenitor cells[211]. Neutropenia is therefore common during neonatal sepsis, where progenitor cell pools in bone marrow are diminished by persistent inflammatory stimulation that results in immune suppression[212]. The absolute blood cell counts of monocytes, DCs, PMNs, NK cells and lymphocytes are decreased in preterm compared to term cord blood due to lower volumes of blood in preterm neonates[207, 213]. Overall, preterm cord blood cell populations are decreased as cellular development occurs from smaller pools of progenitor cells[39] and preterm CBMCs produce decreased GCSF and GMCSF(which typically foster cell development)[214, 215]. As such, GCSF administration is shown to restore neutrophil numbers and their function in murine

models of sepsis[216, 217]. Clinical trials have administered GCSF and GMCSF to septic neonates with neutropenia and as a prophylactic measure. However, failed to reduce the incidence[218] or severity of sepsis[219] despite increasing neutrophil numbers[218], that potentially highlights functional deficits by neutrophils themselves.

While neonatal neutrophils show the same affinity for chemokines as adults, their ability to migrate along a chemotactic gradient is significantly impaired[220]. Chemotaxis is the recruitment of cells to a particular tissue site and has a crucial role in inflammation. It is driven by chemokines such as IL8 and Monocyte Chemotactic Protein-1 (MCP1), whose aberrant expression can lead to the accumulation of immunocytes. Increased IL8 is therefore associated with inflammatory diseases in neonates such as sepsis[221] and inflammatory lung disease[222]. Further, bronchoalveolar lavage samples from neonates that develop CLD show an accumulation of neutrophils compared to neonates with RDS[223], which is also associated with respiratory failure[224]. Conversely, deficient chemotaxis can contribute to neutropenia and exacerbate conditions such as sepsis in neonates[225]. Therefore, regulation of chemokine expression and neutrophil accumulation (through apoptosis) may be critical to a normal immune response by neonates. Typically, chemokine expression is positively correlated with gestational age and is tissue-dependent[226]. Overall, the chemotaxis of leukocytes to the site of inflammation appears attenuated by preterm neonates and may underlie an inability to localise inflammation.

Neutrophils are polymorphonuclear cells (PMNs) that make up 70% of circulating leukocytes and drive pro-inflammatory responses. Prematurity also decreases neutrophilic function throughout the neonatal period, where oxidative burst (which is required for phagocytosed pathogen killing) is consistently decreased in cord and peripheral blood[227, 228]. Reductions in neutrophil 'peak activity' are therefore associated with severe infection[229]. Preterm PMNs stimulated with *Staphylococci* show intact phagocytic activity, but impaired killing capacity, regardless of showing comparable superoxide production compared to term neonates[86, 230, 231]. In fact, neonatal neutrophils produced higher levels of reactive oxygen intermediates than adult neutrophils, which is normally indicative of persistent tissue damage in chronic inflammatory disorders[231, 232]. Reactive oxygen intermediates are important for neutrophil intracellular killing following phagocytosis, however, they are harmful to native cells because they oxidize

membranes, proteins and nucleic acids[233]. During the development of sepsis, endotoxins initially cause local inflammation, but subsequently aberrant production of cytokines and ROS becomes sustained and can lead to systemic inflammation[234]. In the second phase of sepsis, a 'compensatory anti-inflammatory response' occurs, characterised by increased regulatory cytokine expression, but it does not completely resolve inflammation and instead prolongs a systemic response[235]. Peripheral blood from preterm neonates with sepsis therefore shows increased pro-inflammatory IL6, IFNγ and TNFα with increased anti-inflammatory IL4 and IL10 compared to preterm neonates with culture-negative sepsis screens[236]. Although it classically has anti-inflammatory roles, IL10 is associated with septic shock[236] and poor outcomes during sepsis[237]. Together, these findings support a global upregulation of inflammatory pathways during sepsis and highlight the importance of effective immunoregulatory mechanisms in clearing infection.

1.4. THE INNATE IMMUNE RESPONSE IN NEONATAL BLOOD

The constitutive expression of innate immune receptors, signalling molecules and therefore cytokines, is comparable between cord blood and adult peripheral blood. Specifically, cord blood and adult monocytes transcribe comparable levels of mRNA for *TLRs1-10*, and their signalling molecules *CD14*, *MyD88*, *TIRAP* and *IRAK4*[62, 211, 238-243]. While these genes are similarly expressed in neonates and adults, differences in immune function become apparent clinically (increased susceptibility to infection) and experimentally (following *in vitro* immune stimulation).

Cord blood is commonly used to assess the neonatal innate immune function as it is readily available and reflects the status of the neonate at birth. The administration of TLR agonists to cord blood *in vitro* is also common as it enables the study of specific functional aspects of innate immune function. Many authors have used both TLR agonists and whole, heat-killed bacteria to characterise cytokine expression by neonates compared to adults in isolated cell populations and whole blood. These studies demonstrate varied findings and are summarised in Table 1.1.

Most studies show a concurrent increase in tolerogenic cytokines (IL10 and TGFβ[48, 52, 53, 202, 244, 245]) and early-responding cytokines (TNFα[62, 202, 246]), but attenuated expression of anti-viral cytokines (IFNs and IL12[48, 52, 202, 244-251]) in cord blood compared to adult blood stimulated with immune ligands *in vitro*. Notably, disparities observed by other studies can be explained by differences in experimental methods. For example, compared to adults, expression of the chemokine, IL8, is increased by CBMCs stimulated with whole GBS[203], but decreased in cord blood DCs stimulated with endotoxin[202]. Alternately, CBMCs stimulated with GBS express decreased IL12 compared to singular TLR7,8 or 9 stimulation[250]. This may be due to differences in cell populations, cell interactions or antigen complexity. Hartel et al. have shown that singular TLR stimulation in whole preterm cord blood or CBMCs decreases IL10, but stimulation with *S. epidermis* demonstrates comparable IL10 expression compared to term cord blood[252]. Tatad et al. are the only authors that present increased IL10 expression following GBS exposure and comparable expression between preterm and term cord blood following stimulation with *E. coli* or *S. epidermis*[204]. These conflicting results may arise from increased levels of IL10 in preterm cord blood at baseline, the

use of complex ligands or an inconsistent resting period of these cells following birth, prior to *in vitro* stimulation. This highlights a need to consider methodological differences in experiments when examining neonatal immune function.

The innate immune system of the neonates appears more sensitive to specific classes of bacteria. A study compared the response of CBMCs to 11 species of whole, heat-killed bacteria and observed increased IL6, IL12 and TNFα, and comparable IL10 expression compared to adult mononuclear cells[253]. Specifically, they showed gram-negative bacteria increased IL12, IL6 and TNFα, and expressed similar IL10 in CBMCs compared to adult peripheral mononuclear cells. This response was not observed in response to gram-positive bacteria. The authors suggest this is a pathological pro-inflammatory bias towards commensal bacteria. Such a bias could predispose neonates to aberrant inflammatory responses during commensal colonisation *ex utero* and highlights the importance of immuno-regulatory mechanisms. Overall, the complexity of ligands and cell populations (i.e. isolated cells versus whole blood) should be considered in every analysis, given that neonatal immunity does not show generalised attenuation and only specific aspects may be immature.

Table 1.1. ***A summary of studies on cord blood cytokine expression following immune stimulation compared to adult peripheral blood.*** The expression of select cytokines and chemokines in cord blood relative to adult blood following *in vitro* stimulation with different immune agonists ('↑' expression is increased in neonates compared to adults, '↓' expression is decreased in neonates compared to adults and '↔' expression is comparable between neonates and adults).

Cytokine/ chemokine	Cord blood expression compared to adult blood	Role in immunity	Cell type	Agonist used for *in vitro* stimulation (citation)
IFNα	↓ ↓ ↓ ↓ ↔ ↓	Anti-viral, precedes IFNγ expression	CBMCs Monocytes pDCs Whole blood Whole blood Whole blood	TLR 9 agonist[52] TLR 7/8, 9 agonist[48] TLR9 agonist[247] TLR7 agonist[254] TLR9 agonist[254] TLR3 agonist[244]
IFNγ	↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓	Anti-viral, macrophage activation, adaptive immune responses	CBMCs CBMCs CBMCs CBMCs CBMCs DCs Monocytes Whole blood & DCs Whole blood	TLR2-4, 7, 9 agonist[52] TLR4 agonist[249] TLR4 agonist +PHA[246] GBS[250, 251] ConA[255] TLR9 agonist[202] TLR1-4, 7-9 agonist[48] TLR4 agonist[248] TLR4 agonist[202]

Cytokine/ chemokine	Cord blood expression compared to adult blood	Role in immunity	Cell type	Agonist used for *in vitro* stimulation (citation)
IL1β	↔ ↔ ↔ ↓ ↔	Fever response, inflammation	CBMCs CBMCs Whole blood Whole blood Whole blood	GBS, TLR2,4 agonist[203] LPS agonist +PHA[246] TLR4 agonist[202] TLR4 agonist[256] TLR2 agonist[256]
IL6	↑ ↑ ↔ ↑ ↑	Acute phase response, systemic inflammation, fever response	CBMCs CBMCs CBMCs Monocytes Whole blood Whole blood	IFN + TLR4 agonist[201] HSV[257] GBS, TLR2,4 agonist[203] TLR3,4,5,9 agonist [258] TLR4 agonist[202] Heat-killed bacteria[204]
IL8	↑ ↓ ↔	Neutrophil chemotaxis and activation	CBMCs DCs Whole blood	GBS, TLR2,4 agonist[203] TLR4 agonist[202] *S. epidermis*[204]
IL10	↑ ↑ ↑ ↓ ↑ ↑ ↓ ↑	Inhibits inflammatory cytokine production	Whole blood CBMCs Whole blood Whole blood CBMCs CBMCs Whole blood Whole blood	TLR1-4, 7-9 agonist[52] TLR2,3,7-9 agonist[52] TLR3-4 agonist[244] TLR3-4 agonist[259] TLR4 agonist +IFN, TLR3,7,9 agonist[260] TLR4, 7/8, 9 agonist[48] GBS[204] TLR4 agonist[202]

Cytokine/ chemokine	Cord blood expression compared to adult blood	Role in immunity	Cell type	Agonist used for *in vitro* stimulation (citation)
IL12	↓ ↑ ↑ ↑ ↓ ↓ ↓	Th1 differentiation, expression of IFNγ	CBMCs whole blood CBMCs whole blood CBMCs Whole blood Whole blood	GBS[250] TLR3,4,7/8,9 agonist[52] TLR 7/8, 9 agonist[52] TLR3, 4 agonist[244] TLR4 agonist[201] TLR3 agonist[260] TLR4 agonist[202]
IL23	↑ ↔ ↑	Autoimmune inflammation	CBMC DCs Whole blood	TLR2,7/8,8 agonist[52] TLR2-4, 7/8 agonist[261] TLR2,7/8,8 agonist[52]
TNFα	↑ ↓ ↑ ↔ ↑ ↔ ↓ ↑ ↔ ↓	Fever and early phase inflammatory response	CBMCs CBMCs CBMCs CBMCs CBMCs monocytes monocytes Whole blood Whole blood Whole blood	TLR1-3, 7/8,9 agonist[52] TLR4 agonist +PHA[246] GBS[262] TLR4 agonist +IFN[201] GBS, TLR2,4 agonist[203] TLR7/8 agonist[62] BLP, TLR4,7 agonist[62] TLR1-4, 9 agonist[52] TLR2 agonist[256] TLR4 agonist[202, 256]
GCSF	↓	Increased granulocyte proliferation and monocyte production	CBMCs	PHA[263]

Cytokine/ chemokine	Cord blood expression compared to adult blood	Role in immunity	Cell type	Agonist used for *in vitro* stimulation (citation)
GMCSF	↔	Stimulates granulocyte and monocyte differentiation	CBMCs	TLR4 agonist +PHA[246]
TGFβ	↑	Decreases macrophage activation, inhibits Th1 response, Treg maturation	DCs	RSV[53]

1.4.1. The Poly-functionality of Cord Blood Immune Cells

Differences in poly-functionality (i.e. the ability to express different cytokines simultaneously) between adult and neonatal cells may explain disparate results presented between studies on *in vitro* cord blood stimulation. Flow cytometry has shown that isolated CBMCs stimulated with TLR agonists *in vitro* are less capable of producing multiple cytokines simultaneously than cells from stimulated whole blood[52]. Cord blood monocytes show a similar degree of poly-functionality compared to adults, but flow cytometry of whole cord blood shows a decreased ability to express certain cytokines simultaneously (e.g. IL12p40 and TNFα) following TLR2, 4 or 7/8 stimulation compared to adults. It has therefore been suggested neonatal Th1 immune attenuation (see 1.4) can be restored through TLR co-stimulation. Cord blood antigen presenting cells (APCs) show robust TNFα and IL12 expression (comparable to adults) when stimulated with TLR7/8 at the same time as TLRs 2 or 4[264]. This has also been demonstrated in animal models of neonatal sepsis, where TLR priming results in decreased duration of inflammation and therefore, decreased disease severity and mortality[92]. Murine *RAG1*$^{-/-}$ neonates with polymicrobial sepsis that have been pre-treated with TLR4 and TLR7/8 agonists show enhanced neutrophil recruitment, phagocytic ability and oxidative burst, and therefore less severe disease than unprimed mice[56, 92]. In humans, patients with sepsis are administered IFNγ to increase TNFα production and improve phagocytosis[107, 306]. This treatment aims at resolving inflammation through pathogen clearance[94, 265]. This suggests cord blood immune cells may be less poly-functional due to deficiencies in their ability to concurrently express different cytokines compared to adults.

1.4.2. Neonatal Plasma

Cord blood plasma supports a tolerogenic bias through decreased constitutive expression of TNFα, IL12p70 and IFNγ[238, 266], and increased anti-inflammatory IL4, IL10, IL13 and TGFβ compared to adult plasma[267]. Adult mononuclear cells cultured in cord blood plasma and stimulated with TLR3, 4 and 8 ligands, show decreased IL12p70 and TNFα, and increased IL10 expression compared to adult mononuclear cells cultured in autologous plasma[62, 245, 268]. The use of different TLR agonists in this study

also indicates both MyD88-dependent and independent pathways of TLR signalling are supported by plasma-associated factors[245].

Studies on isolated immune cells allow for closer mechanistic analysis, but ignore cellular cross talk and could discard the influence of other soluble autologous factors found in blood[267]. Blood plasma consists of organic molecules such as proteins and hormones[269] and is central to shaping the immune response. Plasma is necessary for rapid immune signalling throughout the body, but its mechanisms remain largely uncharacterised, particularly in the neonate[270]. Blood plasma contains many immune modulating proteins including IgG (11% of its protein content[271]), anti-inflammatory albumin (50% of its protein content) and adenosine, which regulates immunity[270]. Cord blood plasma contains increased concentrations of adenosine compared to adult plasma[272] and metabolises less of the nucleoside than adult plasma[273]. Adenosine represses ROS expression by neutrophils and increases IL10 expression in macrophages, which then represses IL12 and TNFα expression (reviewed in[274]). Increased cord blood adenosine could contribute to the tolerogenic bias compared to adults observed in some stimulation studies.

When neonatal haemocytes are cultured in adult plasma, their TNFα expression is comparable to adults (except following TLR7/8 stimulation)[62]. This response is dose-dependent, where higher concentrations of LPS induce similar expression of TNFα between cord blood and adult mononuclear cells[241]. This 'repaired' response is thought to be due to the higher levels of immune-active plasma proteins (such as platelet activating factors) in adult plasma[241]. These findings highlight the importance of whole blood cell culture in characterising neonatal immunity due to differences between the way that cells are exposed to or interact with exogenous factors in plasma. In fact, it may be a combination of cellular robustness and serum-based factors that contribute to differences in neonatal immunity, although the precise mechanisms remain unclear.

1.4.3. The Neonatal Innate Immune Response to Viral Stimulation

Anti-viral cytokine expression (IFNs and IL12) by cord blood *in vitro* is impaired, independently of gestational age, cell type or immune agonist (see Table 1.1). Even when CBMCs are primed with IFNγ prior to TLR4 stimulation *in vitro*, they express

decreased IL12, IL8, IL23 and IFNs compared to adult blood[201]. This is significant as IFNγ typically activates STAT1 which this leads to microbial killing, antigen presentation and increased pro-inflammatory cytokine expression[275]. IFN production also induces an anti-viral state in adjacent cells which downregulates protein expression, and enhances NK cell, cytotoxic T cell and dendritic cell activation[73, 276]. Notably, the addition of exogenous IFNγ does not rescue ROS release and pathogen killing capacity in neonatal macrophages because STAT1 expression is decreased and unresponsive to the IFNγ relative to adults[257]. The lack of anti-viral mediators and responsiveness by cord blood supports clinical evidence that neonates are susceptible to viral infections including influenza and RSV that lead to increased mortality compared to adults[277].

An absence of IFNγ expression by neonates may be evidence of immune tolerance. When cord blood leukocytes exposed to LPS are co-stimulated with IFNγ, they demonstrate increased expression of TNFα, IL6 and IL10[82] that exceeds 2 month old and 1 year old peripheral blood[201]. Additionally, CBMCs and whole cord blood express increased TNFα compared to adult blood following bacterial and viral TLR stimulation[52], and GBS stimulation[203, 262]. Conversely, co-stimulation of CBMCs with LPS and IFNγ show comparable TNFα expression compared to adults[201]. Studies from rodents support these findings as *IFNγ*$^{-/-}$ rodents are resistant to endotoxic shock[278]. These rodents are also protected against NEC compared to WT strains because they exhibit reduced inflammatory signalling[279]. Altogether, the impaired neonatal IFN response impacts innate immunity towards both viruses and bacteria.

Decreased IFN expression by neonates may be explained by downstream adaptor molecules in TLR signalling. p38MAPK is involved in PMN degranulation, adhesion and respiratory burst and its constitutive expression between neonates and adults is similar[211, 280]. Following LPS stimulation, however, cord blood shows decreased p38, ERK and IκBα phosphorylation, which results in decreased cytokine expression compared to adult blood[35, 62, 201, 211, 241, 256]. The attenuated translocation of IRF7 into the nucleus of cord blood pDCs following TLR7 and TLR9 stimulation[281], and decreased constitutive IRF3 expression[248] also suggest that MyD88-independent signalling may be defective in neonates. Notably, when CBMCs are stimulated with multiple ligands, they

increase phosphorylation of NF-κBp65, ERK1/2 and Akt above adult levels, compared to stimulation with single ligands[282]. This could indicate neonates are less sensitive to viral antigens and need more stimulatory signals to achieve a robust response to viral stimulation.

1.4.4. The Innate Immune Response in Preterm Compared to Term Neonates

Emerging studies are challenging the traditional paradigm of immune attenuation in preterm neonates. Strunk et al.'s aptly titled review 'innate immunity human newborn infants: prematurity means more than immaturity' contrasts a robust ability by preterm neonates to phagocytose and kill pathogens in spite of attenuated cytokine responses[283]. This suggests preterm neonates do not show consistent attenuation in all aspects of their innate immune function. In fact, studies have shown that despite decreased populations of monocytes and neutrophils in preterm cord and peripheral blood[284] and reduced opsonophagocytic activity in plasma[82], phagocytic capacity is increased in early preterm cord blood compared to term[284, 285]. Additionally, previous work from our laboratory has demonstrated preterm cord blood expresses increased IL6 without any compensatory anti-inflammatory response (IL10 or TGFβ) following TLR2 and 4 stimulation *in vitro*[153]. A meta-analysis of very preterm deliveries has also demonstrated a genetic profile in umbilical cord tissue that is similar to deliveries characterised by FIRs, suggesting that lower gestational age predisposes neonates to a pro-inflammatory phenotype[286].

Term and preterm cord blood show comparable constitutive cytokine production, with the exception of the chemokines IL8 and MCP1, which are higher in preterm neonates at birth[148, 287]. Alternatively, *in vitro* studies on TLR stimulation have consistently demonstrated decreased pro-inflammatory cytokine production in preterm compared to term cord blood (see Table 1.2). Further, the expression of IL6 and TNFα are consistently decreased in preterm cord blood compared to term, following stimulation with TLR1-9 agonists[213, 288] or whole bacteria[252, 289]. Studies using flow cytometry have, however, shown conflicting results. Some authors have demonstrated that stimulation with LPS increases the frequency of IL6 and IL8 positive cells in preterm compared to

term cord blood[194], while stimulation with *S. epidermis* decreases TNFα^{+} and IL6^{+} cell counts[252]. Despite these decreased cell counts, stimulation with TLR7-9 agonists or *S. epidermis* shows an increased IL8 response by preterm cord blood PMNs and whole blood compared to term[204, 290]. Alternately, stimulation of whole cord blood or PMNs with TLR1-5 agonists demonstrates comparable or decreased expression of IL8 compared to term[82, 290]. In comparison to stimulation with whole heat killed bacteria, single TLR stimulation produces decreased cytokine responses in preterm cord blood compared to term[204]. These findings support preterm neonates are less sensitive to simultaneous stimulation by the multiple ligands that are present on whole bacteria. Such reduced sensitivity to pathogens may underlie preterm neonates' susceptibility to contracting infection.

In addition to an increased susceptibility to infection, preterm neonates demonstrate differences in regulatory immunity compared to term. The constitutive expression of anti-inflammatory IL10 and TGFβ is increased in preterm cord blood compared to term. Following LPS stimulation, however, preterm and term cord blood both express decreased IL10 and TGFβ compared to adult blood[291]. Further, when cord blood monocytes are supplemented with recombinant IL10 or TGFβ and are stimulated with TLR4, their inhibition of pro-inflammatory cytokine expression is impaired compared to adult monocytes. Anti-inflammatory cytokines limit inflammation through antagonising pro-inflammatory pathways and enhancing immunosuppressive mechanisms and tissue repair. The suppressive effects of anti-inflammatory cytokines are dependent on their ratios relative to pro-inflammatory mediators and the respective bioactivity of the molecules. Therefore, increased constitute cord blood IL10 levels may not be entirely protective against dysregulated inflammation in preterm neonates.

Table 1.2. ***A summary of studies on preterm cord blood cytokine expression compared to term following immune stimulation*** **in vitro.** Cytokine expression may be increased (↑),decreased (↓) or comparable (↔) in preterm compared to term cord blood following immune stimulation of the indicated cell types with specified agonists *in vitro*.

Cytokine/ chemokine	Expression in preterm compared to term cord blood	Agonist	Cell type (citation)
IFNα	↓ ↓	RSV TLR7,9	Monocytes[292] pDCs[213]
IL1β	↓ ↓ ↔	TLR4 TLR2 *S. epidermis*	CBMCs[288] Monocytes[35] CBMCs[46]
IL6	↔ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↔ ↓	*S. epidermis* IL1 TLR2, 4 TLR4 GBS RSV TLR2 *S. epidermis* TLR4 *E. coli, S. aureus* *S. epidermis* *E. coli, Listeria monocytogenes, S. pneumonia*	CBMCs[46] CBMCs[293] CBMCs[213] CBMCs[288] CBMCs, whole blood[289] Monocytes[292] Monocytes[35] Whole blood[252] Whole blood[252] Whole blood[82] Whole blood[82] Whole blood[294]
IL8	↔ ↓ ↔ ↑ ↓	*S. epidermis* TLR2 TLR2-5 TLR7/8, 9 TLR2, 4	CBMCs[46] Monocytes[35] PMNs[290] PMNs[290] Whole blood[82]
IL10	↔ ↓ ↔ ↔ ↑ ↔	*S. epidermis* TLR4 TLR2-9 TLR4 GBS TLR4	CBMCs, whole blood[252] CBMCs, whole blood[252] CBMCs[213] Whole blood[291] Monocytes[204] Whole blood[177]
IL12	↓	TLR2-9	CBMCs[213]

Cytokine/ chemokine	Expression in preterm compared to term cord blood	Agonist	Cell type (citation)
TNFα	↔ ↓ ↓ ↓ ↓	*S. epidermis* TLR2-9 TLR4 GBS *S. epidermis*	CBMCs[46] CBMCs[213] CBMCs[288] CBMCs, whole blood[289] CBMCs, whole blood[252]

1.4.5. Neonatal TLR Signalling

Neonatal TLR signalling is immature compared to adults. In mice, TLR4 deficiency in mice leads to LPS hypo-responsiveness, resulting in increased microbial loads and higher rates of mortality[295, 296]. *TLR2*/*TLR4*$^{-/-}$ mice exposed to *S. typhimurium* are more susceptible to infection and show an increased bacterial load and rate of mortality. Similarly, *IRAK1*$^{-/-}$ animals are resistant to endotoxic shock because inflammatory signalling is downregulated[297]. In humans, IRAK4 deficiency is negatively correlated with age[298], making children more vulnerable to higher incidences of infection and severe complications compared to adults[299]. The attenuated expression of these early signalling molecules could therefore place neonates at an increased risk of infection.

Although there is a wealth of data which suggests that TLR expression is altered by preterm delivery[48, 82, 288, 300], the largest body of literature has focused on TLR4. Seminal work by Forster-Waldl and colleagues found decreased expression of TLR4 in preterm cord blood of very low birth weight infants compared to term controls, with an associated decrease in pro-inflammatory cytokines following TLR4 stimulation[288]. Similarly, other studies have shown a positive correlation between gestational age and TNFα cytokine production following TLR4 stimulation, indicating increased immune responsiveness by preterm neonates with advancing gestational age at delivery[35, 62, 82, 288, 301]. Conversely, constitutive expression of *TLR2* does not appear to differ significantly across gestational age in isolated cord blood monocytes[35]. Preterm monocytes also show comparable expression of constitutive *TLR2, TLR4* and *TLR6* compared to term and adult monocytes[86]. Alternately, other studies have shown that protein expression of TLR2 and TLR4 on monocytes and neutrophils is positively correlated with gestational age[302]. Deficient TLR4 expression could be analogous to mutations seen within the *TLR4* gene itself, which lead to susceptibility to bacterial infection and decreased responsiveness to LPS[288, 303, 304].

Regulating TLR signalling is central to immune tolerance. For example, murine models show blocking NF-κB signalling in IECs decreases systemic inflammation while increasing local inflammation[61]. Other studies have also demonstrated NF-κB activation in IECS is protective against mucosal injury for this reason[305, 306]. Ultimately, increased NF-κB and decreased IκBα contribute to severity of inflammation and increase the risk

of neonatal diseases such as NEC[307]. It is therefore unsurprising that breast milk stimulates intestinal epithelial cells' expression of IκBα, resulting in reduced NF-κB translocation[308]. The very presence of this inhibitor in breastmilk highlights the potential for immune compound supplementation to protect the neonate from inflammation.

1.5. THE REGULATION OF INFLAMMATION

Inflammation requires gradual down-regulation to avoid constant defensive signalling that leads to wide-spread tissue damage. Immune regulation can involve the expression of anti-inflammatory mediators, networks of genetic response signalling, and physiological feedback systems (e.g. via the HPA axis or secondary lymphoid organs)[309]. These mechanisms recruit Tregs, repress inflammatory genes and induce sickness-like behaviours that assist in the process of tissue healing. Notably, the down-regulation of a pro-inflammatory response causes less tissue damage than increased anti-inflammatory mediator expression[310], while uncontrolled inflammation causes more tissue damage than infection itself and can establish a fertile ground for pathogen growth or autoimmunity.

As TLR signalling can be activated by both exogenous and endogenous ligands, it needs to be tightly regulated to avoid such pathological inflammation. Regulatory mechanisms active at different stages of the signalling pathway have distinct effects on inflammatory outcomes depending on the complexes they target. TLR signalling can be decreased in a temporal manner according to the gradual activation of inhibitors stimulated at various stages of the cascade. Examples of TLR regulation include the upregulation of direct inhibitors such as IκBα or post-transcriptional repressors of mRNA transcripts which control the signalling cascade. A key class of intracellular mediators induced by cytokines are inhibitory Suppressor of Cytokine Signalling (SOCS) proteins. These are proteins that regulate transcription of Janus Kinases (JAKs) that activate Signal Transducer and Activators of Transcription (STATs). SOCS proteins therefore inhibit the transcription of cytokines[311]. *SOCS1*$^{-/-}$ mice are therefore hyper-responsive to LPS exposure and produce higher levels of TNFα, IL12 and IFNγ than WT mice, resulting in decreased survival caused by excessive inflammation[312-314]. SOCS1 is also an important regulator of the p65 subunit, as it degrades this transcription factor and preventing cytokine expression[315]. SOCS proteins are responsive to cytokine production and are therefore important regulators of inflammation, impacting different components of the TLR signalling pathway.

Other regulators of inflammation target gene expression and can include microRNAs (miRs), gene promoters, inhibitory proteins and competitive binders. MiRs have emerged as master regulators of inflammation[316] as they are responsive to different cues during inflammation and regulate gene expression post-transcriptionally. Dysregulated patterns of miR expression have also been associated with the development of chronic inflammatory conditions[317]. The role of miRs in regulating inflammation may therefore be critical to a better understanding of neonatal innate immunity.

1.5.1. MicroRNAs

MiRs are 18-22 nucleotide, single-stranded, non-coding RNAs which repress gene expression post-transcriptionally[318]. They are responsive to temporal and tissue-specific cues and therefore, regulate many cellular processes[319, 320]. The post-transcriptional action of miRs may explain the mechanisms behind differences in inflammatory gene and protein expression in studies of cord blood, and could be key to understanding dysregulated innate immune responses in preterm neonates.

MiRs are 'promiscuous' as each can have hundreds of mRNA targets that impact networks of genes. *In silico* analyses of microarray expression data estimate that miRs regulate approximately 30% of the human genome[321]. As such, they are shown to influence fundamental cellular processes including cell differentiation, proliferation, migration and apoptosis. Regulation by miRs is complex because different miRs can have multiple mRNA targets, act on many pathways simultaneously or act on different components of a common pathway. Similarly, mRNAs or entire genetic pathways can be targeted by many miRs at once to achieve a single effect, such as the downregulation of inflammation. These effects are commonly explored using microarray analyses, and *in vitro* miR transfection or knockdown studies.

MiRs are evolutionarily-conserved and encoded by the genome such that they are regulated by their own transcription factors and promoters[319]. They are encoded as primary transcripts or as introns alongside protein sequence exons[322]. MiRs are transcribed by RNA Polymerase II from long primary transcripts (pri-miRNAs), which are subsequently cut by the RNaseIII, Drosha, into 60 base-pair precursors also known as 'pre-miRNAs'[323, 324] (Fig 1.2). Another RNase III, Dicer, then cuts the pre-miR into

an asymmetrical 22bp miR duplex containing the mature miR and its star-form complement[325]. Mature single-stranded miRs are exported to the cytoplasm and loaded onto an RNA-inducing silencing complex (RISC), which guides their targeted recognition of mRNA transcripts. MiRs bind complementarily to the 3' untranslated region (UTR) of their target mRNA transcript and therefore, silence translation or signal transcript degradation[324]. MiR binding results in the adenylation or reduction of mRNA stability to the point of degradation[326, 327], indirectly repressing protein synthesis. MiRs can down-regulate protein synthesis by directly targeting mRNA or indirectly up-regulating protein expression through suppression of negative regulators. For example, *miR-155* upregulates cytokine expression through repressing the suppressor of cytokine signalling *SOCS1*[328, 329].

Approximately 1800 human miRs have been identified[330] and at least 150 of these have known immune implications[331], though their functional roles are not fully characterised[332]. In the context of immunity, tissue-specific or temporal aspects of miR regulation allow for the strict control of inflammation. Inflammation itself upregulates factors that control the expression of miRs, including TGFβ and bone morphogenic protein (BMP), which induce pri-miR processing[333]. A more comprehensive understanding of the regulatory interactions between miRs and inflammation could therefore lead to new avenues of therapy for inflammatory morbidities[334].

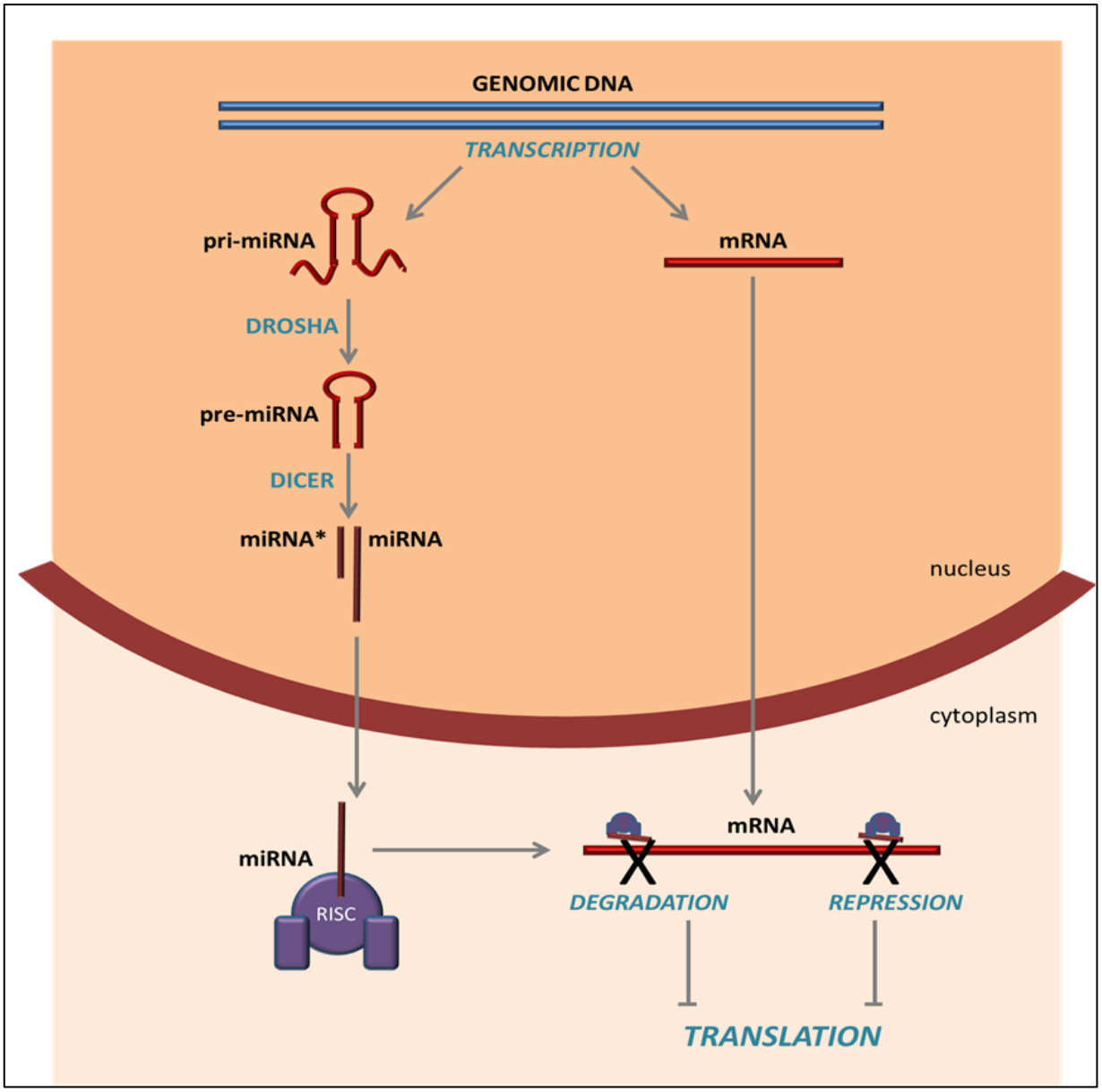

Fig 1.2. ***A diagrammatic representation of miR synthesis and function.*** miRs are encoded in genomic DNA and transcribed as long primary transcripts (pri-miRNAs), which are then cut by Drosha (to form pre-miRNAs) and Dicer (to form a miR duplex consisting of the miR and its asymmetrical star-form partner). The mature miR transcript is then exported into the cytoplasm and loaded onto RISC, which guides its recognition of mRNA transcripts. This complex binds to the mRNA target and either represses the transcript's translation or signals for its degradation.

1.5.1.1. MiRs that regulate TLR signalling

TLR signalling and the induction of pro-inflammatory cytokines can be up- or down-regulated through the actions of miRs (for review see[335]). Approximately 29% of genetic transcripts for cytokines and chemokines contain miR targets[336]. MiRs are also responsive to cytokines: for example, IL10 downregulates *miR-155* release by bone marrow macrophages following LPS stimulation[337]. Human decidual cells stimulated with IL1β demonstrate differential expression of 428 transcripts (containing six miRs) which repress genes in TLR and MAPK signalling pathways[338]. In fact, a majority of miR regulation occurs downstream of TLR receptors to 'dampen' rather than eliminate TLR signalling. This allows for the elicitation of a balanced and appropriate immune response to a pathogen without prolonging inflammatory signalling.

The temporal nature of miR induction means that miRs are expressed as part of early and late response mechanisms. As such, *miR-155* induction occurs within two hours of LPS exposure, while *miR-21* is induced after 8 hours[335, 339]. These temporal cues form a regulatory loop where *miR-21* represses PDCD4, a repressor of *IL10,* and therefore delays an increase in IL10 expression needed to represses *miR-155* later during inflammatory signalling[337]. When *miR-155* is downregulated, it no longer represses *SHIP1* or *SOCS1* genes, allowing these proteins to downregulate the pro-inflammatory TLR4 response[340]. This network of signalling presents an antagonistic relationship between two miRs that rely on temporal cues during inflammatory signalling. As such, it highlights the role of miRs in 'buffering' rather than eliminating inflammation.

TLR-induced inflammation consists of many adaptor molecules and transcription factors that can be regulated throughout the signalling cascade. TLRs themselves have few 3'UTRs, indicating the presence of conserved target sites for regulation by miRs[335]. At the receptor level, *let-7e* represses the expression of *TLR4 mRNA*, restricting signalling downstream of the receptor[335, 341]. *let-7e* is increased with LPS stimulation as demonstrated in macrophages *in vitro*, where silencing the gene upregulates cytokine expression following LPS stimulation. *miR-9* is also upregulated by the activation of TLR2,4 and 7/8 in a MyD88- and NF-κB-dependent manner[332]. This is demonstrated by *in vitro* stimulation of human monocytes and neutrophils, where activation of NF-κB through TLR4 increases *miR-9* expression and subsequently, *miR-9* induces a negative-

feedback response by downregulating NF-κB. It is therefore postulated that most TLR regulation occurs during or after transcription.

1.1.1.1.4. miR-106a

MiR-106a is an indirect pro-inflammatory regulator as it has a binding site on the anti-inflammatory *IL10* gene[339]. In human cell lines, transfection with an IL10 luciferase transporter gene results in decreased *IL10* expression when *miR-106a* is increased[342]. Further, transfection of *miR-106a* into a bladder cancer cell-line increased p38 and JNK phosphorylation during TLR signalling[343]. Murine models of allergic airway inflammation have also demonstrated that the knockdown of *miR-106a* is associated with an upregulation of IL10 expression in the lung[344]. These mice also show increased airway inflammation in a dose-dependent manner to *miR-106a* expression. Together, these studies highlight *miR-106a* as an upregulator of TLR signalling through the repression of *IL10.*

1.1.1.1.5. The miR-146 *family*

The MiR-146 family are negative regulators of inflammation[90, 323, 345, 346] consisting of *miR-146a* and *miR-146b.* Their expression is highest 24 hours following LPS exposure in human monocytes *in vitro*[323, 346] and mice *in vivo*[347]. The miR146 family represses NF-κB, MAPK and downstream EGR transcription factors in the TLR signalling cascade[348, 349]. While *miR-146a* and *miR-146b* differ by two base-pairs, they are both associated with repression of IRAK1 and TRAF6[323, 350, 351]and are regulated by NF-κB[323, 352].

miR-146a expression can be induced by LPS, TNFα and IL1β in a NF-κB-dependent manner[323]. It also shows cell-specific function, decreasing *TRAF6* and *IRAK1* in monocytes[323] and *STAT1* in Tregs[353]. In mice, *miR-146a* deficient Tregs are associated with the formation of autoimmune inflammatory lesions, indicating that the miR is crucial for these cells' tolerogenic roles[353]. Human Langerhans cells also show increased constitutive expression of *miR-146a* compared to dendritic cells, which may be related to the induction of tolerance on the skin and mucosa towards the external, pathogen-rich environment[354]. In pDCs, TLR7 or TLR9 stimulation leads to increased *miR-146a* expression, which decreases NF-κB activation and therefore impairs TLR

signalling[355]. Overall, *miR-146a* clearly has context-dependent roles in inflammatory suppression.

miR-146a may also have a role in conferring endotoxin tolerance, although the precise mechanisms remain unclear. *miR-146a*$^{-/-}$ mice are hyper-sensitive to LPS, developing acute vascular responses following exposure to the endotoxin[353, 356]. Further, neonatal mice upregulate *miR-146a* expression to establish enteric homeostasis in intestinal epithelia[347]. Extensive mucosal damage is observed in mice where *miR-146a* expression is ablated by anti-miR-146a antibodies[347], particularly as *miR-146a* is also involved in the induction of gene sets central to cell survival, differentiation and homeostasis[347, 351]. Tolerance towards LPS through the down-regulation of *IRAK1* remains evident in the murine gut until 3 weeks of age, when *IRAK1* expression is restored[347, 357]. These findings highlight that *miR-146a* expression in the gut is age-specific and that miRs have many roles in supporting a tolerogenic neonatal immune system.

1.1.1.1.6. miR-155

miR-155 is an important pro-inflammatory mediator that regulates many inflammatory targets and influences entire networks of inflammatory genes. The anti-inflammatory action of IL10 has been shown to repress *miR-155* during LPS stimulation in human and murine cell lines[337, 358], highlighting the significance of its role during inflammation. The best characterised target of *miR-155* is *SOCS1*, where the over-expression of *miR-155* in mice directly down-regulates the expression of *SOCS1* and therefore, upregulates cytokine synthesis[328, 341, 359, 360]. *miR-155* expression is increased in mononuclear leukocytes following stimulation with Poly I:C and IFNβ, as it can be induced by both MyD88- and TRIF-dependent signalling pathways [323, 361]. *miR-155* induction is also associated with increased TNFα expression and therefore, its over-expression in mice makes them more vulnerable to septic shock than wild types[362]. Further, the knockdown of *miR-155* is associated with decreased numbers of Th1 and Th17 cells and mild experimental autoimmune encephalitis in mice[363].

miR-155 expression is upregulated by TLR2, 3, 4 and 9 signalling (reviewed in[335]). Cancer and embryonic kidney cell lines have also shown that *miR-155* targets *MyD88* during *Helicobacter pylori* exposure[364]. The knockdown of *miR-155* therefore leads to

increased *MyD88* expression in human macrophage cells in response to oxidised low density lipoprotein (representing oxidative stress). Downstream of MyD88, *miR-155* has also been shown to repress IKKε (an inhibitor of NF-κB binding) and is associated with upregulated cytokine expression. *H. pylori* infection of gastric epithelial cells and human embryonic kidney cells shows that the over-expression of *miR-155* leads to decreased IKKε[365]. Overall, *miR-155* is ubiquitously expressed and has multiple roles during TLR signalling that increase inflammation.

1.6. MIR EXPRESSION IN THE PLACENTA

Placentation is a complex process influenced by epigenetic regulation that is responsive to environmental cues throughout gestation. The placenta is a dynamic organ that requires temporal regulation as it develops and therefore, the differential expression of epigenetic regulators play a critical role during pregnancy. As miRs influence vast networks of cellular processes, it is unsurprising that they are emerging as key regulators of placental gene expression.

Microarray analyses have revealed that the human placenta expresses approximately 600 different miRs, including those exclusive to gestational tissues[366-369]. MiRs that are involved in proliferation, differentiation, cell death and metabolism are abundant in the placenta[366, 370]. Placental miR biogenesis was first confirmed in trophoblasts which demonstrated the expression of proteins specific to facilitating miR synthesis and function, including Argonaute2 and Dicer[371, 372]. In fact, the knockdown of Dicer in human placental explants is associated with the over-proliferation of cytotrophoblast cells due to the absence of regulation by miRs[372]. Murine models have also shown that the knockout of miR biogenesis machinery in the placenta causes impaired angiogenesis and compromised embryonic survival[373]. Together, these studies highlight the role of miRs in regulating placental development and function.

Interestingly, the preterm placenta has a unique miR profile, showing differential expression of 20 placenta-specific miRs compared to term[374]. Further, placental and decidual miRs are differentially expressed in preterm compared to term deliveries with chorioamnionitis[375, 376], suggesting that inflammatory regulation occurs differently at different gestational ages. Deep-sequencing has shown that preterm villous trophoblasts differentially express 7 inflammatory miRs with intra-amniotic infection compared with iatrogentic preterm birth[377]. Notably, there is no difference observed in the decidua of these placentae, highlighting the tissue-specific nature of miR-based regulation.

Unique clusters of miRs are differentially expressed throughout gestation in the placenta[378]. MiRs associated with angiogenesis and cell survival are dominant in first trimester placentae, while those associated with cell differentiation are upregulated in

third trimester placentae[378]. A majority of placental miRs are encoded on the C19MC chromosomal cluster, comprising the largest known cluster of human miRs[321, 378]. These miRs become differentially expressed during pathological pregnancies including hypoxia-induced fetal growth restriction[379, 380], pre-eclampsia[374, 381] and preterm delivery[374]. A microarray targeting 762 miRs expressed in term and first trimester trophoblasts and cell lines has identified several miR clusters exclusive to the placenta and 27 miRs that are differentially expressed according to gestational age[382]. This suggests there are diverse miR 'fingerprints' which are unique to gestational age and can influence the phenotype of placental cells.

The differential expression of placental miRs may contribute to altered placental function, however a majority of such evidence remains correlational. For example, decreased placental expression of *miR-16* and *miR-21* is positively correlated with IUGR and adverse pregnancy outcome[383]. These findings are extrapolated from previous studies which suggest that *miR-21* regulates cell proliferation and migration[384], and *miR-16* is involved in apoptosis and the regulation of the cell cycle[385]. Such analysis is open to interpretation as miRs can have tissue-specific patterns of expression and function[386] and the pathogenesis of IUGR is multifactorial. Studies which transfect or interfere with miRs are therefore required to elucidate precise bioactive pathways or pathological mechanisms. As such, though different placental miRs have been identified by array analyses, their specific function is yet to be elucidated and are currently limited to *in silico* analyses. Further, there are few studies on the involvement of miRs in regulating TLR signalling. As miRs have been demonstrated to play critical roles in regulating networks of inflammation, it is important to characterise their roles in the regulation of placental inflammation and how their expression many be associated with the regulation of neonatal immunity.

1.7. THE REGULATION OF NEONATAL INNATE IMMUNITY BY MIRS

Our understanding of miR expression in neonatal tissue is limited, although it may be key to understanding differences in immune regulation compared to adults. The differential expression of miRs that regulate TLR signalling may also explain differences between term and preterm cord blood cytokine responses to immune stimulation (see section 1.5). In adults, miRs play an important role in the development and differentiation of different immune cells (for review see[359]) and given their influence on developmental processes (e.g. angiogenesis and cell differentiation), they are also likely contributors to immune ontogeny.

Literature regarding neonatal expression of immune-regulatory miRs is particularly scarce. The decrease in TNFα expression demonstrated by cord blood monocytes following LPS stimulation compared to adult monocytes (with no difference in NF-κBp65 expression) may result from post-transcriptional regulation by miRs[387]. In fact, decreased *miR-125b* is associated with increased TNFα in cord blood monocytes following *in vitro* LPS exposure, while overexpression of *miR-125b* represses TNFα[387]. Most other studies on cord blood miR expression lack such mechanistic insight. For example, Charrier et al. associated increased *miR-146a* and decreased *miR-155* expression in cord blood pDCs with dysfunctional responses to viral stimulation relative to adult pDCs[388], but did not confirm any changes in target mRNA expression. Similarly, 20 differentially expressed miRs were reported by a study comparing cord blood and adult mononuclear cells[389], but their functional effects were not defined.

In a seminal study on cord blood miR responses to endotoxin, Lederhuber et al. found the constitutive expression of *miR-146a* and *miR-146b* was comparable between cord blood and adult blood[242]. Following LPS stimulation, they observed a time-dependent upregulation of *miR-146a* and *miR-146b* in cord and adult blood, however, at 24 hours post-LPS exposure, there was a significant, two-fold upregulation of *miR-146a* in cord blood compared to adult blood. *miR-146a* therefore appears to be essential for the negative regulation of TLR4 signalling in both adults and neonates. This extended

period of increasing *miR-146a* expression observed in neonates may suggest an increased reliance on tolerogenic mechanisms. Notably, this study only characterised *TLR4* expression alongside *miR-146a*/*b* and saw no difference between adult and cord blood responses, which is contrary to previous literature on stimulated TLR4 responses (Table 1.1). Further, known targets of *miR-146a/b*, *IRAK1* or *TRAF6* were not characterised.

Microarrays of peripheral neonatal blood has shown that inflammation such as chorioamnionitis alters the preterm transcriptome, including miR expression[390]. Preterm neonates exposed to chorioamnionitis differentially express 488 genes compared to unexposed preterm neonates, with one of the top pathway regulators being *miR-155* (which was upregulated). Similarly, exposure to maternal smoking during pregnancy has been associated with increased cord blood *miR-223* expression and decreased Treg numbers compared to cord blood from non-smoking mothers[391]. As *miR-223* is mainly expressed on leukocytes and has roles in granulocyte development and function, it may directly repress Treg development. Further, the concentration of cigarette smoke toxins in maternal urine is associated with a dose-dependent decrease in cord blood *miR-155* expression. This suggests pro-inflammatory insults during pregnancy may alter post-natal immune regulation, at least as mediated by miRs. Together, these findings begin to clarify a role for miRs in top-down regulatory networks of inflammatory gene regulation in neonates, though they require validation studies to confirm their mechanistic influence.

1.7.1. REGULATION OF PRETERM NEONATAL INNATE IMMUNITY BY MIRS

Currently, there is no literature assessing the impact of preterm delivery on immune miR expression in neonates. Differential expression of miRs according to gestational age could be a key factor contributing to differences between the preterm and term neonates' immune function. Given that LPS-stimulated *TLR4* expression is correlated with gestational age[35, 288], it is likely that the expression of *let-7e* is also age-dependent. Existing studies on miRs expressed by preterm peripheral blood or placenta use microarray techniques that are more exploratory for the sake of establishing biomarkers

(for review see[392]). For example, peripheral blood from neonates with BPD shows increased *miR-152*, *miR-30a*, *miR-133b* and *miR-7* expression compared to healthy neonates[393]. These findings could, however, be part of a more generalised inflammatory response.

Though studies have provided observational insights into differential miR cluster expression in preterm tissues, none have offered mechanistic evidence as to how these miRs are altered in preterm neonates and what impact this has on innate immune function. For example, Rogers et al. identified differential expression of the *miR14~92* gene cluster in autopsies of preterm infants who died from severe BPD[394]. The cluster is typically associated with normal lung development[395] but in the lung tissue of deceased infants with BPD, it was downregulated[394]. The controls for this study were deceased term infants that did not suffer from non-respiratory mortalities, which questions the validity of their use as a control group. To consolidate their findings, the authors showed decreased *miR-17* and *miR-19b* in serum from preterm infants who later developed BPD. They postulated that because these miRs typically downregulate TGFβ expression, their downregulation would predispose preterm neonates to the aberrant inflammation observed in BPD sufferers. While it authors suggest these miRs could be used as biomarkers for screening BPD in vulnerable preterm infants, these findings do not confirm a precise mechanism for their role in aberrant respiratory inflammation. Identifying such mechanisms is critical, because it is likely that whole networks of inflammatory genes are impacted by the pathogenesis of BPD. Although miRs can be correlated with particular disease states or increased under experimental conditions, there often remains the need to demonstrate causality[396].

The difficulty in identifying miRs that regulate neonatal innate immunity is that miR expression can influence entire networks of genes. Preterm birth encompasses multiple phenotypes and in the context of inflammation, genetic alterations affect entire networks of physiology with both scientific and clinical implications. MiRs that are differentially expressed between term and preterm neonates may contribute to perturbed maturity of this system. Currently, knowledge of miR expression and the regulation of inflammatory responses by neonates remains in its infancy. Characterising differential

miR expression could therefore improve diagnostic and prognostic phenotyping or identify potential therapeutic targets for managing inflammation in preterm neonates.

1.8. SYNOPSIS

Preterm neonates are at higher risk of developing inflammatory morbidities compared to their term-born counterparts. TLR signalling is a critical component of inflammation and *in vitro* cord blood studies have shown that it is impaired in preterm neonates. As such, decreased expression of TLRs and cytokines following innate immune stimulation is observed in preterm compared to term cord blood. Preterm neonates are also more susceptible to developing neutropenia and demonstrate decreased phagocytic and cell-mediated killing capacities compared to term neonates. Such attenuation of innate immune function typically increases the likelihood of contracting infection. Preterm neonates are, however, also susceptible to developing conditions characterised by aberrant inflammation including neonatal sepsis, RDS and NEC. The mechanisms underlying these paradoxes in innate immune dysfunction remain unclear, but may relate to immature regulatory signalling.

MiRs have recently emerged as critical regulators of inflammation, but their expression remains poorly characterised in the context of neonatal immunity. Differences in miR expression between preterm and term neonates could contribute to immune dysregulation. To address this gap in immunological research, this thesis will assess the impact of preterm delivery on the expression of miRs and other genes associated with TLR signalling in placenta and cord blood. The identification of mechanisms that predispose preterm neonates to inflammatory conditions could lead to more targeted diagnostic and therapeutic interventions to ameliorate adverse health outcomes.

1.9. AIMS & HYPOTHESES

The aim of this thesis is to investigate TLR signalling pathways in cord blood and placenta to understand factors that may contribute to inflammatory dysregulation in preterm neonates. Specifically, the studies contained in this thesis aim to:

- Characterise the expression of genes associated with TLR signalling, including miRs and their targets (mRNAs) in preterm and term placenta at birth;
- Characterise the expression of miRs and their mRNA targets, and cytokines in preterm and term cord blood at birth;
- Characterise the expression of miRs and mRNAs in preterm and term cord blood following TLR stimulation to mimic exposure to common neonatal pathogens;
    - Identify how this response changes over time *in vitro*; and
- Conduct a bioinformatics-based analysis of TLR signalling molecules to identify network-level alterations in preterm neonates' peripheral blood and identify novel mechanisms and/or targets for further research.

In the context of genes associated with TLR signalling, it is hypothesised that:

- Preterm placenta will show a pro-inflammatory gene expression profile, including decreased anti-inflammatory miRs and increased inflammatory genes;
- Preterm cord blood will show decreased anti-inflammatory miR expression and increased inflammatory gene expression compared to term at birth;
- Following *in vitro* stimulation with TLR2, 3 and 4 agonists, preterm cord blood will show:
    - decreased expression of anti-inflammatory regulators including *let-7e*, *miR-146a* and *SOCS1* compared to term;
    - increased expression of pro-inflammatory mediators including *miR-155*, *miR-106a* and *IL6* compared to term;
    - increased pro-inflammatory gene expression over time compared to term cord blood; and
- Preterm neonates with infection will demonstrate an inflammatory gene network biased towards pro-inflammatory pathways compared to term neonates with infection.

Chapter 2:

General Methods

2.1. SUBJECT RECRUITMENT

Pregnant women (*n*= 152) presenting and delivering at term (control group; ≥ 37 weeks gestation), late preterm (32-37 weeks) or early preterm (≤ 32 weeks) gestation were recruited to this study by the researcher, research midwife or consulting neonatologist. All participants delivered at either the Lyell McEwin Hospital (LMH) or the Women's and Children's Hospital (WCH) between 2012 and 2016 in Adelaide, South Australia. All participants provided informed written consent for participation in this study. Neonates with known congenital malformations were excluded from this study. Clinical data were collected from health records including obstetric history, delivery details and neonatal health outcomes. Birth weight centiles were calculated using the GROW v6.7 Customised Centile Calculator[397]. Intra-uterine growth restriction (IUGR) was defined as birth weight below the 3rd centile, while small for gestational age (SGA) was defined as birth weight below the 10th centile. Chorioamnionitis was confirmed using histopathological reports. Ethical approval for this study was provided by the human research ethics committees of the Lyell McEwin Hospital (Approval: HREC/14/TQEHLMH/158), Women's and Children's Hospital (Approval: REC2198/7/12) and the University of Adelaide, Adelaide, SA, Australia.

2.2. SAMPLE PROCESSING

All reagents and materials are listed in Appendix I.

All cord blood samples (*n*=152) were analysed for baseline gene expression (Chapter 4) and a subset of matched placentae (*n*=60) were analysed for baseline gene expression (Chapter 3). A subset of cord blood samples were also stimulated with TLR agonists *in vitro* for analysis in Chapter 5 (*n*=61). A smaller subset of samples from this group was used for a time course study of *in vitro* TLR stimulation in Chapter 6 (*n*=49). Limitations in cord blood volume were the determinants for sample size in each experimental chapter.

2.2.1. CORD BLOOD

Umbilical cord blood was collected in lithium heparin vials at delivery and processed within one hour of collection. Samples consisted of a mixture of arterial and venous cord blood. One aliquot of whole blood (300µL) was stored at -80°C for baseline analysis of RNA expression. Approximately 6mL of cord blood was used for *in vitro* culture. Remaining cord blood was centrifuged (10 minutes at 3,500 rpm, 4°C) and separated serum was removed and frozen at -20°C for cytokine analysis (section 2.4).

2.2.2. PLACENTAL TISSUE

Placentas were collected within one hour of delivery. Approximately 5mm of surface tissue from the maternal surface was removed prior to sampling to maximise genetic sampling of placental rather than maternal tissue. Samples were dissected from five cotyledons and pooled together. Dissection avoided obvious major vessels, blood clots and calcified or fibrous tissue. Aliquots of the pooled placental tissue (50mg) were snap-frozen in liquid nitrogen and stored at -80°C.

2.3. CORD BLOOD CULTURE AND STIMULATION

2.3.1. WHOLE CORD BLOOD *IN VITRO* TLR STIMULATION

Previous studies have highlighted the importance of cellular cross-talk and plasma-based factors during an immune response (see 1.5.2). Further, previous findings from our laboratory have indicated that CBMCs do not respond as robustly to TLR stimulation as whole cord blood[153]. Whole cord blood was therefore used to investigate gene and protein expression in this thesis.

Replicates of cord blood (100µL) were cultured in flat-bottomed 96-well cell culture plates at 37°C and 5% CO_2. Cultures were rested overnight prior to TLR stimulation to minimise any potential effect of labouring delivery.

TLR agonists were provided as lyophilised powders. PGN from the cell wall component of *S. aureus* and synthetic Poly I:C were reconstituted in sterile water, and LPS derived from *E. coli* was reconstituted in sterile DPBS, as per manufacturer's instructions.

Following an overnight resting period, cord blood was stimulated with PGN (10ng/mL; 10µL), Poly I:C (50ng/mL; 10µL) or LPS (100ng/mL; 10µL). TLR agonist concentrations were determined from existing studies[52, 256] and optimised in our laboratory. Both time and agonist concentration optimisation studies were performed, determining the peak synthesis phase of IL6 and TNFα production according to agonist dose and over time following TLR stimulation[153]. TNFα and IL6 production both peaked and plateaued 6 hours following TLR stimulation. The 6 hour time point was therefore chosen for the analysis of TLR signalling pathways. Cord blood was removed from culture following 2, 6 or 24 hours stimulation, and triplicates were pooled (to achieve a volume of 300µL that would ensure maximal RNA yield) and stored at -80°C.

2.3.1.1. Stimulation of Diluted Cord Blood for Cytokine Analysis

Whole cord blood was diluted in sterile saline (1:4) and plated (100µL) for cytokine analyses. Diluted blood was used in these experiments due to limitations in cord blood volumes available for analysis from preterm deliveries. Cord blood was cultured in 96 well, flat bottom cell culture plates at 37°C and 5% CO_2 and rested overnight prior to agonist exposure. Following resting, cord blood was stimulated with either PGN (100ng), Poly I:C (5µg) or LPS (1µg). Following stimulation, duplicate wells were pooled and collected at 2, 6 or 24 hours. Pooled samples were centrifuged at 10,000rpm for 5 minutes at 4°C for the separation of supernatant from haemocytes. Supernatant was collected and stored at -20°C until further analysis.

2.4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

A select panel of pro- and anti-inflammatory cytokines and chemokines were investigated using ELISA. These targets have well-defined roles in TLR signalling (see 1.3-5).

2.4.1. Single-target ELISA

Single target ELISAs (Human Duo Sets) were used to measure TNFα, IL6 and IL8 in cord blood serum to account for their typically increased serum concentration. The lower detection limit for TNFα was 15.625pg/mL, and the lower detection limit for both IL6 and IL8 was 31.25pg/mL. Samples being assayed for IL6 and IL8 were diluted 1:20 in the appropriate reagent diluent prior to analysis. All inter-assay and intra-assay coefficients were below 10%.

2.4.2. Multi-panel ELISA

Multi-panel ELISA was used to determine the concentration of 7 cytokines and chemokines in cord blood serum (Milliplex MAP Kit). These consisted of GM-CSF, IL1α, IL1β, IL1RA, IL10, IL12p70 and MCP1. These cytokines are well-established as key inflammatory mediators[50]. The limitations and coefficients of variation are listed in Table 2.1.

Table 2.1. ***Detection Specifications for the Milliplex MAP Kit used for this thesis.*** Minimum limit of detection (pg/mL), intra-assay %CV and inter-assay %CV for cytokines measured using the Milliplex MAP Kit.

Target	**Minimum Limit of Detection (pg/mL)**	**Intra-assay %CV**	**Inter-assay %CV**
GM-CSF	7.5	3.1	10.1
IL1α	9.4	3.3	12.8
IL1β	0.8	2.3	6.7
IL1RA	8.3	2.1	10.7
IL10	1.1	1.6	16.8
IL12p70	0.6	2.2	16.7
MCP1	1.9	1.5	7.9

2.5. RNA EXTRACTION AND PURIFICATION

2.5.1. RNA Extraction from Pooled Placental Tissue

RNA extraction from placenta was performed as previously described using TRIzol®[398]. Frozen pooled placental tissue (50mg) was homogenised in TRIzol using 2.8mm Precellys Zirconium Oxide Beads and a Precellys Tissue Homogeniser (2x 20 second cycles at 5,500rpm). Chloroform (300µL) was added to homogenised samples and tubes were manually agitated for 15 seconds. Samples were incubated at room temperature for 10 minutes and then centrifuged (10 minutes at 10,000rpm, 4°C). The upper, clear aqueous phase was transferred into a new RNase-free tube and cold isopropanol (4°C, 1mL) was added. Samples were incubated for 10 minutes at room temperature. RNA was pelleted by centrifugation (10 minutes at 10,000rpm, 4°C) and supernatant was discarded. The pellet was resuspended in cold RNase-free ethanol (1.5mL) and centrifuged (5 minutes at 7,500rpm, 4°C). Supernatant was discarded and the pellet was eluted in RNase-free water (100µL).

RNA was purified using the miRNeasy Mini Kit and RNase-Free DNase Set, according to manufacturer's protocol. Purified RNA was eluted in RNase-free water (50µL) and its concentration was determined using a Nanophotometer. RNA integrity was determined using a RNA 6000 Nano kit and Bioanalyser.

Placental RNA with RNA integrity numbers (RINs) ≥ 5 were used for analysis. Low RINs are typically obtained from placental tissue due to the increased density of RNA in these samples which produces a high level of background fluorescence during electropherogram analysis on microfluidic chips. However, placental RNA integrity ≥ 5 has been shown to be sufficient for reliable qPCR analysis[399, 400].

2.5.2. RNA Extraction from Cord Blood

RNA was extracted from whole cord blood using TRIzol LS® Reagent and purified using a miRNeasy Mini Kit, which extracts all RNA transcripts longer than 18 nucleotides. Three volumes of TRIzol LS were added to one volume of cord blood and vortexed for 1 minute to lyse the cells. The homogenised mixture was incubated at

room temperature for 5 minutes to allow for dissociation of nuclear contents. Chloroform (200μL) was added to each sample and agitated for 15 seconds. The RNA extraction and purification procedure from this stage onwards was identical to that previously described for placental tissue (see section 2.5.1.). Cord blood RNA with RINs ≥ 9 were used for qPCR analysis.

2.6. QUANTITATIVE REAL TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-RT-PCR)

2.6.1. qRT-RT-PCR for miRNA

Purified miRNA (10ng) was reverse transcribed using a Taqman MicroRNA Reverse Transcription Kit. The manufacturer's protocol was modified slightly to accommodate for five RT primer targets per reverse transcription reaction. This was performed to enable consistency and the efficient use of reagents. Samples were amplified in reactions containing RNA in Nuclease-free water (10ng in a 5µL volume). Pooled reverse transcription contained a mixture of dNTP mix (0.19µL), Multiscribe enzyme (1.25µL), 10xRT buffer (1.25µL), RNase inhibitor (0.24µL), five RT primers (1.12µL each) and RNA in nuclease-free water (5µL). Seven miR primers were used for cDNA synthesis (*let-7e, miR-155, miR-146a, miR-146b, miR-106a, RNU6B* and *RNU48* (see Table 2.2)). The reaction mixture was adjusted to a total volume of 13.77µL using nuclease-free water. The reaction was incubated on a Thermal Cycler at 16°C for 30min, 42°C for 30min and 85°C for 5min, and terminated at 4°C.

Relative miRNA expression was quantified using Taqman MicroRNA Assays. Each 20µL reaction contained the cDNA template (10ng in 1.33 µL), RNase-free water (7.67µL), the relevant Taqman gene microRNA expression assay (1µL) and Taqman Universal PCR Master Mix II without UNG (10µL). Relative miR expression was determined using the comparative cycle threshold ($2^{-\Delta CT}$) method using the QuantStudio 12K Flex PCR machine. As miRs are short, non-coding RNA transcripts, endogenous snoRNAs are often used as comparative controls during PCR. The snoRNAs *RNU48* and *RNU6B* were therefore identified as candidate housekeeping genes from previous literature investigating cord blood[242] and placental miR expression[386]. The PCR consisted of two holding stages (2 min at 50°C and 10min at 95°C) and two amplification stages (15sec at 95°C and 60sec at 60°C).

Table 2.2. ***A summary of miR primers used for this thesis.*** The miR assay name and assay ID for primers used in this thesis.

miR	Assay name	Manufacturer's Assay ID
let-7e	hsa-let-7e-5p	002406
miR-155	hsa-miR-155	002623
miR-146a	hsa-miR-146a	000468
miR-146b	hsa-miR-146b	001097
miR-106a	hsa-miR-106a-5p	002169
RNU6B	NR_002752	001093
RNU48	NR_002745	001006

2.6.2. qRT-RT-PCR for mRNA Targets

Purified RNA was reverse transcribed using a SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR according to manufacturer's protocol. Briefly, samples were amplified in 20µL reactions containing RNA (50ng) in RNase-free water, 2xRT enzyme mix (10µL) and RT Enzyme mix (2µL), constituted in RNAse-free water. The cDNA synthesis reaction was performed on a Thermal Cycler at 25°C for 10 minutes, 50°C for 30 minutes and 85°C for 5 minutes. Samples were cooled to 4°C and *E.coli* RNase H (1µL) was added to degrade any remaining RNA. Samples were reheated and held at 37°C for 20min. Converted cDNA was stored at -20°C until qPCR.

The PCR reaction was performed using TaqMan® Gene Expression Assays. Each 20µL reaction contained the cDNA template in RNasefree water (100ng in 2µL), the relevant Taqman gene expression assay (1µL each) and 2X Taqman® Gene Expression Fast Master Mix (10µL). Taqman gene expression assays included *β-actin* (Human ACTB, Hs99999903_m1), *IL6* (Hs00985639_m1), *IL10* (Hs00961622_m1), *IRAK1* (Hs01018347_m1), *MyD88* (Hs00182082_m1), *NF-κB1* (Hs00765730_m1), *SOCS1* (Hs00705164_s1), *TLR2* (Hs00610101_m1), *TLR3* (Hs01551078_m1), *TLR4* (Hs01061963_m1) and *TRAF6* (Hs00377558_m1). Relative mRNA expression was determined using the QuantStudio 12K Flex PCR machine. The PCR consisted of two holding stages (2 minutes at 50°C and 20 seconds at 95°C) and an amplification stage (40 cycles of 95°C for 3 seconds and 60°C for 30 seconds). The $2^{-\Delta CT}$ method was used

to determine mRNA abundance relative to the appropriately determined housekeeping gene, *β-actin*[401].

2.7. BIOINFORMATICS

2.7.1. TLR SIGNALLING PATHWAY ANALYSIS

In silico bioinformatics and statistical analyses were used to examine networks of gene expression in preterm neonatal TLR signalling pathways. We analysed two publicly available custom RNA-sequencing (RNA-seq) datasets profiling whole blood from early and late preterm neonates published by other authors[390, 402, 403]. One of these datasets utilised an Illumina HT12 platform validated using the CodeLink gene platform to perform an array on whole peripheral blood collected from neonates admitted to the neonatal unit who were having their blood sampled for clinical reasons including maternal thyroid disease, jaundice or suspected infection[403]. The second dataset was from a study that sought to characterise neonatal sepsis using blood from neonates with suspected sepsis. Gene expression was analysed using an Affymetrix GeneChip GGh3 Transcriptome Array[390, 402].

The NCBI Gene Expression Omnibus (GEO) is a public repository of high-throughput experimental data[404]. The software package GEOquery was used to download the relevant datasets from the GEO repository (GSE69686 and GSE25504). The GEO2R web application was then used to analyse differentially expressed genes within these microarray datasets. Data were normalised using log transformation before analysis. Bioinformatics analyses were performed in collaboration with Dr Jimmy Breen (Bioinformatics core-facility manager, Robinson Research Institute, University of Adelaide) using the R software package, *limma*. *Limma* is a library used to analyse gene expression microarray data by making simultaneous, multiple comparisons between RNA targets[405]. This software package was utilised as it employs empirical Bayesian methods that provide robust analyses where array numbers are low[406]. The R software package was then used to generate heat maps to represent gene expression in counts per million (cpm).

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) is a standardised reference for the definition of biological pathways that uses information from large-scale molecular datasets to define common networks of molecular signalling[407]. Differentially

expressed genes within the TLR signalling pathway (KEGG ID: hsa04620) were identified from the datasets extracted from GEO and these were generated as a heat-map. The top 20 KEGG pathways differentially expressed according to neonatal bacterial infection and neonatal sepsis.

2.7.2. CELL TYPE ENRICHMENT ANALYSIS

The Immuno-Navigator database[408] as used to query target genes in the TLR signalling pathway (defined by KEGG) to identify correlation scores of gene co-expression in specific adult tissues (sorted by significance of enrichment). The correlation network hub prediction tool was used to determine how many networks the listed genes were involved in, for specific immune cell types. The sub-functionalisation of these genes was isolated in healthy, unstimulated adult peripheral blood immune cells. Publicly available data from adult whole blood was used to explore the expression of these genes in specific immune cell types, as there is currently no data derived from neonates. Heat-maps were generated according to the expression of inflammatory genes within the data set and their association with particular cell types.

2.8. STATISTICAL ANALYSES

Data were analysed using the Statistical Package for Social Sciences (SPSS v24). Graphs were generated using GraphPad Prism software v6.00 (GraphPad Software, Inc.). Where normally distributed, data regarding clinical characteristics were analysed using t-tests or ANOVAs and presented as mean ± SD, unless otherwise indicated. Where data were not normally distributed, clinical data were analysed using Mann-Whitney U or Kruskal-Wallis tests. qPCR data were logarithmically transformed (log_{10}) to normalise data for analysis. Raw data were presented as median (25^{th}-75^{th} centile), unless otherwise indicated. All data reporting frequencies were analysed using Pearson's Chi-squared tests. ANCOVAs or MANCOVAs were used to analyse log-transformed qPCR data, with pre-eclampsia, labouring delivery, maternal smoking during pregnancy, birthweight centile, neonatal sex, chorioamnionitis and antenatal betamethasone exposure used as covariates where relevant. Post-hoc comparisons were conducted using t-tests or ANOVAs where appropriate. An *a priori* Bonferroni correction was made to the critical alpha level for post-hoc comparisons. Spearman's correlations were used to assess the relationship between the matched sample expression of untransformed miR and mRNA data. An alpha level of 0.05 was considered statistically significant.

Chapter 3:

The Expression of Genes Associated with TLR signalling in the Placenta

3.1. ABSTRACT

Background

Preterm birth is commonly associated with an inflammatory aetiology. Markers of placental inflammation, such as genes associated with TLR signalling, may therefore be associated with innate immune immaturity in the preterm neonate. TLR signalling is regulated post-transcriptionally by miRs and the expression of these genes may be critical in regulating the *in utero* environment. Intrauterine perturbations caused by maternal physiology such as pre-eclampsia, smoking and growth restriction also affect placental inflammation. This study aimed to characterise the expression of miRs and mRNAs in the TLR signalling cascade in term and preterm placenta.

Methods and Results

Term and preterm placenta were collected and homogenised at delivery. The expression of miRs and mRNAs was quantified using qPCR. There was no difference in the expression of the miRs or mRNAs assessed between term and preterm placenta. Term, but not preterm, placental *miR-155* and *miR-146a* expression were correlated with their respective targets *SOCS1* (p=0.015) and *IRAK1* (p=0.005). Maternal smoking during pregnancy was associated with decreased *let-7e* (p=0.039) and increased *TLR2*, *MyD88*, *IRAK1*, *NF-κB1*, *SOCS1*, *IL6* and *IL10* expression (all $p<0.05$).

Conclusion

An association between miRs and their mRNA targets was evident in term but not preterm placenta, suggesting TLR signalling may not be regulated in the same manner throughout gestation. Further, maternal smoking during pregnancy was associated with decreased regulatory *let-7e* expression and increased expression of genes in the TLR signalling cascade. Together, these data suggest that the regulation of the TLR signalling pathway may be altered by prematurity and/or inflammation, though this requires further investigation.

3.2. INTRODUCTION

Intrauterine inflammation (IUI) is the most common known cause of preterm birth[10, 110, 145]. Acute or prolonged IUI (pathogen driven or sterile) is associated with poor fetal outcomes such as IUGR[409] and fetal death[130, 131]. Despite the increased prevalence of these adverse outcomes, the impact of IUI on the neonatal innate immune system remains poorly characterised.

In animal models of infection, IUI 'programs' the postnatal immune response. Rodents exposed to endotoxin *in utero* show attenuated pro-inflammatory cytokine responses when re-exposed as neonates[101, 103]. These mice also demonstrate decreased leukocyte recruitment and TNFα and IL1β production. In humans, intrauterine infection increases inflammation in the placenta, cord blood and neonatal peripheral blood[139, 409]. It is therefore commonly associated with inflammatory morbidities including sepsis[410], NEC[411] and CLD[412]. Other inflammatory insults such as maternal smoking during pregnancy have also been associated with increased numbers of pro-inflammatory M1 macrophages in the placenta[413] and subsequently, an increased incidence of childhood atopy amongst offspring[414]. Overall, increased inflammatory signalling *in utero* may contribute to adverse pregnancy and neonatal health outcomes.

TLRs are key drivers of an inflammatory response. Their activation by class-specific ligands elicits a cascade of intracellular signalling through adaptor molecules, resulting in increased cytokine production. Increased TLR activation has been associated with parturition in humans[415] and murine models where administering TLR agonists to the uterine horn induces delivery[416]. As such, studies in mice have also demonstrated that inhibiting TLR4 signalling arrests preterm birth normally induced by endotoxin[417, 418]. TLR signalling can be regulated by negative or positive feedback loops involving cytokines, transcription factors and epigenetic regulators, including miRs. MiRs are non-coding mRNAs that repress gene translation post-transcriptionally, and can therefore regulate different stages of TLR signalling. For example, *let-7e* targets and represses the *TLR4* transcript to dampen TLR signal activation, while indirect regulators such as *miR-155* repress *SOCS1* and therefore increase cytokine signalling. Overall, the regulation of TLR signalling in the placenta remains poorly characterised.

Previous studies highlight the key role of miRs during placentation[366, 370]. Altered signatures of miR expression in the placenta and fetal membranes have been associated with poor pregnancy outcomes including preterm birth[374, 377]. Preterm placenta demonstrate increased pro-inflammatory[419, 420] *miR-210* and *miR-223* expression in the amnion compared to term[421]. Additionally, pregnancies conceived by assisted reproductive technology that deliver preterm show increased placental TLR4 signalling and NF-κB activation, and decreased *miR-146a* expression compared to term placenta[422]. Notably, a majority of these studies on placental miR expression have only found associations between miRs and poor pregnancy outcomes, without characterising target genes directly. As such, the impact of differential miR expression on placental TLR signalling pathways remains poorly understood, particularly in association with preterm delivery.

This study aimed to profile components of the TLR signalling cascade (both miRs and their mRNA targets) in term and preterm placenta. Due to previously reported impacts on inflammatory signalling, betamethasone exposure[423], maternal smoking[424], pre-eclampsia[425] and IUGR[426] were also considered in this analysis. It was hypothesised that preterm placenta would show increased expression of pro-inflammatory genes associated with TLR signalling and decreased anti-inflammatory regulators compared to term placenta.

3.3. METHODS

3.3.1. PARTICIPANTS & SAMPLE COLLECTION

60 pregnant women presenting at the LMH and WCH, who delivered at term (≥ 37 weeks) or preterm (<37 weeks) gestation were recruited to this study. Clinical data were obtained from maternal and neonatal health records, including obstetric history and neonatal outcomes (see section 2.1). Following delivery, placenta were immediately collected and tissue was pooled from five cotyledons.

3.3.2. WHOLE RNA EXTRACTION

TRIzol® reagent was used to extract RNA from pooled placental tissue (see section 2.5.1). Briefly, three volumes of TRIzol were added to one volume of placenta and samples were mechanically homogenised. Lysates were separated by centrifugation with chloroform to retrieve the interphase for subsequent washing with isopropanol and ethanol. RNA was eluted in RNase free water and purified using a miRNeasy Mini Kit and DNAse I treatment as per manufacturers' protocol. RINs were assessed using a bioanalyser, where a RIN≥ 5 was considered sufficient for PCR analysis.

3.3.3. qRT-PCR ANALYSIS OF miRNA EXPRESSION

RNA (10ng) was reverse transcribed using a Taqman® MicroRNA Reverse Transcription Kit according to manufacturer's protocol that was slightly modified for optimal cDNA output (see section 2.6.1). This involved pooling a maximum of five RT primers per batch in a single RT mixture. Reverse transcription targeted seven primer sequences: *let-7e, miR-155, miR-146a, miR-146b, miR-106a, RNU6B* and *RNU48*. Relative miRNA expression was quantified using Taqman® MicroRNA Assays in 20µL reactions containing 1.33µL of cDNA. qRT-PCR utilised the comparative cycle threshold ($2^{-\Delta CT}$) method to determine miR abundance relative to the reference genes *RNU48* and *RNU6B.*

3.3.4. qRT-PCR ANALYSIS OF mRNA EXPRESSION

Purified RNA (100ng) was reverse transcribed using a SuperScript™ III First-Strand Synthesis Kit for qRT-PCR according to manufacturer's protocol (see section 2.6.2). qRT-

PCR was used to quantify the relative expression of miR targets including *TLR2, TLR3, TLR4*, *MyD88*, *IRAK1*, *TRAF6*, *NF-κB1, IL6, IL10* and *SOCS1* relative to the housekeeping gene*, β-actin*. qRT-PCR was performed using TaqMan® Gene Expression Assays and each 20μL reaction contained 2μL of the cDNA template.

3.3.5. STATISTICAL ANALYSES

Data were analysed using SPSS v24. Demographic and clinical data are presented as mean ± SD, unless otherwise indicated. Frequency data were analysed using Chi-squared tests. T-tests and Mann-Whitney U tests were used to compare continuous data according to gestational age groups. Gene expression data are presented as median (25^{th}-75^{th} centile), unless otherwise indicated. Data were logarithmically transformed (log_{10}) for subsequent parametric analysis. ANCOVAs or MANCOVAs were used to analyse qPCR data, with pre-eclampsia, labouring delivery, neonatal sex, maternal smoking during pregnancy, birthweight centile, chorioamnionitis and antenatal betamethasone exposure used as covariates. Post-hoc comparisons were made using t-tests where appropriate, with an *a priori* Bonferroni correction made to the critical alpha level. Spearman's correlations were used to assess the relationship between matched sample expression of miR and mRNA data. An alpha level of 0.05 was considered statistically significant.

3.4. RESULTS

3.4.1. PARTICIPANT CHARACTERISTICS

Clinical and demographic characteristics of the term (*n*=38) and preterm (*n*=22) deliveries included in this analysis are shown in Table 3.1. Maternal age, BMI, ethnicity, gravidity, parity, cigarette smoking during pregnancy, mode of delivery and the incidence of multiple pregnancy were not significantly different between preterm and term deliveries. The incidence of pre-eclampsia was more frequently associated with preterm (36%) than term delivery (5%; $\chi^2(1)=9.703$, $p=0.002$). Preterm deliveries had a lower gestational age than term neonates ($t(58)=12.636$, $p<0.001$) and were more frequently exposed to antenatal betamethasone compared to term neonates (58% vs 8%, respectively; $\chi^2(1)=18.675$, $p<0.001$). Birthweight was lower in preterm compared to term neonates ($t(58)=7.060$, $p<0.001$), but birthweight centile, and incidence of SGA and IUGR was comparable between the groups. Birth length and head circumference were lower in preterm compared to term neonates ($t(29.631)=6.901$, $p<0.001$; and $t(31.519)=6.578$, $p<0.001$, respectively). Preterm neonates had lower APGAR scores at 1 ($U=273$, $p=0.009$) and 5 minutes compared to term neonates ($U=245$, $p<0.001$). Placental weight was lower in preterm compared to term deliveries ($t(55)=2.637$, $p=0.013$).

Table 3.1. ***Maternal demographic and neonatal characteristics for preterm and term deliveries of placenta analysed in Chapter 3.*** Values are given as mean± SD or *n* (%), unless otherwise indicated. *P*-values were calculated using Chi-square tests, t-tests or Mann-Whitney U tests, $\boldsymbol{p} \leq 0.05$.

	Term	**Preterm**	***p* value**
	n=38	*n*=22	
Gestational age (completed weeks), median (min-max)	**39 (37-41)**	**35 (30-36)**	**<0.001**
Maternal demographics:			
Age, years	31±4	29±5	0.356
BMI, kg/m^2	31±8	29±8	0.287
Gravidity, median (min-max)	3 (1-9)	2 (1-9)	0.183
Parity, median (min-max)	1 (0-5)	1 (0-5)	0.157
Smoking during pregnancy	9 (24)	4 (18)	0.618
Ethnicity:			
Caucasian	30 (79)	11 (50)	
Indigenous	2 (5)	2 (9)	0.291
Other	6 (16)	9 (41)	
Maternal complications:			
Pre-eclampsia	**2 (5)**	**8 (36)**	**0.002**
Multiple pregnancy	1 (3)	2 (9)	0.269
Labouring delivery	11 (29)	10 (46)	0.196
Mode of delivery:			
Emergency Caesarean section	3 (8)	4 (18)	
Elective Caesarean section	26 (68)	9 (41)	0.108
Vaginal delivery	9 (24)	9 (41)	
Placenta:			
Weight, g	**689 ±184**	**547 ±209**	**0.007**
Histological chorioamnionitis	0 (0)	1 (5)	0.185
Neonatal characteristics:			
Male	20 (53)	9 (41)	0.381
Birthweight, g	**3563 ±592**	**2374 ±689**	**<0.001**
Birth centile, %	52 ±32	41 ±38	0.195
SGA	7 (18)	8 (36)	0.122
IUGR	3 (8)	5 (23)	0.103
Birth length, cm	**50 ±2**	**44 ±3**	**<0.001**
Head circumference, cm	**35 ±1**	**32 ±2**	**<0.001**
Antenatal Betamethasone	**3 (8)**	**13 (58)**	**<0.001**
APGARS, median (min-max):			
1 min	**9 (5-9)**	**8 (2-9)**	**0.009**
5 min	**9 (9-10)**	**9 (6-9)**	**<0.001**

3.4.2. THE EXPRESSION OF HOUSEKEEPING GENES

Two snoRNAs (*RNU48* and *RNU6B*) were identified from the literature as candidate housekeeping genes[242, 386]. Both snoRNAs were expressed in placenta. *RNU48* was more consistently expressed in placenta (median CT 28.6 (IQR=27-30)) compared to *RNU6B* (CT 32.5 (IQR=31-34)). As *RNU48* was less variable than *RNU6B* and did not differ between gestational age groups, it was deemed the most appropriate housekeeping gene for this study.

3.4.3. THE EXPRESSION OF GENES INVOLVED IN TLR SIGNALLING IN THE PLACENTA

There was no difference in the expression of miRs (*let-7e*, *miR-155*, *miR-146a*, *miR-146b* or *miR-106a*) or mRNAs associated with TLR signalling (*TLR2, TLR4, MyD88, IRAK1, NF-κB1, SOCS1, IL6* or *IL10*) between term and preterm placenta, when adjusting for the covariates pre-eclampsia, birthweight centile, neonatal sex, labouring delivery, maternal smoking, chorioamnionitis and antenatal betamethasone (Fig. 3.1-5).

Only 57% of term placentas and 73% of preterm placentas expressed detectable levels of *TLR3* mRNA. The expression of *TRAF6* was not detected in the placenta (Table 3.2).

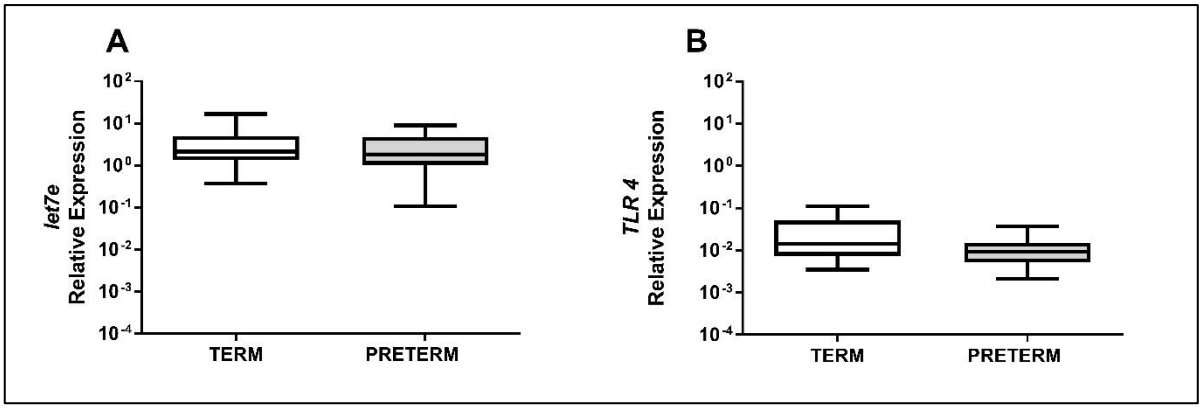

Fig. 3.1. *Placental* let-7e *and* TLR4 *expression following term and preterm delivery.* Placental expression of *let-7e* relative to *RNU48* (A), and *TLR4* expression relative to *β-actin* (B), following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

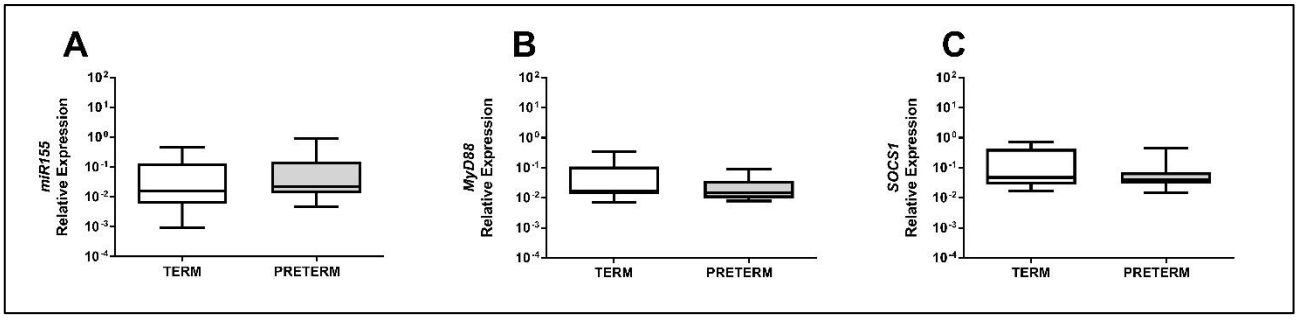

Fig. 3.2. ***Placental*** **miR-155, MyD88** ***and*** **SOCS1** ***expression following term and preterm delivery.*** Placental expression of *miR-155* relative to *RNU48* (A), and *MyD88* (B) and *SOCS1* expression relative to *β-actin* (C), following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

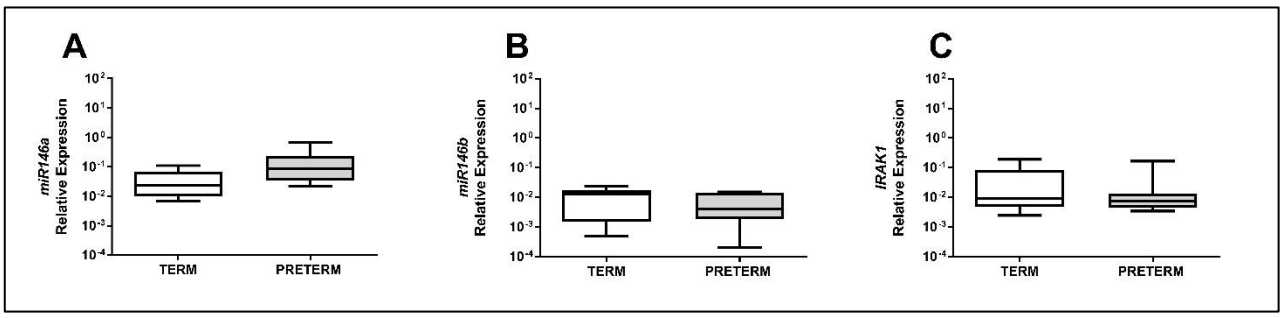

Fig. 3.3. ***Placental*** **miR-146a, miR-146b** ***and*** **IRAK1** ***expression following term and preterm delivery.*** Placental expression of *miR-146a* (A) and *mir-146b* (B) relative to *RNU48*, and *IRAK1* expression relative to *β-actin* (C), following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

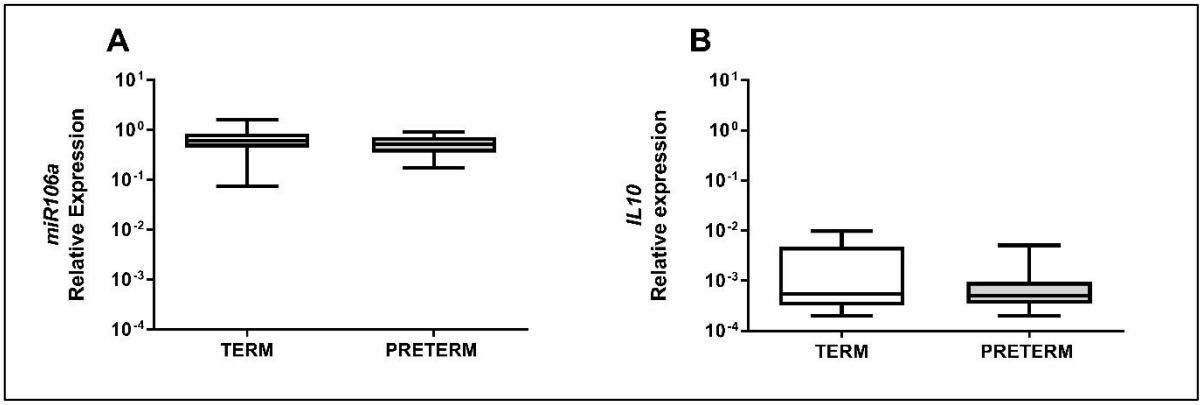

Fig. 3.4. ***Placental*** **miR-106a** ***and*** **IL10** ***expression following term and preterm delivery.*** Placental expression of *miR-106a* relative to *RNU48* (A), and *IL10* expression relative to *β-actin* (B), following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

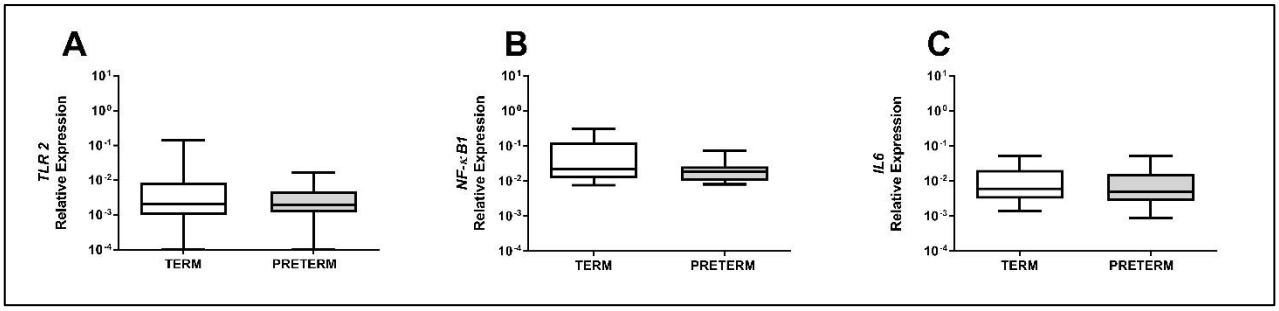

Fig. 3.5. ***Placental*** **TLR2, NF-κB1** ***and*** **IL6** ***expression following term and preterm delivery.*** Placental expression of *TLR2* (A), *NF-κB1* (B) and *IL6* (C) relative to *β-actin,* following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

Table 3.2. ***The frequency of expression for genes associated with TLR signalling in term and preterm placenta.*** The frequency of expression of genes (mRNA) in term and preterm placenta. Data are expressed as the frequency (*n*, %), $\boldsymbol{p} \leq 0.05$.

mRNA	**Term placenta with positive expression** *n*=21	**Preterm placenta with positive expression** *n*=15	***p***
TLR2	21 (100%)	14 (93%)	0.456
TLR3	12 (57%)	11 (73%)	0.402
TLR4	21 (100%)	15 (100%)	0.422
MyD88	21 (100%)	14 (93%)	0.420
IRAK1	21 (100%)	14 (93%)	0.420
TRAF6	0 (0%)	0 (0%)	-
NF-κB1	21 (100%)	15 (100%)	0.422
IL10	18 (86%)	13 (87%)	0.450
IL6	21 (100%)	14 (93%)	0.420
SOCS1	21 (100%)	15 (100%)	0.422

Within our statistical model, the contribution of maternal smoking was identified as having a significant, independent effect on *let-7e* expression. Post-hoc analyses showed that maternal smoking was associated with decreased placental *let-7e* expression (p=0.017), but there was no associated change in the expression of its target, *TLR4* (Fig.3.6). Maternal smoking was also associated with increased placental *TLR2* (p=0.033), *MyD88* (p<0.001), *IRAK1* (p<0.001), *NF-κB1* (p=0.001), *SOCS1* (p<0.001), *IL6* (p=0.014) and *IL10* expression (p=0.043; Fig.3.7). There was no effect of maternal smoking on the expression of any other miRs examined.

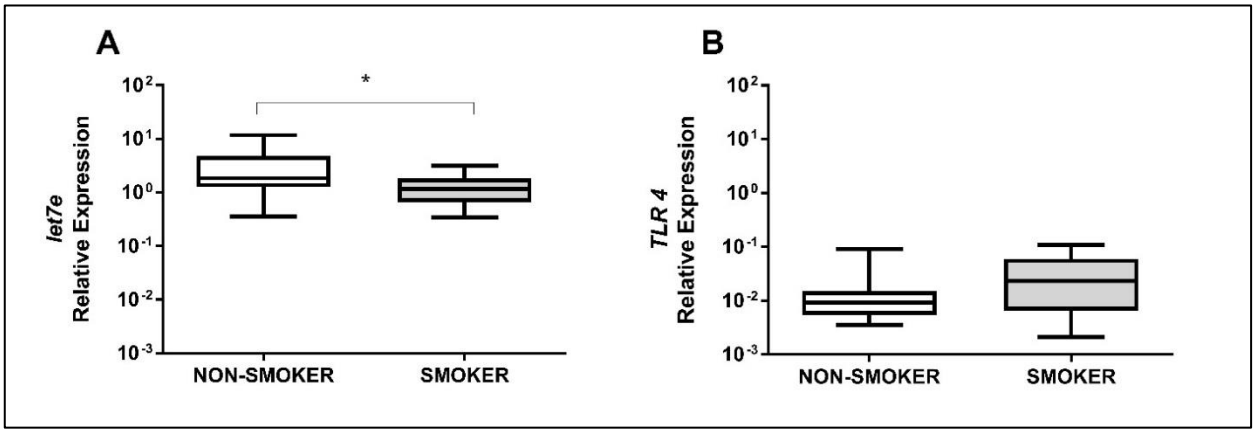

Fig. 3.6. ***Placental* let-7e *and* TLR4 *expression according to maternal smoking during pregnancy.*** Placental expression of *let-7e* relative to *RNU48* (A), and *TLR4* expression relative to *β-actin* (B) in mothers who smoked and mothers who did not smoke cigarettes during pregnancy. Note: y-axis presented on a logarithmic scale, *p<0.05.

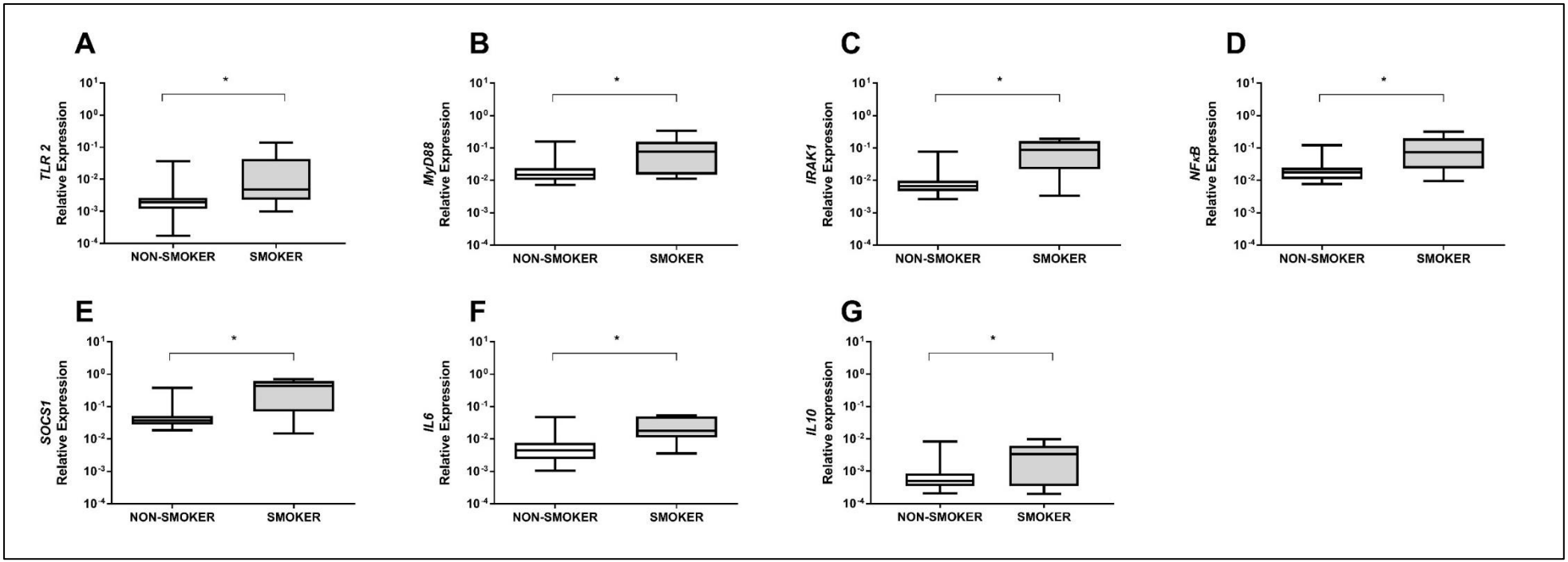

Fig. 3.7. ***The expression of genes associated with TLR signalling in the placenta according to maternal smoking during pregnancy*.** The expression of *TLR2* (A), *MyD88* (B), *IRAK1* (C), *NF-κB1* (D), *SOCS1* (E), *IL6* (F) and *IL10* (G) relative to *β-actin* in placenta from mothers who smoked and mothers who did not smoke during pregnancy, independently of gestational age. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

3.4.4. THE CORRELATION BETWEEN PLACENTAL miR AND TARGET mRNA EXPRESSION

Placental *miR-155* and *SOCS1* expression were positively correlated (ρ=0.377, *p*=0.028). When data were split by gestational age, the correlation was significant in term (ρ=0.535, *p*=0.015), but not preterm placenta (Fig. 3.8).

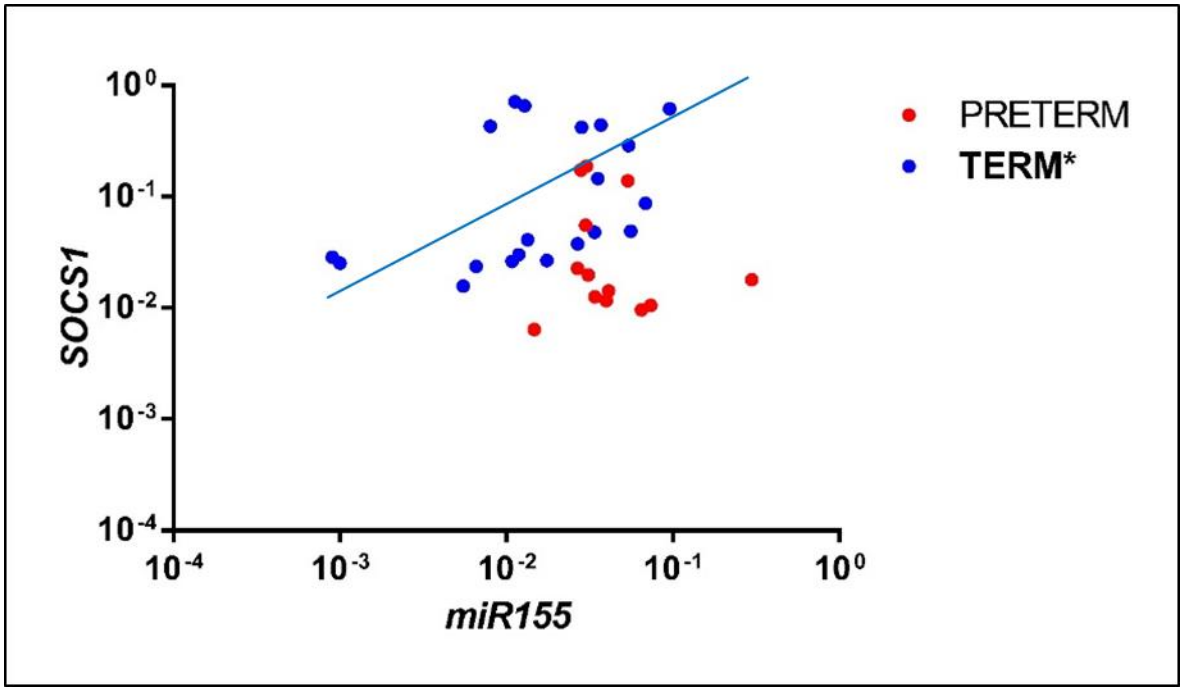

Fig. 3.8. ***A scatterplot of*** **SOCS1** ***and*** **miR-155** ***expression in term and preterm placenta.*** The relative expression of *miR-155* correlated with its target, *SOCS1* in preterm and term placenta. Note: y-axis presented on a logarithmic scale, **p*<0.05.

Placental *miR-146a* and *IRAK1* expression were positively correlated (ρ=0.37, *p*=0.031). When data were split by gestational age, the correlation was significant in term (ρ=0.584, *p*=0.005) but not preterm placenta (Fig 3.9).

Placental *let-7e, miR-146b* and *miR-106a* were not correlated with the expression of their targets *TLR4, IRAK1* or *TRAF6*, or *IL10*, irrespective of the gestational age groups.

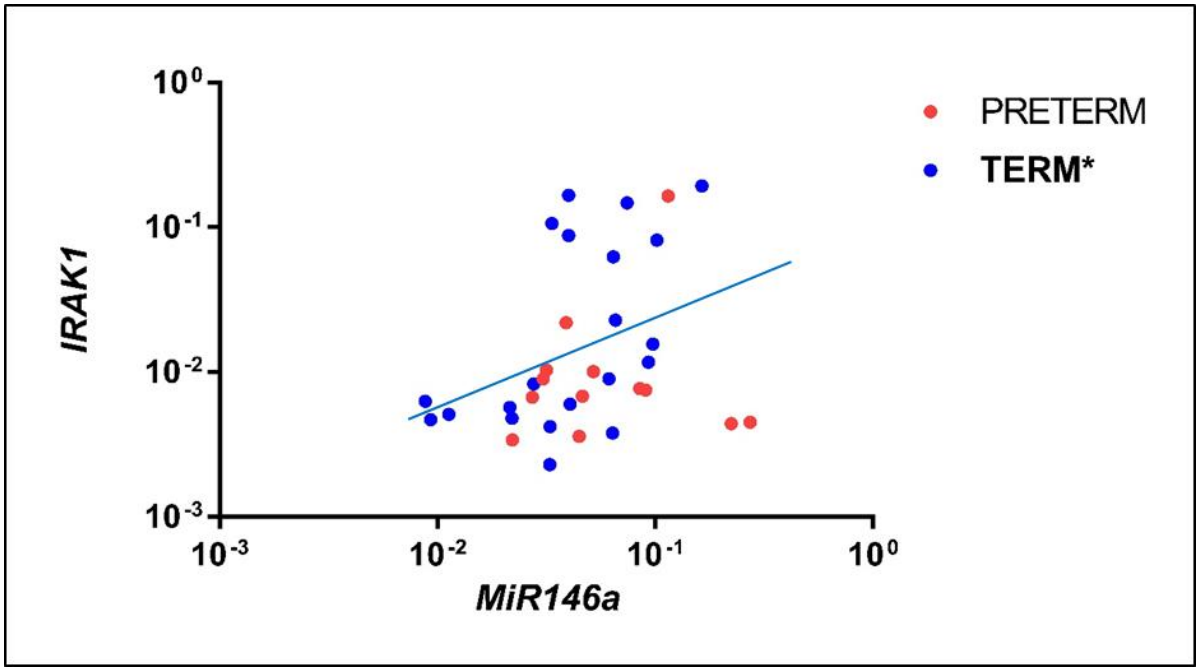

Fig 3.9. ***A scatterplot of IRAK1 and miR-146a expression in term and preterm placenta.*** The relative expression of *miR-146a* correlated with its target *IRAK1,* in preterm and term placenta. Note: y-axes are presented on a logarithmic scale, **p*<0.05.

3.5. DISCUSSION

This study sought to characterise the expression of miRs that regulate the TLR signalling cascade and their targets in term and preterm placenta. No difference in miR or mRNA expression was observed between term and preterm placenta. Correlational relationships between miR and target mRNA expression were identified more frequently in term compared to preterm placenta. The presence of these relationships begins to suggest the post-translational regulation of mRNAs by miRs may be more mature in term compared to preterm placenta. Additionally, maternal smoking was associated with decreased regulatory *let-7e* and increased *TLR2*, *MyD88*, *IRAK1, NF-κB1, SOCS1*, *IL6* and *IL10* expression independently of gestational age, suggesting a pro-inflammatory placental phenotype.

The current study assessed gene expression using homogenised term and preterm placenta. A homogenous cell population was used to provide a more accurate reflection of *in utero* conditions, as others have shown cytotrophoblasts from first trimester placenta express *TLR2* and *TLR4*, while syncytiotrophoblasts do not[427]. Similarly, our analysis of homogenised tissue found no difference between term and preterm placental expression of *TLR2*, *TLR4* or *TLR4*'s regulator, *let-7e* at birth. These findings are in line with previous studies that have shown second[428] and third trimester placental trophoblasts express comparable *TLR1-10 mRNA*[429]. These authors have also shown that following TLR stimulation, third trimester trophoblasts from preterm placenta expressed decreased IL6 and TNFα expression compared to term[429]. They therefore postulated that differences in term and preterm placental immunity may be due to post-transcriptional regulation, which is supported by our correlational analyses.

Relationships between miRs and their targets provide preliminary insights into active inflammatory regulation. As such, we found a positive correlation between *miR-146a* and *IRAK1* in term, but not preterm placenta. *miR-146a* typically downregulates MyD88-dependent signalling through targeting *IRAK1* and *TRAF6* in trophoblast cell lines, ultimately decreasing IL6 and IL10 production[422]. This also supports other authors' observations that preterm trophoblast cytokine expression is decreased compared to term trophoblasts[429]. Overall, these findings suggest that during inflammation, the preterm placenta has diminished regulatory capacity on inflammatory gene expression. Inflammatory

regulation is, however, a complex process that involves networks of gene signalling and therefore these findings should be interpreted with caution. Ultimately, the relationship between these placental miRs and their targets needs to be confirmed using tissue explant stimulation, knockdown cell-lines or luciferase assays.

Term placenta also showed a positive correlation between *miR-155* and its target *SOCS1*, while preterm placenta did not. This again suggests immune-regulatory relationships may be immature in the preterm placenta or that other genes play a more important role in regulating SOCS1 expression. *miR-155* is associated with increased cytokine signalling through the repression of its target, *SOCS1*[341]. Early TLR signalling is typically associated with decreased *let-7e* to allow for the upregulation of *TLR4.* As inflammation proceeds, *miR-155* is then upregulated to repress *SOCS1*, indirectly increasing cytokine signalling[341]. Later during inflammation, IL10 increases to form a negative feedback loop and dampen the immune response, which includes the targeted suppression of *miR-155*[337] to restore *SOCS1* expression and decrease cytokine signalling. While TLR signalling is poorly characterised in studies on placental tissue, it has been demonstrated that placental SOCS1 expression exceeds that of the spleen[430]. It is therefore postulated that placental expression of SOCS1 is integral to maintaining a tolerogenic state *in utero*. As our analysis preliminarily shows no association between miRs and their targets in the preterm placenta, this could indicate a predisposition towards IUI.

Maternal smoking during pregnancy impairs cytotrophoblast development[431] and is also associated with increased oxidative stress[432] and inflammation[413]. Smoking therefore has the potential to affect immunomodulation at the feto-maternal interface. The present study shows that maternal smoking was associated with decreased *let-7e* expression and increased expression of *TLR2*, *MyD88*, *IRAK1*, *NF-κB1*, *SOCS1*, *IL6* and *IL10*. This increase in inflammatory signalling molecules alongside decreased (*let-7e*) or unchanged regulatory miR expression (*miR-155*, *miR-146a* and *miR-106a*) suggests smoking alters the inflammatory profile of the placenta. These findings could indicate perturbed immune regulation in association with maternal smoking, which supports other studies' evidence of a pro-inflammatory *in utero* environment driven by increased ROS production and cellular apoptosis[154, 433]. In a sample of term placentae, Maccani et al. found an association between maternal smoking and decreased *miR-146a* expression, but did not confirm the expression of its target, *IRAK1*[434]. Conversely, our data did not observe any association between smoking

and placental *miR-146a* expression, but confirmed an increase in *IRAK1* expression. Maternal smoking during pregnancy is also a risk factor for preterm birth[435], which is commonly associated with premature inflammatory activation *in utero*[436]. While we could not determine a direct interaction between gestational age and maternal smoking, increased inflammatory gene expression in conjunction with increased anti-inflammatory *IL10* and *SOCS1* expression in our sample could be evidence of the placenta attempting to restore the tolerogenic cytokine balance *in utero*. Ultimately this requires further investigation using a larger sample size recruited with maternal smoking as a primary outcome, though it remains evident maternal smoking is associated with altered inflammatory gene expression.

Glucocorticoids are immunosuppressive and have been shown to decrease placental cytokine expression *in vitro*[437]. However, the same authors found no difference in placental explants' baseline cytokine production between asthmatic mothers who did and did not use inhaled glucocorticoids[438]. Similarly, our sample did not find any difference in placental inflammatory gene expression according to antenatal glucocorticoid exposure. We also observed no change in the expression of these genes in association with pre-eclampsia or chorioamnionitis, which have previously been associated with the differential miR expression in the context of microarrays[374, 376]. Although these conditions are typically associated with inflammation, it is likely we did not observe any effect because these exposures co-occur within our sample. Further studies are therefore required to separate their independent effects.

Interestingly, we found no difference in the expression of the genes measured according to neonatal sex. Previous studies have identified sex-specific expression of both the placental glucocorticoid receptor[439] and cytokines[437]. Notably, these studies were conducted using asthmatic mothers, which indicates that the fetus responds differently to an inflammatory *in utero* environment depending on its sex. Others have confirmed that trophoblasts from pregnancies carrying a male fetus increased pro-inflammatory and decreased anti-inflammatory cytokine production in response to LPS *in vitro*[440]. The authors postulated this response could be a mechanism for the increased incidence of males being born preterm[441]. Our results, however, do not indicate any significant interaction between fetal sex and unstimulated gene expression in homogenised placental tissue samples, irrespective of gestational age. A different cohort may therefore be necessary to investigate sex-differences in placental TLR signalling.

A major strength of the current study is that it is the first to use qPCR to assess miR expression and the expression of previously validated targets in the preterm and term placenta. Although our findings are limited by a lack of functional studies, they have indicated associations between miRs and their targets in term but not preterm placental tissue. These data are important as they suggest different modes of TLR regulation across gestational age. While previous studies have looked at differential miR expression during pathological pregnancies, they present variable results that may be due to differences in experimental protocol. For example, *miR-20a* and *miR-17* were be upregulated in pre-eclamptic placentas when analysed using qPCR, but the same study observed no change in their expression when they used microarray methods[442]. This supports the idea that microarray analyses should be used as explorative tools and reinforced by subsequent qPCR, experimental animal or cell line models once targets are identified.

Although preterm delivery is frequently associated with pathological inflammation, our data have shown that preterm placenta shows similar constitutive expression of genes associated with TLR signalling compared to the term placenta. Notably, we found term placenta showed significant correlations between miRs and their mRNA targets, while preterm did not. In addition to this, maternal smoking, as a risk factor for preterm birth itself, was associated with increased inflammatory gene expression. Overall, the potential lack of a relationship between miR regulators and their targets found in the preterm placenta could suggest an inability to regulate inflammation on immune challenge, though this requires further investigation.

Chapter 4:

The Expression of Genes Associated with TLR-signalling in Preterm Cord Blood

4.1. ABSTRACT

Background

Preterm delivery accounts for 75% of neonatal deaths, half of which are related to inflammatory conditions. Studies on preterm neonates demonstrate decreased expression of Toll-like Receptors (TLRs) and pro-inflammatory cytokines, which does not explain their susceptibility to developing these morbidities. This study aimed to characterise the expression of genes associated with TLR signalling, including microRNAs (miRs), according to gestational age at delivery.

Methods and Results

Term and preterm cord blood was collected at delivery. miR and mRNA expression was quantified using qPCR. Cord blood serum cytokine expression was analysed using ELISA. *let-7e* and *miR-155* expression was decreased in early preterm compared to late preterm and term cord blood (p=0.046 and p=0.018, respectively). There was no difference in the expression of genes associated with TLR signalling (mRNAs) or cytokines between early and late preterm, and term cord blood. However, maternal smoking was associated with decreased *miR-146b* and increased *TLR2*, *TLR4*, *MyD88*, *IRAK1*, *NF-κB1*, *SOCS1*, *IL6* and *IL10* expression (all p<0.05).

Conclusion

These findings suggest early preterm neonates have reduced immuno-regulatory capacity, as influenced by attenuated miR expression. Further, maternal smoking was associated with decreased expression of regulatory *miR-146b* and increased expression of inflammatory TLR-signalling genes. This may predispose neonates to upregulated inflammatory pathways at birth. Overall, the decreased constitutive expression of regulatory miRs by preterm cord blood may contribute to altered innate immune function in early preterm neonates.

4.2. INTRODUCTION

Preterm birth is associated with an increased risk of neonatal morbidity. A majority of these conditions are characterised by aberrant inflammation, including NEC[100], CLD[222] and neonatal sepsis[443]. This suggests preterm neonates are unable to localise or appropriately regulate inflammatory signalling. Previous studies have found decreased numbers of leukocytes[284] and shown decreased opsonophagocytic capacity in preterm compared to term blood[82]. Though these findings suggest preterm neonates are immunosuppressed, others have also demonstrated increased phagocytic capacity in preterm compared to term blood following exposure to whole bacteria *in vitro*[284, 285]. Nonetheless, previous literature supports there are definite differences in preterm immune system function compared to term infants.

TLRs are ubiquitously expressed innate immune receptors that activate pro-inflammatory signalling. When TLRs are ligated by different classes of pathogen-associated molecular patterns, they signal through adaptor molecules including MyD88, TRAF6 and IRAK1. This leads to the translocation of transcription factors, such as NF-κB, into the nucleus to induce pro-inflammatory cytokine transcription. MiRs are post-transcriptional regulators of the TLR signalling that can repress the translation of TLRs, signalling molecules, cytokines and transcription factors. For example, *let-7e* interacts with the *TLR4* mRNA 3'UTR during LPS stimulation, resulting in the down-regulation of TLR activity[341]. Neonatal mice increase *miR-146a/b* expression in the gut to down-regulate excessive and damaging inflammatory responses to harmless commensal bacteria[347]. This occurs because the miR146 family downregulates TLR signalling through repressing *IRAK1* and *TRAF6*[323, 444]. Additionally, human cord blood constitutively expresses increased *miR-146a* and *miR-146b* compared to adult blood[242, 445], suggesting the neonatal immune system maintains a tolerogenic state *ex utero*. Other miRs regulate inflammation by repressing inhibitory molecules. An example is *miR-155*, which is increased with NF-κB induction, and binds to the 3' UTR of *Suppressor of Cytokine Signalling-1 (SOCS1)* gene transcript to indirectly upregulate inflammation[341]. Despite evidence of altered inflammatory regulation relative to adults, miR expression remains sparsely characterised in neonatal immunity.

Cord blood provides a snapshot of the state of immune system at birth. Investigating the TLR signalling pathway in cord blood therefore offers valuable insight into neonatal

immunity. Existing studies on preterm cord blood have yielded inconsistent results on the constitutive expression of TLRs, signalling co-factors and inflammatory cytokines[48, 82, 288]. Some have shown that *TLR4* mRNA is comparable between term and preterm cord blood[82, 86], while others show that both protein and mRNA are decreased in preterm cord blood[288, 302]. The discrepancy between data examining mRNA and protein expression could therefore be related to post-transcriptional regulation.

This study aims to examine the expression of miRs that regulate TLR signalling and their mRNA targets in term and preterm cord blood. Cytokine production was also characterised in cord blood serum from these deliveries to determine post-translational differences in inflammatory gene expression. It was hypothesised that preterm cord blood would show decreased anti-inflammatory miRs and increased inflammatory gene expression compared to term neonates at birth.

4.3. METHODS

4.3.1. PARTICIPANTS & SAMPLE COLLECTION

Pregnant women presenting at the LMH and WCH (*n*=152), who delivered at early preterm (<32 weeks), late preterm (32-36 weeks) or term gestation (≥ 37 weeks) were recruited to this study. Clinical data were obtained from maternal and neonatal health records, including obstetric history and neonatal outcomes (see section 2.1). Cord blood was collected into lithium heparin vials at delivery and stored at -80°C for subsequent analysis.

4.3.2. WHOLE RNA EXTRACTION

TRIzol LS® reagent was used to extract RNA from cord blood (see section 2.5.2). Briefly, three volumes of TRIzol LS were added to one volume of cord blood and samples were mechanically agitated. Lysates were separated by centrifugation with chloroform to retrieve the interphase for subsequent washing with isopropanol and ethanol. RNA was eluted in RNase free water and purified using a miRNeasy Mini Kit and DNAse I treatment as per manufacturers' protocol. RINs were assessed using a bioanalyser, where a RIN≥ 9 was considered sufficient for PCR analysis.

4.3.3. qRT-PCR ANALYSIS OF miRNA EXPRESSION

RNA (10ng) was reverse transcribed using a Taqman® MicroRNA Reverse Transcription Kit according to manufacturer's protocol that was slightly modified for optimal cDNA output (see section 2.6.1). This involved pooling a maximum of five RT primers per batch in a single RT mixture. Reverse transcription targeted seven primer sequences: *let-7e, miR-155, miR-146a, miR-146b, miR-106a, RNU6B* and *RNU48*. Relative miRNA expression was quantified using Taqman® MicroRNA Assays in 20µL reactions containing 1.33µL of cDNA. qRT-PCR utilised the comparative cycle threshold ($2^{-\Delta CT}$) method to determine miR abundance relative to the reference genes, *RNU6B* and *RNU48*.

4.3.4. qRT-PCR ANALYSIS OF mRNA EXPRESSION

Purified RNA (50ng) was reverse transcribed using a SuperScript™ III First-Strand Synthesis Kit for qRT-PCR according to manufacturer's protocol (see section 2.6.2). qRT-PCR was used to quantify the relative expression of miR targets including *TLR2, TLR3,*

TLR4, MyD88, IRAK1, TRAF6, NF-κB1, IL6, IL10 and *SOCS1* relative to the endogenous house-keeping gene, *β-actin*. qRT-PCR was performed using TaqMan® Gene Expression Assays and each 20µL reaction contained 2µL of the cDNA template.

4.3.5. CORD BLOOD SERUM PROTEIN ANALYSES

Single-target ELISAs were used to measure TNFα, IL6 and IL8 (Human Duo Sets, R&D Systems) in cord blood serum, as per manufacturer's instructions (see section 2.4.1). The lower detection limit for TNFα was 15.625pg/mL, and the lower detection limit for both IL6 and IL8 was 31.25pg/mL. All inter-assay and intra-assay coefficients were below 10%.

Multi-panel ELISA was used to determine the concentration of 7 cytokines and chemokines in cord blood serum according to manufacturer's instructions (see section 2.4.2). These included GM-CSF, IL1α, IL1β, IL1RA, IL10, IL12p70 and MCP1. The limitations and sensitivities of these analytes are listed in Table 2.1.

4.3.6. STATISTICAL ANALYSES

Data were analysed using SPSS v24. Demographic and clinical data are presented as mean ± SD, unless otherwise indicated. Frequency data were analysed using Chi-squared tests. Kruskal-Wallis tests or ANOVAs were used to compare continuous data according to gestational age groups. Post-hoc analyses of this data were conducted using Mann-Whitney U tests or t-tests as required. Gene expression and cytokine data are presented as median (25^{th}-75^{th} centile), unless otherwise indicated, and were logarithmically transformed (log_{10}) to normalise data for parametric analysis. ANCOVAs or MANCOVAs were used to analyse qPCR and ELISA data, with pre-eclampsia, labouring delivery, maternal smoking during pregnancy and birthweight centile used as covariates for term and preterm cord blood analyses; and chorioamnionitis and antenatal betamethasone were used as additional covariates where separate preterm cord blood analyses were conducted. Post-hoc comparisons were made using t-tests where appropriate. An *a priori* Bonferroni correction was made to the critical alpha level for post-hoc comparisons. Spearman's correlations were used to assess the relationship between matched sample expression of miR and mRNA data. An alpha level of 0.05 was considered statistically significant.

4.4. RESULTS

4.4.1. PARTICIPANT CHARACTERISTICS

Clinical characteristics from term (n=78), late preterm (n=54) and early preterm (n=20) deliveries are shown in Table 4.1. Gestational age was significantly different between the groups (F(2,151)=574.028, p<0.001). Maternal age, BMI, cigarette smoking during pregnancy, gravidity and ethnicity were not significantly different according to these gestational age groups. Parity was significantly different between the gestational age groups (χ^2(2)=10.104, p=0.006), with post-hoc tests indicating that it was lower in mothers who delivered late preterm compared to term (p=0.006). Pre-eclampsia was more frequently observed in late (17%) and early preterm deliveries (35%) compared to term (8%; χ^2(2)=11.845, p=0.003). Multiple pregnancies delivered more frequently at early (15%) or late preterm (11%) than term gestation (3%; χ^2(2)=7.133, p=0.028). Mode of delivery was significantly different between the groups (χ^2(3)=23.624, p<0.001): emergency Caesarean sections were more frequent in early preterm (40%) and late preterm (31%) compared to term deliveries (9%); while term deliveries (64%) were more frequently delivered via elective Caesarean section compared to early (50%) and late preterm deliveries (30%; χ^2(4)=23.624, p<0.001). Labour was more frequently associated with late preterm delivery (56%) compared to early preterm (25%) or term delivery (31%; χ^2(2)=9.360, p=0.009).

Early preterm neonates (85%) were exposed to antenatal betamethasone more frequently than late preterm (57%) and term neonates (10%; χ^2(2)=54.994, p<0.001). Birthweight was significantly different between the gestational age groups (F(2,150)=136.03, p<0.001). Post-hoc tests showed that it was lower in early compared to late preterm (p<0.001), early preterm compared to term (p<0.001), and late preterm compared to term neonates (p<0.001). Birthweight centile was significantly different between the groups (F(2,150)=9.335, p<0.001). Post-hoc analyses showed it was lower in early preterm compared to term (p<0.001) and in late preterm compared to term neonates (p=0.007). The incidence of SGA and IUGR were more frequent in early preterm compared to late preterm and term neonates (χ^2(2)=14.962, p=0.001; and χ^2(2)=22.872, p<0.001, respectively). Birth length was significantly different between the groups (F(2,143)=139.922, p<0.001) and post-hoc analyses revealed that it was lower in early compared to late preterm, early preterm compared to term and late preterm compared to term neonates (all p<0.001). Head

circumference was significantly different between the groups ($F(2,143)=36.912$, $p<0.001$) and post-hoc analyses showed it was lower in early compared to late preterm ($p=0.001$), early preterm compared to term ($p<0.001$) and late preterm compared to term neonates ($p=0.001$). APGARS at one minute were significantly different between the groups ($\chi^2(2)=20.160$, $p<0.001$) and post-hoc analyses showed they were lower in early compared to late preterm ($p=0.01$), early preterm compared to term ($p<0.001$) and late preterm compared to term neonates ($p=0.016$). APGARS at five minutes were significantly different between the groups ($\chi^2(2)=27.808$, $p<0.001$) and post-hoc analyses showed they were lower in early compared to late preterm ($p=0.005$), early preterm compared to term ($p<0.001$) and late preterm compared to term neonates ($p=0.002$).

Placental weight was significantly different between the gestational age groups ($F(2,137)=58.135$, $p<0.001$) and post-hoc analyses showed it was lower in early compared to late preterm, early preterm compared to term and late preterm compared to term neonates (all $p<0.001$). The incidence of chorioamnionitis was more frequent in early and late preterm deliveries compared to term (10% vs 6% and 0%, respectively; $\chi^2(2)=22.872$, $p<0.001$).

Table 4.1. ***Maternal demographics and neonatal characteristics of term, late and early preterm deliveries for cord blood analysed in Chapter 4.*** Values are given as mean± SD or *n* (%), unless otherwise indicated. *p-values* presented in the table were calculated using ANOVAs or Kruskal-Wallis tests. Post-hoc tests were conducted using t-tests or Mann-Whitney U tests and are represented within each gestational age group column using the following symbols: *$p \leq 0.05$ compared to both other groups; $^{\#}p \leq 0.05$ compared to term.

	Term	**Late Preterm**	**Early Preterm**	***p* value**
	n=78	*n*=54	*n*= 20	
Gestational age (completed weeks), median (min-max)	**39 (37-41)***	**35 (33-36)***	**29 (25-32)**	**<0.001**
Maternal demographics:				
Age, years	30 ±5	28 ±6	30 ±5	0.717
BMI, kg/m^2	31 ±8	28 ±7	27 ±7	0.174
Gravidity, median (min-max)	3 (1-10)	2 (1-9)	3 (1-7)	0.089
Parity, median (min-max)	**1 (0-5)**	**0 (0-6)$^{\#}$**	**1(0-4)**	**0.006**
Smoking during pregnancy	17 (22)	10 (19)	3 (15)	0.818
Ethnicity:				
Caucasian	57 (73)	30 (56)	11 (55)	
Indigenous	3 (4)	4 (7)	2 (10)	0.189
Other	18 (23)	20 (37)	7 (35)	
Maternal complications:				
Pre-eclampsia	**6 (8)**	**9 (17)**	**7 (35)**	**0.003**
Multiple pregnancy	**2 (3)**	**6 (11)**	**3 (15)**	**0.033**
Labouring delivery	**24 (31)**	**30 (56)**	**5 (25)**	**0.009**
Mode of delivery:				
Emergency Caesarean section	**7 (9)**	**17 (31)**	**8 (40)**	
Elective Caesarean section	**50 (64)**	**16 (30)**	**10 (50)**	**<0.001**
Vaginal delivery	**21 (27)**	**21 (39)**	**2 (10)**	
Placental				
Weight, g	**681 ±162***	**531 ±176***	**245±89**	**<0.001**
Histological chorioamnionitis	**0 (0)**	**3 (6)**	**2 (10)**	**<0.001**
Neonatal characteristics:				
Male	40 (51)	21 (39)	11 (55)	0.380
Birthweight, g	**3497 ±609***	**2470 ±653***	**1119±440**	**<0.001**
Birth centile, %	**54 ±33**	**43 ±34**	**20±25***	**<0.001**
SGA	**11 (14)**	**14 (26)**	**11 (55)**	**<0.001**
IUGR	**3 (4)**	**10 (19)**	**9 (45)**	**<0.001**
Birth length, cm	**49.5 ±2.3***	**44.9 ±3.7***	**33.7±6.9**	**<0.001**
Head circumference, cm	**35.3 ±2.6***	**32.5 ±2.1***	**29.1±5.7**	**<0.001**
Antenatal Betamethasone	**8 (10)**	**31 (57)**	**17 (85)**	**<0.001**
APGARS, median (min-max):				
1 min	**9 (2-10)***	**8 (3-9)***	**6 (2-9)**	**<0.001**
5 min	**9 (7-10)***	**9 (6-10)***	**7 (5-9)**	**<0.001**

4.4.2. CORD BLOOD GENE EXPRESSION

Due to limitations in available volumes of cord blood, gene expression analyses were conducted in a subset of samples (*n*=53 term, *n*=32 late preterm and *n*=7 early preterm).

The low frequency of chorioamnionitis meant that its independent contribution to gene expression could not be analysed, however expression data for neonates exposed to chorioamnionitis were within the IQRs for their respective gestational age groups. Chorioamnionitis remained in the final analysis as a covariate when analysing preterm gene expression.

4.4.3. THE EXPRESSION OF HOUSEKEEPING GENES

Two snoRNAs (*RNU48* and *RNU6B*) were identified from the literature as candidate reference genes[242, 386]. Both snoRNAs were expressed in cord blood. *RNU48* was expressed more consistently (median CT 27.5 (IQR=27-28)) compared to *RNU6B* (median CT 31.6 (IQR=30-34)). As *RNU48* was less variable than *RNU6B* and did not differ between gestational age groups, it was deemed the most appropriate reference gene for miR characterisation in this study.

4.4.4. THE EXPRESSION OF miRs THAT REGULATE TLR SIGNALLING IN CORD BLOOD

Cord blood *let-7e* expression was significantly different between term, late preterm and early preterm deliveries after adjusting for labouring delivery, maternal smoking, pre-eclampsia and birthweight centile ($F(2,78)=3.200$, $p=0.046$; Fig. 4.1A). Post-hoc analyses showed *let-7e* expression was increased in term compared to early preterm cord blood ($p=0.021$) and late preterm compared to early preterm cord blood ($p=0.015$), with no difference between late preterm and term cord blood expression.

Cord blood *miR-155* expression was significantly different between term, late preterm and early preterm deliveries ($F(2,78)=4.225$ $p=0.018$; Fig. 4.2A). Post-hoc analyses revealed *miR-155* expression was increased in term compared to early preterm cord blood ($p=0.005$) and in late preterm compared to early preterm cord blood ($p=0.022$), with no difference between late preterm and term cord blood expression.

There was no significant difference between early preterm, late preterm and term cord blood expression of *miR-146a* (Fig.4.3A), *miR-146b* (Fig.4.3.B) or *miR-106a* (Fig. 4.4A). No significant independent contribution of antenatal betamethasone exposure or chorioamnionitis was observed in early or late preterm cord blood miR expression.

Within the MANCOVA model, maternal smoking was identified as having a significant, independent effect on gene expression. This was assessed further using post-hoc ANOVAs, with gestational age groups and smoking status included as independent factors. We found no main effect between gestational age group and smoking, however, a significant main effect of smoking was found in association with increased *miR-146b* expression in cord blood from mothers who smoked during pregnancy (p=0.022; Fig.4.6A).

4.4.5. THE EXPRESSION OF mRNAs INVOLVED IN TLR SIGNALLING IN CORD BLOOD

The expression of mRNA associated with TLR signalling was assessed in a smaller subset of samples due to the low volumes of early preterm cord blood available. The analysis was split according to term (n=18) and preterm cord blood (a pooled group consisting of n=14 late preterm and n=2 early preterm deliveries). Only 13% of preterm cord blood expressed detectable levels of *TRAF6* and the expression of *TLR3* was not detected in any cord blood (Table 4.2).

There was no difference between term and preterm *TLR2* (Fig.4.5A), *TLR4* (Fig.4.1B), *MyD88* (Fig.4.2B), *IRAK1* (Fig.4.3C), *NF-κB1* (Fig.4.5B), *IL6* (Fig.4.5C), *IL10* (Fig.4.4B) or *SOCS1* (Fig.4.2C) expression in cord blood after adjusting for the effect of pre-eclampsia, antenatal betamethasone, birthweight centile, chorioamnionitis and labour.

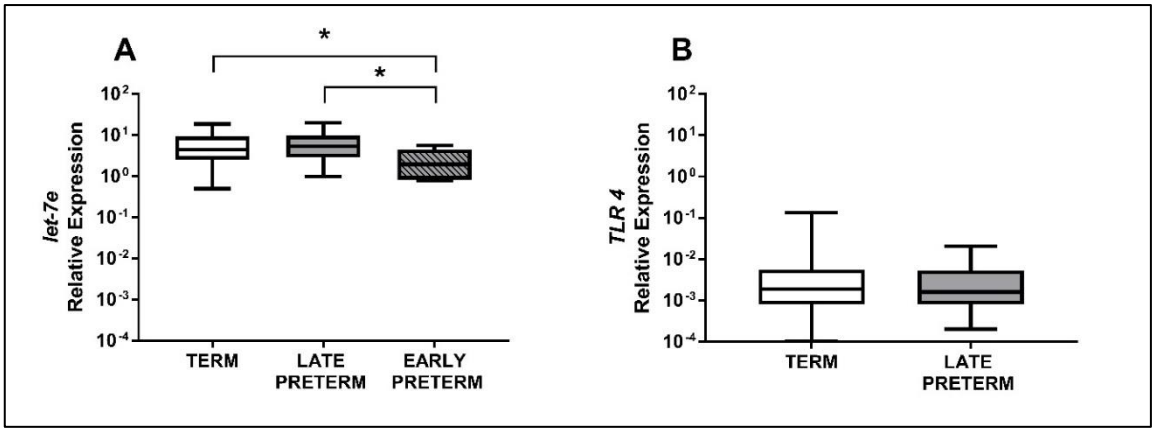

Fig. 4.1. ***Cord blood*** **let-7e** ***and*** **TLR4** ***expression following term and preterm delivery.*** Cord blood expression of *let-7e* relative to *RNU48* (A), and *TLR4* expression relative to *β-actin* (B), following term and preterm delivery. Note: y-axes are presented on a logarithmic scale, **p*<0.05.

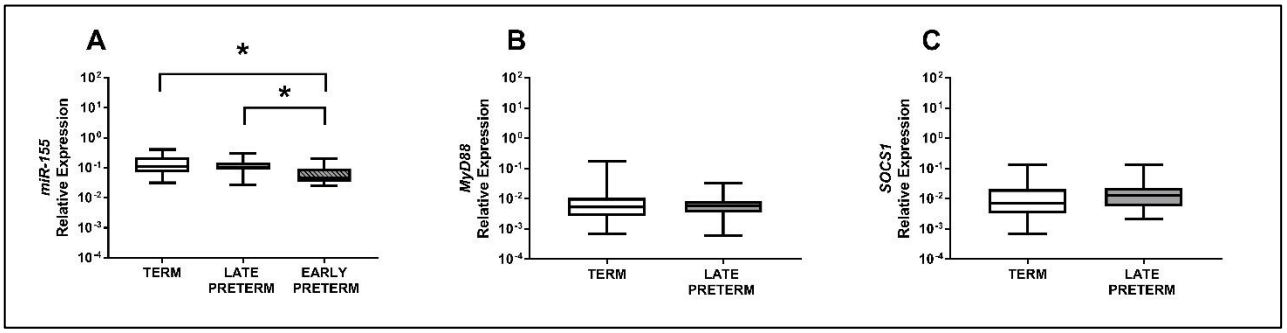

Fig. 4.2. ***Cord blood*** **miR-155, MyD88** ***and*** **SOCS1** ***expression following term and preterm delivery.*** Cord blood expression of *miR-155* relative to *RNU48* (A), and *MyD88* (B) and *SOCS1* (C) expression relative to *β-actin*, following term and preterm delivery. Note: y-axes are presented on a logarithmic scale, **p*<0.05.

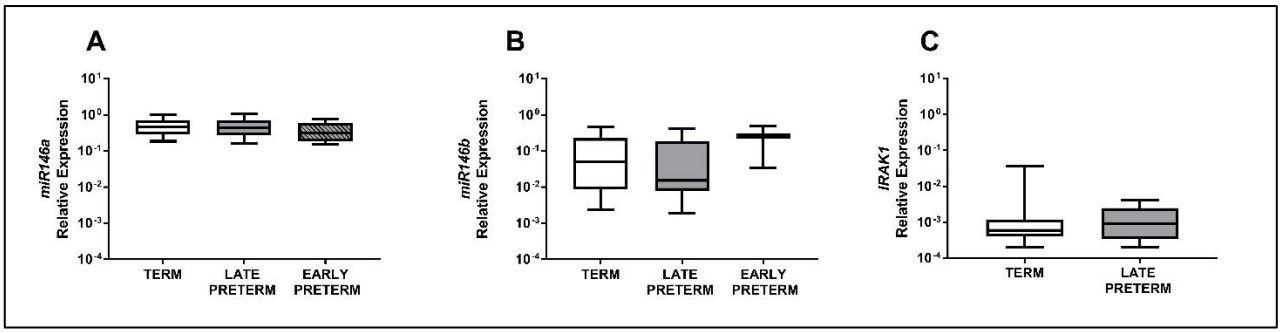

Fig. 4.3. ***Cord blood*** **miR-146a, miR-146b** ***and*** **IRAK1** ***expression following term and preterm delivery.*** Cord blood expression of *miR-146a* (A) and *miR-146b* (B) relative to *RNU48*, and *IRAK 1* expression relative to *β-actin* (C), following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

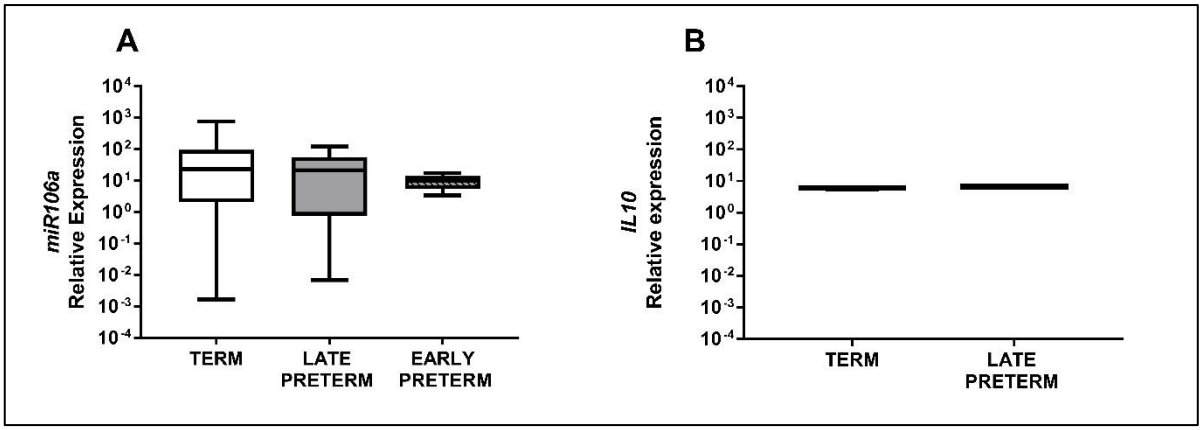

Fig. 4.4. ***Cord blood*** **miR-106a** ***and*** **IL10** ***expression following term and preterm delivery.*** Cord blood expression of *miR-106a* relative to *RNU48* (A), and *IL10* expression relative to *β-actin* (B), following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

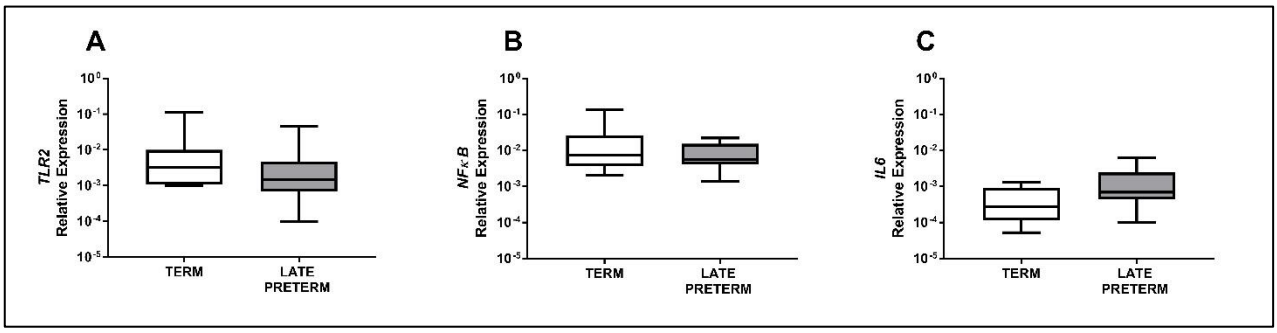

Fig.4.5. ***Cord blood*** **TLR2, NF-κB1** ***and*** **IL6** ***expression following term and preterm delivery.*** Cord blood expression of *TLR2* (A), *NF-κB1* (B) and *IL6* (C) relative to *β-actin,* following term and preterm delivery. Note: y-axes are presented on a logarithmic scale.

Table 4.2. ***The frequency of expression of genes associated with TLR signalling in term and preterm cord blood.*** The frequency of detectable mRNA expression (*n*, %) in term and preterm cord blood.

mRNA	**Term** *n*=18	**Preterm** *n*=16	***p***
TLR2	15 (83%)	14 (88%)	0.413
TLR3	0 (0%)	0 (0%)	-
TLR4	17 (94%)	16 (100%)	0.375
MyD88	16 (89%)	15 (94%)	0.419
IRAK1	14 (78%)	15 (94%)	0.281
TRAF6	0 (0%)	2 (13%)	-
NF-κB1	17 (94%)	15 (94%)	0.419
IL10	8 (44%)	6 (38%)	0.343
IL6	6 (33%)	8 (50%)	0.387
SOCS1	17 (94%)	15 (94%)	0.420

A post-hoc ANOVA was used to analyse the effect of gestational age group and maternal smoking on cord blood mRNA expression. This analysis showed no interaction between gestational age group and smoking. Maternal smoking was associated with increased cord blood *TLR2* (p=0.010), *TLR4* (p=0.007), *MyD88* (p=0.010), *IRAK1* (p=0.012), *NF-κB1* (p=0.038) and *SOCS1* expression (p=0.006; Figs.4.6B and 4.7).

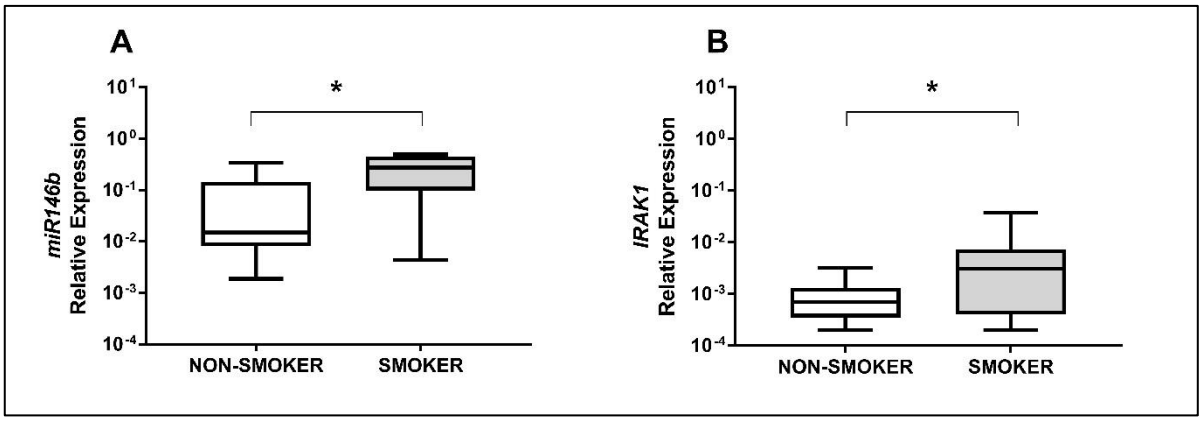

Fig. 4.6. ***Cord blood*** **miR-146b** ***and*** **IRAK1** ***expression according to maternal smoking during pregnancy.*** Placental expression of *miR-146b* relative to *RNU48* (A) and *IRAK1* relative to *β-actin* (B), from mothers who smoked and mothers who did not smoke cigarettes during pregnancy. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

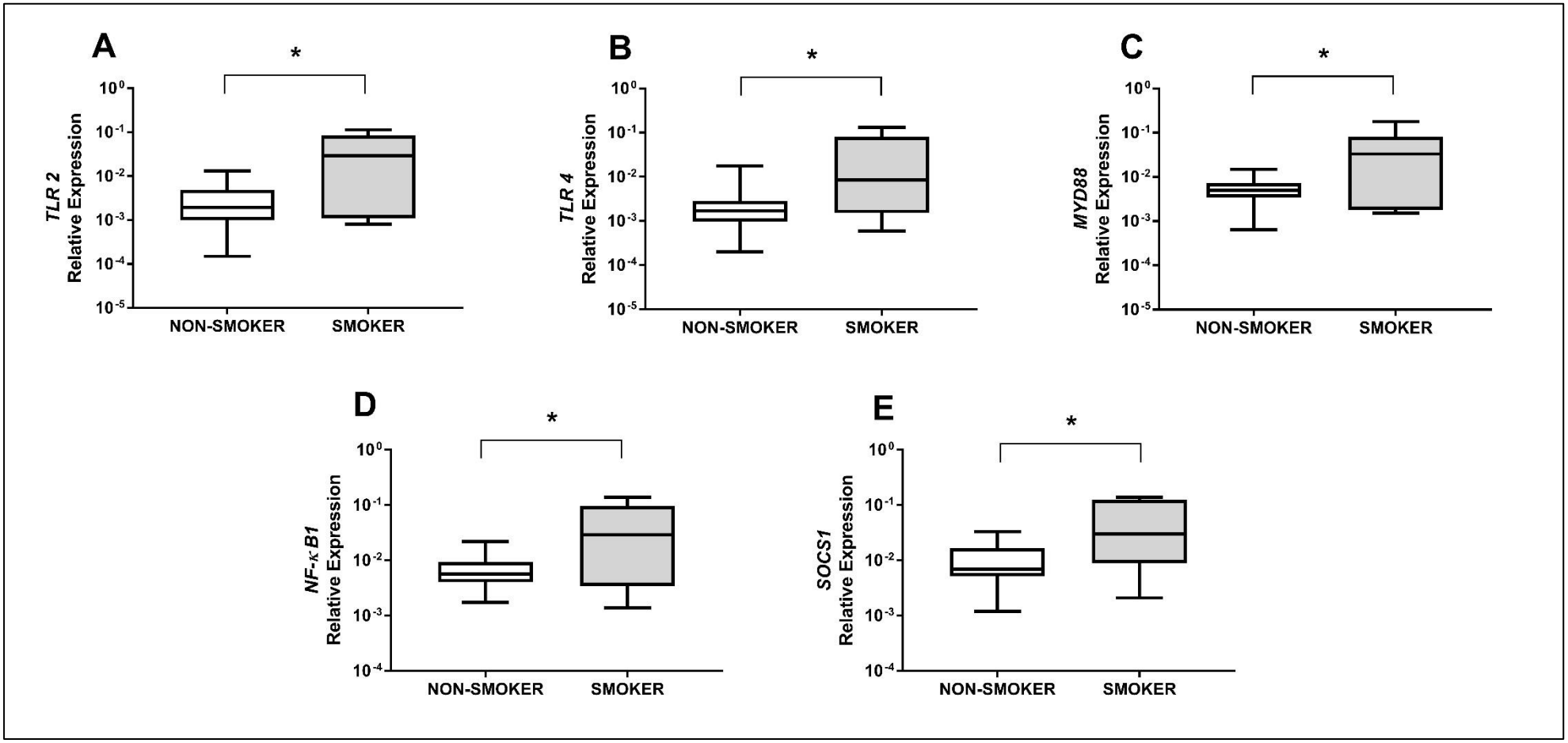

Fig 4.7. ***The expression of genes associated with TLR signalling in cord blood according to maternal smoking during pregnancy*.** The expression of *TLR2* (A), *TLR4* (B), *MyD88* (C), *NF-κB1* (D) and *SOCS1* (E) relative to *β-actin* in cord blood from mothers who smoked and mothers who did not smoke during pregnancy. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

4.4.6. CORRELATIONS BETWEEN miRs AND THEIR TARGET mRNAs IN CORD BLOOD

The expression of *let-7e, miR-155, miR-146a, miR-106a* and their respective targets: *TLR4, SOCS1, IRAK1 and TRAF6 or IL10* were not correlated in term or preterm cord blood. *NF-κB1* was not correlated with the expression of any miRs.

4.4.7. CORRELATIONS BETWEEN CORD BLOOD AND PLACENTAL miRs AND mRNAs

A subset of matched placenta and cord blood samples were analysed for correlating miR expression within gestational groups (n=31 term and n=18 preterm deliveries).

4.4.7.1. Correlations between Matched Genes in Cord Blood and Placenta

The expression of *let-7e, miR-155, miR-146a, miR-146b* and *miR-106a* was not correlated between matched cord blood and placental tissue (Table 4.3).

Table 4.3. ***The correlation between cord blood and placental miR expression following term and preterm delivery***. The correlation (ρ) between miR expression in cord blood and placenta according to term and preterm delivery calculated using untransformed (raw) data for relative gene expression.

miR	**Term** n=31		**Preterm** n=18	
	ρ	***p*-value**	**ρ**	***p*-value**
let-7e	0.246	0.183	0.117	0.645
miR-155	-0.100	0.614	-0.003	0.990
miR-146a	0.151	0.417	-0.102	0.687
miR-146b	0.204	0.629	0.000	1.000
miR-106a	-0.127	0.626	0.151	0.715

A subset of matched samples were analysed using Spearman's correlations to determine the relationship between mRNA expression in cord blood and placenta within gestational groups (n=11 term and n=7 preterm deliveries). Placental and cord blood *MyD88* expression showed a positive correlation following preterm but not term delivery (ρ=0.886, p=0.019; Fig. 4.8). The expression of *TLR2, TLR4, IRAK1* or *NF-κB1*were not correlated in matched cord blood and placental tissue, following term or preterm delivery (Table 4.4). There were insufficient data points to match correlations for the expression of *IL6, IL10, TRAF6* or *TLR3* split according to gestational age.

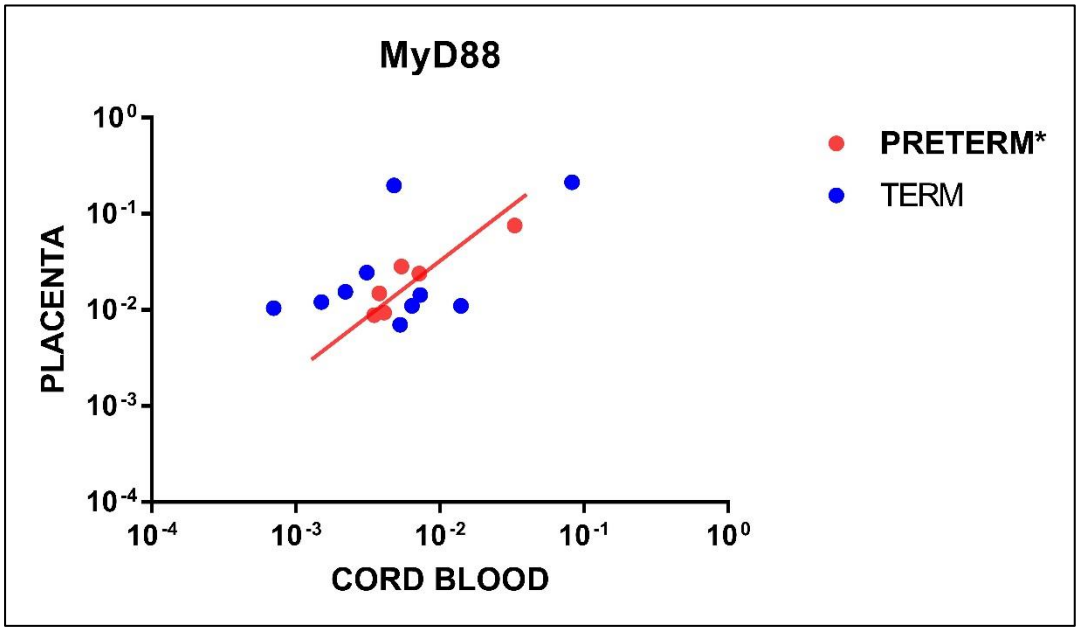

Fig. 4.8. ***A scatterplot of cord blood and placental*** **MyD88** ***expression following term and preterm delivery.*** The correlated expression of *MyD88* between matched placenta and cord blood following term and preterm delivery. Note: y-axis presented on a logarithmic scale, *$p \leq 0.05$.

Table 4.4 ***The correlation between cord blood and placental mRNA expression following term and preterm delivery***. The correlation (ρ) between mRNA expression in cord blood and placenta according to term and preterm delivery calculated using untransformed (raw) data for relative gene expression, $p \leq 0.05$.

miR	**Term** *n*=11		**Preterm** *n*=7	
	ρ	***p*-value**	**ρ**	***p*-value**
TLR2	-0.481	0.190	0.029	0.957
TLR4	-0.318	0.340	0.055	0.908
MyD88	0.213	0.555	**0.886**	**0.019**
IRAK1	-0.370	0.327	-0.657	0.156
NF-κB1	0.321	0.365	0.600	0.208
SOCS1	0.218	0.519	0.250	0.589

4.4.7.2. Correlations between miRs and their target mRNAs in Cord Blood and Placenta

Matched samples were used to analyse whether the expression of placental miRs was correlated with the expression of their respective targets in cord blood. This analysis was repeated according to the expression of cord blood miRs and their mRNA targets' expression in matched placenta. Within term and preterm groups, the expression of *let-7e* and its target, *TLR4* were not correlated between cord blood and placental tissue (Table 4.5). The expression of *miR-155* and its targets, *SOCS1* or *MyD88* was not correlated between cord blood and placental tissues (Tables 4.6-7). The expression of *miR-146a* and its target, *IRAK1* was not correlated between cord blood and placental tissues (Table 4.8). The expression of *miR-106a* and its target, *IL10* was not correlated between cord blood and placental tissues (Table 4.9).

Table 4.5. ***The correlation between cord blood and placental*** **let-7e** ***and*** **TLR4** ***expression in matched samples following term and preterm delivery***. The correlation (ρ) between cord blood *let-7e* and placental *TLR4* expression, and between placental *let-7e* and cord blood *TLR4* expression according to term and preterm delivery calculated using untransformed (raw) data for relative gene expression.

ρ		Placenta	
		let-7e	*TLR4*
Term			
Cord blood	*let-7e*	0.246	0.173
	TLR4	0.573	-0.318
Preterm			
Cord blood	*let-7e*	0.117	0.173
	TLR4	-0.595	0.055

Table 4.6. ***The correlation between cord blood and placental*** **miR-155** ***and*** **SOCS1** ***expression in matched samples following term and preterm delivery***. The correlation (ρ) between cord blood *miR-155* and placental *SOCS1* expression, and between placental *miR-155* and cord blood *SOCS1* expression according to term and preterm delivery calculated using untransformed (raw) data for relative gene expression.

ρ		Placenta	
		miR-155	*SOCS1*
Term			
Cord blood	*miR-155*	-0.100	-0.433
	SOCS1	-0.067	0.218
Preterm			
Cord blood	*miR-155*	-0.003	0.464
	SOCS1	0.107	0.250

Table 4.7. ***The correlation between cord blood and placental*** **miR-155** ***and*** **MyD88** ***expression in matched samples following term and preterm delivery***. The correlation (ρ) between cord blood *miR-155* and placental *MyD88* expression, and between placental *miR-155* and cord blood *MyD88* expression according to term and preterm delivery calculated using untransformed (raw) data for relative gene expression.

ρ		Placenta	
		miR-155	*MyD88*
Term			
Cord blood	*miR-155*	-0.100	0.310
	MyD88	0.417	0.218
Preterm			
Cord blood	*miR-155*	-0.003	0.357
	MyD88	0.600	0.250

Table 4.8. ***The correlation between cord blood and placental*** **miR-146a** ***and*** **IRAK1** ***expression in matched samples following term and preterm delivery***. The correlation (ρ) between cord blood *miR-146a* and placental *IRAK1* expression, and between placental *miR-146a* and cord blood *IRAK1* expression according to term and preterm delivery calculated using untransformed (raw) data for relative gene expression.

ρ		Placenta	
		miR-146a	*IRAK1*
Term			
Cord blood	*miR-146a*	0.151	-0.373
	IRAK1	0.084	-0.370
Preterm			
Cord blood	*miR-146a*	-0.102	-0.600
	IRAK1	0.107	-0.657

Table 4.9. ***The correlation between cord blood and placental*** **miR-106a** ***and*** **IL10** ***expression in matched samples following term and preterm delivery***. The correlation (ρ) between cord blood *miR-106a* and placental *IL10* expression, and between placental *miR-106a* and cord blood *IL10* expression according to term and preterm delivery calculated using untransformed (raw) data for relative gene expression. Note: there were no significant correlations determined by this analysis.

ρ		Placenta	
		miR-106a	*IL10*
Term			
Cord blood	*miR-106a*	-0.127	0.075
	IL10	-0.086	0.000
Preterm			
Cord blood	*miR-106a*	0.151	0.000
	IL10	0.000	1.000

4.4.8. CYTOKINE EXPRESSION IN CORD BLOOD SERUM

Cytokine protein expression was measured in a subset of samples (*n*=49 term, *n*=37 late preterm and *n*=14 early preterm), as determined by the volume of cord blood available. The number of cord blood samples that showed detectable cytokine production was varied (Table 4.10). There was no difference in cord blood cytokine expression between the gestational age groups (Fig. 4.9-10).

The effects of gestational age group and maternal smoking on cord blood cytokine expression were also analysed. There was no main effect of gestational age group or maternal smoking on cord blood GMCSF, IL10, IL12p70, IL1α, IL1β, IL6, IL8, MCP1 or TNFα production.

Table 4.10. ***The frequency of detection of cytokines in cord blood serum.*** The frequency of detection of cytokines in term, late preterm and early preterm cord blood serum (*n*, %).

	Term *n*= 55	**Late preterm** *n*= 37	**Early preterm** *n*= 14	***p***
GMCSF	55 (100%)	55 (100%)	55 (100%)	0.454
IL10	55 (100%)	55 (100%)	55 (100%)	0.212
IL12p70	55 (100%)	55 (100%)	55 (100%)	0.484
IL1α	55 (100%)	55 (100%)	55 (100%)	0.702
IL1β	55 (100%)	55 (100%)	55 (100%)	0.479
IL6	0 (0%)	0 (0%)	0 (0%)	0.793
IL8	41 (75%)	30 (81%)	7 (50%)	0.408
MCP1	55 (100%)	55 (100%)	55 (100%)	0.406
TNFα	0 (0%)	0 (0%)	0 (0%)	0.464

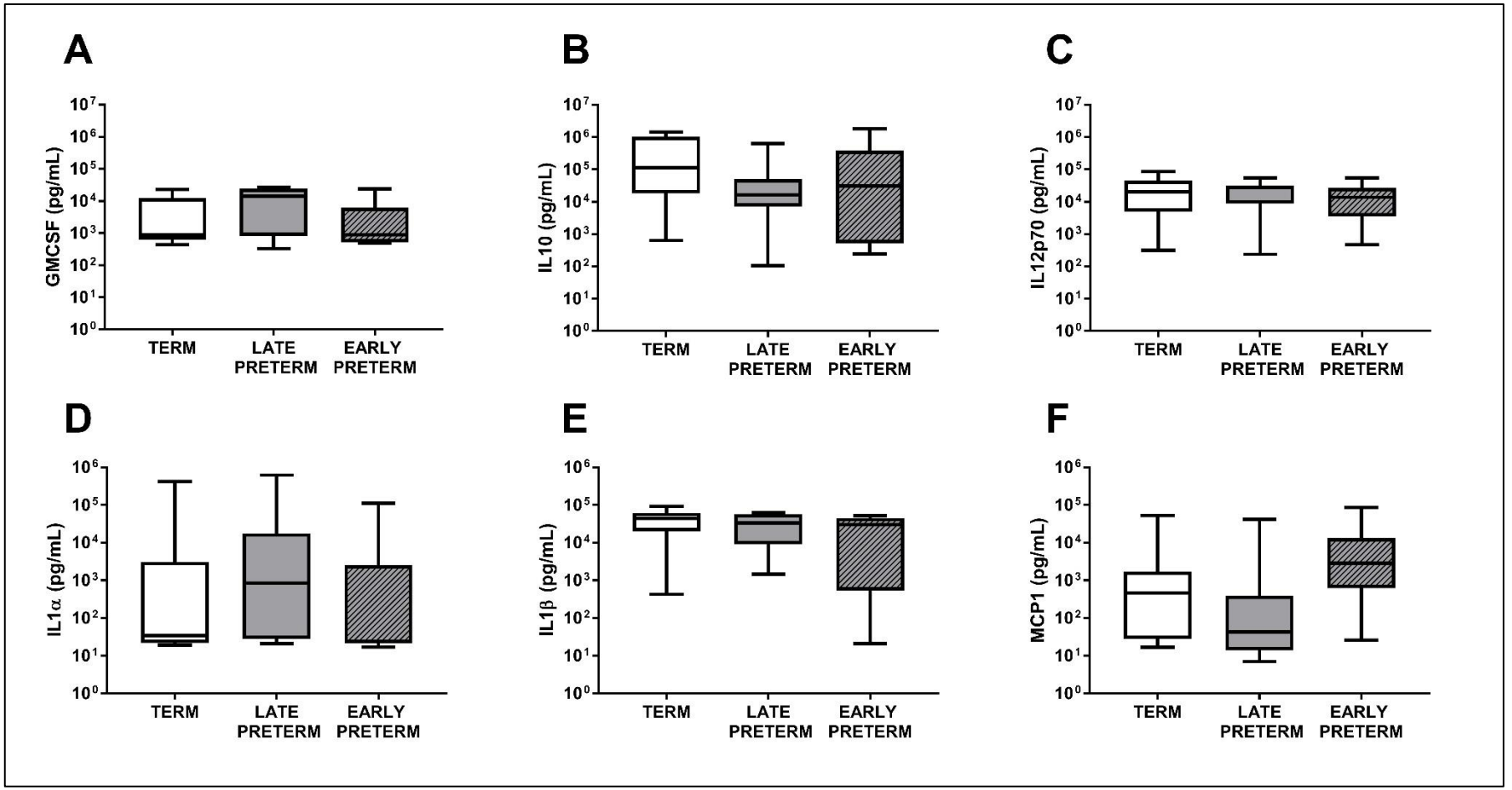

Fig. 4.9. ***Cytokine production in cord blood serum from term, and late and early preterm deliveries.*** The production of GMCSF (A), IL10 (B), IL12p70 (C), IL1α (D), IL1β (E) and MCP1 (F) in cord blood serum from term, and late and early preterm deliveries. Note: the y-axes are presented on a logarithmic scale.

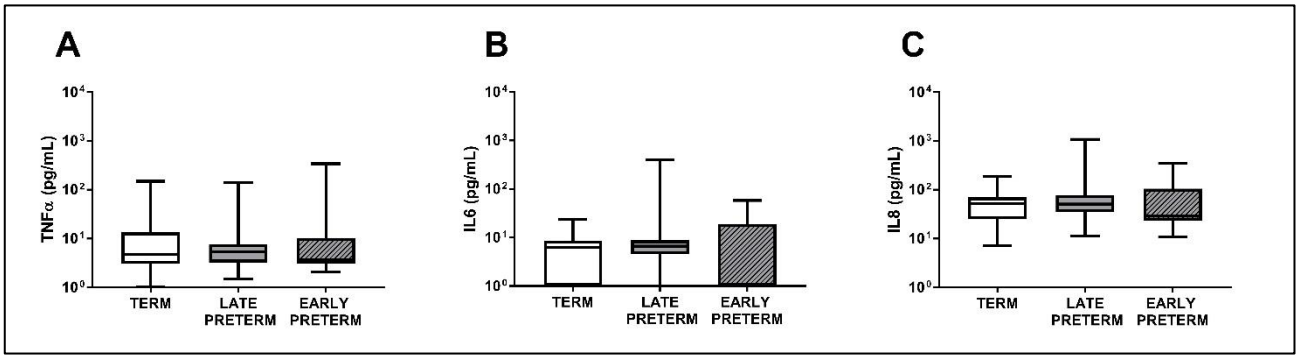

Fig. 4.10. ***TNFα, IL6 and IL8 production in cord blood serum from term, and late and early preterm deliveries.*** The production of TNFα(A), IL6 (B) and IL8 (C) in cord blood serum from term, and late and early preterm deliveries. Note: the y-axes are presented on a logarithmic scale.

4.5. DISCUSSION

This study investigated miRs that regulate TLR signalling in preterm cord blood. Early preterm cord blood showed increased *let-7e* and *miR-155* expression compared to late preterm and term. Interestingly, there was no difference in the expression of *miR-146a*, *miR-146b*, *miR-106a*, mRNAs associated with TLR signalling or cytokine production between early preterm, late preterm and term cord blood. These findings begin to suggest the absence of a regulatory relationship between miRs and mRNA in early preterm cord blood.

Early preterm cord blood expressed decreased *let-7e* with no difference in its target, *TLR4* compared to late preterm and term. The otherwise comparable expression of *TLR2* and *TLR4* mRNA across the gestational age groups agrees with other studies that have measured both mRNA and surface protein expression of these receptors on neonatal monocytes and neutrophils[82, 85, 86]. The decrease in *let-7e* we observed could be associated with the continued upregulation of *TLR4* during inflammation and requires confirmation using *in vitro* stimulation studies. Murine embryos have shown increased let-7 transcription at 10.5 days gestation, however, these transcripts are not processed by the enzyme Drosha and therefore, remain in their precursory, stem-loop form[446]. Other pre-miRs, including those encoded by the Chromosome 19 cluster, are also highly expressed in human embryonic stem cells without being transcribed[447]. Additionally, activity of the secondary miR processing molecule, Dicer is decreased in undifferentiated murine embryonic cell lines compared to differentiated cells[448]. Alternately, mechanisms that inhibit pre-miR transcription may be an attempt at maintaining an undifferentiated state in fetal cells[449]. As such, Lin28b is another negative regulator of *let-7e* and is highly expressed in fetal T cells[449]. As we did not measure pre-miRs (as they are biologically inactive), either of these mechanisms could be an underlying cause for decreased *let-7e* and *miR-155* expression in early preterm cord blood.

MiRs often exert their effects in the context of gene networks or signalling pairs. Although *let-7e* and *miR-155* do not interact directly, they regulate the TLR signalling pathway in opposite directions and are shown to be involved in similar feedback loops. For example, the protein kinase, Akt, is activated by LPS exposure to upregulate *let-7e* and downregulate *miR-155* simultaneously[341]. The overexpression of *let-7e* accompanied by *miR-155* repression in $Akt^{-/-}$ murine macrophages therefore leads to LPS tolerance during repeated LPS stimulation[341]. As we found decreased *let-7e* and *miR-155* expression in early preterm

cord blood, this suggests regulatory feedback loops could be disturbed and that miR-mediated regulation may be attenuated in early preterm neonates. The state of these TLR signalling feedback loops could be investigated using time course studies on inflammatory stimulation in cord blood.

Placental and cord blood *MyD88* expression were correlated in association with preterm but not term delivery. This could indicate underlying inflammatory activation leading to preterm parturition, though it would typically be associated with the co-expression of other inflammatory genes[450] and therefore, requires more direct investigation. Our analysis did not observe any other correlations in gene expression between matched miRs or their targets in and between cord blood and placental tissue. Although ovine studies have found placental exosomes contain miRs which can regulate gene targets in cord blood[451], this cannot be confirmed using the current experimental paradigm. Future studies using murine models and radiolabelled miRs during pregnancy may therefore be useful in determining whether placental miRs regulate neonatal immunity at delivery.

Irrespective of gestational age, cord blood serum cytokine production was inconsistent. Previous studies have also reported between 30-70% of cord blood samples (*n*=370) do not express detectable IL6, IL8 or IL10[452, 453]. Similarly, those that have detected cytokine expression, reported no difference in the constitutive expression of IL6, IL8 and IL10 in monocytes, lymphocytes and granulocytes between preterm and term cord blood[204]. Overall, as we found no difference in *IL6* or *IL10* mRNA expression, it is not surprising that a difference in cytokine expression was not seen.

Maternal smoking during pregnancy is a recognised risk factor for preterm delivery[14] and growth restriction[155]. Our analysis showed maternal smoking was associated with decreased *miR-146b* expression in cord blood and increased *TLR2*, *TLR4*, *MyD88*, *IRAK1*, *NF-κB1*, *IL6*, *IL10* and *SOCS1*, indicating increased inflammatory activation. This agrees with previous studies[432, 433] and findings from Chapter 3 that show smoking during pregnancy contributes to a pro-inflammatory *in utero* environment. In contrast to this, others have observed that term CBMCs from mothers who smoke express decreased constitutive IL6, TNFα and IL10 compared to non-smokers[161]. These findings may differ with ours because of post-transcriptional regulation or the use of isolated cell populations. Alternately, it is possible that *in utero* inflammation programs neonatal tolerance in an attempt to adapt to a pro-inflammatory environment as perceived by the fetus. A limitation of the current cohort

is our inability to separate the effects of gestational age and smoking, while we observed no apparent interaction between these factors, it is likely this occurred due to a smaller sample size. Overall, it is apparent that maternal smoking during pregnancy alters neonatal immunity and our support that dysregulated expression of genes the TLR signalling pathway may contribute to this.

In summary, the current data show early preterm cord blood expresses decreased constitutive *let-7e* and *miR-155*, while the expression of the miRs' mRNA targets remains comparable to late preterm and term cord blood. Decreased regulatory miR expression suggests that late preterm and term neonates may have a more mature innate immune system that better regulates inflammation during infection. Notably, our findings provide a snapshot of neonatal innate immunity at delivery and it remains unclear whether these differences affect their ability to respond to inflammatory challenge. Further research is therefore required to assess the impact of these differences on functional responses in cord blood.

Chapter Five:

Cord Blood Gene Expression Following TLR Stimulation

5.1. ABSTRACT

Background

A majority of health conditions affecting preterm neonates have an inflammatory aetiology. However, *in vitro* studies on preterm cord blood show decreased expression of inflammatory mediators following immune stimulation compared to term. Toll-like Receptor (TLR) signalling results in inflammation and can be regulated by microRNAs (miRs). This study therefore aimed to determine the expression of miRs and mRNAs in cord blood following TLR stimulation.

Methods and Results

Cord blood was collected from term (n=29), late preterm (n=17) and early preterm deliveries (n=15) and stimulated *in vitro* with ligands for TLR2 (PGN), TLR3 (Poly I:C) and TLR4 (LPS). The expression of miRs and mRNA in the TLR signalling pathway was quantified using qPCR. Term cord blood increased *mir-155* in response to PGN (p=0.031), and increased *let-7e* (p=0.008), *miR-155* (p<0.001), *miR-106a* (p=0.05), *NF-κB1* (p=0.023) and *IL6* (p=0.007) in response to LPS. Late preterm cord blood increased *let-7e* (p=0.008), *TLR2* (p=0.008) and *SOCS1* (p=0.024) in response to LPS. Early preterm cord blood only increased *NF-κB1* (p=0.029) and *IL6* (p=0.01) in response to LPS. There was no difference observed in the expression genes associated with TLR signalling by cord blood in response to Poly I:C stimulation.

Conclusion

This study suggests an absence of miR expression may contribute to diminished regulation of TLR signalling during inflammation in early preterm cord blood *in vitro*. Dysregulated innate immune signalling could underlie a susceptibility towards uncontrolled inflammation in early preterm neonates.

5.2. INTRODUCTION

Preterm neonates are more susceptible to developing severe inflammatory conditions than their term counterparts[4, 25]. These conditions are characterised by a bias towards unresolved inflammation and increased expression of pro-inflammatory mediators. Inflammation can be driven by innate immune TLR signalling. TLRs are activated by the ligation to specific classes of PAMPs. TLR2 recognises PGN that forms the cell wall of gram-positive bacteria; TLR3 recognises viral PAMPs including synthetic Poly I:C; and TLR4 recognises LPS, which is a component of the gram-negative bacterial membrane. TLRs activate a series of intracellular adaptor molecules (e.g. MyD88 and IRAK1) to initiate the translocation of nuclear factors that induce inflammatory cytokine transcription (e.g. NF-κB). The expression of these genes can be regulated at different levels of signalling by miRs. Inflammation must be tightly regulated to avoid prolonged or aberrant tissue damage.

MiRs have important roles in regulating TLR signalling during inflammation. They respond to physiological cues and act post-transcriptionally, resulting in a 'dampening' of the inflammatory response. In response to inflammatory cues, the miR, *let-7e*, inhibits *TLR4* expression, while *miR-146a* inhibits expression of the signalling molecule *IRAK1*, and both miRs therefore down-regulate TLR signalling. Other miRs increase inflammatory activation, including *miR-155*, which represses the suppressor of cytokine signalling (*SOCS1*) gene, and *miR-106a* that represses *IL10*.

Preterm neonates show decreased leukocyte counts[207] and attenuated expression of pro-inflammatory cytokines following LPS stimulation compared to their term-born counterparts[288]. Specifically, preterm cord blood expresses decreased IL6 and TNFα following stimulation with TLR agonists[82, 213, 288] or whole bacteria compared to term[252, 289]. Other aspects of TLR signalling have, however, demonstrated comparable responses to immune stimulation *in vitro*. For example, preterm CBMCs exhibit comparable[86, 302] or increased[454] phosphorylation of NF-κB following stimulation with whole bacteria compared to term. As NF-κB phosphorylation results in pro-inflammatory cytokine transcription[76], this suggests inflammatory cytokine expression is either be comparable or increased between term and preterm cord blood. Discrepancies between NF-κB phosphorylation and cytokine expression in cord blood

studies may be explained by differences in post-transcriptional regulation of TLR signalling.

Alterations in the regulation of TLR signalling may also explain preterm neonates' susceptibility to developing inflammatory morbidities. Currently, little is known about miR-related regulation of neonatal immunity. Studies to date have focused on miR arrays that explore patterns of miR expression in association with preterm delivery[374]. Even fewer studies have validated miR expression in cord blood. Charrier et al., demonstrated increased *miR-146a* and decreased *miR-155* expression in cord blood pDCs that expressed decreased *IRAK1* and *MyD88* compared to adult pDCs following TLR9 stimulation[388]. Similarly, cord blood monocytes are shown to increase *miR-146a* expression after 24 hours LPS stimulation *in vitro* compared to adult monocytes[242]. These data support that term neonates exhibit a tolerogenic immune bias during LPS stimulation compared to adults.

It is unclear whether miRs are differentially expressed in preterm compared to term neonates, which could contribute to the differential responses to TLR stimulation observed between them (see Table 1.2). This study therefore aimed to characterise expression of miRs and their gene targets following TLR stimulation in term and preterm cord blood. Previous findings from our laboratory have shown increased *IL6* expression with TLR stimulation, without any change in anti-inflammatory cytokines[153]. It was therefore hypothesised that preterm cord blood would exhibit decreased expression of anti-inflammatory *let-7e, miR146a* and *SOCS1* and increased expression of pro-inflammatory *miR-155, miR-106a* and *IL6* compared to term cord blood following TLR stimulation.

5.3. METHODS

5.3.1. PARTICIPANTS AND SAMPLE COLLECTION

Pregnant women presenting at the LMH and WCH (*n*=61), who delivered at early preterm (<32 weeks), late preterm (32-36 weeks) or term gestation (≥ 37 weeks) were recruited to this study. Clinical data were obtained from maternal and neonatal health records, including obstetric history and neonatal outcomes (see section 2.1). Cord blood was collected into lithium heparin vials at delivery for *in vitro* culture.

5.3.2. CORD BLOOD CULTURE AND TLR STIMULATION

Whole cord blood was plated in volumes of 100µL in flat-bottomed 96-well cell culture plates and cultured at 37°C and 5% CO_2 overnight. Following this 'resting' period, one set of wells were left unstimulated (untreated 'controls') and remaining wells were stimulated with either PGN (TLR2 agonist; 100pg), Poly I:C (TLR3 agonist; 500pg), or LPS (TLR4 agonist; 1ng). Wells were collected and pooled in triplicate after 6 hours *in vitro*.

5.3.3. WHOLE RNA EXTRACTION

TRIzol LS® reagent was used to extract RNA from cord blood (see section 2.5.2). Briefly, three volumes of TRIzol LS were added to one volume of cord blood and samples were mechanically agitated. Lysates were separated by centrifugation with chloroform to retrieve the interphase for subsequent washing with isopropanol and ethanol. RNA was eluted in RNase free water and purified using a miRNeasy Mini Kit and DNAse I treatment as per manufacturers' protocol. RINs were assessed using a bioanalyser, where a RIN≥ 9 was considered sufficient for PCR analysis.

5.3.4. qRT-PCR ANALYSIS OF miRNA EXPRESSION

RNA (10ng) was reverse transcribed using a Taqman® MicroRNA Reverse Transcription Kit according to manufacturer's protocol, that was slightly modified for optimal cDNA output (see section 2.6.1). This involved pooling a maximum of five RT primers per batch in a single RT mixture. Reverse transcription targeted five primer sequences: *let-7e, miR-155, miR-146a, miR-106a* and *RNU48*. Relative miRNA

expression was quantified using Taqman® MicroRNA Assays in 20µL reactions containing 1.33µL of cDNA. qRT-PCR utilised the comparative cycle threshold ($2^{-\Delta CT}$) method to determine miR abundance relative to the housekeeping gene *RNU48*.

5.3.5. qRT-PCR ANALYSIS OF mRNA EXPRESSION

Purified RNA (50ng) was reverse transcribed using a SuperScript™ III First-Strand Synthesis Kit for qRT-PCR according to manufacturer's protocol. qRT-PCR was used to quantify the relative expression of miR targets including *TLR2, TLR4, MyD88, IRAK1, NF-κB1, IL6, IL10* and *SOCS1* relative to the reference gene*, β-actin*. qRT-PCR was performed using TaqMan® Gene Expression Assays and each 20µL reaction contained 2µL of the cDNA template.

5.3.6. STATISTICAL ANALYSES

Data were analysed using SPSS v24. Demographic and clinical data are presented as mean ± SD, unless otherwise indicated. Frequency data were analysed using Chi-squared tests. Kruskal-Wallis tests or ANOVAs were used to compare continuous data according to the gestational age groups. Post-hoc analysis was conducted using Mann-Whitney U tests or t-tests where necessary. Gene expression data are presented as median (25^{th}-75^{th} centile), unless otherwise indicated, and were logarithmically transformed (log_{10}) to normalise data for parametric analysis. ANCOVAs or MANCOVAs were used to analyse qPCR data, with pre-eclampsia, labouring delivery, maternal smoking during pregnancy and birthweight centile used as covariates for term and preterm cord blood analyses; and chorioamnionitis and antenatal betamethasone were used as additional covariates for analyses concerning preterm cord blood. Post-hoc comparisons were made using t-tests where appropriate. An *a priori* Bonferroni correction was made to the critical alpha level for post-hoc comparisons. Spearman's correlations were used to assess the relationship between matched sample expression of miR and mRNA data. An alpha level of 0.05 was considered statistically significant.

5.4. RESULTS

5.4.1. PARTICIPANT CHARACTERISTICS

Clinical characteristics from term (*n*=29), late preterm (*n*=17) and early preterm (*n*=15) deliveries are shown in Table 5.1. Maternal age, BMI, smoking during pregnancy, ethnicity and gravidity were not significantly different between the gestational age groups. Gestational age at delivery (completed weeks) was significantly different between the groups ($F(2,60)=297.701$, $p<0.001$). Parity showed a trend towards being different between the gestational age groups, but this did not reach statistical significance ($p=0.056$). Pre-eclampsia was more frequently associated with early preterm deliveries (33%) compared to late preterm (12%) and term (7%; $\chi^2(2)=7.861$, $p=0.020$). Mode of delivery was significantly different between the groups ($\chi^2(3)=9.687$, $p=0.046$), where emergency Caesarean sections were more frequent in early preterm (47%) and late preterm (33%) compared to term deliveries (10%); and both early preterm (60%) and term deliveries (62%) were more frequently delivered via elective Caesarean section compared to late preterm deliveries (35%). The frequency of labouring deliveries did not differ between the groups.

Early (87%) and late preterm neonates (82%) were more frequently exposed to antenatal betamethasone compared to term neonates (3%; $\chi^2(2)=40.182$, $p<0.001$). Birthweight was significantly different between the gestational age groups ($F(2,60)=116.08$, $p<0.001$) and post-hoc analyses showed that it was lower in early preterm compared to term, early preterm compared to late preterm and late preterm compared to term neonates ($p<0.001$ for all). Birthweight centile was significantly different between the groups ($F(2,60)=11.057$, $p<0.001$) and post-hoc analyses showed that it was lower in early preterm compared to term ($p<0.001$) and early preterm compared to late preterm neonates ($p=0.04$). The incidence of SGA and IUGR occurred more frequently in early preterm compared to late preterm and term neonates ($\chi^2(2)=15.822$, $p<0.001$; and $\chi^2(2)=19.028$, $p<0.001$, respectively). Birth length was significantly different between the groups ($F(2,54)=94.461$, $p<0.001$) and post-hoc analyses showed it was lower in early preterm compared to term, early preterm compared to late preterm and late preterm compared to term neonates ($p<0.001$ for all). Head circumference was significantly different between the groups ($F(2,54)=14.875$,

$p<0.001$) and post-hoc analyses showed that it was lower in early preterm compared to term ($p<0.001$) and late preterm compared to term neonates ($p=0.014$). APGARS at 1 minute were significantly different between the groups (U=76, $p=0.001$) and post-hoc tests showed they were lower in early preterm compared to term ($p=0.001$) and early preterm compared to late preterm neonates ($p=0.04$). APGARS at 5 minutes were significantly different between the groups (U=81.5, $p=0.002$) and post-hoc tests showed that they were lower in early preterm compared to term ($p=0.002$) and early preterm compared to late preterm ($p=0.024$).

Placental weight was significantly different between the gestational age groups ($F(2,47)=32.112$, $p<0.001$) and post-hoc analyses showed that it was lower following early preterm compared to late preterm ($p=0.003$), early preterm compared to term ($p<0.001$) and late preterm compared to term delivery ($p=0.003$). The incidence of chorioamnionitis was higher in early preterm deliveries (20%) compared to late preterm (12%) and term (0%; $\chi^2(2)=13.866$, $p=0.001$).

Table 5.1. ***Maternal demographics and neonatal characteristics of term, late and early preterm deliveries for cord blood analysed in Chapter 5.*** Values are given as mean± SD or *n* (%), unless otherwise indicated. *p-values* presented in the table were calculated using ANOVAs or Kruskal-Wallis tests. Post-hoc tests were conducted using t-tests or Mann-Whitney U tests and are represented within each gestational age group column by the following symbols: $^{*}p \leq 0.05$ compared to both other groups; $^{\#}p \leq 0.05$ compared to term.

	Term	**Late Preterm**	**Early Preterm**	***p*-value**
	n=29	*n*=17	*n*=15	
Gestational age (completed weeks), median (min-max)	**39 (37-41)***	**32 (32-36)***	**30 (25-31)**	**<0.001**
Maternal demographics:				
Age, years	30 ±4	29 ±6	31 ±6	0.672
BMI, kg/m^2	30 ±8	24 ±4	29 ±8	0.104
Gravidity, median (min-max)	3 (1-6)	2 (1-7)	3 (1-7)	0.521
Parity, median (min-max)	1 (0-4)	0 (0-5)	1 (0-4)	0.056
Smoking during pregnancy	4 (14)	1 (6)	3 (20)	0.515
Ethnicity:				
Caucasian	20 (69)	9 (53)	9 (60)	
Indigenous	0 (0)	1 (6)	2 (13)	0.561
Other	9 (31)	7 (41)	4 (27)	
Maternal complications:				
Pre-eclampsia	**2 (7)**	**2 (12)**	**5 (33)**	**0.020**
Multiple pregnancy	2 (7)	4 (24)	3 (20)	0.130
Labouring delivery	11 (38)	9 (53)	3 (20)	0.159
Mode of delivery:				
Emergency Caesarean section	**3 (10)**	**8 (47)**	**5 (33)**	
Elective Caesarean section	**18 (62)**	**6 (35)**	**9 (60)**	**0.046**
Vaginal delivery	**8 (28)**	**3 (18)**	**1 (7)**	
Placenta:				
Weight, g	**693 (±171)***	**477 (±224)***	**241 (±94)**	**<0.001**
Histological chorioamnionitis	**0 (0)**	**2 (12)**	**3 (20)**	**<0.001**
Neonatal characteristics:				
Male	11 (38)	7 (41)	8 (53)	0.613
Birthweight, g	**3582 (±516)***	**2291 (±581)***	**1142 (±421)**	**<0.001**
Birth centile, %	**62 (±31)**	**39 (±33)$^{\#}$**	**18 (±26)$^{\#}$**	**<0.001**
SGA	**3 (10)**	**4 (24)**	**10 (67)**	**<0.001**
IUGR	**0 (0)**	**3 (18)**	**8 (53)**	**<0.001**
Birth length, cm	**50 (±2)***	**44 (±3)***	**33 (±6)**	**<0.001**
Head circumference, cm	**35 (±1)**	**32 (±2)$^{\#}$**	**29 (±6)$^{\#}$**	**<0.001**
Antenatal Betamethasone	**1 (3)**	**14 (89)**	**13 (87)**	**<0.001**
APGARS, median (min-max):				
1 min	**9 (7-9)**	**9 (3-9)**	**6 (2-9)***	**0.004**
5 min	**9 (7-10)**	**9 (6-10)**	**7 (5-9)***	**0.001**

5.4.2. CORD BLOOD miR EXPRESSION FOLLOWING PGN (TLR2) STIMULATION

PGN stimulation significantly increased *miR-155* expression compared to controls (unstimulated samples) in term cord blood after adjusting for the covariates pre-eclampsia, labouring delivery, maternal smoking during pregnancy and birthweight centile (Fig. 5.1, F(1,48)=4.910, p=0.031). There was a trend towards increased *miR-106a* expression with PGN stimulation compared to controls in term cord blood, however, this did not reach statistical significance (p=0.094; Fig. 5.4). Term cord blood showed no difference in *let-7e* or *miR-146a* expression between unstimulated and PGN stimulated samples (Figs. 5.2-3).

Late and early preterm cord blood showed no difference in the expression of any miR between PGN stimulated and unstimulated samples after adjusting for the covariates pre-eclampsia, labouring delivery, maternal smoking during pregnancy, birthweight centile, chorioamnionitis and antenatal betamethasone exposure (Figs. 5.1-4).

There was no difference in the magnitude of *let-7e, miR-155, miR-146a* or *miR-106a* expression with PGN treatment between early preterm, late preterm and term cord blood.

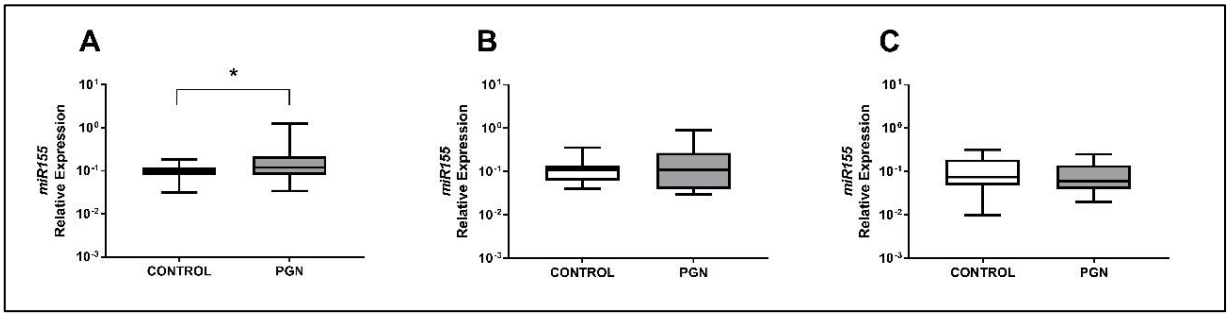

Fig. 5.1. ***Cord blood*** **miR-155** ***expression with and without PGN stimulation.*** The expression *miR-155* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following PGN stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

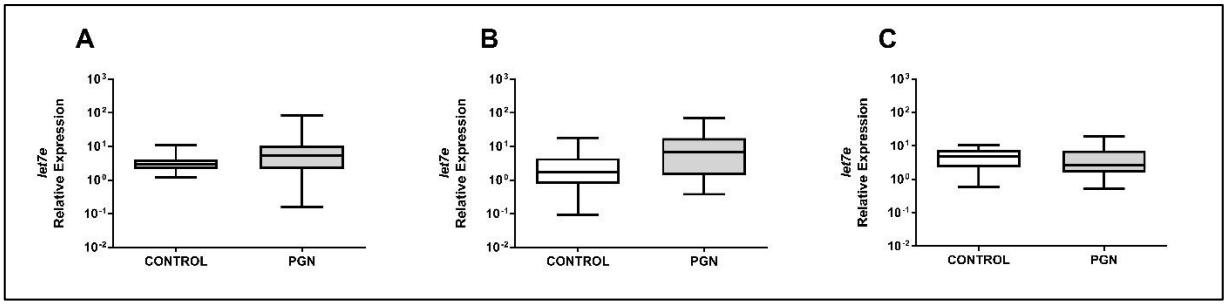

Fig. 5.2. ***Cord blood*** **let-7e** ***expression with and without PGN stimulation.*** The expression *let-7e* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following PGN stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

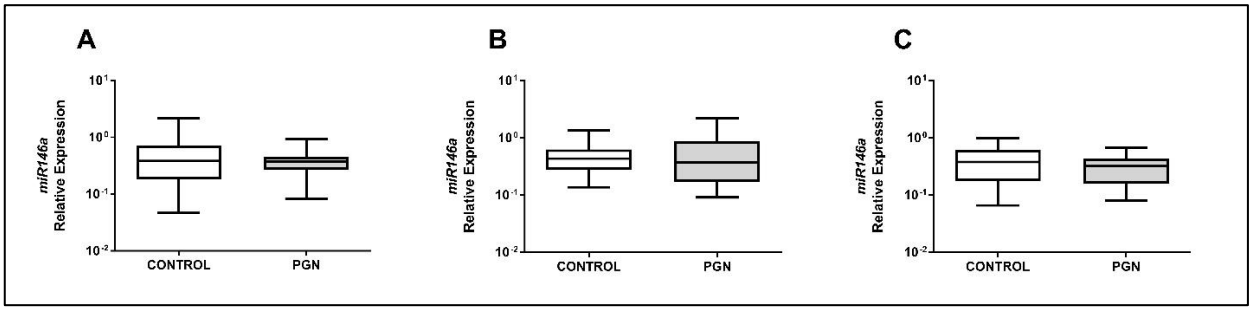

Fig. 5.3. ***Cord blood*** **miR-146a** ***expression with and without PGN stimulation.*** The expression *miR-146a* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following PGN stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

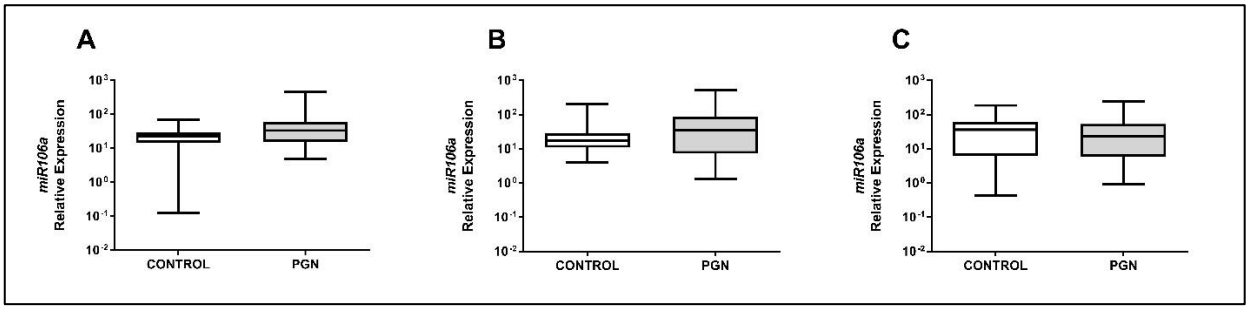

Fig. 5.4. ***Cord blood*** **miR-106a** ***expression with and without PGN stimulation.*** The expression *miR-106a* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following PGN stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

5.4.3. CORD BLOOD miR EXPRESSION FOLLOWING Poly I:C (TLR3) STIMULATION

There was a trend towards increased *let-7e* expression with Poly I:C stimulation compared to unstimulated conditions in late preterm cord blood after adjusting for pre-eclampsia, labouring delivery, maternal smoking, birthweight centile, chorioamnionitis and antenatal betamethasone, however this trend did not reach statistical significance (p=0.072; Fig. 5.5). There was no difference in *let-7e* expression in term or early preterm cord blood between control and Poly I:C stimulated samples. No difference was observed in *miR-155, miR-146a* or *miR-106a* expression with Poly I:C stimulation in term, late preterm or early preterm cord blood (Fig. 5.6-8).

There was no difference in the magnitude of *let-7e, miR-155, miR-146a* or *miR-106a* expression induced by Poly I:C stimulation between early preterm, late preterm and term cord blood.

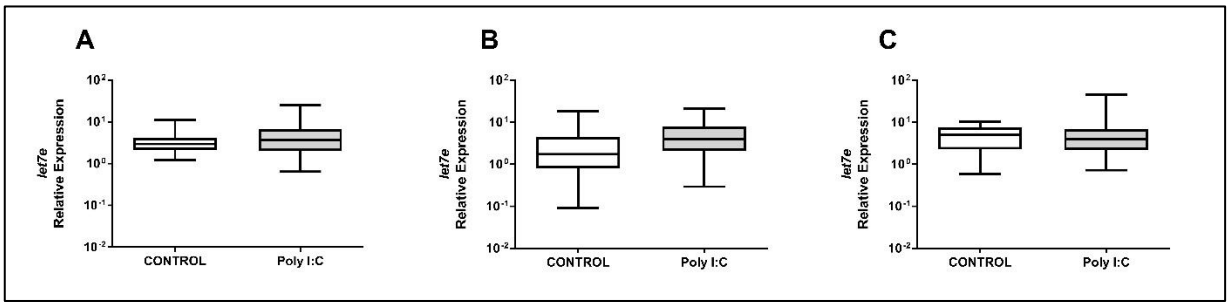

Fig. 5.5. ***Cord blood*** **let-7e** ***expression with and without Poly I:C stimulation.*** The expression *let-7e* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following Poly I:C stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

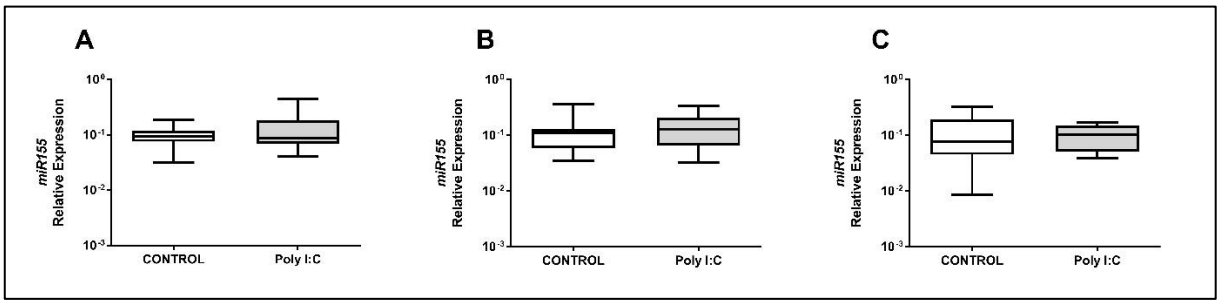

Fig. 5.6. ***Cord blood*** **miR-155** ***expression with and without Poly I:C stimulation.*** The expression *miR-155* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following Poly I:C stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

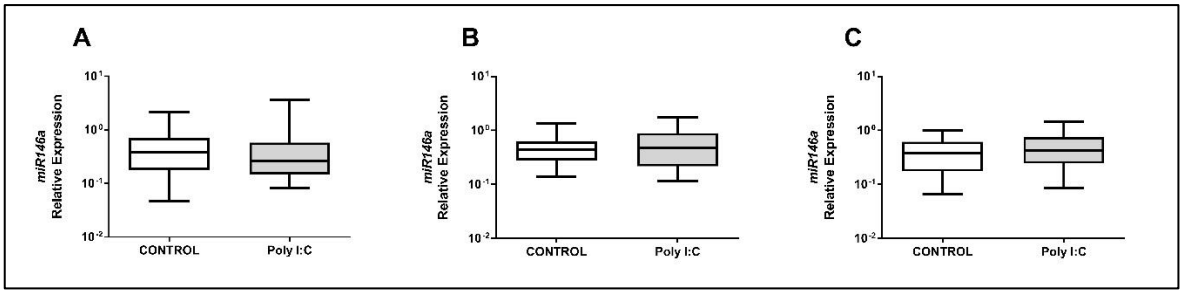

Fig. 5.7. ***Cord blood*** **miR-146a** ***expression with and without Poly I:C stimulation.*** The expression *miR-146a* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following Poly I:C stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

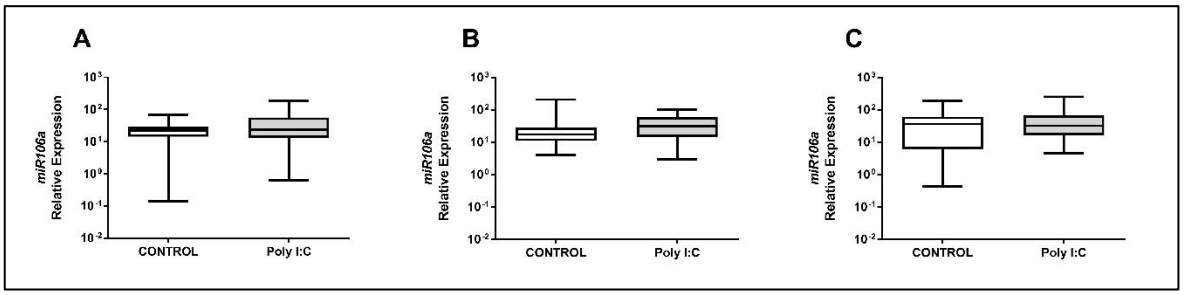

Fig. 5.8. ***Cord blood*** **miR-106a** ***expression with and without Poly I:C stimulation.*** The expression *miR-106a* relative to *RNU48* in (A) term, (B) late preterm and (C) early preterm cord blood at 6 hours *in vitro* following Poly I:C stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

5.4.4. CORD BLOOD miR EXPRESSION FOLLOWING LPS (TLR4) STIMULATION

LPS stimulation significantly increased term cord blood *let-7e* ($F(1,47)=7.653$, $p=0.008$; Fig. 5.9A), *miR-155* ($F(1,47)=25.143$, $p<0.001$; Fig. 5.9D) and *miR-106a* expression ($F(1,47)=4.040$, $p=0.050$; Fig. 5.9J) compared to control term cord blood samples, after adjusting for the covariates pre-eclampsia, labouring delivery, maternal smoking during pregnancy and birthweight centile. No change in *miR-146a* expression was observed between LPS-stimulated and control samples in term cord blood.

Late preterm cord blood increased *let-7e* expression with LPS stimulation compared to unstimulated samples ($F(1,24)=5.816$, $p=0.024$; Fig. 5.9B). There was no difference observed in *miR-155, miR-146a* or *miR-106a* expression by late preterm cord blood between control and LPS-stimulated samples (Fig. 5.9 E, H, K).

There was no difference in expression of any of the miRs in early preterm cord blood between control and LPS-stimulated samples (Fig. 5.9 C, F, I, L).

There was no significant difference observed in the magnitude of LPS-stimulated *miR-155* and *miR-146a* responses between gestational age groups, however a trend towards increased *mir-155* expression ($p=0.067$) and *mir-146a* expression ($p=0.085$) was observed in term cord blood compared to both preterm groups. There was no difference observed in the magnitude of *let-7e* or *miR-106a* expression with LPS stimulation between the groups.

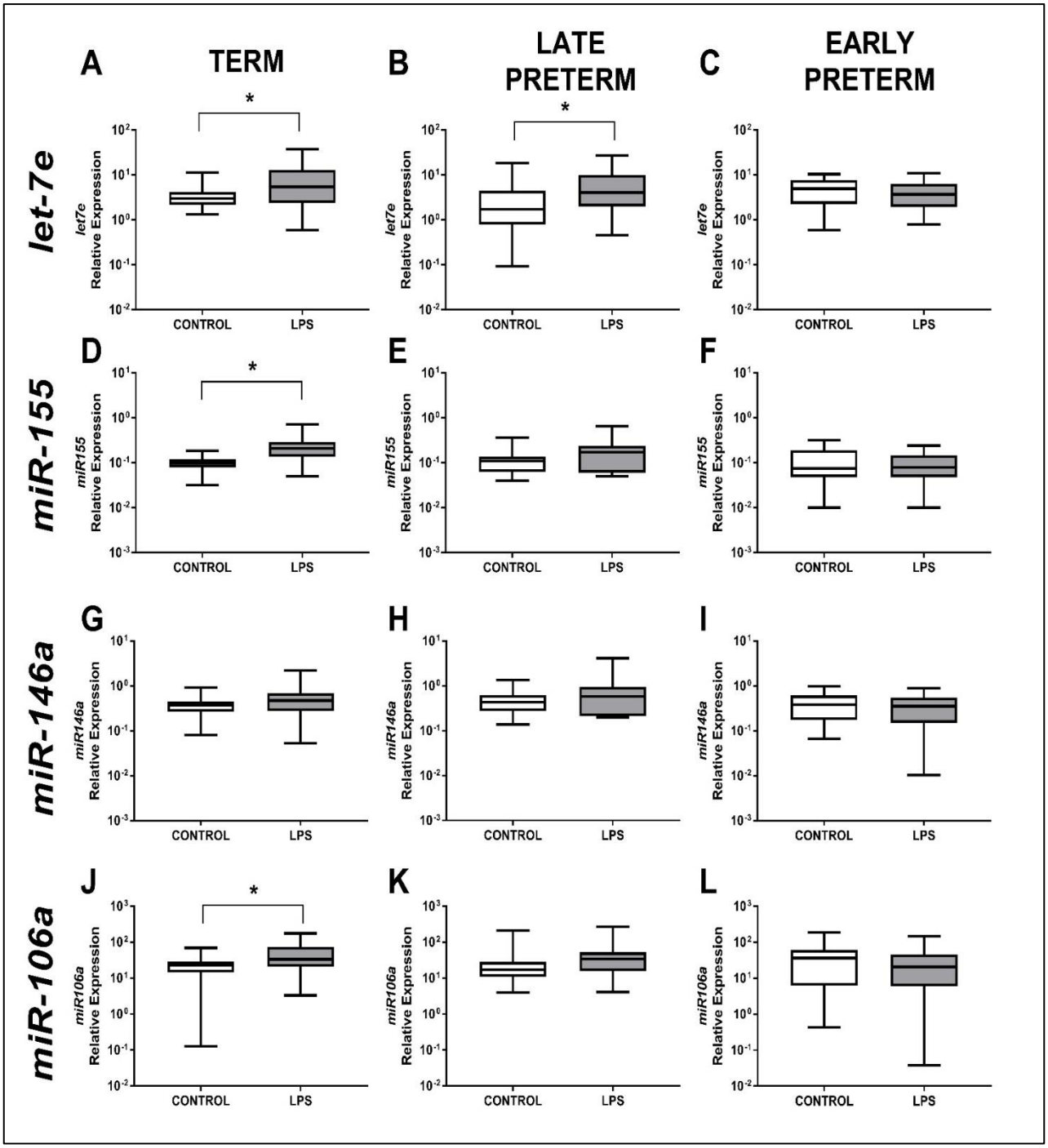

Fig. 5.9. ***Cord blood miR expression with and without LPS stimulation.*** The expression of *let-7e* (A-C), *miR-155* (D-F), *miR-146a* (G-I) and *miR-106a* (J-L) relative to *RNU48* in term (A, D, G, J), late preterm (B, E, H, K) and early preterm (C, F, I, L) cord blood at 6 hours *in vitro* following LPS stimulation or under control conditions. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

5.4.5. CORD BLOOD mRNA EXPRESSION FOLLOWING LPS (TLR4) STIMULATION

The expression of genes associated with TLR signalling (mRNA) was collected for a smaller subset of samples and the analysis was split according to term (*n*= 15), late preterm (*n*= 10) and early preterm (*n*= 10) cord blood. Based on the miR results following LPS stimulation compared to PGN or Poly I:C, our analysis of mRNA expression focused only on responses to LPS stimulation. This strategy prioritised experiments based on limited blood and therefore RNA volumes available following preterm delivery.

The frequency of mRNA expression by LPS-stimulated cord blood was not significantly different according to the gestational age groups (Table 5.2).

Table 5.2. ***The frequency of gene expression (mRNA) in LPS-stimulated cord blood.*** The frequency (*n*, %) of TLR-associated gene expression (mRNA) in LPS-stimulated cord blood from term, late and early preterm deliveries.

	Term *n*= 15	**Late preterm** *n*= 10	**Early preterm** *n*= 10	***p***
TLR2	14 (93%)	10 (100%)	10 (100%)	0.227
TLR4	15 (100%)	9 (90%)	8 (80%)	0.406
MyD88	14 (93%)	7 (70%)	7 (70%)	0.171
IRAK1	14 (93%)	7 (70%)	10 (100%)	0.785
NF-κB1	15 (100%)	6 (60%)	10 (100%)	0.226
IL10	14 (93%)	9 (90%)	7 (70%)	0.403
IL6	12 (18%)	9 (90%)	8 (80%)	0.668
SOCS1	15 (100%)	10 (100%)	10 (100%)	0.410

Late preterm cord blood showed increased *TLR2* expression following LPS stimulation after adjusting for covariates pre-eclampsia, labouring delivery, maternal smoking during pregnancy and birthweight centile (F(1,10)= 10.582, p=0.008; Fig. 5.10). There was a trend towards increased *TLR2* expression in term cord blood following LPS stimulation compared to unstimulated samples, however, this did not reach statistical significance (p=0.077). Early preterm cord blood showed no change in *TLR2* expression following LPS stimulation.

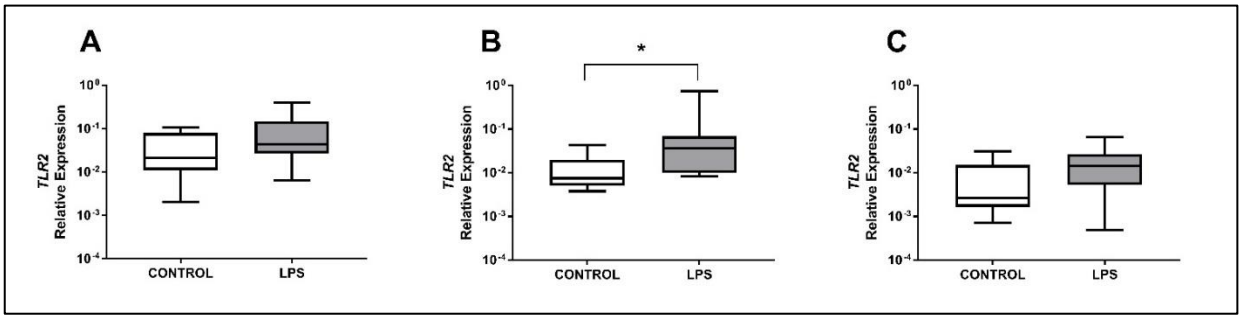

Figure 5.10. ***The expression of*** **TLR2** ***in cord blood with and without LPS stimulation.*** The expression of *TLR2* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood in control samples and following LPS stimulation at 6 hours. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

NF-κB1 expression was increased in term cord blood (F(1,21)= 5.968, p=0.023; Fig. 5.11) and early preterm cord blood following LPS stimulation (F(1,12)= 6.145, p=0.029). Late preterm cord blood showed no difference in *NF-κB1* expression following LPS stimulation.

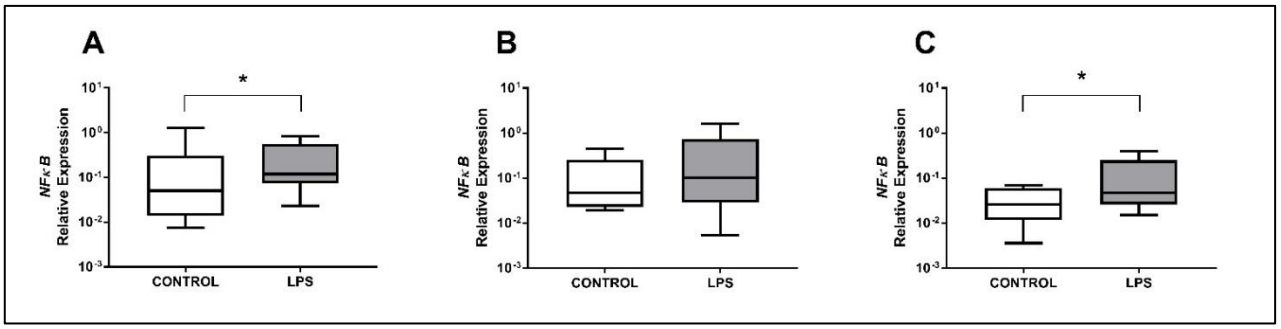

Figure 5.11. ***The expression of*** **NF-κB1** ***in cord blood with and without LPS stimulation.*** The expression of *NF-κB1* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood in control samples and following LPS stimulation at 6 hours. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

IL6 expression was increased following LPS stimulation in term cord blood (F(1,22)= 13.120, p=0.002), late preterm (F(1,10)=99.283, p<0.001) and early preterm cord blood (F(1,11)= 14.693, p=0.003; Fig.5.12).

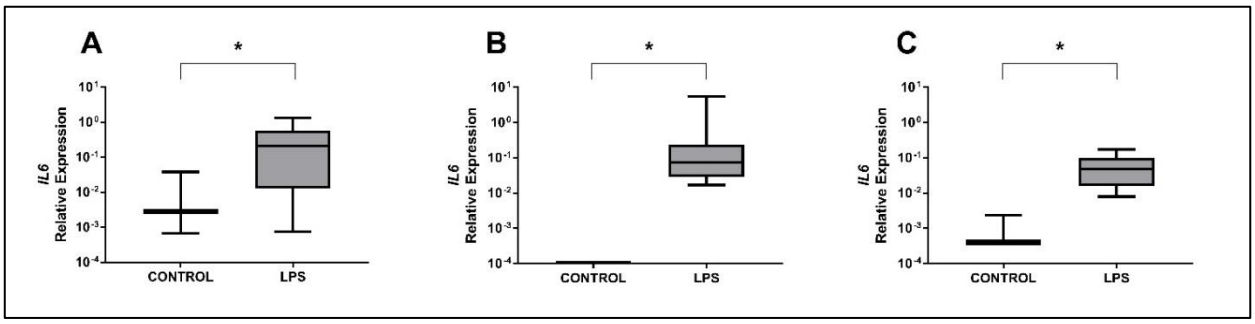

Fig. 5.12. ***The expression of*** **IL6** ***in cord blood with and without LPS stimulation.*** The expression of *IL6* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood following 6 hours in control samples and following LPS stimulation at 6 hours. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

Late preterm cord blood increased *SOCS1* expression following LPS stimulation (F(1,11)=6.861, p=0.024; Fig.5.13). Term and early preterm cord blood showed no difference in *SOCS1* expression following LPS stimulation.

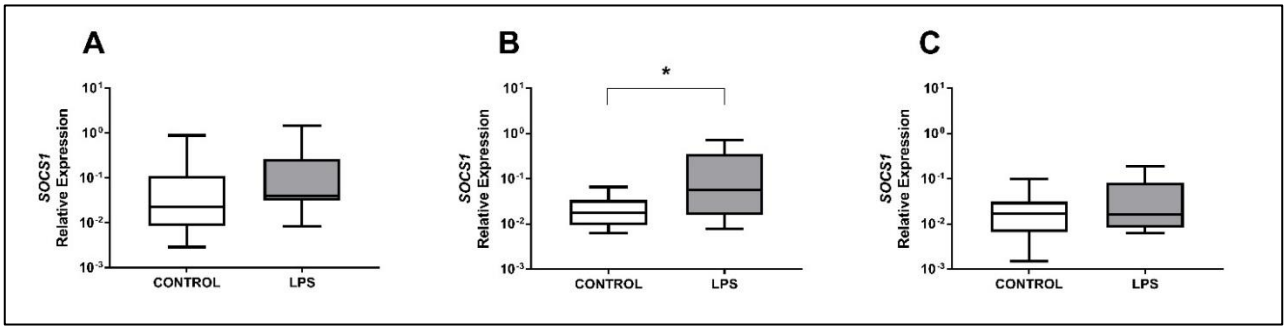

Fig. 5.13. ***The expression of*** **SOCS1** ***in cord blood with and without LPS stimulation.*** The expression of *SOCS1* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood following 6 hours in control samples and following LPS stimulation at 6 hours. Note: the y-axes are presented on a logarithmic scale, *p<0.05.

There was no difference observed in *TLR4*, *MyD88*, *IL10* or *IRAK1* expression following LPS stimulation within the gestational age groups (Fig. 5.14-17).

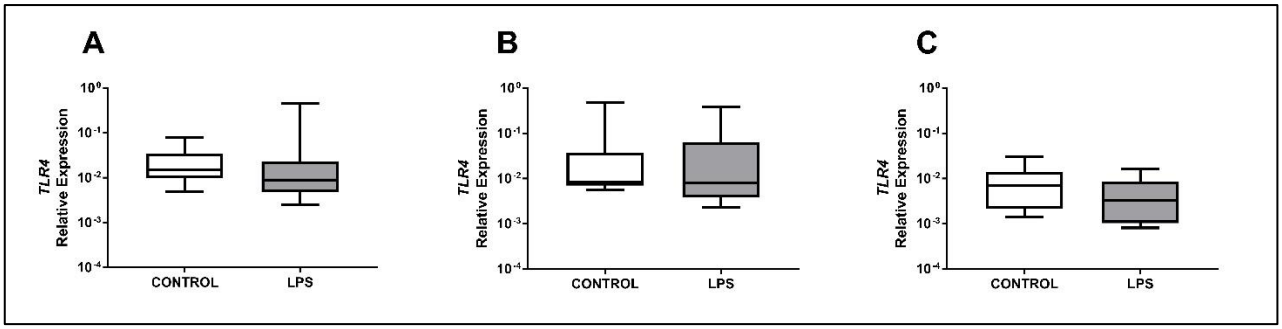

Fig. 5.14. ***The expression of*** **TLR4** ***in cord blood with and without LPS stimulation.***
The expression of *TLR4* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood following 6 hours under control conditions and following LPS stimulation. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

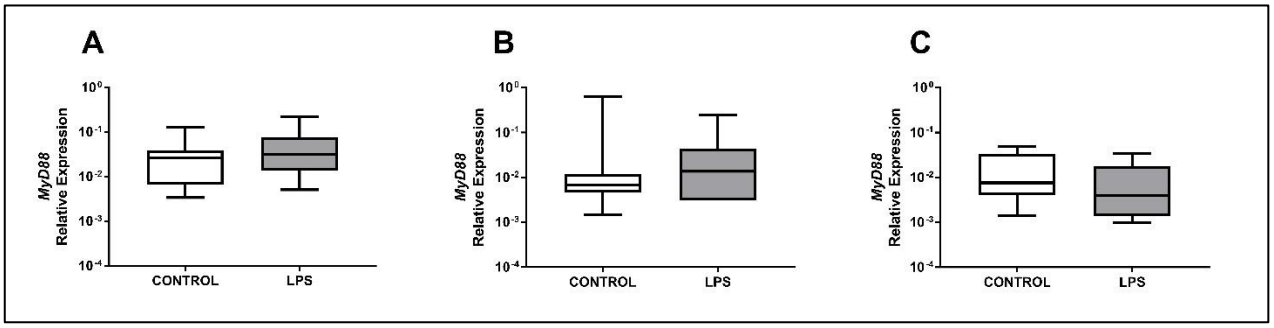

Fig. 5.15. ***The expression of*** **MyD88** ***in cord blood with and without LPS stimulation.***
The expression of *MyD88* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood following 6 hours under control conditions and following LPS stimulation. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

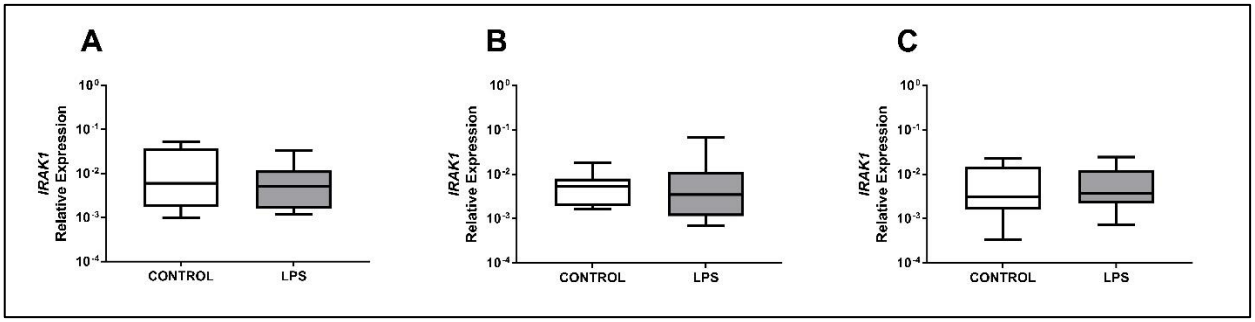

Fig. 5.16. ***The expression of*** **IRAK1** ***in cord blood with and without LPS stimulation.***
The expression of *IRAK1* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood following 6 hours under control conditions and following LPS stimulation. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

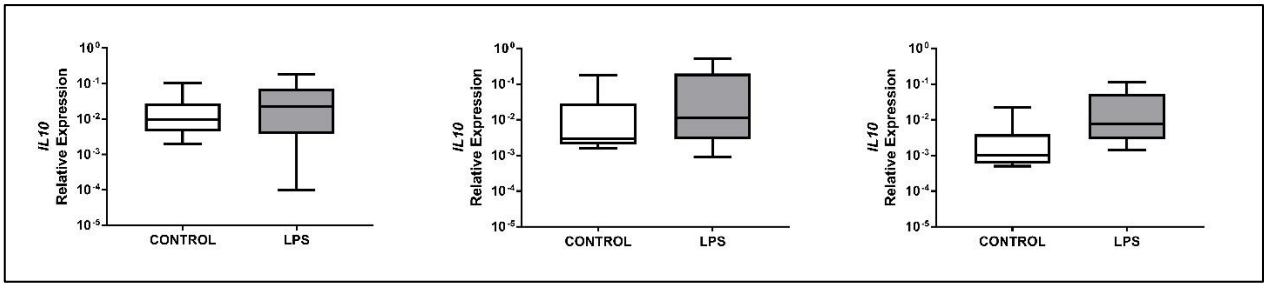

Fig. 5.17. ***The expression of*** **IL10** ***in cord blood with and without LPS stimulation.*** The expression of *IL10* relative to *β-actin* in in (A) term, (B) late preterm and (C) early preterm cord blood following 6 hours under control conditions and following LPS stimulation. Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

There were no correlations between the expression of *let-7e, miR-155, miR-146a, miR-106a* and their respective targets: *TLR4, SOCS1, IRAK1* and *TRAF6* or *IL10* in term, late preterm or early preterm cord blood. As a common regulator of miRs, *NF-κB1* was not associated with miR expression in these samples.

5.5. DISCUSSION

This study is the first to show there is a disparity in TLR-stimulated miR expression between term, late preterm and early preterm cord blood. Further, *IL6* was the only gene investigated that was consistently increased in cord blood following LPS stimulation by all gestational age groups. In term cord blood, LPS or PGN exposure increased *miR-155.* LPS stimulation also increased *let-7e, miR-106a* and *NF-κB1* expression in term cord blood. Similarly, late preterm cord blood increased *let-7e*, *TLR2* and *SOCS1* with LPS stimulation. In contrast to this, early preterm cord blood stimulated with LPS increased *NF-κB1* without any change in miR expression. No changes in cord blood gene expression were observed in any gestational age group following Poly I:C stimulation. These data demonstrate an absence of miR expression following TLR stimulation in early preterm cord blood despite increased expression of pro-inflammatory *NF-κB1* and *IL6*.

The lack of a miR response to TLR stimulation in early preterm cord blood may be associated with immature miR transcript processing. A previous study analysed 2,108 term cord blood samples for the expression of the miR processing complex, Dicer[455]. Interestingly, *Dicer* mRNA expression was decreased in cord blood from neonates who later developed severe RSV infections during the neonatal period compared to healthy neonates. The authors of this study postulated that in this scenario, decreased Dicer expression led to an increased viral load, as it has previously been associated with the inhibition of viral expression in the context of Influenza A[456]. While our findings are not specific to viral immunity, decreased Dicer expression could contribute to the absence of a miR response to TLR stimulation in early preterm cord blood. This would not only predispose early preterm neonates to contracting viral infections, but also underlie an inability to control the inflammatory response to other pathogens.

Both term and late preterm cord blood increased *let-7e* expression in response to TLR stimulation. *let-7e* is best known for targeting *TLR4* and therefore, downregulating TLR signalling[341]. Term and late preterm cord blood therefore show evidence of a capacity to regulate inflammatory gene expression, while early preterm cord blood does not. Our study showed simultaneous increases in inflammatory and regulatory genes as we

investigated the peak synthesis phase of cytokine expression (6 hours[201]). Increased expression of the pre-miR, let7 has been associated with decreased IL10 and IL6 expression in human and murine macrophages stimulated *in vitro* with *Salmonella* and *E. coli*[457]. We therefore postulate that as TLR signalling progresses, term and late preterm neonates eventually express enough regulatory *let-7e* to downregulate cytokine expression. In contrast, early preterm neonates do not show this regulatory miR response, at least with respect to the TLR ligands we used for this study.

Interestingly, we observed no difference in miR expression following TLR3 stimulation with Poly I:C in any gestational age group. These results are unsurprising as *TLR3* mRNA was undetectable in cord blood at birth (Chapter 4). Others have also found CBMCs do not express cytokines in response to Poly I:C[78] or TLR7/8 viral agonist stimulation[48]. The only studies that have been able to demonstrate a cytokine response to viral stimulation have used high concentrations of Poly I:C in conjunction with IFNγ[254], which is normally decreased in cord blood compared to adult blood[201]. It is likely the absence of cytokine expression following viral stimulation *in vitro* also reflects an increased susceptibility by neonates towards viral infections such as RSV[277].

The expression of *IRAK1* and *MyD88* did not change following LPS stimulation in term or preterm cord blood, and neither did IRAK1's negative regulator, *miR-146a*[323]. Others have observed increased *miR-146a* expression after 24 hours LPS stimulation *in vitro* in term cord blood monocytes[242]. While their findings support previous evidence that *miR-146a* is regulated by NF-κB expression during terminal TLR signalling[458], we characterised an earlier time-point which may exclude these effects. In fact, *miR-146a* expression is typically shown to increase up to 100 fold between 24-48 hours *in vitro* in association with decreased *IRAK1* and cytokine expression[89, 90]. The effect of increased *NF-κB1* expression on *miR-146a* expression in term and early preterm cord blood are not evident in the current analysis.

Increased *NF-κB1* expression in term and early preterm cord blood stimulated with LPS suggests active inflammatory cytokine transcription. Interestingly, preterm cord blood monocytes have shown increased NF-κB phosphorylation following bacterial stimulation compared to term[454]. Additionally, intestinal epithelial cells from preterm neonates also show increased NF-κB activation and cytokine expression in response to flagellin (a TLR5 agonist), and decreased expression of anti-inflammatory IκB,

compared to adults[459]. Similarly, rodent models of NEC show increased NF-κB activation and decreased IκB expression[307], leading to inflammatory injury that precedes NEC[61]. Together, these studies indicate that over-expression of NF-κB by TLR-stimulated early preterm cord blood, in the absence of miR expression, could be a contributing mechanism to uncontrolled inflammation in these neonates.

Although *miR-106a* is known to target *IL10*[339], term cord blood showed increased *miR-106a* expression and no difference in *IL10* with LPS stimulation. Conversely, early preterm cord blood showed no change in the expression of *miR-106a,* but increased *IL10* with LPS stimulation. The concurrent increase in *IL10* and *IL6* in early preterm cord blood suggests a 'genomic storm' scenario, where inflammatory transcription is increased without resolving inflammation. While IL10 is an anti-inflammatory cytokine, its increased expression has been associated with septic shock where patients increase the expression of both pro- and anti- inflammatory genes[460]. Furthermore, peripheral blood from term neonates with sepsis expresses increased IL10 and pro-inflammatory IL8, IL6 and IL1β[461]. Therefore, the increase in IL10 and IL6 without any change in regulatory miRs could be evidence of a system that is hyper-responsive to inflammatory stimulation.

Neonatal immune development is shaped by the *in utero* environment (see section 1.3.2). For example, transcriptome-wide analyses of neonatal peripheral blood have also shown that exposure to chorioamnionitis is associated with a unique pattern of inflammatory gene expression compared to unexposed neonates[390]. Maternal smoking during pregnancy has also been associated with altered inflammatory signalling in cord blood, where CBMCs show decreased IL6, TNFα and IL10 expression following TLR stimulation *in vitro* compared to non-smoking mothers[162, 163]. We therefore used these factors as covariates in our analysis to adjust for the potential influence they may have on stimulated immune responses. In the current study, we observed no significant interaction effects between smoking and gestational age. The ability to discriminate the independent effects of these exposures would be interesting, however, would require a larger sample size. Further, many *in utero* insults can increase the risk of preterm birth and co-occur with different clinical management strategies. This would make it difficult to isolate the impact of these exposures, even in a larger sample size. Animal studies and *in vitro* modelling may be the best way to understand these independent effects.

A major strength of our study is that we stimulated whole cord blood. This method allowed for a holistic insight into neonatal immune function. Further, previous findings from our laboratory have shown that isolated CBMCs do not respond as robustly to TLR stimulation over time or increasing doses of TLR agonists[153]. Cord blood leukocytes are immature compared to adults and likely require support from other factors in whole blood to mount effective immune responses. Whole blood also contains platelets, which express TLRs and can contribute to inflammatory signalling[462]. Notably, miRs show cell-specific function which is a limitation of the current study. As such, we cannot determine whether specific cells are responsible for the upregulation of certain genes that could explain the observed gestational age-specific responses to TLR stimulation. For example, Tregs numbers are increased in preterm cord blood compared to term[463, 464]. The FOXP3 protein is specific to Tregs and ensures their survival and proliferation[465] and has been shown to suppress *miR-155* expression in Tregs[466]. Therefore, if preterm cord blood has increased numbers of Tregs, they may be suppressing any increases in TLR-induced *miR-155* expression in early preterm cord blood. Future studies could confirm this using flow cytometry targeting cell-specific miR expression.

In summary, the coordinated increase in pro-inflammatory and anti-inflammatory genes observed in term but not preterm cord blood suggests the key miRs we measured may not regulate inflammation as effectively in preterm neonates. This deficiency in miR-based immune regulation could contribute to chronic inflammatory signalling that precedes inflammatory disease. Overall, our novel findings support that preterm neonatal inflammatory responses are not intrinsically immature, rather, their coordination and regulation of TLR signalling may be dysfunctional.

Chapter 6:

Temporal Changes in Cord Blood Gene Expression Following TLR Stimulation

6.1. ABSTRACT

Background

The process of inflammation changes over time under the influence of genetic regulators such as microRNAs (miRs). MiRs exhibit time-sensitive expression, forming feedback loops with other genes involved in inflammatory Toll-like Receptor (TLR) signalling. In Chapter 5, term and late preterm cord blood increased miR expression following TLR stimulation *in vitro* while early preterm cord blood did not; however, this finding was limited by the analysis of a single time-point. This study therefore aimed to identify temporal differences in TLR signalling between term and preterm cord blood to characterise potential disruptions in inflammatory feedback mechanisms.

Methods and Results

Term, late and early preterm whole cord blood were collected and rested *in vitro* overnight. A rested sample was collected as a control measure and then cord blood was stimulated with TLR2, 3 or 4 agonists for 2, 6 or 24 hours before collection. Expression of miRs and mRNA associated with TLR signalling was quantified using qPCR. Cord blood cytokine production was quantified using ELISA. There was no change in the expression of any miRs or mRNAs over time in control cultures. In response to PGN, preterm cord blood increased IL6 (p=0.004) and TNFα production over time (p=0.002). In response to LPS, preterm cord blood increased IL6 (p=0.002) and TNFα over time (p<0.001). Term cord blood increased TNFα in response to PGN (p<0.001) and LPS (p=0.004) over time.

Conclusion

Preterm cord blood upregulates pro-inflammatory cytokine expression over time following TLR stimulation without changing the expression of anti-inflammatory genes associated with TLR signalling. The lack of a temporal response to inflammatory stimulation by preterm cord blood may contribute to uncontrolled inflammation in preterm neonates.

6.2. INTRODUCTION

Inflammation involves a dynamic series of processes which change in response to gene expression over time. It begins with the recruitment of leukocytes to the site of antigen recognition followed by pathogen killing, inflammatory resolution and repair of host tissues. These processes require regulation so they occur sequentially to avoid prolonged, pathological inflammation. Inflammatory signalling is therefore increased or decreased through positive and negative feedback loops created by genetic regulators such as miRs.

TLR signalling is a key inflammatory mechanism that involves the sequential recruitment of adaptor molecules over time. This process can be coordinated by miRs, which repress inflammatory gene expression over time. For example, approximately two hours after LPS stimulation *in vitro*, murine macrophages decrease *TLR4* mRNA expression, which leads to decreased expression of the pro-inflammatory cytokine, TNFα[296, 490]. At a similar time point, an increase in SOCS1 expression is observed[467], which contributes to the downregulation of cytokine signalling through NF-κB inhibition[313]. These sequential processes highlight time-dependent changes in gene expression that regulate inflammation.

In the context of TLR signalling, *miR-146a* and *miR-155* play important roles. *miR-146a* forms part of a negative feedback loop as it targets *IRAK1* and *TRAF6*, therefore contributing to decreased signalling along the TLR cascade. The expression of *miR-146a* is NF-κB-dependent, as its promoter region is located downstream of NF-κB binding sites. *miR-146a* expression is therefore upregulated with NF-κB signalling and peaks later during the inflammatory response (approximately 8 hours following pathogen exposure)[323]. This delayed timing is critical to allow the initial inflammatory response to occur. *miR-155* also exhibits time-specific expression, which has implications for expression of its target, *SOCS1*[341]. Murine macrophages show increased *miR-155* expression as early as 30 minutes following TNFα exposure, followed by a decrease in *miR-155* at 1 hour *in vitro*[362]. The expression of *miR-155* is repressed by both NF-κB[468] and IL10[337]. Further, *IL10* mRNA is regulated by *miR-106a*, highlighting the intricate network of temporal regulation of the TLR signalling cascade at miR, mRNA and protein levels.

Chapter 5 demonstrated concurrent increases in pro- and anti-inflammatory genes by term cord blood at 6 hours following LPS stimulation and we suggested term neonates exhibit inflammatory regulation through miRs. Alternately, the lack of a response by early preterm

cord blood suggests its miR expression is unresponsive to inflammatory stimuli. Previous studies have characterised temporal responses to LPS stimulation in cord blood, showing that neonates exhibit a delayed, but increased response in *miR-146a* expression over 24 hours compared to adults[242]. This chapter therefore aimed to assess a time-course of miR and mRNA expression following TLR stimulation to examine if and when these genes were inducted by term and preterm cord blood. It was hypothesised that preterm cord blood would show increased pro-inflammatory and decreased anti-inflammatory gene expression over time.

6.3. METHODS

6.3.1. PARTICIPANTS & SAMPLE COLLECTION

Pregnant women (*n*=49) presenting at the LMH and WCH, who delivered at early preterm (<32 weeks), late preterm (32-36 weeks) or term gestation (≥ 37 weeks) were recruited to this study. Clinical data were obtained from maternal and neonatal health records, including obstetric history and neonatal outcomes (see section 2.1). Cord blood was collected into lithium heparin vials at delivery for *in vitro* culture.

6.3.2. CORD BLOOD CULTURE AND TLR STIMULATION

Whole cord blood was plated in volumes of 100µL in flat-bottomed 96-well cell culture plates and cultured at 37°C and 5% CO_2 overnight. Following this 'resting' period, one set of wells were left unstimulated (the untreated 'control') and remaining wells were stimulated with either PGN (TLR2 agonist; 100pg), Poly I:C (TLR3 agonist; 500pg), or LPS (TLR4 agonist; 1ng). Wells were collected and pooled in triplicate after 2, 6 or 24 hours *in vitro*.

Additional replicates of whole cord blood were diluted in sterile saline (1:4) and plated (100µL) for subsequent cytokine analyses. Following resting, wells were stimulated with either PGN (100ng), Poly I:C (5µg) or LPS (1µg). Wells were collected and pooled in duplicate following 2, 6 or 24 hours *in vitro.* Replicates were centrifuged to extract culture supernatant.

6.3.3. WHOLE RNA EXTRACTION

TRIzol LS® reagent was used to extract RNA from cord blood (see section 2.5.2). Briefly, three volumes of TRIzol LS were added to one volume of cord blood and samples were mechanically agitated. Lysates were separated by centrifugation with chloroform to retrieve the interphase for subsequent washing with isopropanol and ethanol. RNA was eluted in RNase free water and purified using a miRNeasy Mini Kit and DNAse I treatment as per manufacturers' protocol. RINs were assessed using a bioanalyser, where a RIN ≥ 9 was considered sufficient for PCR analysis.

6.3.4. qRT-PCR ANALYSIS OF miRNA EXPRESSION

RNA (10ng) was reverse transcribed using a Taqman® MicroRNA Reverse Transcription Kit according to manufacturer's protocol, that was slightly modified for optimal cDNA output (see section 2.6.1). This involved pooling a maximum of five RT primers per batch in a single RT mixture. Reverse transcription targeted five primer sequences: *let-7e*, *miR-155*, *miR-146a*, *miR-106a* and *RNU48*. Relative miRNA expression was quantified using Taqman® MicroRNA Assays in 20µL reactions containing 1.33µL of cDNA. qRT-PCR utilised the comparative cycle threshold ($2^{-\Delta CT}$) method to determine miR abundance relative to the reference gene *RNU48*.

6.3.5. qRT-PCR ANALYSIS OF mRNA EXPRESSION

Purified RNA (50ng) was reverse transcribed using a SuperScript™ III First-Strand Synthesis Kit for qRT-PCR according to manufacturer's protocol. qRT-PCR was used to quantify the relative expression of miR targets including *TLR2, TLR4, MyD88, IRAK1, NF-κB1, IL6, IL10* and *SOCS1* relative to the house-keeping gene*, β-actin*. qRT-PCR was performed using TaqMan® Gene Expression Assays and each 20µL reaction contained 2µL of the cDNA template.

6.3.6. CORD BLOOD SERUM PROTEIN ANALYSES

Single-target ELISAs were used to measure TNFα and IL6 in cord blood supernatant, as per manufacturer's instructions (see section 2.4.1). The lower detection limit for TNFα was 15.625pg/mL and the lower detection limit for IL6 was 31.25pg/mL. All inter-assay and intra-assay coefficients were below 10%.

6.3.7. STATISTICAL ANALYSES

Data were analysed using SPSS v24. Demographic and clinical data are presented as mean ± SD, unless otherwise indicated. Frequency data were analysed using Chi-squared tests. Kruskal-Wallis tests or ANOVAs were used to analyse continuous data. Post-hoc analyses were conducted using Mann-Whitney U tests or t-tests as required, using the Bonferroni adjustment to the critical alpha level. Gene and cytokine expression are presented as median (25^{th}-75^{th} centile), unless otherwise indicated, and were logarithmically transformed ($\log_{10}$) to normalise data for parametric analysis.

Repeated measures ANCOVAs were used to interpret transformed data, using pre-eclampsia, labouring delivery, maternal smoking during pregnancy, chorioamnionitis and antenatal betamethasone as covariates where required. Where assumptions of sphericity were violated, degrees of freedom were corrected using the Greenhouse Geisser estimates. Post-hoc comparisons were made using t-tests where appropriate. An *a priori* Bonferroni correction was made to the critical alpha level for post-hoc comparisons. An alpha level of <0.05 was considered statistically significant.

6.4. RESULTS

6.4.1. PARTICIPANT CHARACTERISTICS

Clinical characteristics from term (n=26), late preterm (n=13) and early preterm (n=10) deliveries are shown in Table 6.1. Maternal age, BMI, cigarette smoking during pregnancy, pre-eclampsia, gravidity and ethnicity were not significantly different between gestational age groups. Gestational age at delivery (completed weeks) was significantly different between the gestational age groups ($F(2,48)=319.239$, $p<0.001$). Parity was significantly different between the groups ($\chi^2(2)= 21.127$, $p=0.009$). Post-hoc tests showed that parity was higher in mothers who delivered late preterm compared to term ($p=0.009$) and between mothers who delivered late preterm compared to early preterm ($p=0.011$). Mode of delivery and labour were equally distributed between the groups.

Early (90%) and late preterm neonates (77%) were more frequently exposed to antenatal betamethasone compared to term neonates (12%; $\chi^2(2)=24.446$, $p<0.001$). Birthweight was significantly different between the gestational age groups ($F(2,48)=97.995$, $p<0.001$) and post-hoc tests showed it was lower in early preterm compared to late preterm and term, and between late preterm compared to term ($p<0.001$ for all). Birthweight centile was also significantly different between the groups ($F(2,48)=9.021$, $p<0.001$), with post-hoc tests showing it was lower in early preterm compared to term ($p=0.001$). The incidence of SGA and IUGR was more frequent among early preterm neonates compared to late preterm and term ($\chi^2(2)=12.772$, $p=0.002$; and $\chi^2(2)=14.763$, $p=0.001$, respectively). Birth length was significantly different between the groups ($F(2,44)=93.539$, $p<0.001$) and post-hoc tests revealed it was significantly lower between early preterm and term, early preterm and late preterm, and late preterm and term neonates ($p<0.001$ for all). Neonatal head circumference was significantly different between the groups ($F(2,44)=13.142$, $p<0.001$). Post-hoc tests showed it was significantly lower in early preterm compared to term ($p<0.001$) and late preterm compared to term ($p=0.035$). APGARS at 1 and 5 minutes were significantly different between the gestational age groups ($\chi^2(2)=11.007$, $p=0.004$; and $\chi^2(2)=13.783$, $p=0.001$, respectively). Post-hoc tests showed APGARS at one minute ($p=0.002$) and at five minutes ($p=0.006$) were lower in early preterm compared to term neonates.

Placental weight was significantly different between the gestational age groups ($F(2,38)=34.384$, $p<0.001$). Post-hoc tests showed it was lower in early preterm compared to

term (p<0.001), early preterm compared to late preterm (p=0.016) and late preterm compared to term deliveries (p=0.001). The incidence of chorioamnionitis was more frequent in early preterm deliveries (30%) compared to late preterm (8%) and term (0%; $\chi^2(2)=8.676$, p=0.013).

Table 6.1. ***Maternal demographics and neonatal characteristics of term, late and early preterm deliveries for cord blood analysed in Chapter 6.*** Values are given as mean± SD or *n* (%), unless otherwise indicated. *p-values* presented in the table were calculated using ANOVAs or Kruskal-Wallis tests. Post-hoc tests were conducted using t-tests or Mann-Whitney U tests and are represented within each gestational age group column by the following symbols: *$p \leq 0.05$ compared to both other groups; $^{\#}p \leq 0.05$ compared to term.

	Term	**Late Preterm**	**Early Preterm**	***p* value**
	n=26	*n*=13	*n*=10	
Gestational age (completed weeks), median (min-max)	**38 (37-41)***	**34 (32-36)***	**30 (27-31)**	**<0.001**
Maternal demographics:				
Age, years	30 ±4	28 ±7	30 ±7	0.301
BMI, kg/m^2	30 ±8	24 ±4	30 ±8	0.113
Gravidity, median (min-max)	2 (1-6)	2 (1-4)	3 (1-7)	0.250
Parity, median (min-max)	**1 (0-4)**	**0 (0-1)***	**2 (0-4)**	**0.009**
Smoking during pregnancy	4 (15)	1 (8)	2 (20)	0.628
Ethnicity:				
Caucasian	17 (65)	8 (62)	7 (70)	
Indigenous	0 (0)	0 (0)	1 (10)	0.391
Other	9 (35)	5 (38)	2 (20)	
Maternal complications:				
Pre-eclampsia	2 (8)	5 (39)	2 (20)	0.093
Multiple pregnancy	1 (4)	3 (23)	2 (20)	0.158
Labouring delivery	9 (35)	6 (46)	2 (20)	0.426
Mode of delivery:				
Emergency Caesarean section	5 (19)	6 (46)	3 (30)	
Elective Caesarean section	15 (58)	4 (31)	6 (60)	0.366
Vaginal delivery	6 (23)	3 (23)	1 (10)	
Placenta:				
Weight, g	**787 (±172)***	**446 (±160)***	**229 (±94)**	**<0.001**
Histological chorioamnionitis	**0 (0)**	**1 (8)**	**3 (30)**	**0.013**
Neonatal characteristics:				
Male	12 (46)	4 (31)	7 (70)	0.173
Birthweight, g	**3618 (±521)***	**2273 (±534)***	**1110 (±394) 16 (±23)***	**<0.001 <0.001**
Birth centile, %				
SGA	**63 (±31)***	**38 (±35)***	**7 (70)**	**0.002**
IUGR	**3 (14)**	**2 (15)**	**5 (50)**	**0.001**
Birth length, cm	**0 (0)**	**1 (8)**	**34 (±6)**	**<0.001**
Head circumference, cm	**50 (±2)* 35 (±1)***	**44 (±3)* 32 (±1)**	**29 (±7)**	**<0.001**
Antenatal betamethasone, *n* (%)	**3 (12)**	**10 (77)**	**9 (90)**	**<0.001**
APGARS, median, (min-max):				
1 min	**9 (6-9)**	**9 (3-9)**	**6 (2-9)$^{\#}$**	**0.004**
5 min	**9 (7-10)**	**9 (6-9)**	**7 (5-9)$^{\#}$**	**0.001**

6.4.2. THE EXPRESSION OF GENES ASSOCIATED WITH TLR SIGNALLING IN CORD BLOOD OVER TIME

To ensure that time alone did not alter gene expression, miR expression was compared in matched unstimulated samples of term and late preterm cord blood. Early preterm samples were not used for this analysis due to limitations in available cord blood. Although whole blood was cultured over 24 hours, RNA integrity was lost by the 24 hour time point. RNA integrity was, however, maintained at 2 and 6 hours (RIN ≥ 9). This meant that only qPCR data from 0, 2 and 6 hour time-points were available for analysis. No change was observed in miR or mRNA expression in unstimulated cultures over time (Figs. 6.1-10). There were insufficient data points on *IL6* and *IL10* expression in rested cultures, which may relate to the previously discussed issue of inconsistent constitutive cytokine gene expression (see section 4.5)

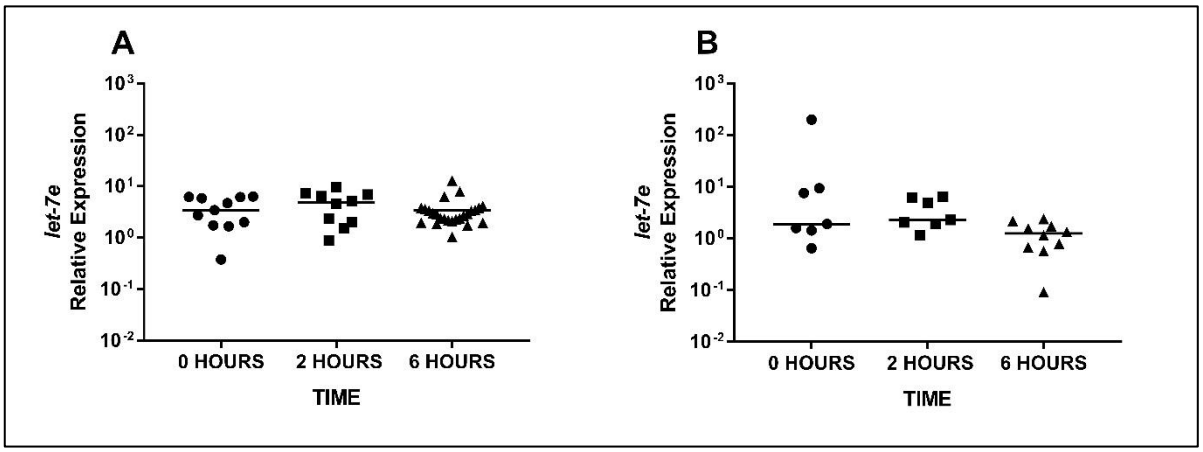

Figure 6.1. ***The expression of*** **let-7e** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *let-7e* relative to *RNU48* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

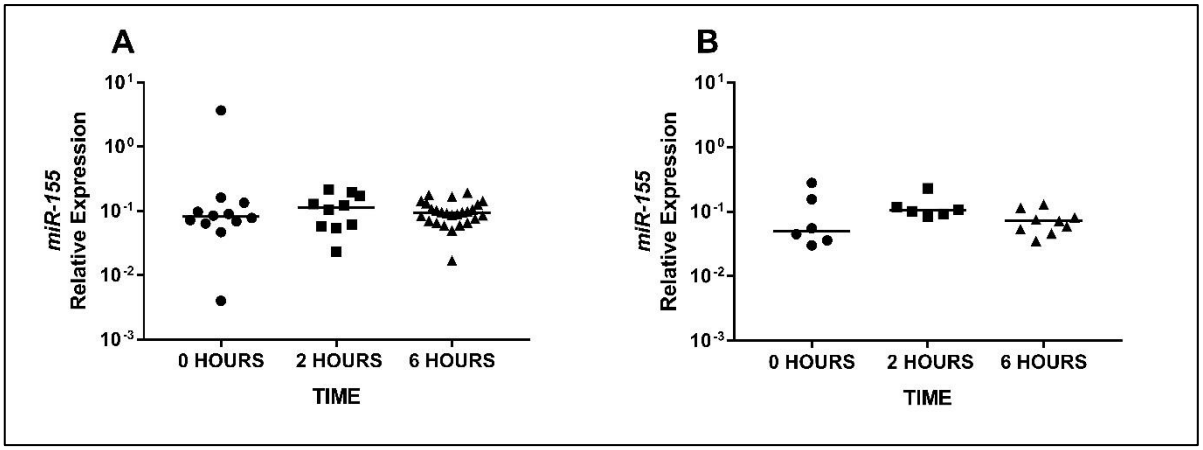

Figure 6.2. ***The expression of*** **miR-155** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *miR-155* relative to *RNU48* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

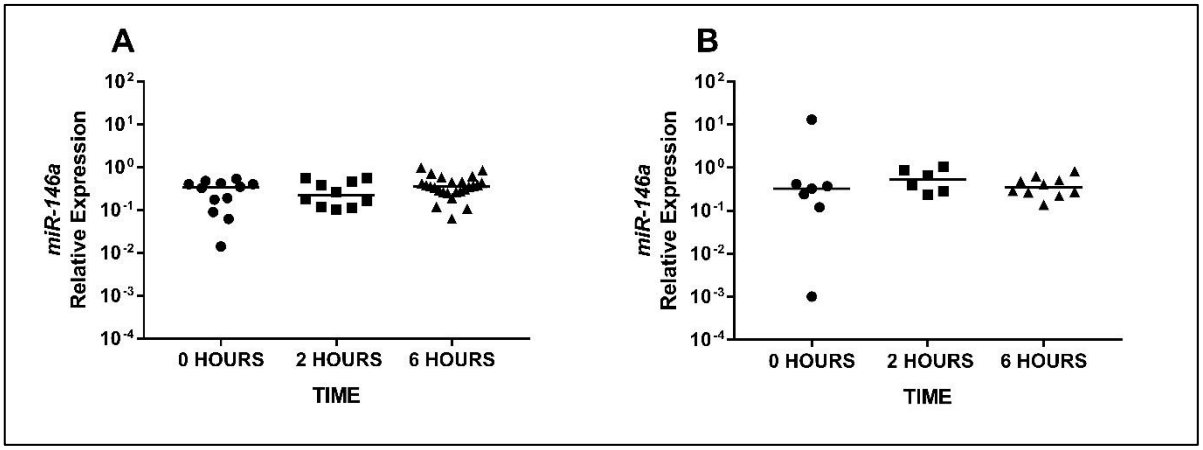

Figure 6.3. ***The expression of*** **miR-146a** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *miR-146a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

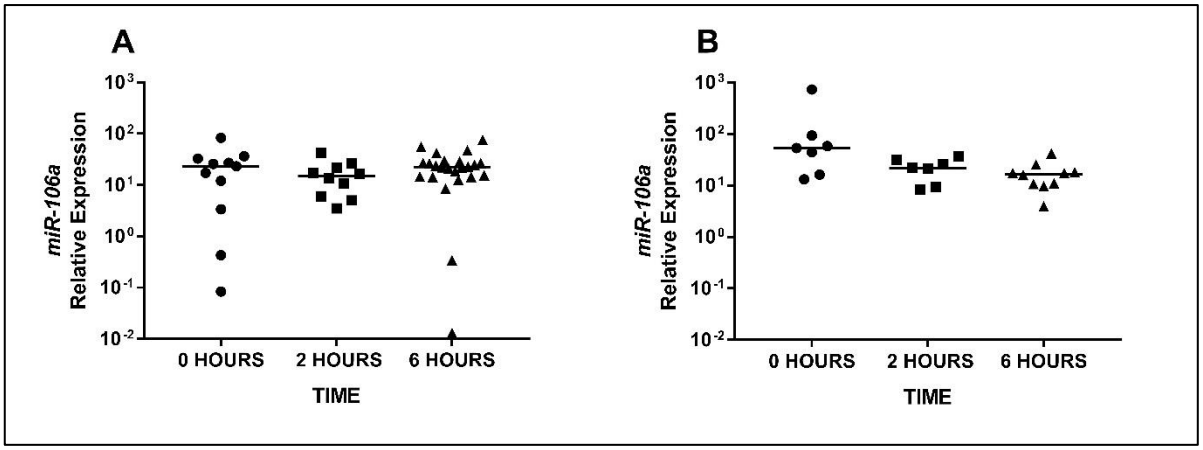

Figure 6.4. ***The expression of*** **miR-106a** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *miR-106a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

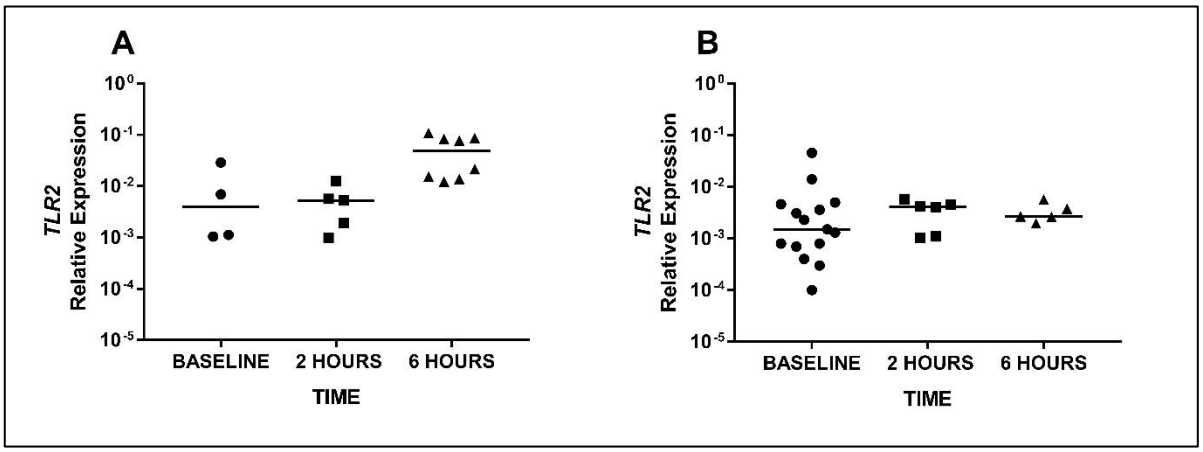

Figure 6.5. ***The expression of*** **TLR2** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *TLR2* relative to *β-actin* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

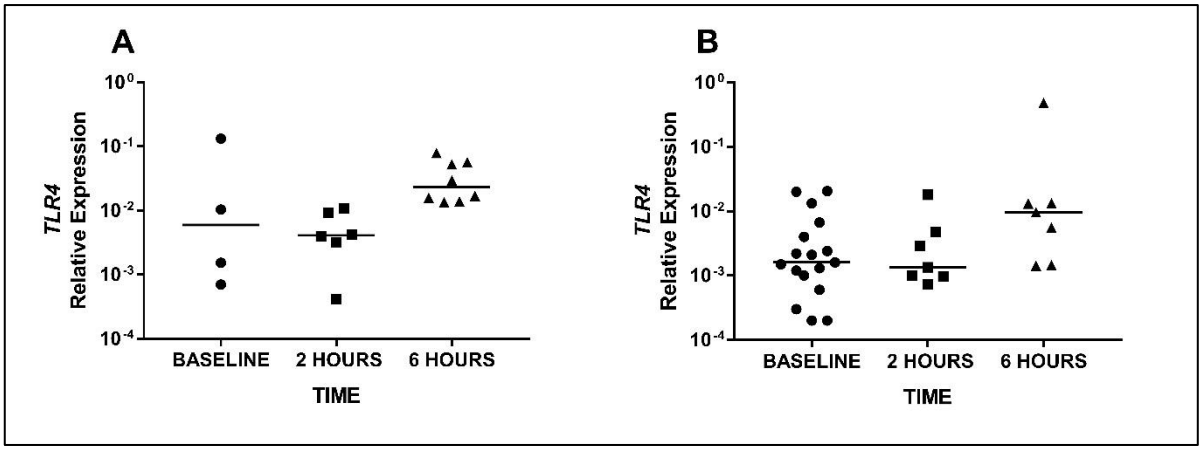

Figure 6.6. ***The expression of*** **TLR4** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *TLR4* relative to *β-actin* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

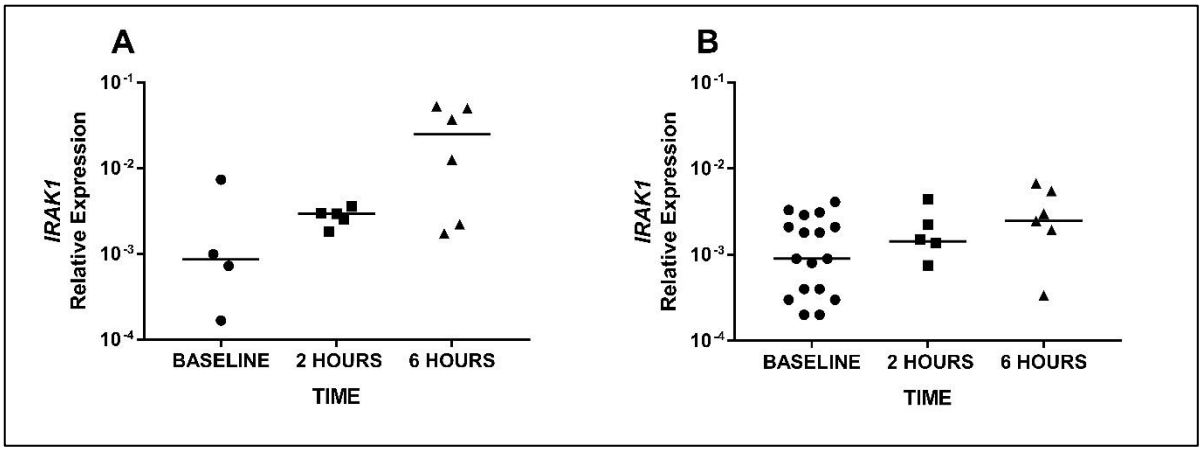

Figure 6.7. ***The expression of*** **IRAK1** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *IRAK1* relative to *β-actin* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

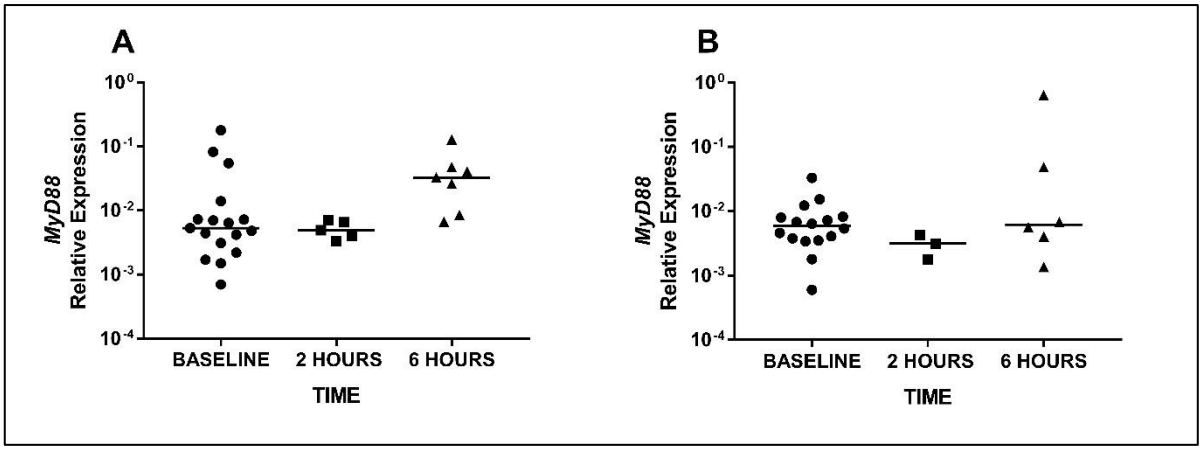

Figure 6.8. ***The expression of*** **MyD88** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *MyD88* relative to *β-actin* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

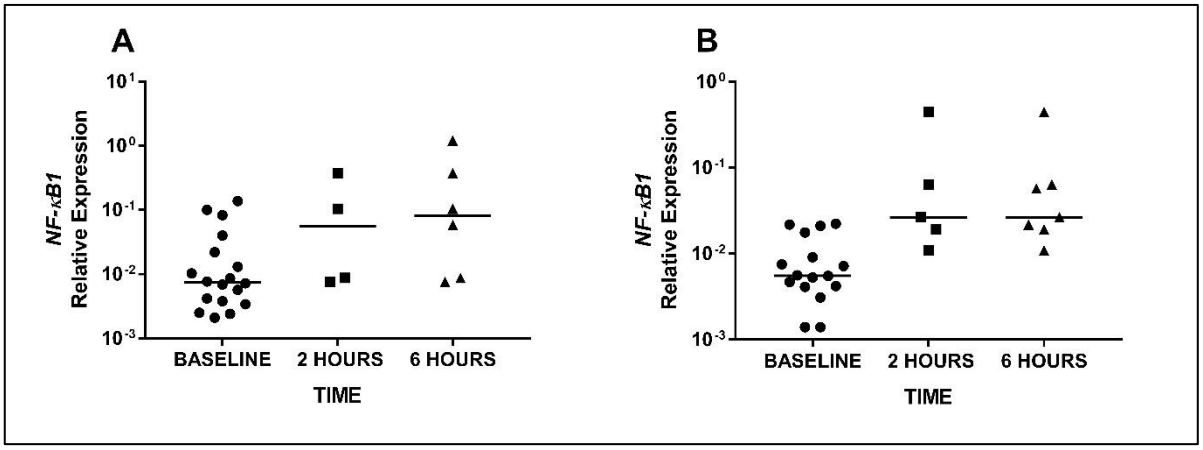

Figure 6.9. ***The expression of*** **NF-κB1** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *NF-κB1* relative to *β-actin* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

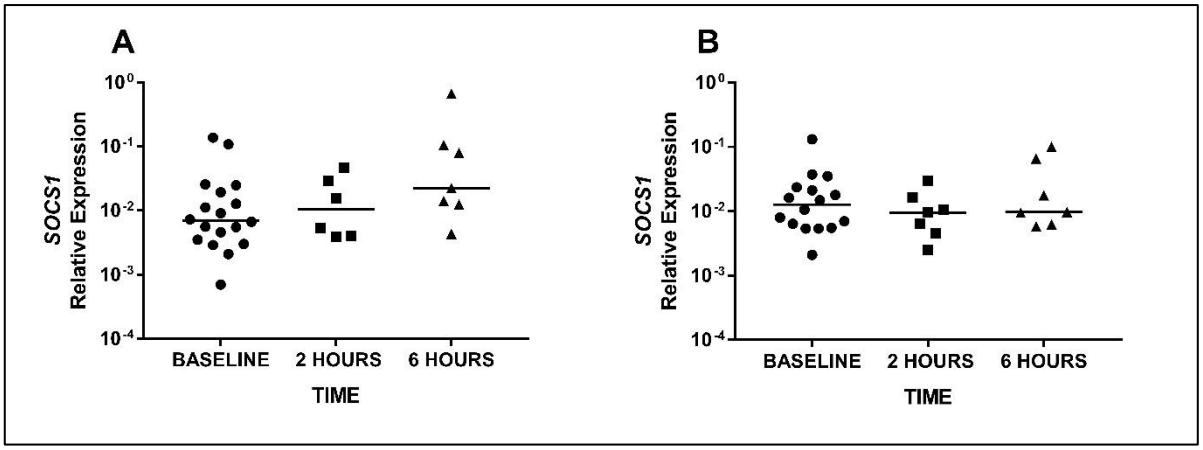

Figure 6.10. ***The expression of*** **SOCS1** ***in term and late preterm cord blood over time*** **in vitro.** The expression of *SOCS1* relative to *β-actin* in term (A) and late preterm cord blood (B) over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

6.4.2.1. TLR2 (PGN) Stimulation Over Time

MiR expression was analysed in term, late preterm and early preterm cord blood over time (between rested cultures, and 2 and 6 hours following PGN stimulation). There was no interaction between time and gestational age group in our analysis of cord blood *let-7e, miR-155* or *miR-146a* expression after adjusting for pre-eclampsia, maternal smoking, labour, chorioamnionitis and betamethasone exposure (Figs. 6.11-13). Similarly, no significant interaction effect of gestational age and time was observed for expression of *miR-106a* following stimulation, however a non-significant trend towards increased *miR-106a* expression in term cord blood was observed at 6 hours (*p*=0.058; Fig. 6.14).

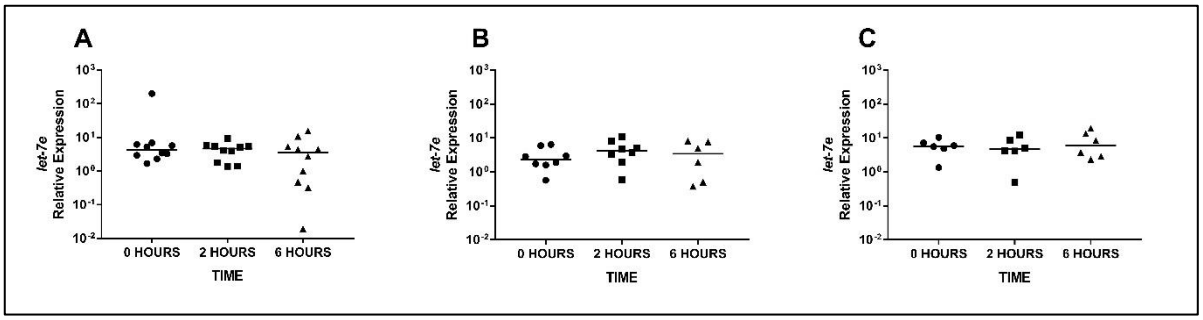

Figure 6.11. ***The expression of*** **let-7e** ***in term and late preterm cord blood over time following PGN stimulation.*** The expression of *let-7e* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following PGN stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

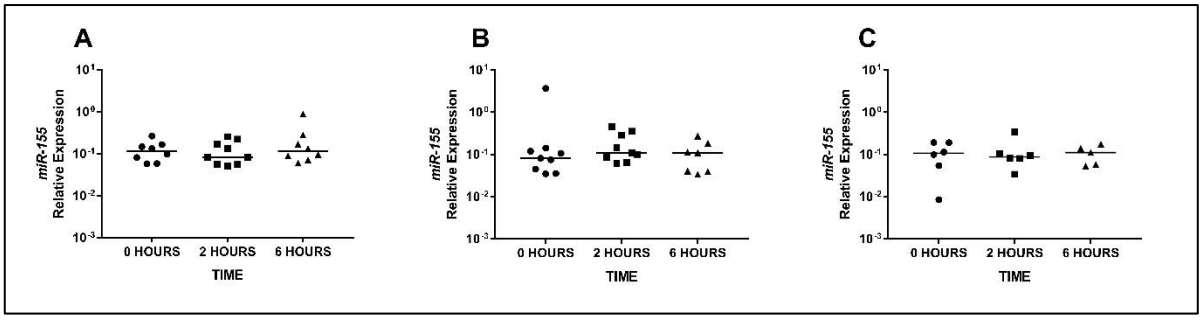

Figure 6.12. ***The expression of*** **miR-155** ***in term and late preterm cord blood over time following PGN stimulation.*** The expression of *miR-155* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following PGN stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

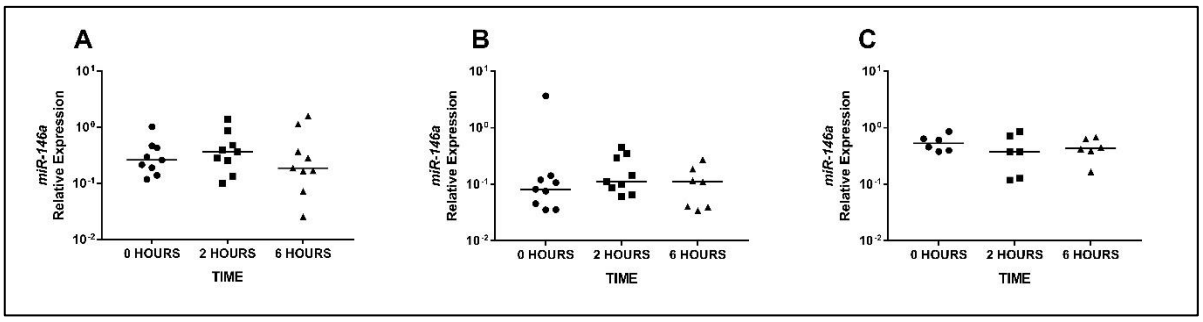

Figure 6.13. ***The expression of*** **miR-146a** ***in term and late preterm cord blood over time following PGN stimulation.*** The expression of *miR-146a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following PGN stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

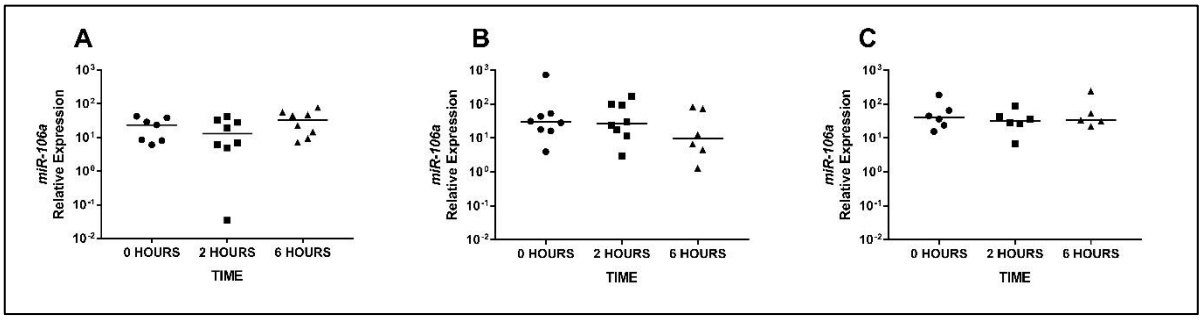

Figure 6.14. ***The expression of*** **miR-106a** ***in term and late preterm cord blood over time following PGN stimulation.*** The expression of *miR-106a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following PGN stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

6.4.2.2. TLR3 (Poly I:C) Stimulation over time

There was no interaction between time and gestational age in cord blood miR expression following Poly I:C stimulation over time after adjusting for pre-eclampsia, labour, maternal smoking, chorioamnionitis and antenatal betamethasone (Figs. 6.15-18).

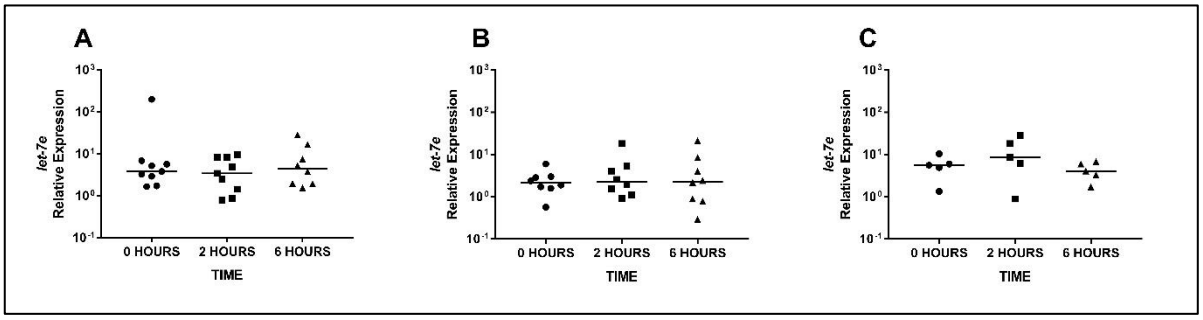

Figure 6.15. ***The expression of*** **let-7e** ***in term and late preterm cord blood over time following Poly I:C stimulation.*** The expression of *let-7e* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following Poly I:C stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

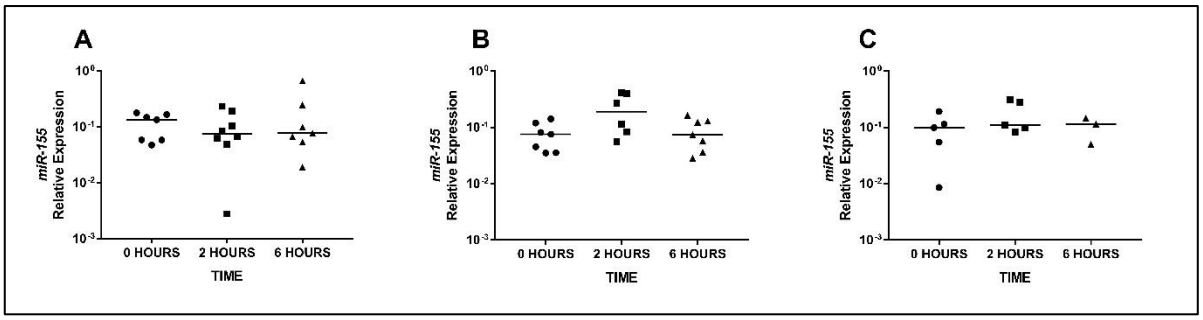

Figure 6.16. ***The expression of*** **miR-155** ***in term and late preterm cord blood over time following Poly I:C stimulation.*** The expression of *miR-155* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following Poly I:C stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

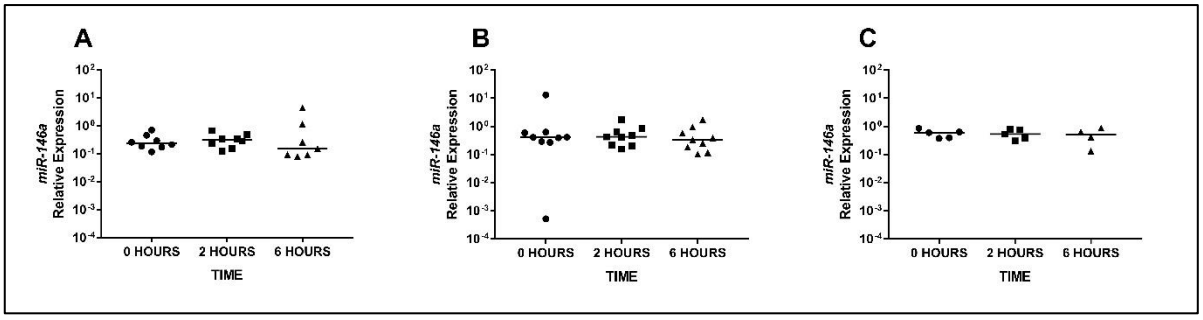

Figure 6.17. ***The expression of*** **miR-146a** ***in term and late preterm cord blood over time following Poly I:C stimulation.*** The expression of *miR-146a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following Poly I:C stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

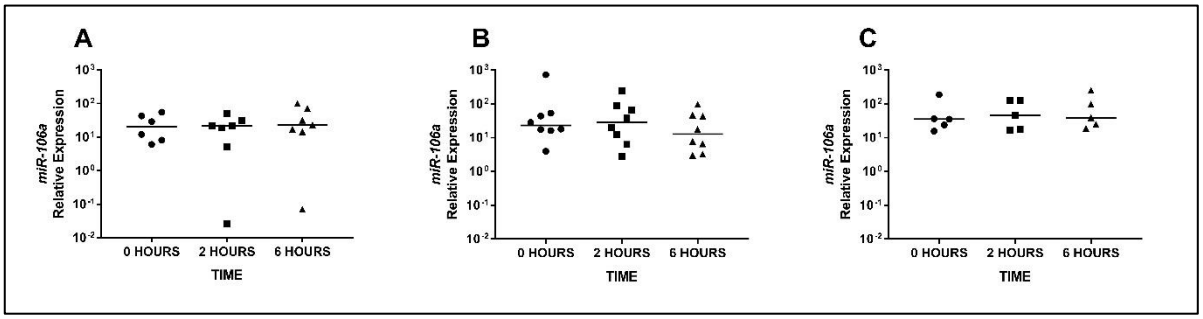

Figure 6.18. ***The expression of*** **miR-106a** ***in term and late preterm cord blood over time following Poly I:C stimulation.*** The expression of *miR-106a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following Poly I:C stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

6.4.2.3. TLR4 (LPS) Stimulation Over Time

There was no interaction between time and gestational age in cord blood miR expression following LPS stimulation over time after adjusting for pre-eclampsia, labour, maternal smoking, chorioamnionitis and antenatal betamethasone (Figs. 6.19-22).

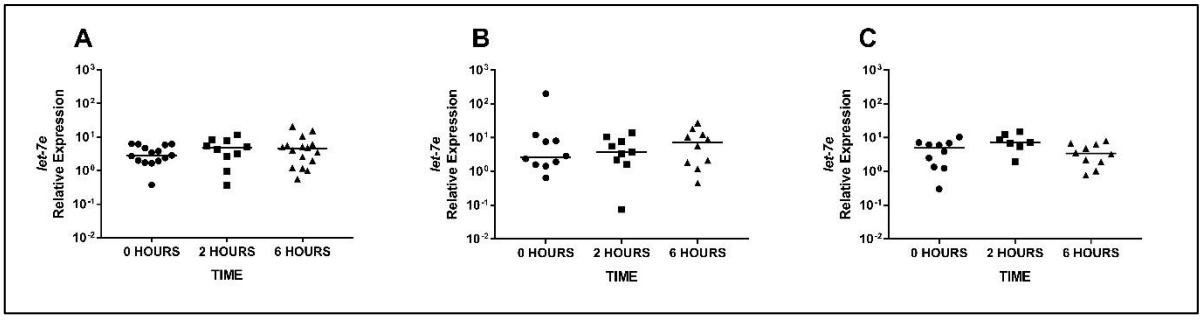

Figure 6.19. ***The expression of*** **let-7e** ***in term and late preterm cord blood over time following LPS stimulation.*** The expression of *let-7e* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following LPS stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

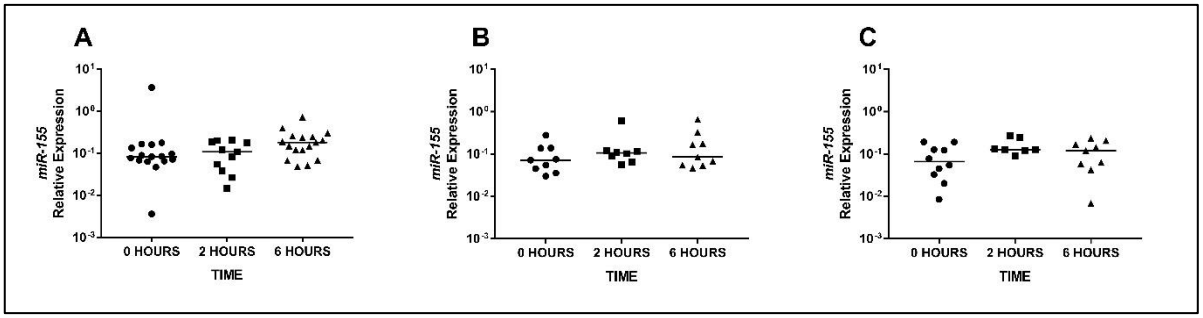

Figure 6.20. ***The expression of*** **miR-155** ***in term and late preterm cord blood over time following LPS stimulation.*** The expression of *miR-155* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following LPS stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

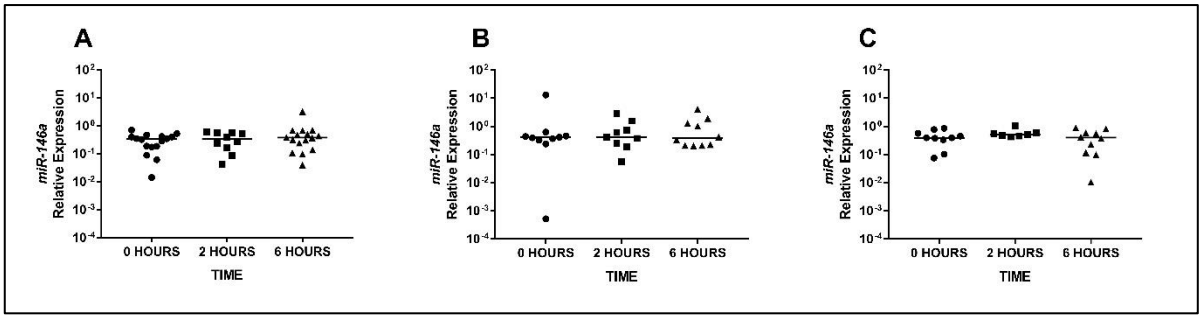

Figure 6.21. ***The expression of*** **miR-146a** ***in term and late preterm cord blood over time following LPS stimulation.*** The expression of *miR-146a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following LPS stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

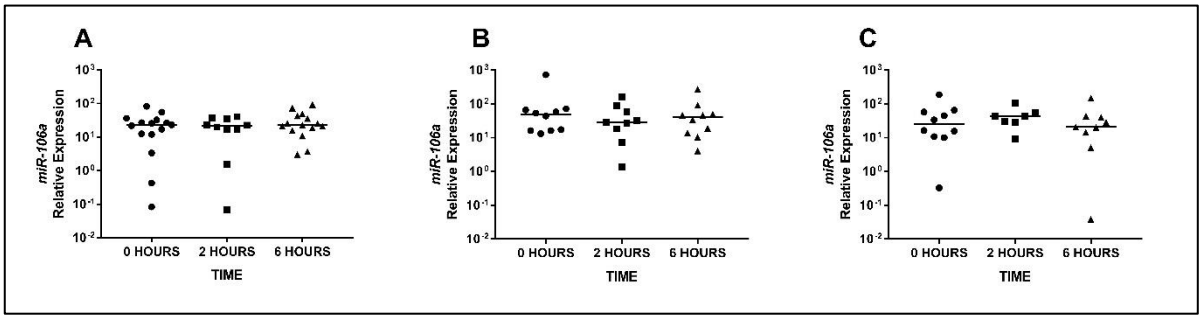

Figure 6.22. ***The expression of*** **miR-106a** ***in term and late preterm cord blood over time following LPS stimulation.*** The expression of *miR-106a* relative to *RNU48* in term (A) and late preterm cord blood (B) over time following LPS stimulation *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

6.4.2.3.1. The expression of genes associated with TLR signalling (mRNA) following LPS stimulation over time

A subset of samples were analysed for mRNA expression associated with TLR4 signalling in term (n=6) and late preterm (n=8) cord blood.

Late preterm cord blood increased *IL6* expression over time (F(2,2)=121.312, p=0.008; Fig. 6.23). Post-hoc analyses revealed that *IL6* expression was increased between baseline and 6 hours (p=0.038), but not between baseline and 2 hours or 2 and 6 hours in preterm cord blood following LPS stimulation. Term cord blood did not show any change in *IL6* expression over time following LPS stimulation.

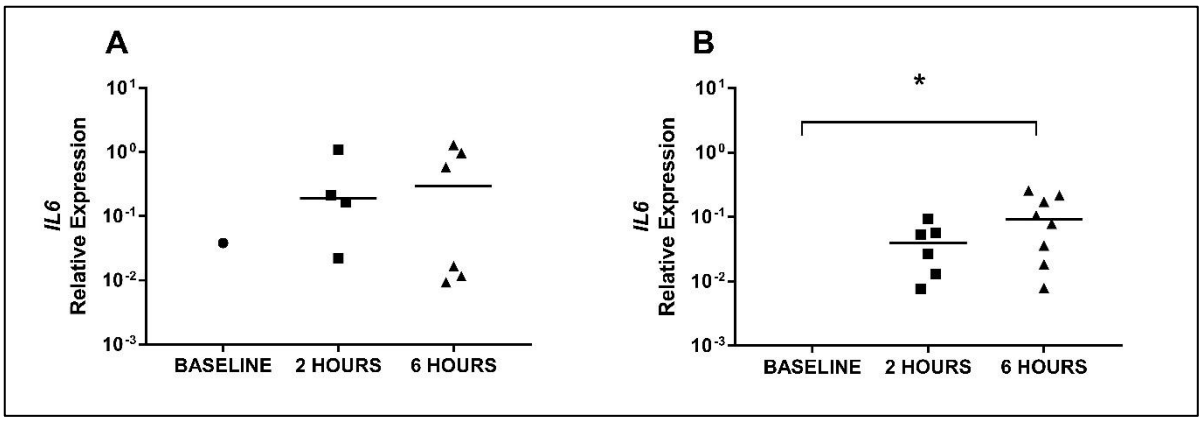

Figure 6.23. ***The expression of*** **IL6** ***in term and late preterm cord blood over time following LPS stimulation.*** The expression of *IL6* relative to *β-actin* in term (A) and late preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, *p<0.05.

There was no interaction between time and gestational age group in cord blood *TLR2* (Fig.6.24), *TLR4* (Fig.6.25), *MyD88* (Fig.6.26), *IRAK1* (Fig.6.27), *NF-κB1* (Fig.6.28), *IL10* (Fig.6.29) or *SOCS1* expression (Fig.6.30) following LPS stimulation after adjusting for the covariates.

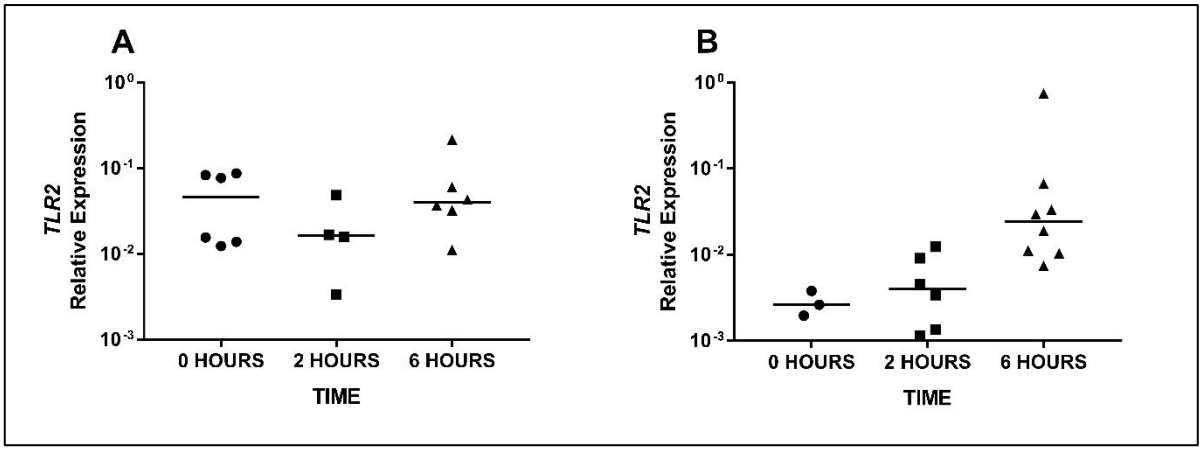

Figure 6.24. ***The expression of*** **TLR2** ***in term and late preterm cord blood following LPS stimulation over time.*** The expression of *TLR2* relative to *β-actin* in term (A) and preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

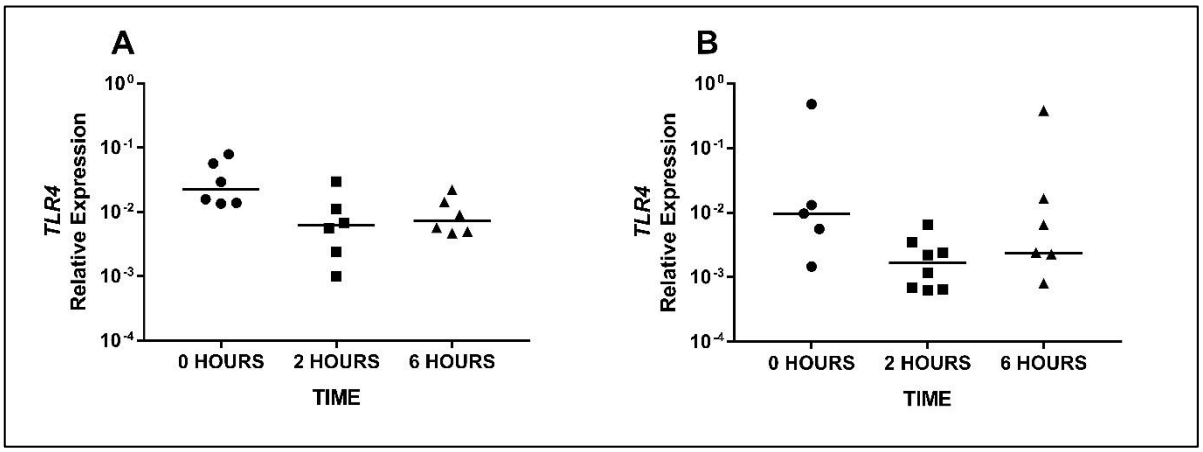

Figure 6.25. ***The expression of*** **TLR4** ***in term and late preterm cord blood following LPS stimulation over time.*** The expression of *TLR4* relative to *β-actin* in term (A) preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

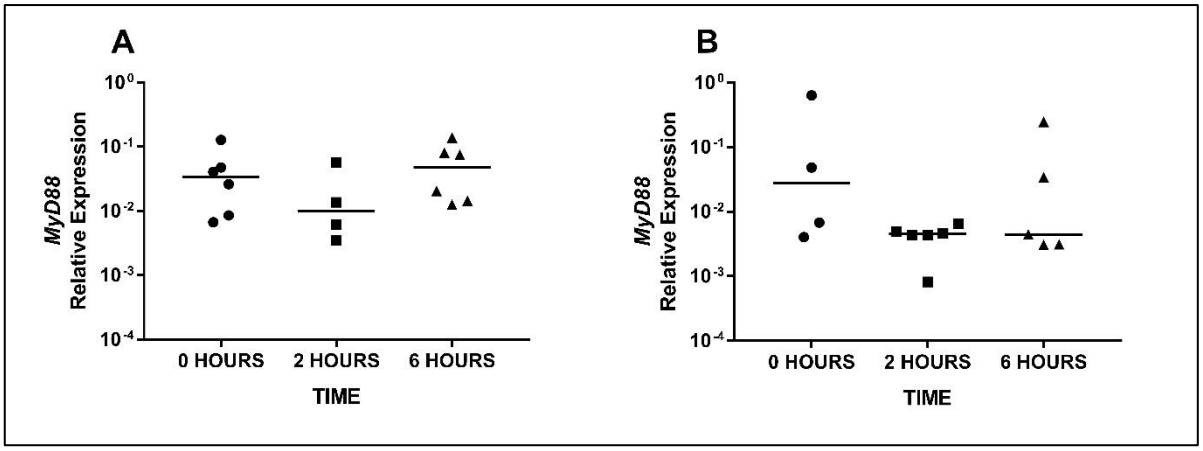

Figure 6.26. ***The expression of*** **MyD88** ***in term and late preterm cord blood following LPS stimulation over time.*** The expression of *MyD88* relative to *β-actin* in term (A) and preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

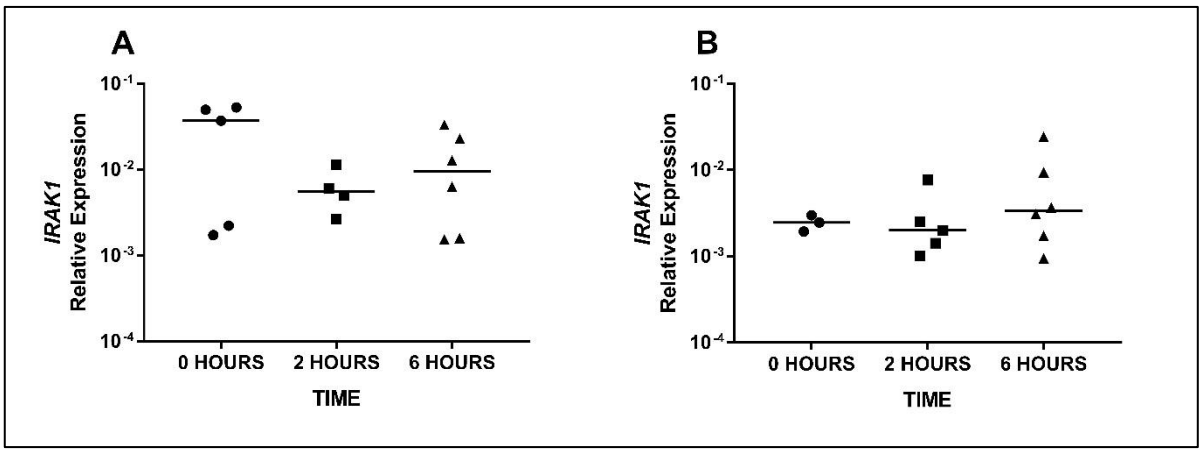

Figure 6.27. ***The expression of*** **IRAK1** ***in term and late preterm cord blood following LPS stimulation over time.*** The expression of *IRAK1* relative to *β-actin* in term (A) and preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

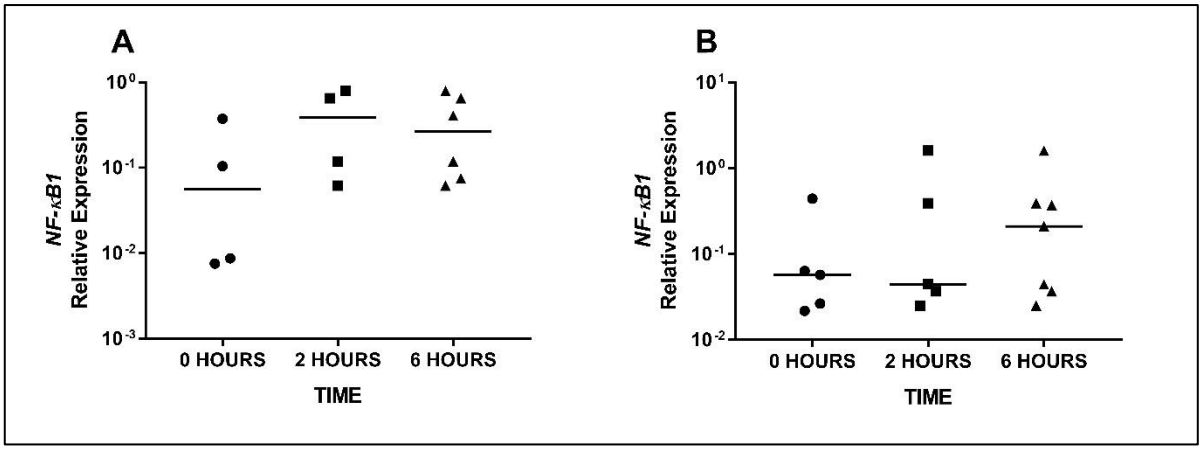

Figure 6.28. ***The expression of*** **NF-κB1** ***in term and late preterm cord blood following LPS stimulation over time.*** The expression of *NF-κB1* relative to *β-actin* in term (A) and preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

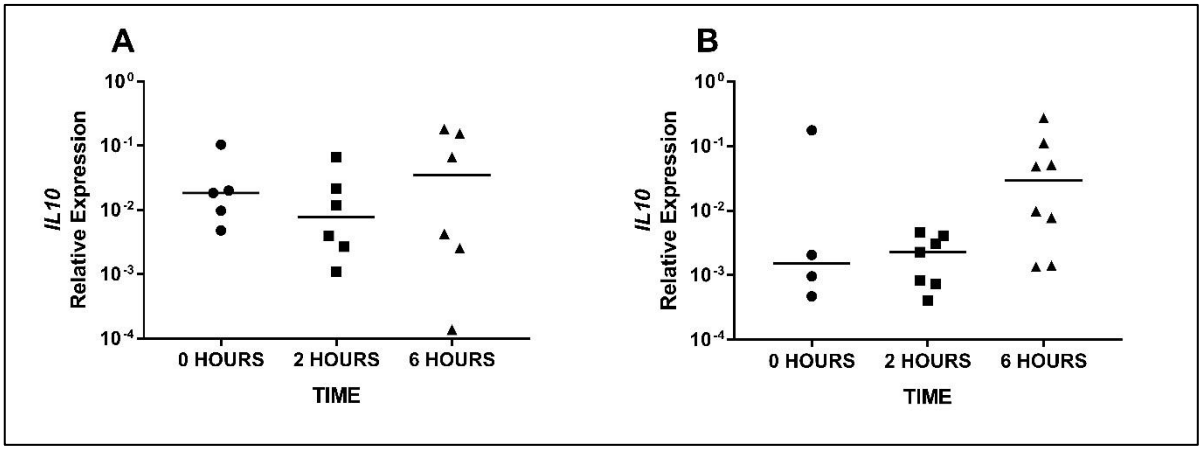

Figure 6.29. ***The expression of*** **IL10** ***in term and late preterm cord blood following LPS stimulation over time.*** The expression of *IL10* relative to *β-actin* in term (A) and preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

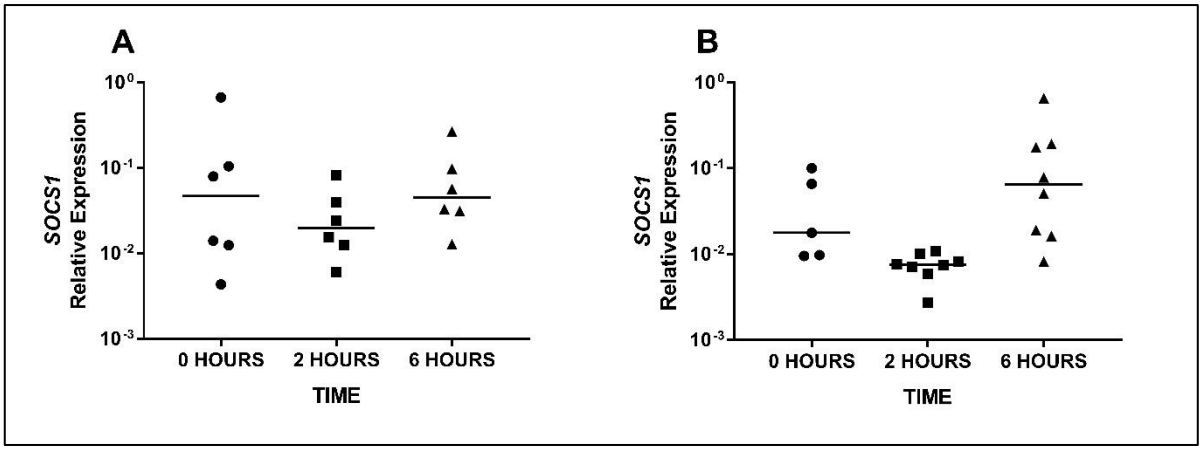

Figure 6.30. ***The expression of*** **SOCS1** ***in term and late preterm cord blood following LPS stimulation over time.*** The expression of *SOCS1* relative to *β-actin* in term (A) and preterm cord blood (B) following LPS stimulation over time *in vitro* (hours). Note: the y-axes are presented on a logarithmic scale, **p*<0.05.

6.4.3. CORD BLOOD CYTOKINE PRODUCTION FOLLOWING TLR STIMULATION OVER TIME

TNFα and IL6 production following TLR stimulation was assessed in a subset of term (*n*=17) and late preterm (*n*=17) cord blood samples due to limitations in volumes available.

6.4.3.1. Cord Blood Cytokine Expression Following PGN Stimulation

Preterm cord blood IL6 production showed a significant interaction with time following PGN stimulation (F(2,24)=6.922, *p*=0.004; Fig. 6.31B). Post-hoc analyses revealed IL6 expression was increased between 2 and 24 hours (*p*=0.001), but not between 2 and 6 or 6 and 24 hours in preterm cord blood. Term cord blood IL6 production did not show an interaction with time following PGN stimulation (Fig. 6.31A.

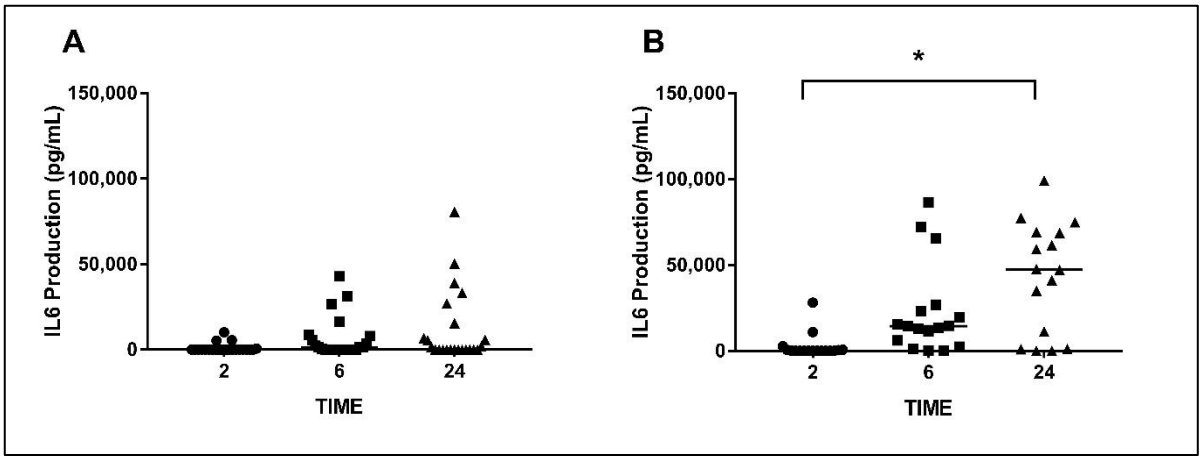

Fig. 6.31. ***Cord blood IL6 production in term and preterm cord blood following PGN stimulation over time*.** IL6 production by term (A) and late preterm (B) cord blood stimulated with PGN over time *in vitro* (hours), **p*<0.05.

Term cord blood TNFα production increased significantly over time following PGN stimulation (F(2,53)=8.811, *p*<0.001; Fig. 6.32A). Post-hoc analyses revealed TNFα expression was increased between 2 and 6 hours (*p*=0.001), and 2 and 24 hours (*p*<0.001) but not between 6 and 24 hours in term cord blood. Late preterm cord blood TNFα showed a significant increase over time following PGN stimulation (F(2,34)=7.219, *p*=0.002; Fig. 6.32B). Post-hoc analyses revealed TNFα expression was increased between 2 and 6 hours (*p*=0.023) and 2 and 24 hours (*p*=0.001), but not between 6 and 24 hours in preterm cord blood.

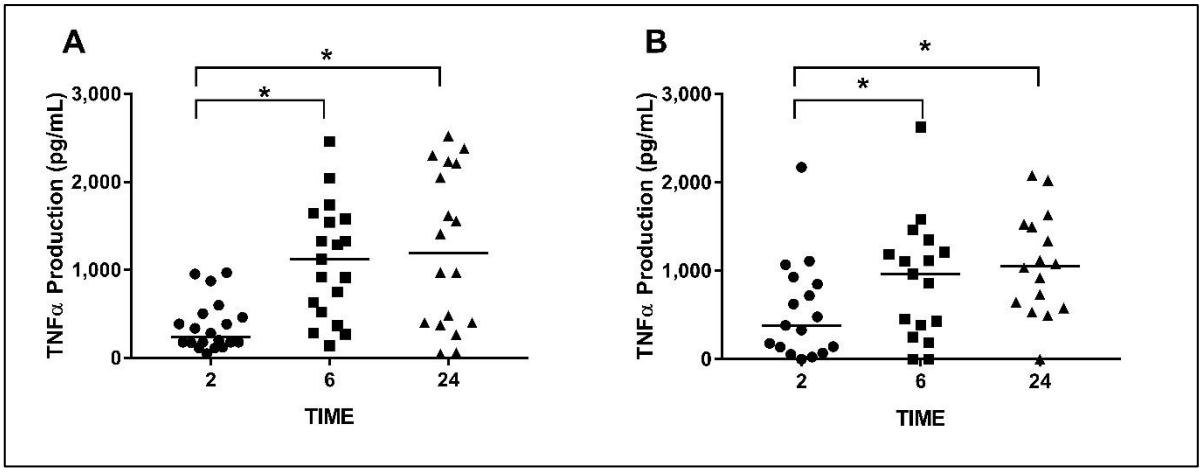

Fig. 6.32. ***Cord blood TNFα production in term and preterm cord blood following PGN stimulation over time*.** TNFα production by term (A) and preterm (B) cord blood stimulated with PGN over time *in vitro* (hours), *$p<0.05$.

6.4.3.2. Cord Blood Cytokine Expression Following Poly I:C Stimulation

There was no change in IL6 or TNFα production over time *in vitro* following Poly I:C stimulation in term or preterm cord blood (Figs. 6.33-4).

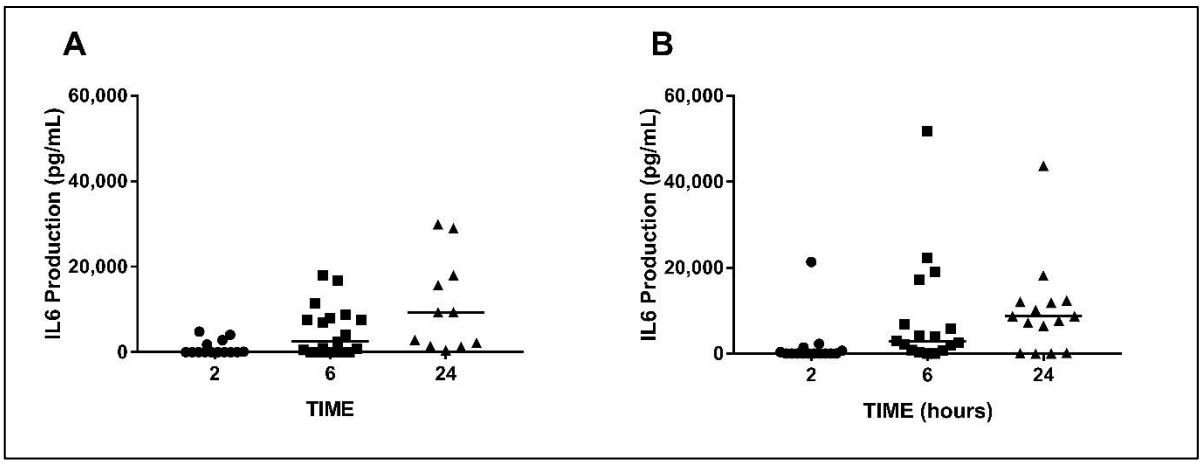

Fig. 6.33. ***Cord blood IL6 production in term and preterm cord blood following Poly I:C stimulation over time*****.** IL6 production by term (A) and late preterm (B) cord blood stimulated with Poly I:C over time *in vitro* (hours), **p*<0.05.

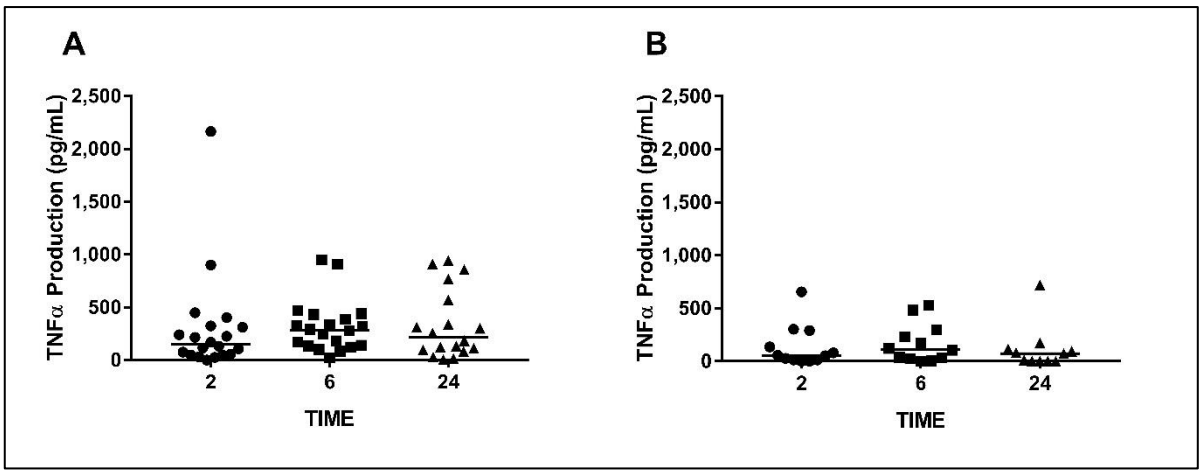

Fig. 6.34. ***Cord blood TNFα production in term and preterm cord blood following Poly I:C stimulation over time*****.** TNFα production by term (A) and preterm (B) cord blood stimulated with Poly I:C over time *in vitro* (hours), **p*<0.05.

6.4.3.3. Cord Blood Cytokine Expression Following LPS Stimulation

Term cord blood IL6 did not increase over time following LPS stimulation (Fig.6.35A). Preterm cord blood IL6 showed a significant increase over time following LPS stimulation ($F(2,21)=8.095$, $p=0.002$; Fig. 6.35B). Post-hoc analyses revealed IL6 expression was increased between 2 and 24 hours ($p=0.001$), and 6 and 24 hours ($p=0.046$), but not between 2 and 6 hours in preterm cord blood.

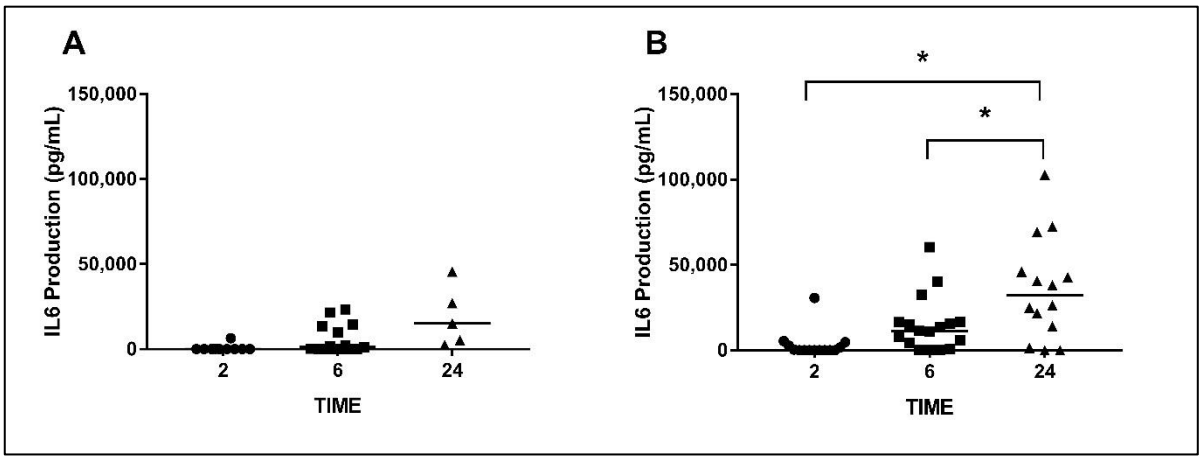

Fig. 6.35. ***Cord blood IL6 in term and preterm cord blood following LPS stimulation over time*.** IL6 production by term (A) and preterm (B) cord blood stimulated with LPS over time *in vitro* (hours), *$p<0.05$.

Term cord blood TNFα production increased over time following LPS stimulation ($F(2,43)=6.383$, $p=0.004$; Fig. 6.36A). Post-hoc analyses revealed TNFα expression was increased between 2 and 6 hours ($p=0.011$) and 2 and 24 hours ($p=0.002$), but not between 6 and 24 hours in term cord blood. Preterm cord blood TNFα production increased over time following PGN stimulation ($F(2,34)=11.162$, $p<0.001$; Fig. 6.36B). Post-hoc analyses revealed TNFα expression was increased between 2 and 6 hours ($p=0.001$) and 2 and 24 hours ($p<0.001$), but not between 6 and 24 hours in preterm cord blood.

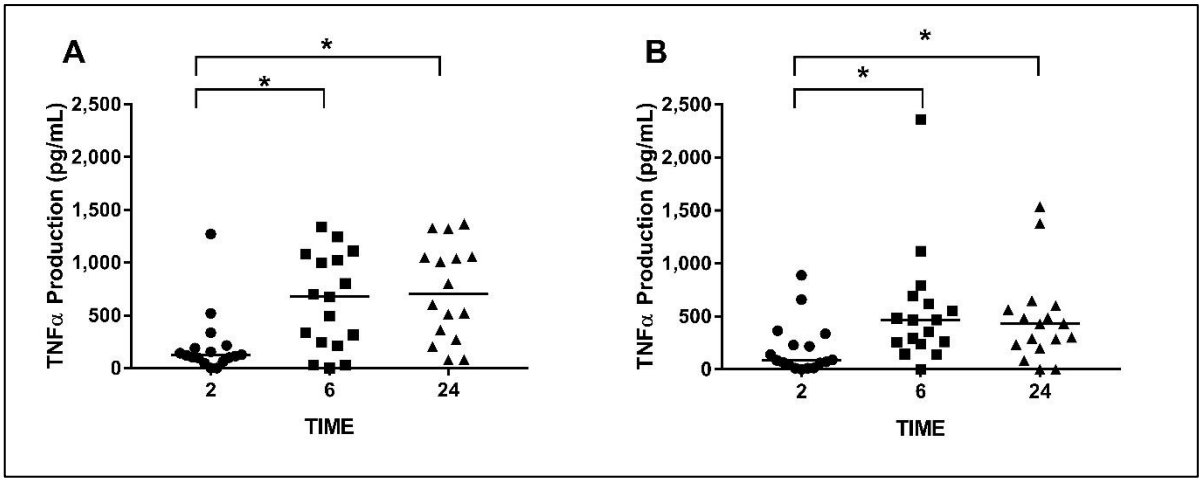

Fig. 6.36. ***Cord blood TNFα production in term and preterm cord blood following LPS stimulation over time*.** TNFα production by term (A) and preterm (B) cord blood stimulated with LPS over time *in vitro* (hours), **p*<0.05

6.5. DISCUSSION

We observed no change in cord blood miR expression between rested cultures, 2 and 6 hours following TLR2, 3 or 4 stimulation. However, preterm cord blood showed time-dependent increases in *IL6* mRNA and cytokine expression following LPS stimulation. We also observed increases in IL6 and TNFα protein following PGN and LPS stimulation in preterm cord blood over 24 hours. Conversely, term cord blood did not change gene expression over time following TLR stimulation, but increased TNFα production following LPS and PGN stimulation. These findings do not support the presence of time-dependent gene regulation in cord blood over the first 6 hours following TLR stimulation. However, the difference between preterm and term cytokine responses suggests differential regulation of the innate immune response may occur between 6 and 24 hours post stimulation. Whether this can be explained by differential miR regulation is beyond the scope of the current data, given that we were unable to assess miR expression at 24 hours due to low RNA integrity.

Previous studies have assessed miR expression in immortalised human monocyte cell lines (THP-1 cells) and shown TNFα expression increases 4-6 hours following LPS stimulation and then decreases when *miR-146a* starts to increase 8 hours following stimulation[323]. *miR-146a* expression then peaks at 18 hours following LPS stimulation, when TNFα is not detected, which the authors suggest is a mechanism for endotoxin tolerance. Our data may also support this, demonstrating a plateau in TNFα expression 6 hours following PGN and LPS stimulation, although we were unable to confirm any change in *miR-146a* expression between 6 and 24 hours. In a similar study to ours, Lederhuber et al. observed a two-fold increase in *miR-146a* expression by LPS-stimulated term cord blood monocytes after 24 hours *in vitro* (exceeding adult levels), but not any earlier during the first 12 hours *in vitro*[242]. These findings suggest miR transcripts accumulate gradually to dampen inflammation, which may be the reason our data did not demonstrate changes in gene expression between rested cultures and 2 and 6 hours following TLR stimulation *in vitro*.

The current data shows TNFα production was increased until 6 hours, without any change in *TLR4* expression, but an increase in *NF-κB1* (see Chapter 5). This could suggest the lack of an effective feedback loop during TLR signalling. Typically, TNFα is classed as an early-response cytokine. In murine macrophages, its expression decreases 2 hours after LPS stimulation due to an associated decrease in *TLR4* expression and NF-κB inhibition[469, 470].

miR-155 expression is also responsive to TNFα expression[362] and therefore, altered by changes associated with NF-κB induction at 30 minute intervals following LPS stimulation[346]. Studies have shown that peak *miR-155* expression in murine macrophages also occurs between 4 and 6 hours following LPS stimulation[341]. As we observed no significant increase in miR expression between 2 and 6 hours following TLR stimulation, we postulate that cord blood demonstrates delayed miR expression or alternative means of gene regulation.

A limitation of the current study was our inability to characterise cord blood miR expression following TLR stimulation at 24 hours *in vitro.* Future characterisation of this time-point is critical as previous studies using term cord blood monocytes have shown *miR-146a* significantly increases after 24 hours LPS stimulation *in vitro*[242]. Others have used immortalised cell lines to investigate temporal feedback loops in the context of LPS-stimulated miR expression[323], which is also a plausible alternative for cord blood characterisation. Notably, however, both of these studies used isolated cell populations that were supplemented with cell culture media. In contrast to this, our model aimed to investigate cord blood as a more realistic analogue for the neonatal TLR response. Future studies could therefore characterise temporal miR responses using whole cord blood supplemented with appropriate cell culture media. This strategy would increase the likelihood of cell survival across the 6-36 hour period following TLR stimulation *in vitro*.

Despite limitations in characterising gene expression, we observed a significant upregulation in IL6 protein expression over 24 hours following PGN and LPS stimulation in preterm but not term cord blood. Therefore, in addition to the lack of miR induction shown by preterm cord blood, our findings support a predisposition towards pro-inflammatory mediator expression over time. IL6 is associated with chronic and acute inflammatory diseases, as it has systemic roles in inflammation, including the secretion of acute phase proteins (e.g. C-reactive protein)[471]. The regulation of IL6 is therefore key in influencing the magnitude, duration and outcome of inflammation[472]. While the robust IL6 response to TLR stimulation may be protective initially, its continued upregulation past 6 hours in preterm cord blood and a lack of regulatory gene expression appears pathological. The consistent upregulation of IL6 could lead to systemic inflammatory signalling that could underlie the increased burden of systemic inflammatory conditions in preterm neonates[473, 474], though this requires more specific investigation.

Aberrant TLR signalling characterised by disrupted feedback loops has been associated with severe inflammatory conditions. As such, the pathogenesis of sepsis results from poorly timed and inappropriate anti-inflammatory mechanisms. Traditionally, sepsis is viewed as a biphasic response consisting of a systemic inflammatory response (SIRS) followed by a compensatory anti-inflammatory response (CARS) that ultimately prolong low-grade inflammatory signalling[475]. Preterm neonates with sepsis show increased numbers of Tregs in their peripheral blood, suggesting that they are downregulating inflammation that could lead to a chronic instead of acute inflammatory response (which would normally clear infection)[476]. More recent reviews, however, suggest no evidence for sequential SIRS and CARS, instead proposing a model for a 'genomic storm' where both pro- and anti-inflammatory genes are upregulated[475, 477]. This occurs to the extent that elevated expression of anti-inflammatory IL10 is associated with higher rates of patient mortality[478]. The current study indicates an absence of temporal-gate switching to allow for changes from pro-inflammatory to anti-inflammatory signalling in preterm cord blood. Further, data from Chapter 5 also support the model of a 'genomic storm', where we observed simultaneous increases in *IL6* and *IL10* mRNA in early preterm cord blood. Therefore, while further studies need to better characterise this feedback loop over longer periods of time, our data begins to suggest that preterm cord blood lacks effective regulatory relationships between inflammatory genes.

In summary, while there was no change in miR or mRNA expression in term or preterm cord blood, preterm cord blood increased IL6 protein production over time following TLR stimulation. This may suggest temporal signalling switches are impaired, predisposing preterm neonates to harmful loops of inflammatory signalling. Future studies are required to confirm this lack of gene regulation extends to the 24 hour time point *in vitro.*

Chapter 7:

A Bioinformatics Analysis of TLR Signalling Pathways in Neonates

7.1. ABSTRACT

Background

Toll-like Receptor (TLR) signalling is regulated by complex networks of inflammatory genes, including microRNAs (miRs). The experiments described in previous chapters demonstrated increased TLR signalling in early preterm cord blood in the absence of regulatory gene expression following immune stimulation. Since miRs are ubiquitously expressed and affect different networks of genes, this chapter aimed to use bioinformatics approaches to identify alternative genes and network-level perturbations that may alter inflammatory signalling in preterm neonates.

Methods and Results

Publicly available microarray data published on the NCBI GEO database was used to assess the expression of genes in the TLR signalling pathway in peripheral blood from neonates with and without infection. Data on healthy adult blood cells from the ImmunoNavigator database was extracted and co-expression profiles of genes associated with TLR signalling were identified and assessed.

Neonatal infection was associated with upregulated innate immune pathways and downregulated of adaptive immune pathways. Preterm neonates with infection upregulated *STAT1* and *CXCL8* compared to term neonates with infection. Co-expression profiles of adult cells found that neutrophils were associated with increased expression of *MyD88* and NF-κB inhibitors compared to other leukocytes and lymphocytes.

Conclusions

Our bioinformatics analysis identified novel gene targets from innate immune signalling pathways. A unique pattern of gene expression was observed in preterm neonates, particularly those with infection, which supports they are biased towards increased inflammatory signalling.

7.2. INTRODUCTION

Preterm neonates are more susceptible to developing systemic inflammatory conditions compared to term neonates. These conditions are associated with high morbidity and mortality, which occur secondary to acute inflammation. Inflammatory regulation is complex and involves networks of genes that can be regulated by miRs, which exhibit time and tissue-specific patterns of expression. These pathways are sparsely characterised in neonatal immunity.

Preceding chapters in this thesis have demonstrated that early preterm cord blood increases pro-inflammatory gene expression following TLR stimulation *in vitro*, which is comparable in magnitude to stimulated term cord blood. In association with this, TLR-stimulated term cord blood upregulates the expression of miRs known to regulate TLR signalling, whereas preterm cord blood does not. This apparent lack of miR expression may have wide-spread effects on neonatal physiology, given that a single miR can target over 200 mRNA transcripts[479] and mRNA targets themselves can be regulated by multiple miRs[480]. To examine such patterns of regulation more broadly, bioinformatics pathway analyses have emerged as important tools for understanding large-scale cellular processes such as inflammation. Pathway analyses provide a wider perspective for the potential effects of gene changes and are useful for recognising the complexity of miR-based regulation and inflammation. Bioinformatics can be used to investigate patterns of gene expression *in silico*, where statistical analyses are driven by specific biological questions.

Bioinformatics has previously been used to assess differential gene expression in neonates who develop infection[390, 402]. The results of genome-wide association studies (GWAS) have demonstrated that term and preterm neonates who develop sepsis less than 72 hours following birth (early-onset neonatal sepsis) show a unique profile of gene expression compared to neonates who develop sepsis at least 72 hours after birth (late-onset neonatal sepsis)[402]. Specifically, early-onset neonatal sepsis was associated with greater upregulation of pro-inflammatory interferons and *IL1α*, genes associated with neutrophil extracellular trap (NET) formation, and decreased expression of apoptotic genes, including *sFAS* (which contributes to inflammatory resolution). This genetic profile suggests early-onset neonatal sepsis is associated with a greater magnitude of inflammation compared to late-onset neonatal sepsis. This is an example of the same condition exhibiting unique transcriptomic

profiles that are dependent on timing (postnatal age) or mode of acquisition. Overall, these studies demonstrate the potential for GWAS in informing prognostic testing or novel methods of intervention.

This chapter aimed to review publicly available microarray expression data examining gene expression in peripheral neonatal blood exposed to pathogens[402, 403]. Specifically, we aimed to assess TLR signalling pathways to identify any differentially expressed genes in neonates with and without infection. We used bioinformatics approaches to characterise pathways of genetic regulation in preterm cord blood to identify potential mechanisms that contribute to differences in inflammatory signalling between preterm and term neonates. Additionally, publicly-available array data generated from adult immunocytes was investigated to identify patterns of cell-specific gene expression in the context of TLR signalling. Overall, it was hypothesised that preterm neonates with infection or sepsis would upregulate inflammatory pathways.

7.3. METHODS

7.3.1. TLR SIGNALLING PATHWAY ANALYSIS

In silico bioinformatics analyses were used to examine networks of gene expression in neonatal peripheral blood (see section 2.7.1). Publicly available custom Affymetrix gene array data profiling whole peripheral blood from neonates was analysed[390, 402, 403]. GEOquery and GEO2R packages were used to download the datasets (GSE69686 and GSE25504). The analysis involving GSE69686 selected individuals that were defined as preterm neonates with or without clinical sepsis. We selected individuals from the GSE25504 dataset that were defined as preterm or term neonates, with or without bacterial infection. Demographic and clinical data are presented as frequencies or mean ± SD for each of these study groups, unless otherwise indicated.

Bioinformatics analyses were performed in collaboration with Dr Jimmy Breen (Bioinformatics core-facility manager, Robinson Research Institute, University of Adelaide) using the R software package, *limma*. Data were normalised using log transformation prior to analysis. Differentially expressed genes within the TLR signalling pathway (KEGG ID: hsa04620) were identified from each dataset and generated as heat-maps (counts per million, cpm). The top 20 KEGG pathways that were differentially expressed according to infection were presented for the data set GSE25504.

7.3.2. CELL TYPE ENRICHMENT ANALYSIS

The Immuno-Navigator database (http://sysimm.ifrec.osaka-u.ac.jp/immuno-navigator/) was used to query target genes in the TLR signalling pathway to identify correlation scores of gene co-expression in specific tissues (sorted by significance of expression enrichment; see section 2.7.2). The correlation network hub prediction tool was used to determine how many networks the listed genes were associated with in healthy, unstimulated adult immunocytes from peripheral blood.

7.4. RESULTS

7.4.1. PATHWAY ANALYSIS OF DATA COLLECTED FROM NEONATES WITH BACTERIAL INFECTIONS

Complete demographics for the dataset are reported in the original publication[403], with samples extracted for our analysis summarised in Table 7.1. The data extracted for this analysis originated from peripheral blood collected from neonates admitted to the NICU, including preterm neonates with confirmed bacterial infection (*n*=22), preterm neonates without infection (*n* =8), term neonates with bacterial infection (*n*=3) and term neonates without infection (*n*=21). Infection was confirmed by positive blood culture results, with the most common culture-positive pathogen being Coagulase Negative *Staphylococcus* (CoNS). White blood cell (WBC) counts were decreased in term neonates with infection compared term neonates without infection, but increased in preterm neonates with infection compared to those without infection. Decreased platelet counts and increased numbers of neutrophils were associated with infection in both groups of neonates.

Table 7.1. ***Clinical characteristics of neonates extracted from the GSE25504 dataset and analysed in Chapter 7.*** Maternal demographics and neonatal characteristics of term and preterm deliveries with and without infection from the dataset GSE25504 (further summarised in the original publication[403]) used for the analysis of peripheral blood gene expression. Values are presented as mean± SD or *n* (%), unless otherwise indicated.

	Term		**Preterm**	
	No infection	**Infection**	**No infection**	**Infection**
	n=21	*n*=3	*n*=8	*n*=22
Gestational age (completed weeks), median (min-max)	40 (37-42)	37 (37-38)	34 (30-36)	29 (25-34)
Neonatal demographics:				
Male sex	11 (52%)	2 (67%)	4 (50%)	12 (55%)
Antibiotics at time of sampling	0 (0%)	0 (0%)	0 (0%)	15 (68%)
Birthweight, g	3483±689	2647±1442	1478±602	962±234
Neonatal blood culture results:				
Negative	21 (100%)	-	8 (100%)	-
GBS positive	-	1 (33%)	-	1 (5%)
CoNS positive	-	1 (33%)	-	13 (59%)
Enterococcus positive	-	0 (0%)	-	5 (23%)
Other pathogen	-	1 (33%)	-	3 (14%)
Reason for sampling:				
Neonatal screen	1 (5%)	-	5 (63%)	-
Jaundice	3 (14%)	-	1 (13%)	-
Maternal thyroid disease	14 (67%)	-	1 (13%)	-
Suspected infection	0 (0%)	3 (100%)	0 (0%)	22 (100%)
Other	3 (14%)	-	1 (13%)	-
Blood counts:				
WBCs (x10^9/L)	16.8±12.0	11.1±4.4	11.3±2.2	14.9±9.6
Neutrophils (K/μL)	4.2±1.9	8.6±5.1	3.2±1.4	10.1±9.5
Platelets (x10^9/L)	288±156	163±84	391±56	159±124

KEGG pathways were compared in peripheral blood from term and preterm neonates with or without infection. Pathways were listed according to the number of differentially expressed (DE) genes between these groups, ranked in order of statistical significance. The top five KEGG pathways that were significantly upregulated according to the presence or absence of infection in this dataset involved the immune system (Table 7.2). Innate immune pathways, including NOD-like receptor (NLR) and TLR signalling pathways showed the greatest number of upregulated genes between infected and uninfected neonates (not differentiated by gestational age).

The top five KEGG pathways that were significantly downregulated with infection in this dataset involved transcription (Table 7.3). These included Ribosomal pathways and RNA degradation pathways. T cell immunity pathways were also downregulated according to infection, including T cell receptor signalling pathways, and Th1, Th2 and Th17 cell differentiation pathways.

Table 7.2. ***The top 20 KEGG pathways upregulated in peripheral blood from term and preterm neonates with or without bacterial infection.*** KEGG pathways upregulated in peripheral blood from term and preterm neonates with infection compared to uninfected neonates from the dataset GSE25504. Pathways are listed according to the number of DE genes ranked in order of statistical significance.

KEGG Pathway ID	Pathway Name	Total *n* genes in pathway	Number of DE genes	*p*-value
hsa04621	NLR signalling pathway	168	54	1.01e-13
hsa05164	Influenza A	173	45	2.43e-08
hsa04380	Osteoclast differentiation	128	35	2.41e-07
hsa04620	Toll-like Receptor Signalling Pathway	104	30	5.01e-07
hsa04137	Mitophagy-animal	65	22	8.63e-07
hsa05162	Measles	134	34	2.35e-06
hsa04216	Ferroptosis	40	15	1.17e-05
hsa05134	Legionellosis	55	18	1.46e-05
hsa04920	Adipocytokine signaling pathway	69	20	3.71e-05
hsa05169	Epstein-Barr virus infection	203	42	4.10e-05
hsa05168	Herpes simplex infection	185	39	4.98e-05
hsa04066	HIF-1 signalling pathway	99	25	5.51e-05
hsa05160	Hepatitis C	131	30	7.57e-05
hsa04062	Chemokine signalling pathway	182	38	7.72e-05
hsa045144	Malaria	49	15	1.76e-04
hsa04920	NF-kappa B signaling pathway	95	23	2.14e-04
hsa04920	Complement and coagulation cascades	79	20	2.89e-04
hsa04920	Leishmaniasis	74	19	3.36e-04
hsa04920	Kaposi's sarcoma-associated herpesvirus infection	173	34	5.93e-04
hsa04920	TNF signalling pathway	108	24	6.17e-04

Table 7.3. ***The top 20 KEGG pathways downregulated in peripheral blood from term and preterm neonates with or without bacterial infection.*** KEGG pathways downregulated in peripheral blood from term and preterm neonates with infection compared to uninfected neonates from the dataset GSE25504. Pathways are listed according to the number of DE genes ranked in order of statistical significance.

KEGG Pathway ID	Pathway Name	Total n genes in pathway	Number of DE genes	*p*-value
hsa03010	Ribosome	154	76	2.92e-28
hsa03040	Spliceosome	134	50	6.81e-13
hsa03018	RNA degradation	77	30	9.06e-09
hsa03008	Ribosome biogenesis in eukaryotes	106	36	2.22e-08
hsa03013	RNA transport	171	49	3.88e-08
hsa04120	Ubiquitin mediated proteolysis	137	38	3.15e-06
hsa00230	Purine metabolism	175	43	2.14e-05
hsa04660	T cell receptor signaling pathway	103	29	3.37e-05
hsa04658	Th1 and Th2 cell differentiation	92	26	7.94e-05
hsa00640	Propanoate metabolism	32	13	9.36e-05
hsa04659	Th17 cell differentiation	107	28	1.85e-04
hsa00280	Valine, leucine and isoleucine degradation	48	16	2.40e-04
hsa01100	Metabolic pathways	1272	205	2.94e-04
hsa03430	Mismatch repair	23	10	3.16e-04
hsa05340	Primary immunodeficiency	37	13	5.12e-04
hsa00062	Fatty acid elongation	25	10	7.05e-04
hsa04110	Cell cycle	124	29	1.08e-03
hsa03015	mRNA surveillance pathway	91	23	1.14e-03
hsa00240	Pyrimidine metabolism	102	25	1.14e-03
hsa03030	DNA replication	36	12	1.42e-03

Analysis using *limma* found *CD14* and *NF-κB1* showed the highest level of upregulation in term and preterm neonates with infection (≥ 11cpm; Fig.7.1). These genes were also upregulated in neonates without infection, but at a lower magnitude (9cpm). Other genes upregulated in most neonates with infection included *TLR1, TLR5, TLR6, TLR8, CCL4, IL1B, MyD88* and *LY96* (≥ 7cpm).

Preterm neonates with infection upregulated *STAT1* (12cpm), *CXCL10* (in 6 samples, approximately 8cpm), *CXCL8* (in 6 samples, 7cpm), *PIK3R2* (≥ 9cpm), *FOS* (≥ 9cpm), *IL1β* (11cpm) and *MAP2K3* (≥ 9cpm) compared to term neonates with infection. Other than these genes, term neonates with infection showed a similar phenotype to preterm neonates with infection.

Neonates without infection upregulated the *MAPK3*, *IKBKG* and *CCL5* gene clusters in both term and preterm samples (9cpm) more frequently than neonates with infection. *TLR2* (≥ 6cpm), *TNF* (≥ 6cpm), *IRAK1* (≥ 6cpm) and *IL6* (7cpm) were comparable, independently of gestational age or infection. *TLR4* was increased in three preterm samples with infection, while *TLR3* was not detected. Viral pathways or interferon gene clusters were not detected in any of the samples analysed.

(Over page) Fig. 7.1. ***A heat-map of genes in the TLR signalling pathway differentially expressed in term and preterm neonates with and without bacterial infection.*** The gene expression profile of TLR signalling pathways in whole blood collected from term and preterm neonates with ("infected") or without bacterial infection ("uninfected"). Individuals are represented along the x-axis: uninfected preterm (purple), uninfected term (green), infected preterm (blue) and infected term neonates (pink). Normalised expression levels are colour-graded for each gene, from red (high expression) to blue (low expression), represented as counts per million (cpm). Bars on the y-axis represent gene clustering according to similar profiles of expression.

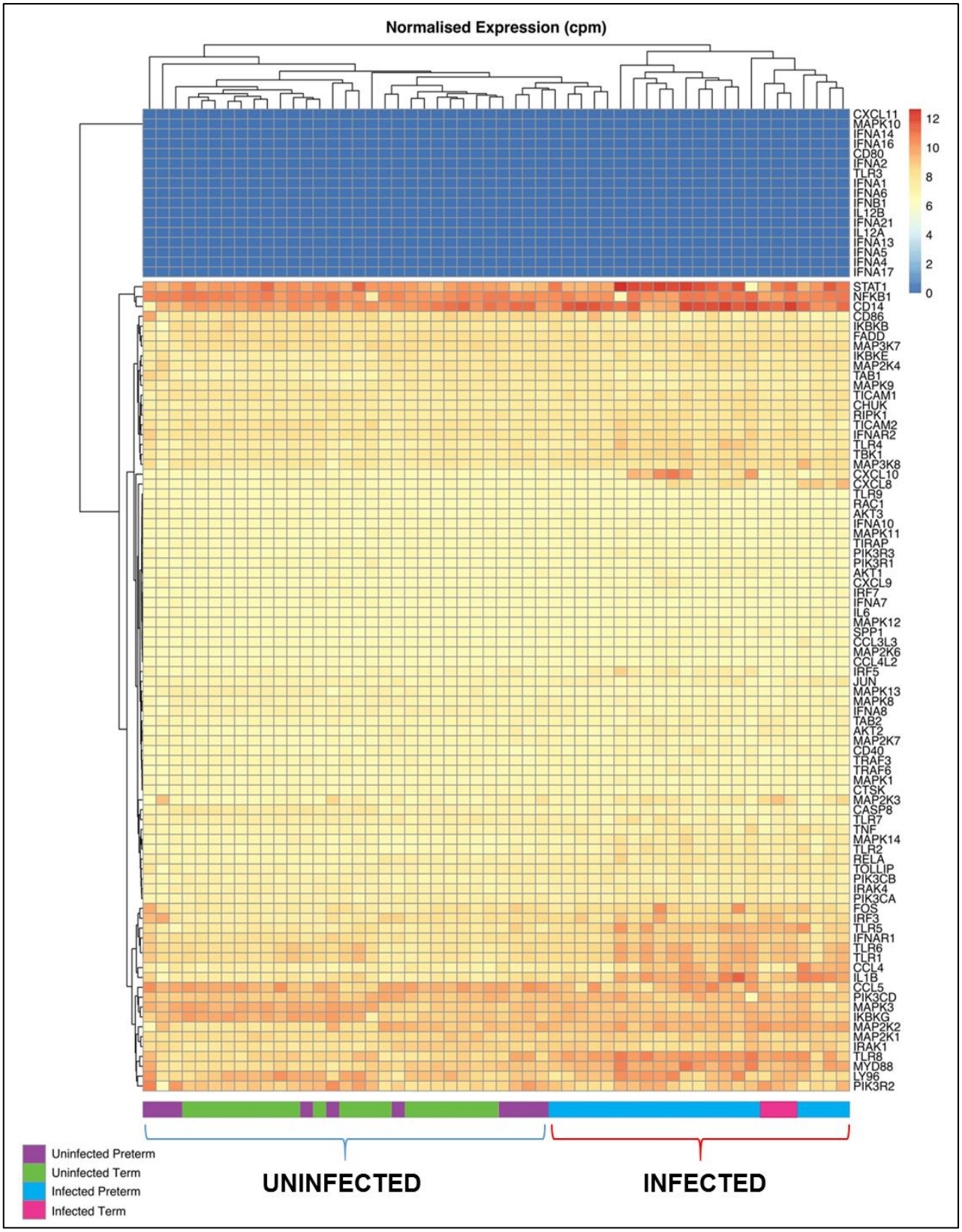

Fig.7.1. ***A heat-map of genes in the TLR signalling pathway differentially expressed in term and preterm neonates with and without bacterial infection.***

7.4.2. PATHWAY ANALYSIS OF GENES EXPRESSED IN PERIPHERAL BLOOD FROM PRETERM NEONATES WITH SEPSIS

Complete demographics for the dataset (*n*=58) are reported by two previous publications[390, 402], with samples extracted for our analysis being summarised in Table 7.4 using all publicly available data. The subset of samples we analysed consisted of peripheral blood from preterm inpatients of the neonatal intensive care unit who suffered from sepsis (*n*=37; "infected") and preterm neonates without sepsis (*n*=21; "uninfected").

Clinical characteristics are reported according to preterm neonates without infection, with clinical sepsis (*n*=22) or with culture-proven sepsis (*n*=15). Mean gestational age was comparable between the groups and a majority of patients were male. A majority of cases of culture-proven neonatal sepsis were associated with *Staphylococci*. Neutropenia was defined as having an absolute neutrophil count of <1500 cells/ mm^3 and occurred most frequently in preterm neonates with culture-proven sepsis. White blood cell counts were decreased at magnitudes greater than 30% over 24 hours (indicating increasing leukopenia) in 40% of preterm neonates with culture-proven sepsis. C-Reactive Protein (CRP) was greater than 45mg/L (indicating active inflammation) in all preterm neonates with clinical or culture-proven sepsis.

Table 7.4. ***Clinical characteristics of neonates extracted from the GSE69686 dataset and analysed in Chapter 7.*** Clinical characteristics (as summarised from the original publication[402]) of preterm neonates that were diagnosed with clinical sepsis, culture-proven sepsis or that had no evidence infection ("uninfected") from the dataset GSE69686 that was used for our analysis of neonatal peripheral blood gene expression. Values are presented as the mean or *n* (%), unless otherwise indicated.

	Uninfected	**Clinical Sepsis**	**Culture-proven Sepsis**
	n=21	*n*=22	*n*=15
Gestational age, completed weeks	29	30	31
Neonatal demographics:			
Male	15 (71%)	15 (68%)	13 (87%)
Neonatal blood culture results:			
Negative	21 (100%)	22 (100%)	-
GBS positive	-	-	5 (33%)
MRSA positive	-	-	5 (33%)
E. coli positive	-	-	5 (33%)
Neonatal blood:			
Neutropenia	1 (5%)	3 (14%)	5 (33%)
>30% drop in WBC over 24 hours	0 (0%)	0 (0%)	6 (40%)
CRP≥ 45mg/L	0 (0%)	22 (100%)	15 (100%)

Our analysis of the GSE69686 dataset focused on genes in the TLR signalling pathway, identifying differences between preterm neonates with or without sepsis (defined as CRP ≥ 45mg/L, because culture-proven sepsis was not delineated by the NCBI GEO repository). A gene expression profile was generated as a heat-map of probe sets specific to genes in the TLR signalling pathway using normalised expression values (Fig. 7.2). Genes were clustered hierarchically based on similar profiles of expression levels in grouped (infected or uninfected) samples.

Gene clusters that were upregulated by neonates with sepsis compared to neonates without sepsis included *MAPK14*, *TLR8*, *STAT1*, *RAC1* (approximately 8.5cpm); *NF-κBIA*, *MyD88* and *FOS* (7cpm); *TLR2*, *IFNAR1*, *LY96*, *TLR5*, *IRAK4* (8cpm); and *MAP2K6*, *PIK3CB*, *TLR1* (7cpm). *TLR4* was also upregulated with sepsis compared to neonates with no sepsis (10cpm). Neonates with no infection showed upregulation of *CCL5* (10cpm) and *AKT2* (8cpm) compared to neonates with sepsis. *NF-κB*, *IRAK1* and *TNF* were comparable between the groups (6cpm), while *IL6* and *IL10* were not upregulated in either group (<4cpm).

(Over page): Fig. 7.2. ***A heat-map of genes in the TLR signalling pathway differentially expressed in neonates with and without sepsis.*** Gene expression profile of TLR signalling pathway genes in whole blood collected from preterm neonates with ("infected") and without sepsis ("uninfected"). The normalised expression of each gene (cpm) is colour graded from red (high expression) to blue (low expression). Bars on the left axis represent gene clustering according to similar profiles of gene expression.

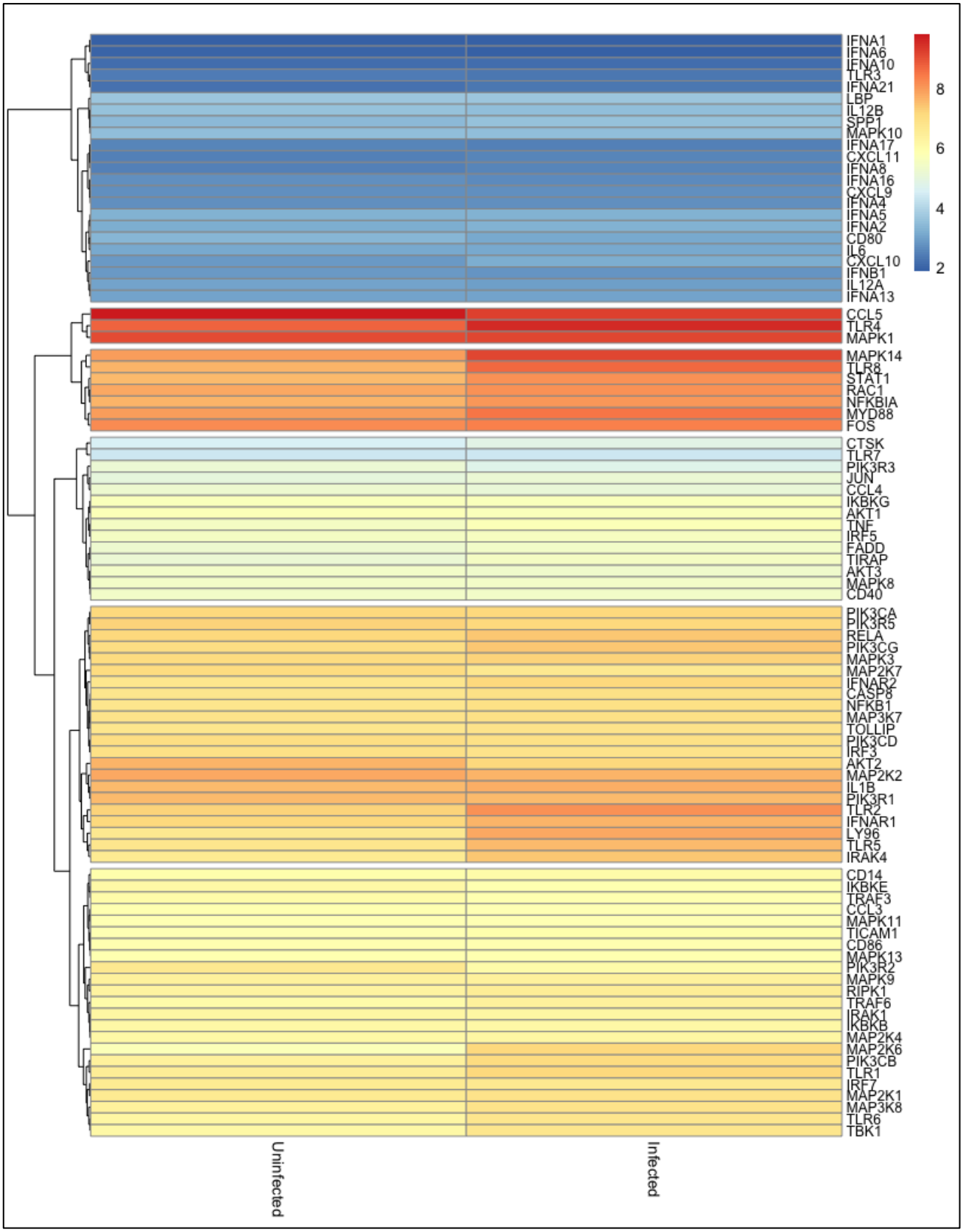

Fig. 7.2. ***A heat-map of genes in the TLR signalling pathway differentially expressed in preterm neonates with and without sepsis.***

7.4.3. CELL TYPE ENRICHMENT ANALYSIS

Select genes from the TLR signalling pathway were analysed using an immune transcriptome-wide repository of data on adult peripheral blood and analysed for differential expression in leukocyte subsets (Fig.7.3). This database collates evidence of the constitutive co-expression of genes from 4,639 healthy human samples and 19 cell types generated by 191 studies to date[408]. We extracted data relating to a subset of leukocytes and assessed the co-expression of genes from the TLR signalling pathway, specifically including those that have been investigated previously in this thesis. We aimed to determine cell-specific expression of these genes, which could inform more targeted analyses of cell types in future studies.

The results of this analysis are presented in Fig 7.3. Mean signals represent the average expression level of a gene per cell type. *TLR2* and *TLR4* were upregulated in neutrophils, monocytes and macrophages. *MyD88* was upregulated in neutrophils. *IRAK1* and *NF-κB1* were consistently upregulated in all immune cell types analysed. *IL6* was expressed at low levels in all cell types, with the highest level of expression observed in conventional dendritic cells (cDCs). *IL10* was most predominantly expressed in macrophages compared to the other cell types analysed. *SOCS1* expression was upregulated in $CD4^+$ T cells, Tregs, cDCs and granulocytes. Genes with mean signals ≤ 5 were not expressed by the represented cell types.

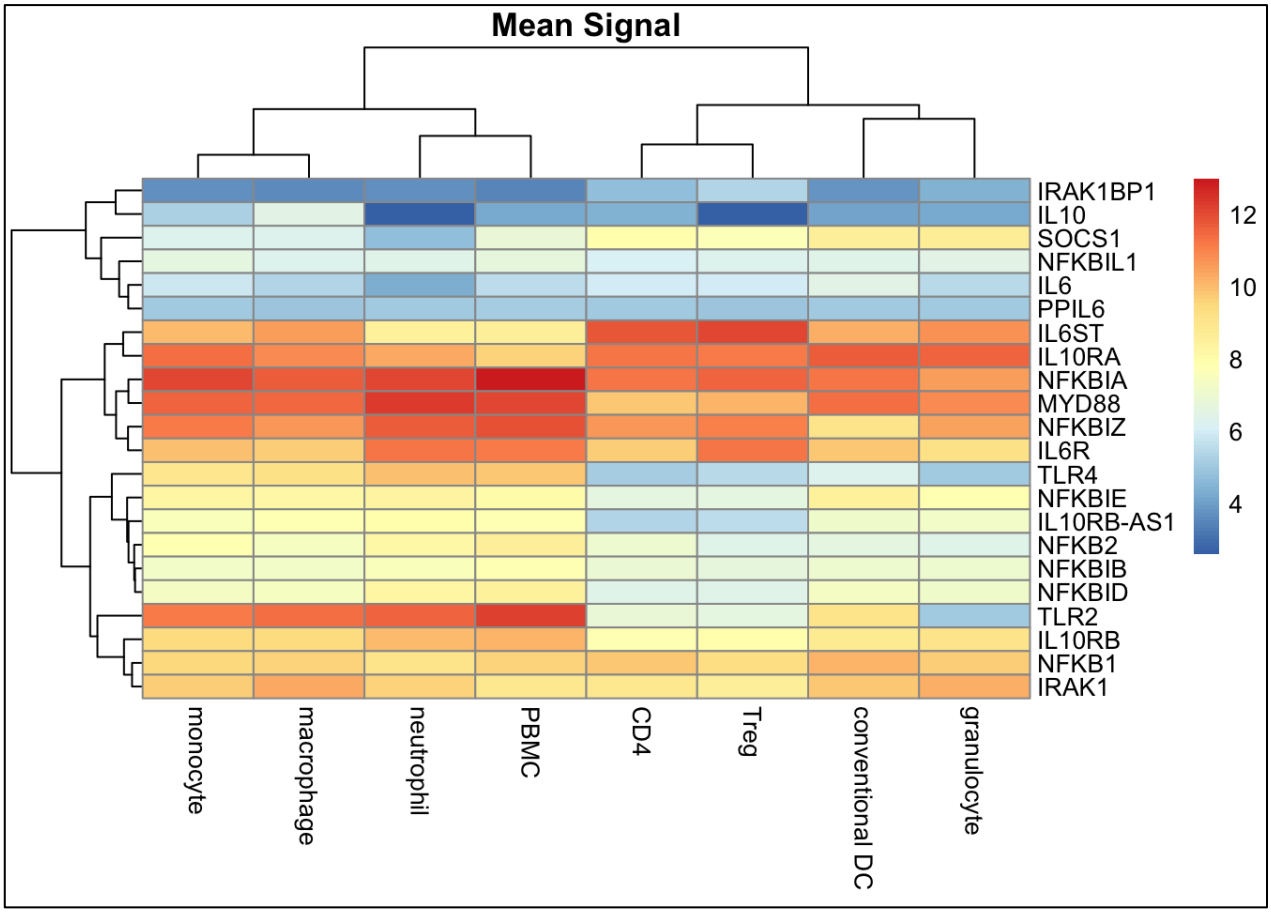

Fig. 7.3. ***A heat-map of genes in adult peripheral blood differentially expressed according to cell type.*** Gene expression profile of select genes from the TLR pathway genes (right axis) in healthy adult peripheral blood according to specified immune cell types (bottom axis). Genes are clustered according to their co-expression (left axis) and cell-specific gene expression is clustered on the top axis (mean signal of expression). Data were extracted from ImmunoNavigator website for analysis.

7.5. DISCUSSION

This study used a bioinformatics approach to explore wider innate immune pathways that were differentially expressed with neonatal infection. We found that neonates with bacterial infection upregulated innate immune KEGG pathways and downregulated pathways associated with transcriptional regulation and T cell immunity, highlighting immature adaptive immunity. Within the TLR signalling pathway, both term and preterm neonates with infection increased *CD14* and *NF-κB* expression. In the same gene cluster, however, preterm neonates with infection upregulated *STAT1* compared to term neonates with infection. In our second analysis, preterm neonates with sepsis increased MAPK pathways and TLR signalling compared to preterm neonates without sepsis, however, other inflammatory pathways were similarly expressed between the groups. Cell-specific analyses using expression data on healthy adult blood showed neutrophils and monocytes were the main source of TLR-associated genes, including *MyD88,* NF-κB inhibitors and the IL6 receptor. This could support a central role for neutrophils in contributing to TLR signalling in whole blood and argues for better characterisation of neonatal neutrophil function. Overall, our analysis supports an exaggerated pro-inflammatory phenotype and highlights specific pathways of activation in preterm neonates with infection.

The current pathway analysis reinforces the significance of innate rather than adaptive immunity in neonates. As such, TLR and NLR pathways were among the top four upregulated pathways associated with neonatal infection, while pathways associated with T cell signalling and Th cell differentiation were downregulated. These findings agree with the original paper by Smith et al., who identified an 'unbalanced homeostatic immune response' characterised by increased myeloid regulatory signalling and inhibited lymphoid signalling in neonates with infections, independently of gestational age at delivery[403]. They found neonates with infection demonstrated an increased presence of monocyte- and neutrophil-specific markers, alongside markers of suppressed T cell mediated immunity. This supports exaggerated innate immune responses and deficient adaptive immunity may both contribute to poor immune homeostasis by preterm neonates. The authors infer that dysregulation may result from an inability of the neonatal immune system to recognise a homeostatic inflammatory threshold has been reached and that regulatory mechanisms must be activated. This phenomenon could underlie the findings of this thesis from Chapter 6, where there was an absence of regulatory gene expression over at least the first 6 hours following TLR

stimulation and inflammatory cytokines continued to be upregulated between 6 and 24 hours. Altogether, this could highlight a more global deficit in immune regulation by preterm neonates, which extends past the targets we have investigated.

Bioinformatics approaches are useful for identifying changes in entire pathways. Our analysis showed infection in preterm neonates was associated with an upregulation of the master regulators *MAPK* and *STAT1* compared to term neonates. MAPKs phosphorylate p38 and JNK, which are essential for pro-inflammatory cytokine transcription. Similarly, STAT1 is a transcription factor that translocates into the nucleus on binding with IFNα, IFNγ or IL6[481]. Although the network analysis indicated IL6 expression was comparable according to gestational age and infection, results from Chapter 6 showed increased IL6 production by preterm cord blood following 24 hours of TLR stimulation *in vitro*. This would suggest differences in JAK-STAT signalling by preterm neonates. Evidence regarding this is currently limited, however a previous study has shown the phosphorylation of STAT1 and STAT3 by IL6 is comparable between term and preterm cord blood monocytes[454]. It would therefore be useful to characterise JAK-STAT signalling in whole blood to determine if it contributes to IL6 dysregulation in preterm neonates.

Smith et al. established a 52-gene invariant signature of gene expression that predicted bacterial infection in neonates. Within this, they found a specific patient phenotype that was associated with increased TLR and IL6 signalling pathways. This specific phenotype could explain why certain preterm neonates are predisposed to developing inflammatory morbidities compared to others. While we could not confirm this in our analysis, the use of microarray analyses could be used by future studies to validate the dysregulation of these pathways. A novel study on preterm cord blood would be advantageous in comparison to the datasets currently available on the GEO repository, as it would avoid sampling unwell neonates as controls. As such, the individuals we analysed as 'controls' from the Smith et al. dataset were sampled due to maternal thyroid dysfunction which could affect gene networks associated with metabolism and adaptive immunity.

Our bioinformatics analysis of peripheral blood from preterm neonates with sepsis reinforced a dysregulated innate immune phenotype. We observed that a majority of genes expressed in the TLR signalling pathway were comparable between preterm neonates with and without sepsis, including key regulators such as NF-κB, IRAK and TNF (that are not typically upregulated in the absence of infection). Although the obvious limitation of our

analysis was that the dataset grouped neonates with and without culture-proven sepsis, all neonates demonstrated CRP levels equal to or greater than 45mg/L. CRP is an acute phase protein expressed by the liver during severe inflammation, indicating that irrespective of pathogenic growth, these neonates reflected a pro-inflammatory state. This was confirmed by our analysis that showed septic preterm neonates increased TLR2, TLR4, FOS (a component of AP-1 that induces cytokine transcription) and MAPK pathways, with only slight increases in anti-inflammatory regulators such as NF-κBIA and IL10. Wynn, et al.'s paper on neonatal sepsis also analysed plasma cytokine production and found increased GCSF, IL8, TNFR1, sFAS and IL1β expression associated with both culture-positive and clinical sepsis. Further, they found that the timing of sepsis affected unique nodes of genes, specifically, leukocyte extravasation, despite activating similar immune pathways. In conjunction with our analysis, this may indicate early stages of TLR signalling are upregulated by preterm neonates with sepsis compared to those without sepsis. Specifically, we found increased expression of TLRs and other early signalling molecules, but comparable terminal molecule upregulation between infected and uninfected groups (i.e. *NF-κB* and cytokines *TNFα, IL6* and *IL10*). The comparable expression of a majority of genes associated with pro-inflammatory TLR signalling between preterm neonates with and without sepsis reinforces the idea that their system may be naturally biased towards a pro-inflammatory state.

As our bioinformatics analyses examined whole blood samples, we also conducted a cell-type enrichment analysis. Investigation of data from healthy adult haemocytes showed that genes associated with TLR signalling are upregulated in innate cell types compared to adaptive immune cells. We used adult cell gene expression as there were no data provided regarding neonatal cell-specific arrays that were publicly accessible, likely due to the constraints in neonatal blood volume available for analysis. Nonetheless, our analysis is applicable to neonates as they demonstrate some aspects of innate immune function that are comparable to adults[202, 203, 258]. Adult neutrophils appeared to be the primary source of *TLR2, IL6ST, IL10RA, IL6R, MyD88* and *NF-κBIA,* which are central to activating TLR signalling and regulating NF-κB. The increased expression of *NF-κB1* in early preterm cord blood following LPS stimulation *in vitro* (Chapter 5) may therefore be attributed to neutrophil activation or accumulation, but this requires further confirmation. Neutropenia is common in preterm neonates and is observed in our cohort of neonates with sepsis who showed less numbers of neutrophils and increased numbers of platelets (Table 7.1). Interestingly,

preterm cord blood neutrophils demonstrate increased phagocytic function compared to term[284]. Our data also observed that preterm neonates with infection increased IL8 expression, which could suggest increased neutrophil chemotaxis. Notably, aberrant IL8 expression has been associated with chronic inflammation, including that observed with neonatal sepsis[221] and inflammatory lung disease[222]. If the neutrophil response is not appropriately regulated by preterm neonates, as our results suggest, neutrophils may accrue at the sites of inflammation and their potent ROS and cytokine expression may become pathological[482, 483].

The two datasets we analysed showed notable discrepancies in neonatal blood cell counts. Term neonates with bacterial infection showed lower WBC counts compared to term neonates without infection, while preterm neonates showed higher white blood cell counts compared to uninfected preterm neonates, and all infected groups showed higher neutrophil counts compared to uninfected neonates. Conversely, in association with sepsis, white blood cell and neutrophil counts were lower. In context of the adult cell analysis, we observed that macrophages and monocytes were associated with increased *TLR2*, *TLR4* and *IL10* expression. Further, studies on cord blood that have used flow cytometry show monocytes are primary producers of IL6 and IL10 in response to stimulation with whole bacteria[204]. This could mean the decreased number of WBCs associated with term infection underlies the observed absence of IL10 expression. Conversely, preterm WBCs increase with infection alongside IL10 and IL6 production that could lead to increased inflammatory activation.

DCs are another important subset of antigen presenting cells that are critical to both innate and adaptive immunity. In the adult cell analysis, cDCs were associated with the highest IL6 and SOCS1 expression. Interestingly, neonates have higher numbers of DCs compared to adults, however these cells show decreased function and TLR4 expression[484]. Further, while pDCs are known to upregulate IL6 in adults, they have a unique phenotype compared to neonate. Unfortunately, there are few studies that have investigated cell-specific cytokine production in neonates to support these findings[48, 201, 485]. Future studies would therefore need to validate the primary source of inflammatory gene expression in order to better characterise the direction of inflammation.

A limitation of analysing large microarray datasets is the restricted ability to determine the contribution of different physiological exposures, particularly given the complexity of

preterm delivery. Notably, it would be difficult to obtain a 'clean' sample of preterm cord blood that has not been exposed to inflammatory perturbations *in utero.* The comparisons made using term peripheral blood in these datasets is therefore not ideal, even though they have been screened for the outcome of interest (in our case, infection). These 'control' samples were collected on the basis of other clinical indications, including jaundice or maternal thyroid disease. Despite not having obvious direct effects on inflammation, such factors may still perturb transcriptomic networks. Nonetheless, arrays are still useful exploratory exercises that can be used to direct future research by characterising larger patterns of gene co-expression and feedback loops. These analyses could identify sets of predictive genes for the development of diagnostic (using peripheral blood) or prognostic tools (using cord blood).

Overall, our analysis presents several pathways for future elucidation in the context of preterm neonates. Future studies could characterise preterm neonatal neutrophil responses, perform microarrays on preterm blood and further investigate STAT and NLR pathways of signalling in order to fully characterise innate immunity. These studies would need to account for inflammatory activation and how different conditions affect gene and cell-specific relationships in different preterm phenotypes. Ideally, cell-separation experiments and miR microarrays should be incorporated to fully analyse the process of innate immune stimulation on preterm blood, as preliminarily investigated by this thesis.

Chapter 8:

General Discussion

The innate immune response provides defence during neonates' vulnerable period of transition to *ex utero* environment. Previous studies have demonstrated that preterm blood exhibits either decreased or comparable expression of pro-inflammatory cytokines compared to term following TLR stimulation (Table 1.2). Nonetheless, preterm neonates remain more susceptible to contracting infections and are unable to clear pathogens adequately, suggesting other mechanisms contribute to a defective innate immune response. As such, deficiencies in immune regulation could influence the increased prevalence of inflammatory conditions among preterm neonates[34].

The overarching aim of this thesis was to understand how preterm birth alters miRs that regulate TLR signalling in neonates. We characterised genes associated with TLR signalling in term and preterm placenta and cord blood, and reported how their expression changed in cord blood following TLR2, 3 and 4 stimulation. We found no difference in gene expression preterm placenta compared to term. Conversely, early preterm cord blood showed decreased constitutive expression of *miR-155* and *let-7e* compared to late preterm and term cord blood, with no difference in other genes involved in TLR signalling. Following TLR2 and TLR4 stimulation, term cord blood increased expression of miRs (*let-7e, miR-155* and *miR-106a*), inflammatory signalling genes (*NF-κB1* and *IL6*) and cytokines (TNFα and IL6); while early preterm cord blood upregulated inflammatory pathways (*IL6, IL10, NF-κB1*) and cytokines (TNFα and IL6) without changing miR expression. Further, our time-course experiment showed preterm cord blood continued to increase IL6 production past 6 hours *in vitro*, while term cord blood IL6 plateaued at 6 hours. Together, our findings suggest the absence of regulatory miR expression by cord blood could be an underlying mechanism for uncontrolled inflammatory signalling in preterm neonates.

8.1. THE SAMPLE POPULATION

Preterm delivery is a complex condition with multiple phenotypes (see section 1.2). Our sample consisted of a single cohort of women that delivered at term and preterm gestation. A majority of samples were analysed in Chapter 4 (baseline cord blood gene expression). These consisted of a homogenous population of smokers, indigenous women and pre-eclamptic women, which are all characteristics associated with an increased risk of preterm delivery. Notably, the incidence of chorioamnionitis or maternal infection (e.g. GBS-positive women) was low in our cohort. While infection is

the most common risk factor for preterm delivery, it would have complicated the functional analysis of cord blood in our sample because antenatal infection such as the exposure to chorioamnionitis is known to affect the neonatal transcriptome[390]. A strength of the current cohort is its relative homogeneity and was random sampling that encompassed conditions such as pre-eclampsia and maternal smoking, with our analysis taking these factors into account as covariates.

8.2. THE EXPERIMENTAL PARADIGM

The TLR signalling cascade provides a useful experimental paradigm for investigating innate immune function. TLRs are evolutionarily-conserved PRRs and alterations in their function can reflect system-level perturbations in immunity. While TLRs demonstrate ubiquitous expression, they are involved in complex pathways of intracellular signalling that provide many checkpoints for regulation. miRs are post-transcriptional regulators with temporal and tissue-specific sensitivity. The discovery of miRs has revolutionised thinking on post-transcriptional regulation of genes and in the context of large signalling networks such as inflammation. Notably, both miRs and TLRs are evolutionarily conserved, which highlights not only their important roles in immunity, but organism survival.

Our experimental model investigated miRs known to regulate TLR signalling and the expression of their known targets, alongside other key genes in the TLR signalling cascade. We aimed to identify mechanisms by which preterm neonates are susceptible to uncontrolled inflammation, which is characteristic of common neonatal morbidities include NEC and neonatal sepsis. We hypothesised that preterm placenta and cord blood would show increased expression of pro-inflammatory genes compared to term placenta and cord blood at birth. It was further hypothesised that following TLR stimulation, preterm cord blood would exhibit increased expression of genes with pro-inflammatory regulatory roles (*miR-155, miR-106a* and *IL6*) and decreased expression of miRs with anti-inflammatory roles (including *let-7e*, *miR-146a* and *SOCS1).* Overall, it was expected that preterm delivery would be associated with a pro-inflammatory phenotype driven by perturbed networks of innate immune gene regulation.

The data presented in this thesis suggest the TLR signalling system is immature in early preterm neonates compared to late preterm and term neonates, where:

1. the association between regulatory miRs and their target mRNAs is not mature in the preterm placenta, which may create a predisposition towards uncontrolled inflammation *in utero*;
2. early preterm cord blood shows decreased expression of regulatory miRs (*let-7e* and *miR-155*) compared to late preterm and term cord blood, which suggests a predisposition towards unregulated expression of their targets (*TLR4* and *SOCS1*) during inflammation;
3. TLR2 and 4 stimulation increases the expression of pro-inflammatory genes, including *IL6* and *NF-κB1* in early preterm cord blood without any change in regulatory miR expression, demonstrating a bias towards pro-inflammatory mediators during a response;
4. following TLR4 stimulation, preterm cord blood increases IL6 production between 6 and 24 hours *in vitro* following TLR stimulation without any change in regulatory miR expression, while term cord blood IL6 expression peaks and plateaus at 6 hours; and
5. preterm neonates with infection show an upregulation in inflammatory signalling pathways compared to term neonates with infection, providing *in vivo* evidence of a pro-inflammatory bias potentially associated with neutrophil dysfunction.

8.2.1. Investigating the placenta as a window into the *in utero* environment

Placental gene expression of TLR pathways was characterised to assess the phenotype of the intrauterine environment. Preterm delivery is most commonly associated with intrauterine inflammation[10-13]. Evidence of this is observed where chorioamnionitis is associated with increased TLR4[428] and *NF-κB1*[486] expression in the preterm placenta. The increased activation of inflammatory pathways *in utero* has been demonstrated to have profound effects on postnatal immune function in neonates. Animal models have shown that antenatal exposure to endotoxin attenuates the progeny's subsequent responses to endotoxin *ex utero*[101-104]. Our analysis was therefore aimed at determining whether the neonate was exposed to an active inflammatory milieu *in utero*.

8.2.2. Investigating cord blood as an analogue for neonatal immune function

In addition to placenta, cord blood provides a snapshot of the *in utero* environment at delivery and neonatal immune status at birth. A majority of existing *in vitro* studies on cord blood have used isolated cells and have shown preterm CBMCs are less capable of mounting a cytokine response than term (Table 1.2). A strength of studying whole cord blood is that it accounts for interactions that more accurately reflect the *in vivo* immune response. Whole blood immune responses consist of a variety of cell-to-cell interactions, platelet activating factors and even unique cellular metabolism. Additionally, cells cultured in whole blood do not alter their phenotype as significantly as isolated cells over time *in vitro*[62]. Previous work within our laboratory has demonstrated an absence of time and dose-dependent changes in response to TLR4 stimulation in CBMCs *in vitro*[153]. These findings contrast the time-dependent upregulation of IL6 and TNFα production by whole cord blood exposed to the same treatments *in vitro* (Chapter 6). The interaction between cells and/or plasma-based factors likely contributes to this robust response and is therefore a better reflection of the *in vivo* immune context.

8.3. THE EFFECT OF GESTATIONAL AGE ON GENES ASSOCIATED WITH TLR SIGNALLING IN THE PLACENTA

Chapter 3 profiled the expression of TLR associated genes in placental tissue from term and preterm deliveries. It was hypothesised the preterm placenta would demonstrate increased expression of pro-inflammatory genes due to sequelae commonly associated with preterm delivery[10-13],[14]. Using murine models, others have demonstrated that preterm delivery is associated with increased TLR4 signalling *in utero*[418]. This suggests the placenta responds differently to inflammatory activation according to gestational age. Notably, our findings did not observe any differences in constitutive miR expression or inflammatory TLR expression between gestational age groups. We did, however, find the lack of a correlation between the miRs and their mRNA targets, which could suggest the way these genes interact with each other (i.e. their regulatory relationship) is immature in the preterm placenta, as these correlations were evident in term placenta. Ultimately, our assessment of total placental cell populations is better

reflective of the *in utero* environment and supports comparable expression of genes associated with TLR signalling according to gestational age at birth.

While the current study focused on the status of the TLR signalling pathway at birth, responses to TLR stimulation may provide closer insight into immune regulation. Increased TLR signalling[418] and IUI[10, 11] are common sequelae to preterm parturition. Increased TLR expression by trophoblasts has also been observed *in vitro* following stimulation with TLR agonists[427, 487, 488] and *ex vivo*, in association with chorioamnionitis[428, 486]. These studies demonstrate the role of the placenta as an immunomodulatory organ that can respond to different pathogens. The lack of differentially expressed miRs observed by our findings may therefore be due to the absence of inflammatory activation at birth in our sample. *In vitro* stimulation of placental explants is likely required to confirm any regulatory dysfunction in relationships between miRs and their targets as postulated by our correlational analyses.

8.3. THE EFFECT OF GESTATIONAL AGE ON BASELINE EXPRESSION OF GENES ASSOCIATED WITH TLR SIGNALLING IN CORD BLOOD

The aim of Chapter 4 was to compare the expression of inflammatory genes involved in TLR signalling in term, late preterm and early preterm unstimulated whole cord blood to understand the immune status of neonates at delivery. Previous studies have found comparable TLR mRNA and protein expression in cord blood according to gestational age[82, 86]. While our data confirmed that term and late preterm cord blood showed comparable expression of genes associated with TLR signalling, early preterm cord blood expressed decreased *let-7e* and *miR-155*. These miRs have opposing roles in regulating inflammation, so their simultaneous decrease may suggest more global immaturity of coordinated regulatory function. This could underlie the lack of any observed difference in the expression of their respective target genes, *TLR4* and *SOCS1*. Further, the fact that miRs with opposing roles are both downregulated could suggest network level perturbations in inflammation, particularly because miR expression did not change with TLR stimulation in early preterm cord blood (Chapter 5). Previous studies have associated decreased miR expression with decreased expression of the processing molecule, Dicer in neonates who develop severe RSV[455]. Our data could

therefore reflect an absence of regulatory miR expression in preterm cord blood due to immature transcript processing.

8.4. THE EFFECT OF GESTATIONAL AGE ON INNATE IMMUNE FUNCTION AND REGULATION

In Chapter 5, we aimed to compare the expression of genes following TLR stimulation in term, late preterm and early preterm cord blood. We hypothesised that preterm cord blood would show increased pro-inflammatory and decreased anti-inflammatory gene expression compared to term cord blood stimulated with TLR agonists.

We demonstrated that TLR2 and TLR4 stimulation increased regulatory miRs (*let-7e*, *miR-155* and *miR-106a*) and pro-inflammatory *IL6* expression in term cord blood. This supports miRs contribute to inflammatory regulation, which is critical for neonates in their adaptation to the *ex utero* environment. Term cord blood has previously been shown to increase *miR-146a* expression 24 hours following LPS stimulation, exceeding that of adult peripheral blood[242]. In a murine model of intestinal inflammation (exposure to *E. coli*), *miR-146a* upregulation was established as a critical factor for developing enteric tolerance in the neonatal intestine. The inhibition of *miR-146a* in this model was associated with increased intestinal epithelial cell apoptosis induced by *E. coli*, which is a characteristic feature of NEC in human infants. The lack of *miR-146a* or any change in expression of other miRs measured following TLR stimulation by early preterm cord blood may contribute to an inability to regulate inflammation and therefore, a decreased ability to develop immune tolerance to the *ex utero* environment.

A majority of existing studies suggest preterm cord blood demonstrates attenuated responses to immune stimulation *in vitro.* These studies have observed decreased immune cell counts[284] and impaired pathogen killing capacity in preterm blood[283]. *In vitro* studies have also shown decreased IL6 and TNFα expression without any change in IL10 in preterm compared to term cord blood in response to stimulation with TLR agonists[35, 213, 288] and whole bacteria[82, 252, 294]. Recent data from our laboratory somewhat disagrees with these findings, demonstrating increased IL6 and no change in IL10 following TLR2 and 4 stimulation[153]. The disparity between these findings may be related to methodology, where previous authors compared gene expression between gestational age groups, but did not investigate the kinetics or change in the response

within gestational age groups. In further support of our postulated pro-inflammatory preterm phenotype, meta-analyses have shown that umbilical cord blood tissue from early preterm deliveries demonstrates a transcriptome reminiscent of FIRS[286]. This predisposition towards a pro-inflammatory bias may therefore explain increased expression of IL6 (that can be used to confirm FIRS[489]) and could also contribute to systemic inflammation by preterm neonates.

Responses to TLR stimulation in late preterm cord blood showed a similar pattern of gene expression to that observed in term cord blood. TLR expression itself was also comparable between the gestational age groups, however, *let-7e* and *SOCS1* were increased in late preterm following TLR stimulation, but not early preterm cord blood. This suggests a switch in maturation of these pathways could occur at around 32 weeks gestation.

IL10 expression is thought to be inversely correlated with gestational age in cord blood responding to whole bacteria[204], however we found *IL10* expression was comparable between term and preterm placenta and cord blood, and did not change with cord blood TLR stimulation. Notably, however, we observed an increase in its regulator *miR-106a* following LPS stimulation of term cord blood. This suggests changes in IL10 expression occur later during the neonatal response to TLR agonists. Further characterisation of IL10 is critical, as it has been shown to decrease up to 20% of genes associated with the body's response to LPS[490]. This may be apparent in the case of other instances of *in utero* inflammation, as our analysis observed an interaction between increased *IL10* expression and maternal smoking during pregnancy in cord blood and placenta. Notably, our bioinformatics has preliminarily suggested that *IL10* is predominantly expressed by macrophages, at least in adults. This may again highlight the possibility of cell-specific functional deficits in cord blood.

Despite a lack of change in miR expression following TLR2 and 4 stimulation, early preterm cord blood upregulated expression of the inflammatory genes *NF-κB1* and *IL6.* Although NF-κB is a super-enhancer of pro-inflammatory gene expression, its p50 subunit expression was only associated with *miR-155* expression in term and not early preterm cord blood. The underlying mechanism for decreased miR expression in early preterm cord blood remains unclear and could be related to a dysfunction or absence of the miR-processing complex, Dicer. Overall, this thesis has demonstrated that early

preterm neonates are able to respond robustly to TLR stimulation through cytokine expression, but the absence of miR expression may mean that this is not regulated sufficiently, resulting in inadequate clearance of pathogens and/ or continuous inflammatory signalling.

8.5. TEMPORAL RESPONSES TO IMMUNE REGULATION ACCORDING TO GESTATIONAL AGE

In Chapter 5, we observed the lack of an association between regulatory miR expression and inflammatory genes in early preterm cord blood. Chapter 6 therefore aimed to identify whether these differences resulted in different temporal responses between gestational age groups. Specifically, we characterised gene expression following a resting period and at 2 and 6 hours after TLR stimulation *in vitro*. As no other studies have assessed this, we chose to examine a time point prior to the 6 hours (i.e. 2 hours), and one after the 6 hours (i.e. 24 hours). These time points correlate with NF-κB inhibition[469] and a respective decrease in TNFα *in vitro*[323]. Again, we expected that preterm cord blood would increase pro-inflammatory and decrease anti-inflammatory gene expression over time following TLR stimulation.

Our study found that gene expression did not appear to change over time, irrespectively of gestational age. However, term cord blood showed increased TNFα and IL6 protein production until 6 hours following TLR2 and 4 stimulation, when it plateaued. In contrast, preterm cord blood increased TNFα production until 6 hours following TLR stimulation and continued to increase IL6 production between 6 and 24 hours *in vitro*. The lack of time-sensitive mRNA or miR expression in response to TLR stimulation in preterm cord blood despite continued IL6 production, could indicate poor regulatory responsiveness by preterm neonates.

These findings suggest IL6 may be uncontrollably upregulated, particularly because the miRs we assessed were not expressed in early preterm cord blood despite being upregulated in term cord blood following TLR stimulation by 6 hours. The regulation of IL6 is important because it is a systemic acting cytokine commonly associated with acute and chronic inflammatory disease. It also recruits mononuclear cells, stimulates endothelial cells and inhibits T cell apoptosis[491]. More notably, IL6 can breach the blood brain barrier during systemic inflammation to cause white matter injury during

NEC[492, 493]. The consequences of dysregulated IL6 signalling are therefore critical in the context of preterm neonatal immunology.

TLR signalling is a time-dependent process that involves the gradual recruitment of adaptor molecules to result in the transcription of pro-inflammatory genes through NF-κB or AP-1. Each stage of molecular recruitment provides a unique checkpoint for regulation. The progression of molecule recruitment, cytokine gene transcription and translation all occur at different times during an inflammatory response. The expression of miRs in the TLR signalling cascade therefore changes across time. For example, LPS increases *miR-146a* in cord blood between 12 and 24 hours following LPS exposure *in vitro*, but not before 12 hours[242]. Unfortunately, we were unable to validate miR expression following 6 hours TLR stimulation due to decreased RNA integrity after this time point. However, given that IL6 was significantly increased following stimulation in preterm cord blood compared to term between 6 and 24 hours, it may be possible that differences in miR expression become evident at later than 6 hours. Future studies should address optimal culture conditions that can enable this to be investigated further, potentially using isolated cell populations as we were unable to pursue this with our limited blood volumes.

MiRs are 'globally stable', with a half-life ranging between 28-220 hours *in vitro* in immortalised murine embryonic fibroblasts or 8 hours in human Tcell lines, with corresponding mRNA transcripts showing a half-life of 20 hours in the same context[494]. Notably, the stability of miRs is context-specific where *miR-21* and *miR-223* are degraded after 3 hours in whole blood at room temperature, but remain stable for up to 24 hours in serum at room temperature[495]. Additionally, LPS challenge decreases the stability of *miR-21* in murine embryonic fibroblasts between 10 and 20 hours following challenge *in vitro*[494]. Unfortunately, there is a paucity of data available regarding the stability of transcripts of the miRs we measured in cord blood *in vitro*. The only study to date that has investigated miRs used CBMCs *in vitro*[242], which likely enhanced cell and therefore, miR transcript survival.

8.6. NETWORKS OF GENES AFFECTED BY IMMUNE REGULATION ACCORDING TO GESTATIONAL AGE

This thesis primarily investigated miR expression in whole blood following TLR stimulation. A limitation of Chapters 4-6 was the inability to assess the context of the select genes investigated or the contributions of individual cell types generating an immune response. The nature of assessing cord blood from very preterm neonates means that cell separation was not a viable option to generate sufficient RNA for analysis. Bioinformatics, however, can be informative in directing research and identifying likely pathways and cell types responsible for the effects we observed. This was the focus of Chapter 7, where we analysed the TLR signalling pathway in context of transcriptomic data generated by microarrays from peripheral neonatal blood during infection.

Bioinformatics is utilised to identify critical pathways that are implicated in biological processes such as inflammation. This is critical to consider when examining miRs, as they can regulate entire networks of genes such that alterations in one miR can alter a whole system. Peripheral blood from preterm neonates with infection upregulated innate immune pathways including TLR, NLR and STAT-based signalling compared to term neonates with infection. Again, these findings highlight the potential bias towards pro-inflammatory signalling by preterm neonates, particularly because IL6 activates STAT1 and therefore, the increased expression of IL6 by preterm cord blood could perpetuate inflammation in more ways than originally thought.

The model of innate immune activation that has been used in the preceding chapters of this thesis used whole cord blood, however identifying the source of cytokine expression may be critical to understanding the direction of inflammation. For example, if antigen-presenting cells show attenuated gene expression, it is likely to have flow-on effects with regards to attenuated adaptive immune development. Previous findings from our laboratory have shown CBMCs are unable to produce a dose or time-dependent response to TLR stimulation *in vitro*, therefore we postulate that cell to cell interactions are critical in analysing the neonatal immune system. To understand the individual contribution of cells to gene expression and cytokine production in TLR signalling, we investigated cell-specific expression using publicly available data collected from healthy adults. We identified that neutrophils upregulated NF-κB-related

signalling genes more than mononuclear cell types (including adaptive immune cells, monocytes and macrophages). Further, preterm neonates with infection increased IL8 in comparison to term despite exhibiting neutropenia. IL8 is key for neutrophil chemotaxis and its aberrant expression can lead to immune cell accumulation. We therefore postulate that a reliance on neutrophil-driven inflammation may predispose neonates to inflammatory diseases such as CLD that are characterised by neutrophil accumulation[222].

Online databases of gene expression have demonstrated the promiscuous nature of miRs, where *miR-155*, for example, has 918 putative gene targets[496]. Others have reported 85 miRs and 41 genes associated with TLR signalling that are differentially expressed in cord blood leukocytes stimulated with LPS compared to and unstimulated leukocytes[497], although this data was not publicly available for our consideration. The contribution of miRs to TLR signalling is therefore quite complex. As such, *miR-155*, is not only associated with increased NF-κB activation, but is also increased with STAT1 activation, as the *miR-155* promoter region shows putative *STAT1* binding elements[498]. Further, SOCS1, which is also downregulated by *miR-155*, inhibits STAT activation. This supports the role of *miR-155* as a master regulator of the innate immune response. Interestingly, *miR-155* has been identified as a top upstream miR in peripheral blood from preterm neonates who are exposed to chorioamnionitis but did not develop postnatal infections, compared to those who did (where *miR-155* was not identified)[390]. This could suggest the existence of a unique preterm phenotype that is unable to mount miR-based responses to inflammation and is therefore more susceptible to infection and increased pro-inflammatory gene expression. Future microarray analyses of neonatal blood with and without TLR stimulation will be important in separating the effects of decreased miR expression we observed in Chapters 4-6.

8.7. HOW MATERNAL PHYSIOLOGY CONTRIBUTES TO NEONATAL INNATE IMMUNITY

Maternal physiology impacts on the neonatal immune response as a product of immune priming *in utero.* Physiological insults that occur during pregnancy contribute differently to fetal physiology. Others have shown that pre-eclampsia is associated with increased Th1 cytokine expression *in utero*[499] and increased cord blood IL8 and IL10 expression[500]. Additionally, maternal smoking during pregnancy has been associated

with decreased TNFα and IL6 production following TLR2 stimulation of term cord blood *in vitro*[162]. The impact of pre-eclampsia or maternal smoking are not clearly characterised in preterm cord blood, though their effects could compound immature inflammatory regulation. Our analyses therefore incorporated the effects antenatal exposure to betamethasone, labour, pre-eclampsia, maternal smoking during pregnancy and chorioamnionitis into a statistical model. It was hypothesised that typically pro-inflammatory perturbations such as pre-eclampsia, labour, chorioamnionitis and maternal smoking would contribute to a pro-inflammatory cord blood profile, while antenatal betamethasone would have anti-inflammatory effects due to its immunosuppressive properties[452].

Notably, the factors we investigated as covariates within our analysis may have unique 'magnitudes' of inflammatory influence. For example, pre-eclampsia typically develops later in gestation, meaning the fetus is not exposed to its associated inflammatory signalling for the entire duration of pregnancy. Maternal infection or chorioamnionitis are likely to be acute and present towards the end of pregnancy, as they induce labour or are indications for premature delivery[10-13]. Alternately, maternal smoking during pregnancy is a chronic exposure as it occurs prior to and for the entire duration of gestation. Maternal cigarette smoking during pregnancy is also associated with poor perinatal outcomes including preterm delivery[14], growth restriction[155] and an increased risk of childhood atopy[501] and asthma[502]. Smoking creates this pro-inflammatory *in utero* environment through ROS production and cellular apoptosis in the placenta[163, 433]. Therefore, it may shape neonatal immunity in a similar way to chronic infection.

In the placenta, maternal smoking was associated with decreased regulatory *let-7e* expression alongside increased inflammatory *TLR2*, *SOCS1*, *MyD88*, *IRAK1*, *NF-κB1*, *IL6 and IL10* (Chapter 3). This corroborates with a study that found a dose-dependent decrease in *miR-146a* expression in immortalised villous and extra-villous trophoblast cell lines cultured with nicotine and benzo(a)pyrene, which are the main constituents of cigarette smoke[434]. Similarly, our analysis of cord blood at birth showed decreased *miR-146b* and increased all genes associated with TLR signalling (Chapter 4). Following TLR-stimulation, however, cord blood gene expression showed no interaction with maternal smoking in the context of our analysis. Notably, others have previously demonstrated decreased TNFα expression in response to TLR2, 3,4 stimulation *in vitro* and decreased IL6 in response to TLR2 stimulation in term cord blood from mothers

who smoked during pregnancy[162]. This suggests cord blood becomes hypo-responsive after being exposed to a pro-inflammatory *in utero* environment, however, postnatal immunity of these offspring is associated with a hypersensitive Th2 response[503] and atopic or respiratory disease[163, 504]. Overall, while our analyses were independent of gestational age, all of these findings demonstrate some form of maladaptive immune regulation. Future studies should investigate maternal smoking as a sub-phenotype of preterm delivery in the context of neonatal TLR function in order to separate these effects.

This thesis was unable to separate any effects of antenatal betamethasone in association with TLR signalling in preterm placenta or cord blood. Animal models have demonstrated that betamethasone exposure is associated with a state of immunosuppression by the neonate that is marked by decreased lymphocyte expression[196] and decreased TNFα and IL1β expression following LPS challenge[106]. *In vitro* studies also show cord blood decreases pro-inflammatory cytokine expression in response to viral stimulation[165] and inhibits neutrophil chemotaxis during LPS stimulation[189]. *miR-155,* which is normally decreased by glucocorticoid exposure in cell lines stimulated by LPS *in vitro*[191], was therefore expected to decrease inflammatory pathways in cord blood consistent with immune suppression. Nonetheless, we were unable to discern such an effect. A follow-up study with larger numbers of preterm neonates without betamethasone exposure stratified according to gestational age at delivery may be ideal to separate this. Notably, such a cohort would be difficult to obtain given preterm deliveries without betamethasone exposure are more likely to occur spontaneously[505]. Overall, the impact of glucocorticoid exposure should be explored further as it could be an underlying contributor to our current findings.

8.8. LIMITATIONS OF OUR FINDINGS

As the field of neonatal miR regulation is in its infancy, the current findings are highly valuable, but represent only the early stages of such research. While other methods such as microarrays are able to characterise larger volumes of transcriptomic data, we specifically chose to investigate genes within the TLR signalling pathway as we could easily validate them using qPCR to determine how they change with TLR stimulation *in vitro*. Our findings could be expanded upon by focusing on more targets within the TLR

signalling pathway or using specific vectors or murine models to knockdown miR expression *in vitro* to confirm the expression of targets we investigated.

Factors such as smoking, pre-eclampsia and antenatal betamethasone exposure can affect gene expression and although we adjusted for these in the analyses, we could not separate their effects within our sample. Future work is therefore required to stratify each of these factors and their influence on the preterm neonatal immune phenotype. Notably, a strength of the samples analysed by this thesis was that the recruited cohort was largely unexposed to maternal infection, which enabled us to identify TLR function more as a product of prematurity rather than exposure to inflammation *in utero*.

The experimental chapters of this thesis have focused on whole cord blood as an analogue for neonatal immune function. While this strategy has many strengths, it provided limited insight into individual cell-types' function or how their frequency may contribute to an inflammatory response. This may be particularly important, as our bioinformatics analysis suggested neutrophils may play a central role in dysregulated innate immunity. In this case it would have been useful to deem differential cell counts either from clinical testing or within the laboratory using flow cytometry analyses.

8.9. THE ROLE OF INNATE IMMUNITY IN THE PRETERM NEONATE

Preterm delivery occurs most commonly as a product of inflammation. Under these conditions, preterm birth may be an attempt by the fetus to escape adverse *in utero* conditions (such as intrauterine inflammation) to enhance chances of survival (Fig.8.1A, B). Preterm delivery exposes the neonate to an antigen-rich *ex utero* environment at a time when mechanisms of immune regulation may not be fully developed. A consequence of early parturition is therefore the increased risk of infection and inflammation. This thesis has highlighted early preterm neonates show a diminished capacity to regulate inflammation through the actions of miRs, which appears to be mature in term neonates. This included decreased *let-7e* and *miR-155* expression by early preterm neonates, which could indicate a primed state for inflammatory dysregulation (Fig.8.1C, D). As such, following TLR stimulation we postulate this culminates in increased expression of inflammatory genes (i.e. *NF-κB1* and *IL6*) without any change in regulatory miRs (Fig.8.1E). Conversely, late preterm

neonates increased regulatory genes such as *let-7e* and *SOCS1* alongside pro-inflammatory genes and showed a phenotype that was more similar to term neonates who increased *let-7e*, *miR-155* and *miR-106a* on TLR stimulation. These experimental scenarios suggest that term neonates are able to return to immune homeostasis following inflammatory activation, while preterm neonates continue to upregulate inflammatory pathways, as reinforced by our time course analysis (Fig.8.1F).

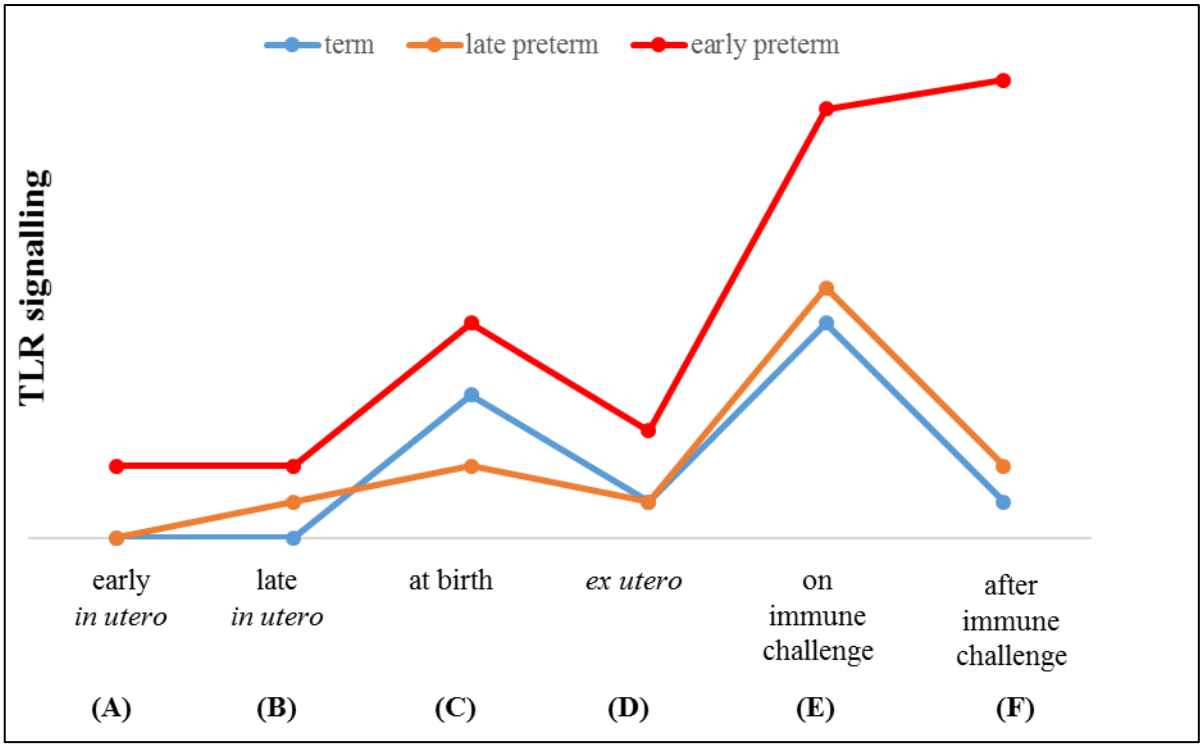

Fig 8.1. ***A theoretical representation of TLR signalling by the fetus/ neonate during gestation and following term, late preterm and early preterm delivery.*** Early preterm neonates are a most commonly a product of an inflammatory *in utero* environment from earlier during gestation (A) than late preterm neonates (B), which results in premature delivery. At birth, early preterm neonates show evidence of poor regulatory capacity (C) that becomes apparent during their adaptation to the *ex utero* environment (D). On immune challenge, early preterm neonates are unable to regulate inflammatory signalling, such that it becomes aberrant (E). Late preterm neonates show an *ex utero* response to immune challenge similar to term cord blood, suggesting a more mature system that shows some evidence of regulatory gene expression *in vitro*. Term fetuses typically encounter inflammation during labouring delivery and are born in an immune-tolerant state, showing increased regulatory miR expression compared to early preterm neonates (C). Following immune challenge *ex utero*, they are able to downregulate inflammatory signalling appropriately, unlike early preterm neonates that perpetuate inflammatory signalling that may predispose them to inflammatory conditions (F).

Preterm neonates are born in a state where immune cell numbers, cell function and miR expression is mitigated, contributing to increased vulnerability to pathogen colonisation. Following immune challenge, they become hyper-responsive and inflammatory signalling exceeds that seen in late preterm and term neonates. Late preterm neonates are more likely a product of indicated delivery (such as pre-eclampsia or growth restriction) or intrauterine inflammation occurring later during gestation[7]. Late preterm neonates demonstrate a miR and mRNA profile of TLR-associated gene expression that was similar to term neonates. This indicates the timing of a maturational switch in TLR signalling pathways occurs later in gestation allowing for more effective inflammatory regulation.

8.9.1. A PROPOSED MODEL FOR OUR FINDINGS

The *in utero* environment preceding preterm delivery may include adverse exposures to inflammation, labour, maternal health (e.g. pre-eclampsia) or maternal health behaviours during pregnancy (e.g. maternal smoking) (Fig. 8.2.A). This thesis has demonstrated that at delivery, the early preterm neonate shows decreased expression of regulatory miRs, with no difference in cytokine expression compared to the term neonate (Fig. 8.2.B). Following exposure to an inflammatory stimulus, this appears to culminate in the dysregulation of inflammatory gene networks, such that inflammatory genes *TLR4*, *IL6*, *IL10* and *NF-κB1* are increased and IL6 production therefore exceeds that of the term neonate (Fig. 8.2.C). This is evidence of a hyper-responsive state following birth that predisposes preterm neonates to uncontrolled inflammation. An inability to regulate inflammation may predispose preterm neonates to acute and/ or systemic responses characteristic of morbidities such as NEC, PVL, neonatal sepsis, CLD and ROP (Fig. 8.2.D).

Over page: Fig. 8.2. ***A proposed model for the development of inflammatory morbidities in preterm neonates.*** Early preterm neonates are exposed to a pro-inflammatory *in utero* environment (A) and show decreased regulatory miR expression at birth (B). On exposure to inflammatory stimuli *ex utero* (when they are prone to contracting infections), their pre-existing dysregulated inflammatory gene networks upregulate pro-inflammatory genes without increasing regulatory genes (C). Preterm neonates that develop infections are prone to developing acute and/ or systemic inflammation that commonly results in the development of morbidities such as PVL, ROP, neonatal sepsis, CLD and NEC (D). Images presented in this figure are self-illustrated or courtesy of open source designs (http://www.servier.com/Powerpoint-image-bank).

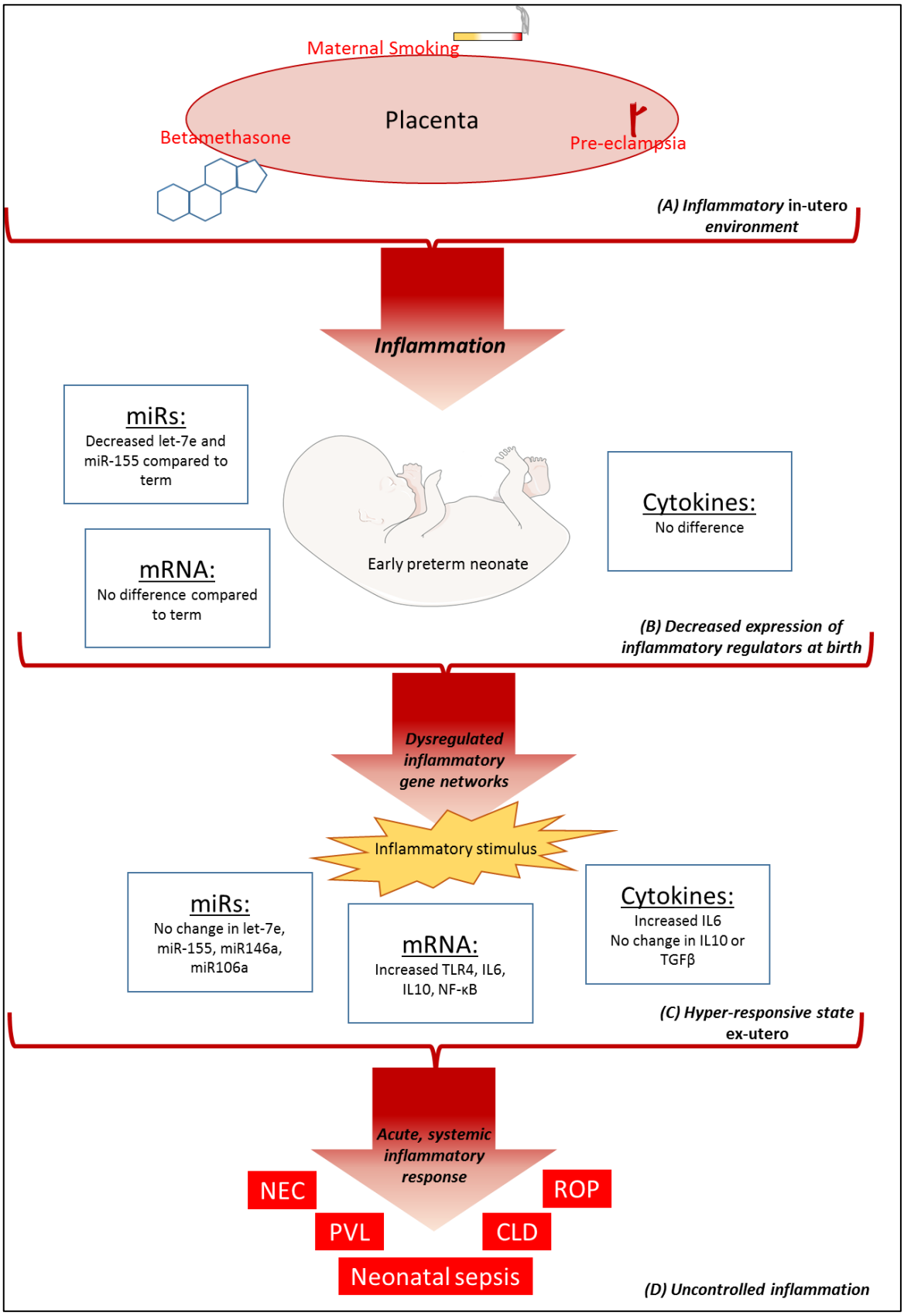

Fig. 8.2. ***A proposed model for the development of inflammatory morbidities in preterm neonates.***

8.10. FUTURE RESEARCH

The current understanding of cord blood miR expression and its role in innate immunity in preterm and term infants remains in its infancy. This field of research still has great potential in informing the clinical management of preterm neonates. TLR signalling is critical to innate immune function and neonatal immunity overall. While miRs have considerable influence on the neonatal immune phenotype, there is still scope for investigation into other inflammatory pathways from signalling networks to cell-specific signalling.

Based on the limitations of this thesis, future studies could involve GWAS to identify perturbations involving other miRs or networks of expression associated with inflammation in preterm neonates. This could lead to the identification of phenotypes that are more susceptible to disease and therefore, direct prophylactic interventions or changes in clinical management. Further, microarrays could be conducted on samples of neonatal peripheral blood similar to our cohort (i.e. in isolation of infection) that are stimulated with LPS *in vitro* to determine the upregulation or perturbation of specific pathways or gene clusters that may contribute to uncontrolled inflammatory signalling in early preterm cord blood compared to late preterm and term. Although neonatal outcomes cannot necessarily be predicted using *in vitro* stimulation studies, larger scale investigations looking for key miRs that were characterised by this thesis (specifically, *miR-155* and *let-7e*) could be used in conjunction with microarray data as proposed in Chapter 7, to assess their value (sensitivity and specificity) as diagnostic or prognostic tools. This research could lead to diagnostic advances such as establishing transcriptomic fingerprints and even sub-analyses for separating risk factors such as maternal smoking during pregnancy. Overall, such tools may be used clinically for prophylactic management (e.g. the use of probiotics) or to develop novel management strategies (e.g. to boost regulatory miR expression).

On a more specific scale, identifying underlying factors that contribute to aberrant inflammatory signalling by immune cells is also necessary. This could include the use of murine or cell-line knockdown models that are valuable for isolating the function of specific molecules or pathways. Small interfering RNAs (siRNAs) can be used *in vitro* to silence mRNA translation. Therefore, introducing them to isolated cord blood cell populations during TLR stimulation would help isolate the precise mechanism for the

lack of miR expression in preterm cord blood observed by this thesis. For example, silencing TLR2 or TLR4 would support our investigation of the TLR signalling cascade as a primary contributor to uncontrolled IL6 production in preterm cord blood. Further, investigation into Dicer expression in cord blood and how it might affect early preterm miR expression could be the underlying mechanism that requires confirmation in this experimental model.

We were also unable to confirm the contribution of different cell types to the preterm inflammatory response *in vitro,* which may reveal particular dysfunctional aspects of innate immunity. The use of flow cytometry to characterise cell-specific gene expression could therefore confirm our theory from Chapter 7, where we found that adult neutrophils were key producers of NF-κB-related genes. To date, the analysis of neonatal neutrophils is sparsely characterised, though they show comparable phagocytic function across gestational age[454]. Overall, neonatal neutrophil characterisation is therefore a key area for further research, particularly because neutropenia is associated with neonatal sepsis.

Although we chose to focus on neonatal outcomes rather than the aetiology of preterm birth, maternal physiology is clearly an important influence on the neonatal immune system. Increased maternal CRP, IL6[506] and IL1β[507] have been proposed as predictors for preterm delivery in amniotic fluid and maternal blood. As a majority of deliveries in our cohort were not exposed to infection or PPROM, data on maternal CRP levels were not consistently available from clinical records. Future studies could incorporate these parameters and also, the expression of miRs in maternal serum to trace the effect of immune regulation and relate it to the neonatal system. In fact, others have associated specific patterns of miR expression in maternal serum with poor pregnancy outcomes, and recommended the use of these targets as diagnostic tools (for review see[508]).

Clinical research is also important for investigating evidence of uncontrolled inflammation and managing it. Currently, the management of chronic or acute inflammation in preterm neonates (including sepsis), involves the use of antibiotics and immunomodulation therapy[509]. However, there remain complex obstacles between the translation of scientific findings and clinical outcomes. Altering cytokine concentrations, such as GMCSF supplementation to improve neutropenia in septic preterm neonates, has not been effective to date[218]. This may be because while early

preterm neonates can produce cytokines (as reinforced by this thesis), they show an inability to regulate their production temporally. Our data hereby highlights the need to manage preterm neonates' immune dysregulation. Therefore, therapies that promote immune regulation and as such, the tolerance of commensal bacteria may be ideal. This could include breastmilk supplementation or the administration of antibodies that neutralise IL6 overexpression.

The clinical management of preterm neonates is challenging. It is not feasible to completely inhibit pro-inflammatory cytokines or increase regulatory miRs, because they have ubiquitous roles in physiology and are simultaneously involved in unique networks of genes. Immunomodulating therapies such as Tocilizumab, which is a monoclonal antibody for IL6 receptor, have been trialled in the context of juvenile arthritis to reduce disease severity[510]. While it has been shown to decrease non-specific markers of inflammation such as CRP, Tocilizumab is associated with neutropenia and infection. Immune mediators such as Pentoxifylline are already used in the NICU to inhibit inflammatory signalling by TNFα and IL1 and decrease oxidative stress during neonatal sepsis and NEC[509]. Therapies involving miRs are yet to be trialled clinically, however, supplements such as curcumin are shown to decrease *miR-155* in mice and protect against sepsis and tissue damage during acute LPS-driven inflammation in this model[511]. Overall, the systemic nature of miR signalling means they may be better used as a diagnostic tool rather than a therapeutic target, at least with our current level of understanding.

8.11. SUMMARY

Preterm neonates are susceptible to adverse health outcomes, a majority of which are associated with inflammatory conditions. Notably, preterm blood shows lower leukocyte counts, attenuated intracellular killing capacity and decreased pro-inflammatory cytokine expression following immune stimulation *in vitro* compared to term blood. To date, studies have been unable to explain how such immune attenuation is associated with aberrant inflammation in preterm neonates. This thesis therefore aimed to characterise inflammatory gene expression and its regulation by miRs in the context of TLR stimulation. We demonstrated that inflammation in early preterm cord blood was not associated with any change in miR expression, but continued to increase pro-inflammatory gene expression. These findings reflect a poorly regulated, hyper-

responsive immune phenotype that could underlie preterm neonates' susceptibility towards developing inflammatory conditions. Further characterisation of miRs and other pathways associated with preterm innate immune regulation is therefore critical. This field of research could ultimately lead to the development of diagnostic tools and targeted therapies to reduce the burden of inflammatory disease among preterm neonates.

Chapter 9:

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Appendix I:

Materials and Equipment

REAGENTS AND CONSUMABLE MATERIALS

Reagent	Supplier	Catalogue Number
2.8mm Zirconium Oxide Precellys Beads	Bertin Technologies, Montigny, FR	03961-1-102
200 Proof Ethanol (C_2H_6O) (RNA grade)	Sigma-Aldrich, St Louis, USA	E7023
2-Propanol (C_3H_8O)	Sigma-Aldrich	I9516-500ML
Agilent RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, USA	5067-1511
Albumin from Bovine Serum (BSA)	Sigma-Aldrich	A7906-100G
Cellstar® Flat Bottom 96 Well Culture Plate (with lid)	Greiner Bio-one, Kremsmunster, AT	655180
Chloroform ($CHCl_3$)	Ajax Finechem, Taren Point, AU	1888
di-Sodium Hydrogen Phosphate (Na_2HPO_4)	Merck KGaA	7558-79-4
Dulbecco's Phosphate Buffered Solution, no calcium, no magnesium	Gibco®, Thermofisher Scientific, Waltham, USA	14190235
Human ACTB Endogenous Control FAM/MGB Probe	Life Technologies, Carlsbad, USA	4352667
Human Cytokine/ Chemokine Magnetic Bead Panel Milliplex MAP Kit	Merck Millipore, Billerica, USA	HCYTMAG-60K-PX29 Control Catalogue #MXH6060-2
Human Duo Set ELISA Development Systems (TNFα, IL6, CXCL8/ IL8)	R&D Systems, Minneapolis, USA	DY210 (TNFα) DY206 (IL6) DY208 (CXCL8/ IL8)

Reagent	Supplier	Catalogue Number
Hydrochloric Acid (HCl)	Merck, Kilsyth, AU	1789
LH Lithium Heparin Vial, 9mL Vacuette®	Greiner Bio-one, Kremsmunster, AT	445084
Lipopolysaccharides from *Escherichia coli* (LPS)	Sigma-Aldrich	L4391
miRNeasy Mini Kit	Qiagen, Limberg, NL	217004
Nuclease-Free Water	Ambion® by Life Technologies Carlsbad, USA	AM9932
Peptidoglycan from *Staphylococcus aeureus*	Sigma-Aldrich	77140-10MG
Polyinosinic:polycytidylic acid sodium salt (γ-irradiated)	Sigma-Aldrich	P0913-10MG
Potassium Chloride (KCl)	Ajax Finechem	A383-500G
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	VWR International, Poole, UKA	102034B
RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, USA	5067-1511
RNase-Free DNase Set	Qiagen, Limberg, NL	79254
Reinforced 2mL tubes and screw caps	Sapphire Bioscience	13119-500
Sodium Chloride (NaCl)	Merck KGaA, Darmstaat, DE	S3014-5KG
SuperScript III First-Strand Synthesis SuperMix for qRT-PCR	Invitrogen™	11752-050
Taqman ® Fast Advanced Master Mix	Applied Biosystems®, Life Technologies, Carlsbad, USA	4444557

Reagent	Supplier	Catalogue Number
Taqman ® MicroRNA Reverse Transcription Kit	Applied Biosystems	4366596
Taqman ® Universal PCR Master Mix II, No UNG	Applied Biosystems	4440040
Taqman ® MicroRNA Assays	Applied Biosystems	4427975
Taqman® Gene Expression Assays	Applied Biosystems	4331182
TMB/E solution (Horseradish Peroxidase)	Millipore, Temecula, USA	E50001-500ML
Tris-base	Amresco, Solon, USA	0497-1KG
TRIzol LS® Reagent	Ambion® by Life Technologies	10296028
TRIzol® Reagent	Ambion by Life Technologies	15596018
Tween®20 viscous liquid (Polyethylene glycol sorbitan monolaurate)	Sigma-Aldrich	P1379-IL

EQUIPMENT

Equipment	Supplier
2720 Thermal Cycler	Applied Biosystems Life Technologies, Carlsbad, USA
Agilent 2100 Bioanalyser	Agilent Technologies, Santa Clara, USA
HERAcell 150i CO_2 Incubator	Thermo Fisher Scientific, Waltham, USA
Heraeus Fresco17 Microfuge	Thermo Fisher Scientific, Waltham, USA
Heraeus Multifuge X3R Centrifuge	Thermo Fisher Scientific, Waltham, USA
Implen Nanophotometer	Thermo Fisher Scientific, Waltham, USA
Luminex® 100/200 System	Luminex Corporation, Austin, USA
Multiskan EX Plate Reader	Thermo Fisher Scientific, Waltham, USA
pH510 pH/mV/°C meter	Eutech Instruments, Thermo Fisher Scientific, Waltham, USA
Precellys 24 Tissue Homogeniser	Bertin Technologies, Montigny, FR
QuantStudio 12K Flex	Applied Biosystems by Life Technologies, Carlsbad, USA