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# Platelets, thrombin generation and extracellular vesicles: the effects of prematurity and COVID-19 on neonatal coagulation

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland,
in fulfilment of the degree of Doctor of Philosophy

Supervisors: Prof. Naomi McCallion
Prof. Patricia Maguire

2022

# Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Doctor of Philosophy is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Date: 18th October 2022

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Word Count: 47,871 (65,842 including bibliography, tables and figures)

# List of Abbreviations

| | |
|---|---|
| 3f-PCC | Three factor prothrombin complex concentrate |
| 4f-PCC | Four factor prothrombin complex concentrate |
| ABB | Annexin binding buffer |
| ADP | Adenosine diphosphate |
| AF-700 | Alexa Fluor 700 |
| APC | Allophyocyanin |
| APTT | Activated partial thromboplastin time |
| ART | Assisted reproductive technology |
| AT | Antithrombin |
| BDNF | Brain derived neurotrophic factor |
| BMI | Body Mass Index |
| BPD | Bronchopulmonary dysplasia |
| CAT | Calibrated automated thrombography |
| CD | Cluster of differentiation |
| CGA | Corrected gestational age |
| CLD | Chronic lung disease |
| CMOS | Complementary metal oxide semiconductor |
| CNTN-2 | Contactin 2 |
| CONS | Coagulase negative staphylococci |
| COVID-19 | Coronavirus Disease 2019 |
| CP | Cerebral palsy |
| CPB | Cardiopulmonary bypass |
| CSF | Cerebrospinal fluid |

| | |
|---|---|
| DA | Ductus arteriosus |
| DCT | Direct Coombs test |
| DLS | Dynamic light scattering |
| DOL | Day of life |
| ECMO | Extracorporeal membrane oxygenation |
| EDTA | Ethylenediaminetetraacetic acid |
| EEVs | Endothelial-derived extracellular vesicles |
| ELBW | Extremely low birth weight (<1000 g) |
| ELISA | Enzyme-linked immunosorbent assay |
| EOS | Early onset sepsis |
| EPCR | Endothelial Protein C receptor |
| ETP | Endogenous thrombin potential |
| EVENT Study | Extracellular Vesicles in Early preterm Neonates and Thrombin generation study |
| EVs | Extracellular vesicles |
| FBC | Full blood count |
| FETO | Fetoscopic endotracheal obstruction |
| FIP | Focal intestinal perforation |
| FITC | Fluorescein isothiocyanate |
| GCP | Good Clinical Practice |
| GDPR | General Data Protection Regulation |
| GP | Glycoprotein |
| GM | Germinal matrix |
| GO | Gene Ontology |
| GSS | Glutathione synthetase |

| | |
|---|---|
| HDN | Haemolytic disease of the newborn |
| HSC-LEVs | Haematopoietic stem cell-derived large extracellular vesicles |
| HSCs | Haematopoietic stem cells |
| hsPDA | Haemodynamically significant patent ductus arteriosus |
| ICAM-1 | Intercellular adhesion molecule 1 |
| ICU | Intensive care unit |
| IQR | Interquartile range |
| ISEV | International Society of Extracellular Vesicles |
| IUGR | Intrauterine growth restriction |
| IVH | Intraventricular haemorrhage |
| KEGG | Kyoto Encyclopaedia of Genes and Genomes |
| LBW | Low birth weight (<2500 g) |
| LDL | Low density lipoprotein |
| LEVs | Large extracellular vesicles |
| LOS | Late onset sepsis |
| LPS | Lipopolysaccharide |
| MERS | Middle Eastern Respiratory Syndrome |
| miRNA/miR | Micro ribonucleic acid |
| MIS-C | Multisystem inflammatory syndrome in children |
| MISEV | Minimal information for studies of extracellular vesicles |
| MIS-N | Multisystem inflammatory syndrome in neonates |
| MN-CMS | Maternal and Newborn Clinical Management System |
| MP | Microparticle |
| MPV | Mean platelet volume |

| | |
|---|---|
| MTCT | Mother to child transmission |
| MUC 4 | Transmembrane mucin receptor (human) |
| NBAT | Neonatal bleeding assessment tool |
| NEC | Necrotising enterocolitis |
| NE | Neonatal encephalopathy |
| NICHD | National Institute of Child Health and Human Development |
| NICU | Neonatal intensive care unit |
| NT | Neonatal thrombocytopenia |
| NTA | Nanoparticle tracking analysis |
| PAMPs | Pathogen associated molecular patterns |
| PBS | Phosphate buffered saline |
| PBS | Pacific blue |
| PCR | Polymerase chain reaction |
| PDA | Patent ductus arteriosus |
| PDEVs | Platelet-derived extracellular vesicles |
| PE | Phycoerythrin |
| PECAM | Platelet endothelial cell adhesion molecule |
| PET | Pre-eclampsia |
| PF4 | Platelet factor 4 |
| PFA | Platelet function analyser |
| PHH | Post-haemorrhagic hydrocephalus |
| PIL | Patient information leaflet |
| PIMS-TS | Paediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 |
| PLAP | Placental-type alkaline phosphatase |

| | |
|---|---|
| PlGF | Placental growth factor |
| PPHN | Persistent pulmonary hypertension |
| PPP | Platelet poor plasma |
| PPROM | Preterm prelabour rupture of the membranes |
| PRP | Platelet rich plasma |
| PS | Phosphatidylserine |
| PT | Prothrombin time |
| PVL | Periventricular leukomalacia |
| RDS | Respiratory distress syndrome |
| rFVIIa | Recombinant FVIIa |
| RNA | Ribonucleic acid |
| ROP | Retinopathy of prematurity |
| ROTEM | Rotational thromboelastometry |
| SARS | Severe Acute Respiratory Syndrome |
| SARS-CoV-2 | Severe Acute Respiratory Syndrome Coronavirus 2 |
| SD | Standard deviation |
| SEVs | Small extracellular vesicles |
| SGA | Small for gestational age |
| SNAP II | Score for neonatal acute physiology II |
| SNAP-PE II | Score for neonatal acute physiology with perinatal extension II |
| SVD | Spontaneous vaginal delivery |
| TA | Tracheal aspirates |
| TEG | Thromoelastography |
| TEM | Transmission electron microscopy |

| | |
|---|---|
| TF | Tissue factor |
| TFCK | Thrombin initiated fibrin clot kinetics |
| TF-EVs | Tissue factor extracellular vesicles |
| TFPI | Tissue factor pathway inhibitor |
| TH | Therapeutic hypothermia |
| TLR | Toll-like receptors |
| TM | Thrombomodulin |
| TRAP | Thrombin receptor agonist protein |
| UCB | Umbilical cord blood |
| UVC | Umbilical vein catheter |
| VEGF | Vascular endothelial growth factor |
| VEGFA | Vascular endothelial growth factor A |
| VEGFR2 | Vascular endothelial growth factor receptor 2 |
| VLBW | Very low birth weight (<1500 g) |
| VLDL | Very low density lipoprotein |
| VOC | Variant of concern |
| VON | Vermont Oxford Network |
| vWF | von Willebrand factor |
| WHO | World Health Organisation |

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# Summary

Premature infants are at increased risk of haemorrhage, have reduced levels of coagulation factors, and hyporeactive platelets *in vitro.* Despite prolonged standard clotting times, thrombin generation is similar or increased in preterm compared with term plasma. Extracellular vesicles (EVs) are tiny particles released from cells and evidence suggests they may have a role in haemostasis.
COVID-19 can cause hypercoagulability and thrombosis. The effects of COVID-19 on pregnant women and the developing fetus are poorly understood.

The EVENT Study, a prospective observational study, aimed to evaluate the impact of platelets on thrombin generation in preterm infants and the release of EVs during perinatal adaption. Umbilical cord blood and postnatal samples were collected from preterm infants (24-31 weeks) and healthy term controls. Coagulation was evaluated using Calibrated Automated Thrombography (CAT) in platelet rich plasma (PRP) and EVs were characterised by nanoparticle tracking analysis, flow cytometry and ELISA.

Secondly, I aimed to evaluate the effects of COVID-19 infection during pregnancy. The neonatal clinical outcomes, of infants born to women with SARS-CoV-2 during pregnancy, were investigated in two retrospective studies, while a prospective case-control study evaluated the haematological impact, measured in cord blood.

101 preterm infants were recruited to the EVENT Study. Thrombin generation in PRP was hypercoagulable in preterm compared with term infants. There were changes in the concentration, size, cellular origin and procoagulant activity of EVs during perinatal adaption in both preterm and term infants.

The clinical outcomes of infants born to women with SARS-CoV-2 were reassuring. There were no haematological abnormalities identified (n=15 cases) and thrombin generation was comparable between groups.

This thesis supports the growing evidence that preterm infants are hypercoagulable, despite reported hyporeactive platelets. I describe changes in EVs during perinatal

adaption. I present reassuring data on the neonatal clinical and haematological outcomes of a maternal COVID-19 infection during pregnancy.

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# Chapter 1 : Introduction I: Prematurity and neonatal haemostasis

## 1.1 Adaption to extrauterine life

After birth, several physiological changes occur in healthy neonates to allow adaption from intrauterine to extrauterine life (1). There is a reduction in pulmonary vascular resistance following the onset of respiration and increase in arterial oxygen concentration (2). From a cardiac perspective, the systemic circulation changes from a low pressure circuit, including the placenta, to a high pressure circuit following clamping of the umbilical cord (1). This increase in systemic vascular resistance causes closure of the two intracardiac shunts, the ductus arteriosus (DA) and the foramen ovale, which were essential for *in utero* survival. There are also changes within the neonatal haematological system during perinatal adaption. Over the first few hours of life, the haematocrit, red cell concentration and neutrophil count all rise, followed by a reduction in the haematocrit over the next few weeks (3, 4). While perinatal adaption occurs in healthy full-term infants, I will discuss the complications of premature birth below.

## 1.2 Introduction to prematurity

Globally, one in ten infants are born preterm and it is the leading cause of death in the first month of life (5). The World Health Organisation (WHO) defines preterm birth as any birth before 37 weeks gestational age, and it is subdivided into extreme preterm (less than 28 weeks gestation), moderate preterm (28 to 32 weeks gestation) and late preterm birth (32-37 weeks gestation) (6). Alternatively, preterm neonates may be classified by birth weight; low birth weight (LBW) <2500g, very low birth weight (VLBW) <1500g and extremely low birth weight (ELBW) <1000g. The complications of prematurity are most severe in the extremely preterm cohort, owing to the interaction between severe physiological immaturity, inflammation, oxygen and the extrauterine environment, and diminish as an infant approaches term.
Preterm birth may have long-term implications for the child, their family and society. Survivors are at increased risk of hospitalisation for infection throughout childhood (7), and of both physical and cognitive disability (8). The parents of infants born

preterm are at increased risk of mental illness, which persists into the early years of their child's life (9). Parents of premature infants are also at higher risk of marital breakdown, particularly if the child has a poor neurodevelopmental outcome or the family is of lower socioeconomic status (10). In Ireland, there are approximately 600 VLBW babies born every year (11) and the average initial NICU hospitalisation of an ELBW infant in Ireland costs between €66,017 and €78,919 per child (2009 data) (12). This does not include personal financial burdens to their families, such as travel, loss of income and accommodation, or include the costs of long-term medical and developmental follow up and early intervention services for ex-preterm infants, which are significantly greater than for their term counterparts (13).

### 1.3 Intraventricular haemorrhage

Intraventricular haemorrhage (IVH) is a common neurological complication of prematurity, caused by haemorrhage within the germinal matrix (germinal matrix). IVH occurs in 32% of preterm infants less than 1500 g (14). A majority of IVHs occur in the first week of life with 78% occurring in the first 72 hours (15). For this reason, preterm infants have routine cranial ultrasounds performed during this period to assess for haemorrhage.

Preterm infants are vulnerable to IVH due to the presence of the GM, immaturity of cerebral autoregulation of blood flow and possibly due to immaturity of neonatal platelets and the coagulation system (16). The GM is a network of blood vessels found in the developing brain, situated between the caudate nucleus and the lining of the lateral ventricles, which involutes by 36 weeks gestation. This is a highly vascularised network which is prone to bleeding.

Studies by Ballabh *et al.* have demonstrated increased vessel density in the GM, with rapid angiogenesis related to increased levels of VEGF (vascular endothelial growth factor) and Angiopoeitin-2 (17), and reduced pericyte coverage of the germinal matrix vasculature (18), which renders them fragile. In animal studies of preterm rabbits, prenatal administration of an inhibitor of VEGF Receptor 2) (VEGFR2) reduced the density of vasculature in the germinal matrix, increased pericyte coverage (18) and reduced the incidence of IVH (17). Prenatal administration of glucocorticoids has also been shown to suppress angiogenesis by

reducing VEGF expression, thus decreasing the vascular density within the GM and increasing pericyte coverage (19).

IVH severity is classified using the Papile grading system (20). Grade 1 and Grade 2 haemorrhages are considered mild, and are either limited to the germinal matrix (Grade 1) or extend into the ventricular cerebrospinal fluid (CSF) (Grade 2). Grade 3 haemorrhage is of moderate severity and causes dilatation of the ventricle, and a Grade 4 haemorrhage is often severe and extends into the parenchymal tissue causing direct neuronal injury.

Over time, these haemorrhages may extend, or may involute and resolve. Where they persist, there is a risk of post-haemorrhagic hydrocephalus as residual blood from the IVH prevents both the circulation of CSF and the re-absorption of CSF from the arachnoid granulations (21). In post-haemorrhagic hydrocephalus, there is progressive dilatation of the ventricles which, if it persists, ultimately requires drainage with serial lumbar punctures or shunt insertion. This occurs in 10% of all ELBW infants who have an IVH, but is most common in those with Grade 3 or Grade 4 IVH (22).

While there is no treatment for IVH, the use of antenatal steroids has been shown to reduce the incidence of IVH (RR=0.55) (23).

In the long-term, there is an increased risk of neurodevelopmental impairment in preterm infants with an IVH, particularly where post-haemorrhagic hydrocephalus develops. In one study, the incidence of moderate or severe neurosensory impairment in infants with Grade 1/Grade 2 IVH was 22% versus 43 % for those with a Grade 3/ Grade 4 IVH (infants with no IVH had an incidence of 12.1%) (24). There was also a higher incidence of cerebral palsy (30.1% v 6.5%) and bilateral hearing loss (8.6% v 2.3%) in those with Grade3/4 IVH compared to those infants without an IVH.

### 1.4 Neonatal thrombosis

In addition to the high incidence of haemorrhage in this cohort, infants born preterm are also at risk of thrombosis. The reported incidence of thrombosis among infants admitted to NICU is 1.4 – 2 per 1000 admissions, with preterm infants at increased risk (25, 26). However, a recent prospective study described thrombosis in 75% of infants following umbilical venous catheter (UVC) insertion, compared to none in

age-matched controls without UVCs (27). While 83% of thrombosis persisted after UVC removal, 80% spontaneously regressed without treatment. The medical management of thrombosis in this cohort is controversial (28), although the NEOCLOT Study has reported a protocol for the suggested management of neonatal thrombosis (29). Interestingly, VLBW infants with a prothrombotic gene mutation (e.g. Factor V Leiden or Prothrombin mutation) are at increased risk of IVH in the neonatal period, thought to be because hypercoagulability may result in venous stasis, thrombosis and infarction (30, 31).

### 1.5 Other complications of prematurity

In addition to the haemorrhagic and thrombotic complications of preterm birth described in *Section 1.3 and 1.4*, preterm infants are at risk of infective and inflammatory pathologies which can affect multiple organ systems as well as long term survival (*Figure 1.1*).

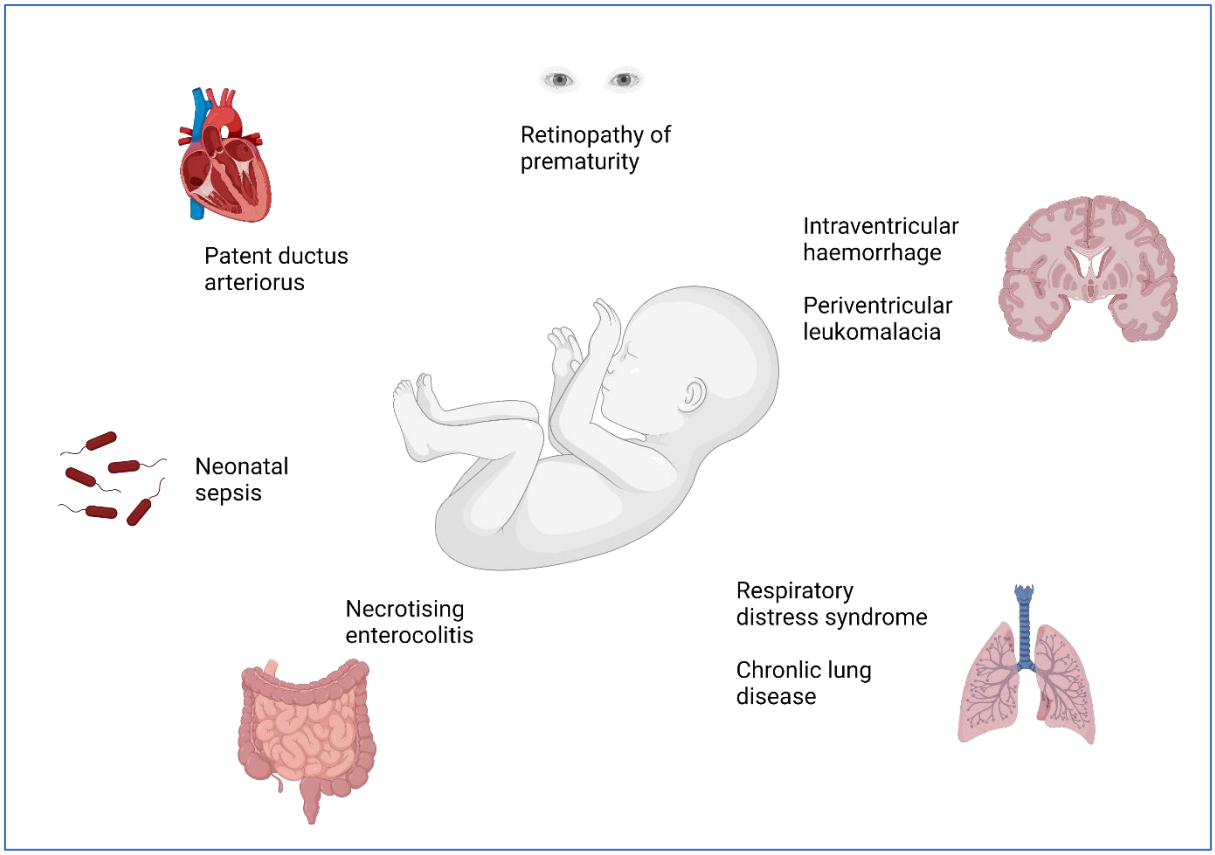


**Figure 1.1 Preterm birth can cause pathologies of all organ systems**

Image created with BioRender.com

### 1.5.1 Respiratory disease

Respiratory distress syndrome (RDS) is caused by surfactant deficiency within the alveoli of the lungs. Surfactant is a substance containing phospholipids and proteins, required to decrease surface tension and hence facilitate gaseous exchange. Surfactant production only begins at 24 weeks, and development continues until 34 weeks gestation (32). The incidence of RDS in ELBW infants is 91-93% (14, 33). The treatment of RDS with exogenous surfactant has been widespread since the early 1990's (34) and new methods to deliver surfactant using less invasive techniques are gaining prominence (35).

Chronic lung disease/ Bronchopulmonary dysplasia (CLD/BPD) is a clinical diagnosis defined as an oxygen requirement at 36 weeks corrected gestational age (or at 28 days of life in infants born greater than 32 weeks) that correlates with both radiographic and histopathological characteristic findings. The incidence of BPD in VLBW infants is 42 -45% (14, 36). Very preterm infants are at risk of BPD due to the

exposure of their developing lungs to mechanical trauma from ventilatory support, oxygen toxicity, infection and inflammation in the postnatal period (37). Exposure to these toxic effects causes arrested alveolarisation, and this results in fewer and larger alveoli with insufficient vascularisation and airflow limitation (38). The introduction of antenatal steroids, postnatal surfactant, caffeine therapy and volume targeted ventilation has reduced the risk of BPD (23, 39-41). Postnatal steroid therapy is the primary treatment option for BPD (42, 43). However, the routine use of postnatal steroids is associated with an increased risk of neurodevelopmental impairment and cerebral palsy (44).

### 1.5.2 Patent ductus arteriosus

The DA is a fetal blood vessel which shunts blood from the high-pressure pulmonary system to the low-pressure systemic circulation and it is essential for the survival of the fetus. Under physiological conditions at term, 90% of DAs close within 48 hours of delivery (45). However, in preterm infants the ductus arteriosus remains patent in 70% of infants less than 28 weeks gestation (46).

The presence of a large patent ductus arteriosus (PDA) can result in dilatation of the left heart, pulmonary over-circulation and a reduction in systemic blood flow to vital organs. This may result in pulmonary haemorrhage, progressive cardiac failure and pulmonary oedema and PDA has been associated with an increased risk of IVH, necrotising enterocolitis (NEC) and BPD, although causality has not been established (46). PDAs may be managed conservatively, medically treated with indomethacin, ibuprofen or paracetamol, or be surgically managed by device closure or open surgery (47-51).

### 1.5.3 Periventricular leukomalacia

Periventricular leukomalacia (PVL) is a structural brain injury involving the periventricular deep white matter. The preterm brain is most susceptible to PVL; the incidence of cystic PVL is 3-4% in extremely preterm infants (14, 52). The principal pre-disposing insults are cerebral ischemia, in-utero or neonatal infection and systemic inflammation in-utero or in the neonatal period. These insults result in downstream excitotoxicity and free radical development, ultimately leading to damage of pre- myelinating oligodendrocytes (53). The motor fibres in the

corticospinal tracts pass through the periventricular white matter and may be damaged by PVL, resulting in cerebral palsy (54). There are no specific treatments for PVL. Prevention strategies are limited to optimising antenatal management with antenatal steroids, magnesium sulphate and *in utero* transfer to a tertiary neonatal centre, avoiding hypotension and minimising exposure to infection in the postnatal period (55).

### 1.5.4 Necrotising enterocolitis

NEC is an ischaemic and inflammatory necrosis of the bowel, usually seen in preterm and critically ill infants (56). The incidence of NEC is 11% in infants born before 28 weeks (14). The pathophysiology of NEC is not completely understood, but there are likely multiple contributing factors including prematurity, ischaemia, pathological bacteria in the gut microbiome and artificial formula, which cause mucosal damage triggering an inflammatory cascade (57). The clinical features of NEC can include abdominal distension, gastrointestinal bleeding, hypotension, thrombocytopenia and metabolic acidosis. Diagnosis is confirmed by x-ray imaging with the identification of pneumatosis, portal venous gas and, in cases of perforation, pneumoperitoneum. The severity of NEC is classified using the Modified Bell's Staging Criteria (58). Treatment involves the interruption of feeds, antibiotics and surgical intervention in severe cases. NEC has a high mortality which ranges from 15-30%, although it may be up to 50% in those requiring surgical intervention (59).

### 1.5.5 Retinopathy of prematurity

Retinopathy of prematurity (ROP) is one of the leading causes of childhood blindness worldwide (60). ROP is caused by the abnormal vascular development of the retina *ex utero*. ROP occurs in 59% of VLBW infants and in its most severe form, results in retinal detachment and blindness. ROP is classified into five stages using an International Classification System (61). 12% of VLBW infants require medical or surgical treatment of ROP(14).

The link between oxygen exposure and the development of ROP has been well described (62). Currently, the target oxygen saturations for extremely preterm infants are maintained between 91-95% to reduce their oxygen exposure and to optimise

their incidence of ROP, while not compromising their survival or neurodevelopmental outcomes (63, 64). The treatment options for clinically significant ROP include Bevacizumab (an anti-VEGF agent), laser therapy and cryotherapy.

#### 1.5.6 Neonatal infections

Preterm infants are at increased risk of neonatal sepsis due to the high incidence of chorioamnionitis (which can trigger preterm birth), their immature immune system and incomplete passive transplacental transfer of maternal antibodies, an immature skin barrier and a high number of invasive procedures. Neonatal sepsis may be early onset (EOS) (<72 hours) or late onset (LOS) (>72 hours). EOS typically presents as a fulminant multi-system disease, and respiratory symptoms are common. The incidence of EOS in very preterm infants is 1-2% (14, 65, 66) and mortality is high among VLBW infants 37-40% (65, 66). LOS occurs in 21-36% of VLBW infants and 28% of these infants had more than one episode of LOS (14, 67). The case fatality rate of LOS was 5% in one study, while the National Institute of Child Health and Human Development (NICHD) reported a mortality rate of 18% in infants who developed LOS versus a rate of 7% in those who did not have LOS (65, 67). Moreover, VLBW survivors of neonatal sepsis have an increased burden of disease, with higher rates of IVH, PVL and BPD (66).

### 1.6 Survival and long term outcomes of preterm infants

The survival of very preterm infants has substantially improved over recent decades with the most significant improvement seen in infants of the lowest gestational age (68). This has predominantly been due to the introduction of antenatal steroids, surfactant and non-invasive ventilation strategies (69). In Ireland, the threshold of viability is now 23 + 0 weeks and guidelines recommend that resuscitation may be offered to infants born at 23 weeks gestation who present in favourable condition (11). According to the most recent Irish data, during the five year period 2014 – 2018, survival at 23 weeks was 33% which increased to 82% at 26 weeks and 96% at 30 weeks' gestation (11).

Despite improvements in the survival of extremely preterm infants, there remain high rates of neuro-disability among survivors. Very preterm infants are at risk of cognitive impairment, cerebral palsy, blindness and deafness. In the EPICURE-2 study, 25%

of survivors between 22 to 26 weeks gestation had a moderate or severe disability, with those at the lowest gestational age having the highest risk (8). Cerebral palsy (CP), a permanent static neurological insult which impairs motor function, occurred in 14% of extremely preterm infants, 39% of which had diplegia, 25% had hemiplegia, 12% had quadriplegia and 24% had other patterns of injury (8). Preterm infants are also vulnerable to hearing loss and the incidence of confirmed hearing loss in infants less than 30 weeks is 5.9% (70). The infants at highest risk are those with the youngest GA and the longest NICU stay. 7% of infants between 22-26 weeks gestation had moderate to severe visual impairment at follow up (8). In addition, high rates of autism spectrum disorders (6-20%) have been described in preterm infants (71).

At school-age, ex-preterm infants display lower scores in reading and mathematics, poorer executive and academic function and in school, language problems and overall school difficulties (72-74).

The medical complications of premature birth continue into adulthood and it is a risk factor poorly recognised by adult services (75). There is growing awareness of the long-term respiratory (76), cardiac (77, 78) and psychological (79) sequelae of preterm birth (80). Regarding the social development of ex-preterm infants, a recent study showed that adults born preterm were less likely to have romantic relationships or become parents than their full-term peers (81). In a large Norwegian study, ex-preterm infants had a higher need for social security benefits, lower rates of school and university completion and were more likely to be in lower paid jobs than their full-term peers (82). However, they were no more likely than full-term infants to have had a criminal conviction.

Preterm birth has life-long sequelae for the child, family and society and the optimisation of early neonatal management is essential to optimise the quality of their lives.

### 1.7 Haemostasis

Haemostasis is the process by which blood loss is arrested at the site of an injury to a blood vessel (83, 84). The primary haemostatic response includes vascular spasm, platelet adhesion (predominantly mediated by von Willebrand factor) and aggregation to form a platelet plug. Platelets become activated from their quiescent state at the site of vessel injury by interactions with subendothelial tissue, von Willebrand factor, fibrinogen and agonists including thrombin. Secondary haemostasis results in thrombus formation via the coagulation cascade at the site of injury. Under physiological conditions, coagulation is initiated by the extrinsic pathway, following the exposure of sub-endothelial tissue factor (TF) to circulating activated factor VII, resulting in thrombin formation via the common pathway, in turn activating the intrinsic pathway. Fibrinogen is cleaved to fibrin by thrombin which stabilises the evolving clot.

There are several physiological mechanisms to limit haemostasis to the site of vessel injury, including the release of nitric oxide and prostacyclin by healthy endothelium to maintain platelets in their quiescent state (83). There are also several anti-coagulant pathways including Tissue Factor Pathway Inhibitor (TFPI), Antithrombin (AT) and Protein C (85), in addition to the fibrinolytic pathway.

Pathology can occur due to abnormalities in platelet number or function, abnormalities in pro- or anti-coagulant activity or in fibrinolysis, resulting in excessive bleeding or thrombosis and, in some conditions, both occurring simultaneously (86). Haemostasis is critically important in preterm infants due to their increased risk of both haemorrhage and thrombosis (27, 87, 88).

Below, I will discuss the role of platelets and developmental haemostasis in more detail, to further understand the differences between neonatal and adult haemostasis.

### 1.8 Neonatal platelets

Platelets are small anuclear circulating fragments of megakaryocytes produced in the bone marrow. The production of platelets begins by 8-9 weeks gestation and platelets reach adult levels of >150 x 10^9/L by the end of the second trimester (89). The classical role of platelets is in primary haemostasis as discussed in *Section 1.7*. At the site of vessel injury, platelets are exposed to subendothelial collagen and

circulating von Willebrand factor, to which they adhere via glycoprotein (GP) receptors (90). GPIIb/IIIa (Cluster of differentiation [CD]) CD41/CD61) is uniquely expressed in platelets and binds to fibrinogen, while GPIb (CD42b) binds to von Willebrand factor (91).

The platelets become activated, change shape and degranulate, which facilitates aggregation and formation of a platelet plug. Platelets contain alpha granules (containing p-selectin, platelet factor 4, von Willebrand factor, tumour necrosis factor, thrombospondin, fibrinogen and factor V), dense granules (containing adenosine diphosphate [ADP], serotonin, and calcium) and lysosomes (92, 93).

Platelets also contribute to secondary haemostasis by providing a phospholipid surface for the enzymatic complexes (tenase and prothrombinase complexes) of the coagulation cascade. Platelets also release many of the circulating extracellular vesicles (94) which will be discussed later in *Chapter 2: Section 2.1.*

In addition to their role in haemostasis, platelets are an important component of the innate immune system, displaying Toll-like receptors (TLR) which recognise pathogen associated molecular patterns (PAMPs) (95, 96). The activation of TLRs results in production of type 1 interferons and inflammatory cytokines (97). These pro-inflammatory cytokines facilitate communication between platelets and leukocytes.

In neonates, platelets may contribute to the closure of the ductus arteriosus. In 2010, a study of a murine model of PDA demonstrated that platelets migrate to the DA and cause a thrombotic occlusion of the duct very shortly after delivery, a function mediated by multiple platelet receptors (98). Moreover, there is evidence that thrombocytopenia on the first day of life is an independent predictor of PDA closure failure (99). However, a recent study by Kumar *et al.* failed to demonstrate improved rates of PDA closure following the liberal transfusion of platelets to thrombocytopenic infants receiving medical treatment of a PDA (100).

Neonatal thrombocytopenia (NT) is defined as a platelet count less than 150 x 10^9/L and severe NT as less than 50 x 10^9/L. NT occurs in 22-35% of infants admitted to NICU (101). The differential diagnosis is extensive and includes intrauterine growth restriction, perinatal asphyxia, neonatal alloimmune thrombocytopenia and sepsis/NEC.

The haemorrhagic consequences of NT are poorly understood. One observational study (PlaNeT-1) described a 9% incidence of major haemorrhage in infants with severe NT and showed that platelet number was a poor predictor of bleeding (102). At present, platelet transfusions are the treatment of choice for NT, although they have not been shown to reduce the risk of haemorrhage. The PlaNeT-2 Study demonstrated a higher incidence of death or a major bleed within 28 days, when a transfusion threshold of 50 x 10^9/L compared with 25 x 10^9/L was used (103). Interesting, there was also a significantly higher rate of BPD in survivors in the ”50 group” (63% v 54%). Similar findings, of an increased incidence of IVH, were also noted in the liberal transfusion arm of the study by Kumar *et al.* investigating the role of platelets in PDA closure, although it was not a primary outcome of this study (100).

The current evidence supports the use of restrictive thresholds for prophylactic platelet transfusions in preterm neonates. The reason for the apparent harms of liberal platelet transfusions may be related to the differences between adult (used in transfusion products) and neonatal platelets.

Neonates have higher circulating levels of thrombopoietin, are more sensitive to thrombopoietin at lower levels, and have smaller megakaryocytes with lower levels of ploidy that mature more quickly (104). Although there was no difference in the platelet count between preterm, term infants and adults, the mean platelet volume (MPV) was significantly lower in preterm compared with term infants (105). Differences in the composition of glycoprotein receptors on the platelet membrane have also been described. Preterm and term infants had reduced levels of GPIIb/IIIa (CD41a) compared with adults, and very preterm infants (<30 weeks) had reduced levels of GPIIIa (CD61) compared with both term infants and adults (105). There was no difference in surface GPIb (CD42b) between neonates and adults, or preterm and term neonates. In contrast, a study by Gruel *et al.* demonstrated similar levels of GPIIb/IIIa on fetal platelets (collected on cordocentesis from fetus between 18 and 26 weeks) and adults, and antibody binding to GPIb was higher in fetuses than adults (106).

Neonatal platelets are hyporeactive to multiple agonists compared with adult platelets. Several studies have shown no difference in platelet activation between neonatal and adult platelets at baseline, measured using flow cytometry (105, 107, 108). Following stimulation with thrombin receptor agonist protein (TRAP), neonatal

platelets showed significantly reduced expression of p-selectin and PAC-1 binding (markers of platelet activation) compared with adults, suggesting a reduction in platelet degranulation in neonates (105). Over the first twelve days of life, the platelet responsiveness to stimulation (measured by p-selectin production) increased significantly in term infants, although it did not reach adult levels by day 12. Using alternative platelet agonists, such as thrombin, ADP/epinephrine and thromboxane A2, multiple studies have demonstrated this hypo-responsiveness of neonatal platelets in preterm and term infants (107, 108). Interestingly, stimulation with ristocetin, a co-factor which causes von Willebrand factor to bind to the GPIb platelet receptor, resulted in increased aggregation in neonatal PRP compared to maternal samples (109).

In spite of this reported hypo-reactivity of neonatal platelet aggregation *in vitro*, clinical tests to evaluate primary haemostasis in neonates are typically enhanced compared to adults (91). Term neonates have a similar or shorter “Bleeding Time” than adults (110), and this is thought to be due to an increased concentration of von Willebrand factor and a higher haematocrit. However, preterm infants had prolonged bleeding times compared to full-term infants and bleeding times shortened in all groups over the first month of life (111). Moreover, the “PFA (Platelet Function Analyzer)-100 Closing Time” is also shorter in neonates, likely due to higher concentrations of von Willebrand factor in neonatal plasma (112). When PFA-100 was compared between preterm and term cord samples, a significant inverse relationship was seen between gestational age and closure time. Moreover, closure times in postnatal neonatal samples were significantly prolonged when compared to cord samples (113). Deschmann *et al.* demonstrated that in thrombocytopenic preterm infants, the closure time, using PFA-100, was associated with an infant’s Bleeding Score (obtained from the Neonatal Bleeding Assessment Tool [NBAT] (114)), unlike platelet count, which was not (115).

One *in vitro* study evaluated the effect of transfusing adult versus neonatal platelets to thrombocytopenic cord blood samples (116). Impedance aggregometry, PFA-100 and thromboelastography were used to assess the effect of adult and neonatal platelet transfusions on haemostasis. It was shown that adult platelet transfusions to thrombocytopenic cord blood samples resulted in increased platelet aggregation and shorter PFA-100 closure times after stimulation, while the clot strength and firmness measured using Thromboelastography (TEG) was also greater compared with

neonatal platelet transfusions. This experiment demonstrates the significant differences between the effects of adult and neonatal platelets. Although the hypercoagulable effect of adult platelets may seem desirable in the prevention of haemorrhage in preterm neonates, their prophylactic use appears to produce undesirable results (117).

It is possible that these differences between adult and neonatal platelets could result in a post-transfusion release of large amounts of prothrombotic and pro-inflammatory mediators contained within platelet granules, resulting in adverse neonatal outcomes.

### 1.9 Developmental haemostasis and compensatory neonatal haemostatic mechanisms

There are developmental changes in the coagulation system between neonates and adults and between preterm and term neonates. Clotting factors are present in the fetus from 10 weeks gestation, and concentrations rise with increasing fetal maturity (118).

Neonatal plasma has reduced levels of pro-coagulant factors, including the vitamin k-dependent factors II, VII, IX and X, compared with adults, with the exception of factor VIII levels which are similar or increased in neonates (118-120). Preterm plasma also has reduced levels of clotting factors compared to full-term infants (119, 121, 122).

However, there are several compensatory mechanisms to counteract these reduced pro-coagulant factors levels. Von Willebrand factor (vWF) levels are similar or increased in neonates compared to adults (119, 120), in addition to the presence of larger vWF polymers in neonates (123). Anti-coagulant factor levels such as Protein S, Protein C and Antithrombin are reduced in term infants compared to adults but are further reduced in preterm compared with term infants (119, 121, 122). Most coagulation factors reach adult levels by six months of age in preterm and term infants (119, 120). In addition to these differences, neonates have a higher haematocrit (124) and reduced fibrinolytic activity (125).

It is hypothesised that the reduction in pro-coagulant factor levels is countered by these compensatory mechanisms, and evidence suggests that preterm infants are in fact hypercoagulable compared to term infants (126).

## 1.10 Evaluation of coagulation

### 1.10.1 Standard clotting tests

In clinical practice, prothrombin time (PT) and activated partial thromboplastin time (APTT) are the standard laboratory tests used to measure the ability of the blood to clot. PT measures the activity of the extrinsic and the common pathway, specifically the function of factor V, VII, X, prothrombin and fibrinogen (127). It is carried out by the addition of tissue factor (thromboplastin) and calcium to patient plasma and calculating the time from the addition of the reagents to the initiation of clot formation. This value (in seconds) is compared to a normal reference range. APTT measures the activity of the intrinsic pathway and common pathway, including factors XII, XI, IX, X, VIII, V, prothrombin and fibrinogen (127). Calcium, kaolin (or other contact pathway activator) and phospholipid are added to the patient's plasma and the time to the initiation of clot formation is measured.

It has previously been demonstrated that standard clotting times (PT and APTT) are prolonged in preterm compared with term neonates (119, 121), and most prolonged in the extremely preterm group <28 weeks (128). However, there was no correlation between baseline PT/APTT and the risk of developing an intraventricular haemorrhage in the first week of life (121, 128). One study has demonstrated a potential role of standard clotting tests in the first week of life for the prediction of adverse neurological outcome in infants with neonatal encephalopathy (129).

The disadvantages of PT and APTT are that they merely measure the initiation of coagulation. They do not describe clot formation, maximum thrombin generation or the fibrinolytic system (130). Moreover, standard clotting tests do not evaluate for hypercoagulability in neonates (131).

The use of gestational age appropriate reference ranges for standard clotting tests in preterm infants is essential, given the prolongation seen (119, 121, 128). However, given the limitations to the information provided by these tests, and their inability to predict risk of haemorrhage, their use is limited in the preterm neonatal population (131).

### 1.10.2 Calibrated Automated Thrombography (CAT)

CAT is a global assay of coagulation first described by Hemker *et al.* in 2002 (132). It measures the amount of thrombin produced in a sample, in real-time, following the incubation of plasma with a thrombin specific fluorogenic substrate. CAT has the advantage of measuring both the pro- and anti-coagulant pathways, particularly important in neonates given that factor levels in both pathways are reduced (118, 120, 122).

CAT may be performed in platelet-poor plasma (PPP) or PRP. CAT in PRP allows evaluation of the effect of platelets on thrombin generation. In adult PRP, thrombin generation is dependent on platelet count when the latter is less than 100 x 10^9/L but there is no relationship between platelet count and thrombin generation parameters with platelet counts above 100 x 10^9/L, suggesting that while there may be a minimum platelet count required for effective thrombin generation, the absolute count is not a major determinant (133, 134).

Plasma is incubated with the appropriate reagent, containing TF and phospholipid (PPP reagent), TF only (PRP reagent) or phospholipid only (Microparticle reagent) (132). To initiate the reaction, calcium and the fluorescent substrate are added to plasma. A fluorimeter measures the fluorescence produced by cleavage of the thrombin substrate and this is converted to a thrombin concentration.

A Thrombogram is produced with the key parameters described in *Figure 1.2*. These include lag time (time from the beginning of the experiment until 10nM of thrombin is produced (135)), peak thrombin generation (maximum amount of thrombin produced), time to peak thrombin and endogenous thrombin potential (ETP) (total amount of thrombin produced during the coagulation process; area under the thrombin generation curve). It has previously been shown that ETP is the parameter most predictive of the risk of thrombosis or bleeding (136). Features suggestive of a hypercoagulable state include a shortened lag time and time to peak in conjunction with an increase in ETP/peak thrombin, while a prolonged lag time and time to peak in addition to a reduction in ETP/peak thrombin are suggestive of a hypocoagulable state (136).

CAT has been used to demonstrate increased thrombin generation in hypercoagulable states such as pregnancy (137) and venous thromboembolism (138). Several studies have demonstrated reduced ETP in patients with bleeding

disorders such as Factor VIII and Factor IX deficiency and von Willebrand's disease, most pronounced in those with severe disease although the association between depressed ETP and clinical risk of haemorrhage is less clear (139-141).

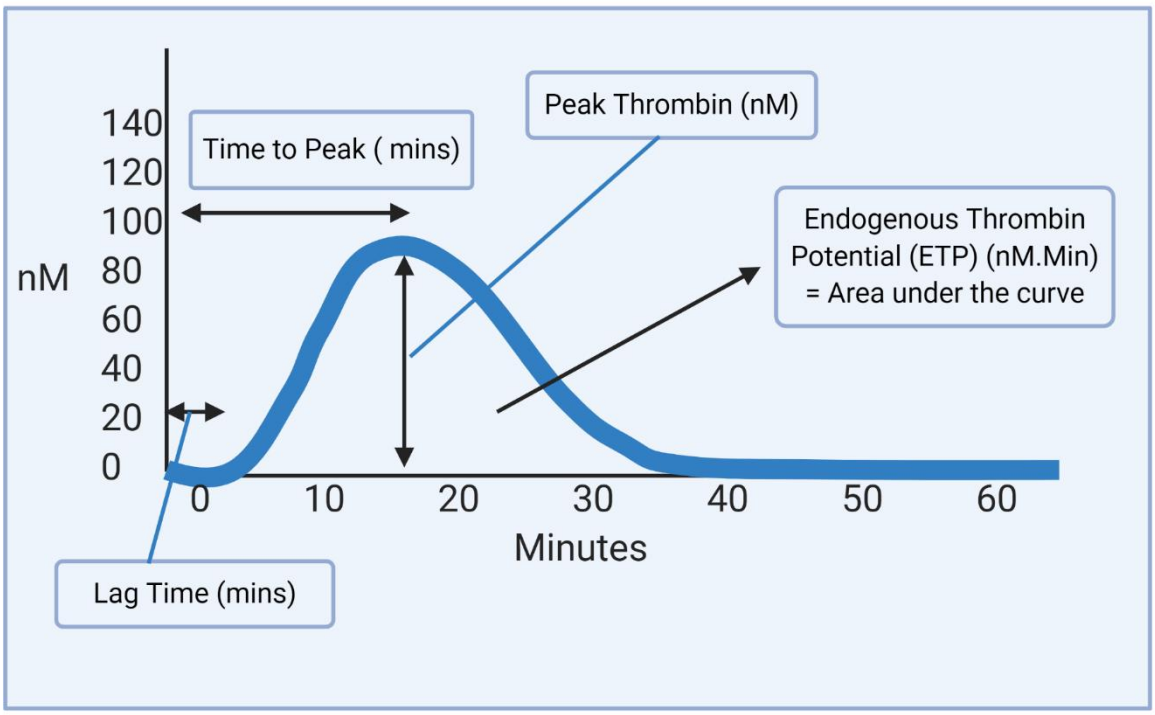


**Figure 1.2: A standard thrombin generation curve, depicting the lag time, time to peak thrombin, endogenous thrombin potential and peak thrombin**

Image created with BioRender.com and first published in the European Journal of Paediatrics (142)

### 1.10.3 Thromboelastography

Thromboelastography (TEG) (and Rotational thromboelastometry (ROTEM)) are viscoelastic techniques used to assess haemostatic function (143). TEG is a global assay of the coagulation system, measuring both primary and secondary haemostasis, in addition to fibrinolysis (144). This test is performed in whole blood which allows the assessment of all the cellular components of the blood on coagulation. TEG measures the viscoelastic properties of clot formation. It is performed by the addition of whole blood to an oscillating cup with a vertical pin. The rotation of the pin is transduced and a graphical representation of clot formation and degradation is produced.

TEG reference ranges have been developed for both healthy term and preterm infants (145-150). Interestingly, no significant differences were found in TEG parameters between healthy term and preterm infants, except in clot lysis parameters, which were enhanced in preterm infants (147, 150).

The advantages of TEG include the small blood volume required for testing (340 µl) and the use of whole blood which reduces the time and sample manipulation required for the preparation of plasma in other assays (144). TEG can be performed at the bedside and allows assessment of the cellular component of blood and the fibrinolytic system. Finally, heparinase is used in some studies, to extinguish the effect of heparin, often present in samples collected from heparinised arterial lines (147).

There are some limitations to TEG. It does not evaluate the role of the endothelium in coagulation and it is not possible to evaluate individual components of the coagulation system. Finally, the use of TEG in neonates has been limited by the lack of treatment algorithms to allow targeted rational prescribing of blood products, but these are beginning to emerge (151). The results of some of the TEG studies will be further discussed in our *Chapter 6: Discussion*.

## 1.11 The role of CAT in neonatal studies

This section (*Section 1.11*) pertaining to the current knowledge derived from the use of CAT in the neonatal population, was published in the *European Journal of Paediatrics* in 2021 (142).

### 1.11.1 Haemostasis in healthy term neonates

Six studies used CAT to characterise thrombin generation in PPP from term newborns compared with adults (152-157). All demonstrated a significantly shortened lag time and time to peak in neonates. However, neonates had significantly reduced ETP and peak thrombin compared with adults, although one study showed no difference (155). Similar findings were described in neonatal platelet rich plasma (158).

It is hypothesised that this reduction in lag time and time to peak in neonates (suggestive of a hypercoagulable state) is due to reduced TFPI, and that the reduction in ETP and peak thrombin (suggestive of a hypocoagulable state) is due to lower levels of pro-coagulant factors (particularly factor II) (156, 159), which itself might be further offset by a reduction in physiological anti-coagulant factors such as antithrombin (120).

### 1.11.2 Haemostasis in the preterm infant

Preterm infants are at high risk of haemorrhage and thrombosis (160, 161) and have reduced levels of procoagulant (FIX, FXI, FXII and fibrinogen) and anti-coagulant factors (Antithrombin, Protein C and Protein S) compared with term neonates (119). Three studies evaluated CAT in PPP from preterm compared with term infants. In preterm infants > 30 weeks gestation, ETP was higher than term controls (122). Neary *et al.* demonstrated a significantly shorter lag time and time to peak in umbilical cord blood in preterm infants (24-30 weeks gestation), but found no difference in ETP or peak thrombin between groups (121).

Most recently, Tripodi *et al.* characterised thrombin generation in peripheral blood in VLBW infants <1500g. VLBW infants had higher ETP than term controls (126). However, infants < 30 weeks gestation had significantly lower ETP than infants > 30

weeks (unfortunately, no comparison to term ETP was provided). There was no difference in ETP between small for gestational age (SGA) and appropriately grown infants. These findings in SGA infants, replicate findings by Sokou *et al.* using TEG, an alternative global coagulation assay (162).

Using thrombomodulin (TM), a key regulator of the protein C pathway, ETP-TM ratio was higher in preterm infants, suggesting a resistance to Protein C and thus a procoagulant imbalance in preterm plasma (126). Interestingly, the presence of a procoagulant imbalance in preterm plasma may predispose to IVH, possibly due to an increased risk of venous infarction and venous haemorrhage. This hypothesis is supported by data describing increased IVH risk associated with hereditary thrombophilia (30). Moreover, a study using TEG, demonstrated a trend towards hypercoagulability in premature infants with IVH, compared to those without (163). While these findings are not conclusive, they highlight the inability of standard clotting tests (PT/APTT) to accurately reflect the true complexity of haemostatic balance *in vivo*.

### 1.11.3 Evaluating the effect of neonatal platelets

CAT in PRP is performed using a reagent which contains tissue factor only (without a source of exogenous phospholipids). This renders the assay dependent upon the phospholipid content of PRP.

Haidl *et al.* compared thrombin generation in PRP from term cord blood and adults (158). In neonatal PRP, there were no differences in any thrombin generation parameters at platelet counts of 10,000/µL and 100,000/µL, suggesting that neonatal thrombin generation is not dependent on absolute platelet number. In contrast, thrombin generation in adult PRP is dependent on platelet count (133, 139). CAT was evaluated in TFPI-depleted adult PPP, following the addition of high or low concentrations of TFPI, and varying concentrations of platelets (158). Lower levels of TFPI (to represent neonatal plasma) were associated with lower platelet dependency of thrombin generation. Reduced TFPI activity has been reported in neonates, although endogenous TFPI activity levels in cord blood and adult samples were not described in the same studies and would have been useful to confirm the hypothesis (159, 164).

The respective effects of neonatal and adult platelets on thrombin generation were evaluated by CAT following the addition of platelets (neonatal/adult) to PPP (neonatal/adult) (165). Newborn and adult platelets supported thrombin generation comparably. This suggests that CAT parameters were primarily determined by the plasma present (neonatal/adult). These results differ from a similar study using TEG, which found that the "transfusion" of neonatal platelets resulted in a shorter reaction time in both neonatal and adult blood, while the "transfusion" of adult platelets to cord blood resulted in a greater maximal amplitude and clot firmness, compared to neonatal platelets (116).

Schlagenhauf *et al.* demonstrated that upon stimulation, neonatal platelets release fewer inorganic polyphosphates, a pro-coagulant substance released from the dense granules of activated platelets (155). Using CAT, exogenous polyphosphates had a lower relative impact on thrombin generation parameters in neonatal PPP, but exerted their maximal effect at lower concentrations than in adults. Lower TFPI levels rendered neonates more sensitive to the effect of polyphosphate, while limiting its potential impact.

Different PRP preparation techniques were used in the platelet studies, which may explain some variability in the findings. Haidl *et al*. centrifuged whole blood at 200 xg for ten minutes and diluted this with PPP to produce specific platelet counts (10, 50, 75 and 100 x 10^9/L) (158). Peterson *et al.* centrifuged whole blood at 100 xg for ten minutes, before diluting with PPP to achieve a standard platelet count of 50 x 10^9/L (166). Bernhard *et al.* pelleted and washed platelets, before re-suspending them in PPP and adjusting "to similar counts" (165). The International Society on Thrombosis and Haemostasis recommends centrifugation at 200 xg for ten minutes with no brake to produce PRP (167). These recommendations, to reduce red cell contamination and maintain platelet quiescence, derive from an adult study (168). In CAT, PPP is often added to PRP to standardise platelet counts. The study by Haidl *et al.* suggests that in term neonates, PRP platelet counts do not influence thrombin generation parameters (158).

To date, no studies have evaluated the effect of premature platelets on neonatal haemostasis.

### 1.11.4 The effect of extracellular vesicles on haemostasis

Extracellular vesicles (EVs) are nanoparticles (ranging from 50-1000 nm) released from cells (169), surrounded by a lipid bi-layer, and will be discussed further in *Chapter 2: Section 2.1.* EVs may play a role in haemostasis, increasing the phospholipid surface for the enzymatic reactions of the coagulation cascade and potentially increasing the local concentration of TF present (170). Tissue Factor extracellular vesicles (TF-EVs) originate from many cells, including endothelial cells and monocytes (171, 172). Several studies have demonstrated an increase in the number of platelet derived EVs and procoagulant EV activity in neonates compared with adults (164, 173-177).

CAT was used to evaluate the procoagulant effect of EVs in term cord blood compared with adults (154). CAT was performed using *Thrombinoscope BV* PPP reagent (tissue factor and phospholipid) and Microparticle (MP) reagent (phospholipid only). The MP:PPP ratio was used to evaluate the relative effect of TF-EVs on thrombin generation. TF- EVs had a greater impact on thrombin generation in neonates than adults. This increased procoagulant EV activity supports the possible compensatory role of EVs in the neonatal haemostatic system.

### 1.11.5 CAT as a predictor of clinical bleeding in neonates

Numerous studies have evaluated CAT as a predictor of bleeding in adults (141, 178), but few have in neonates. Peterson *et al.* found that CAT, did not predict post-operative bleeding after cardiopulmonary bypass (CPB) (166). Tripodi *et al.* found no difference in ETP measurements at birth between VLBW infants that developed an IVH and those that did not (126). Similarly, Neary *et al.* demonstrated no difference in any thrombin generation parameters between infants who developed a severe (or any) IVH and those that did not (179). The current clinical application of CAT in neonates is limited by a lack of evidence to support CAT as a predictor of bleeding.

### 1.11.6 CAT to evaluate haemostatic therapies in neonates

Sick neonates frequently receive blood products and haemostatic drugs, but few randomised controlled trials have evaluated their use in this population (103, 180). Neonatal doses are frequently extrapolated from adult regimens and guidelines are often consensus agreements. PlaNeT-2 has raised awareness of the potential harms of blood products in neonates (103). Haemostatic drugs must be evaluated in neonates, given the differences in neonatal factor levels (119, 120). CAT has been used as a pre-clinical tool to evaluate the potential haemostatic effects of drugs in neonates.

Cvirn *et al.* evaluated the anti-coagulant effect of Melagatran, a direct thrombin inhibitor, in neonatal cord blood and adult PPP (153). While a similar concentration of Melagatran was required to prolong the lag time and time to peak in both groups, both ETP and peak thrombin were suppressed by over 50% using a much lower drug concentration in cord blood plasma than that required to achieve the same effect in adult plasma. These distinct patterns of sensitivity to the same anticoagulant drug highlight the variability in endogenous haemostatic pathway activity which exists between neonatal and adult plasma, detectable by CAT.

CAT assessed the effect of *ex vivo* addition of NovoSeven® (recombinant factor VIIa [rFVIIa]) or three-factor prothrombin complex (3f-PCC) (containing FII, FIX, FX and a small amount of FVII) to PPP of term infants post CPB (181). While NovoSeven® reduced the lag time only, 3f-PCC also significantly increased peak thrombin and velocity index, above pre-CPB levels.

Franklin *et al.* studied the effect of two “four factor prothrombin complex concentrates” (4f-PCC), one which contained FVII and the other FVIIa, in PPP from term infants who had undergone CPB (142, 152). While both concentrations increased the peak thrombin and velocity index, only the preparation containing FVIIa reduced lag time to pre-CPB levels. The lower dose of both drugs tested was sufficient to enhance thrombin generation in neonates.

The effect of NovoSeven® in umbilical cord blood and adult PRP was investigated (158), due to the high incidence of reported thrombotic adverse events in the neonatal population (182). NovoSeven® altered clot dynamics, it did not alter ETP in either group, but shortened the lag time and reduced the peak height in both groups, most significantly in the neonatal group. The effect of rFVIIa did not appear to be

platelet dependent *in vitro*. Moreover, the dose response to rFVIIa was comparable between the neonates and adult PRP.
CAT cannot replace trials to evaluate the clinical effects of these drugs, but it may provide some insights into the relative effects of haemostatic therapies in supporting normal coagulation, at least *in vitro*.

### 1.11.7 CAT in specific populations

#### *1.11.7.1 Infants undergoing cardio-pulmonary bypass*

Infants who require neonatal surgical correction of cardiac malformations with CPB are at high risk of post-operative haemorrhage, due to a dilution of coagulation factors, exposure to heparin anti-coagulation and activation of blood cells as a result of interactions with extravascular tissue and artificial tubing (183). Neonates typically receive blood products and pro-coagulant drugs to overcome these challenges.
Two studies evaluated thrombin generation in neonates pre- and post-CPB (following the reversal of heparin and the administration of blood products) (152, 181). Both demonstrated a prolonged lag time post-CPB and an increase in peak thrombin compared with pre-CPB samples.
Peterson *et al.* evaluated CAT in PRP compared with other coagulation assays, assessing heparin reversal and rebound effect, in neonates undergoing CPB (166). CAT results were compared with Thrombin initiated Fibrin Clot Kinetics (TFCK). Peak thrombin inversely correlated with high TFCK ratios (blood samples with the highest heparin activity).

#### *1.11.7.2 Factor VIII deficiency*

Neonates with factor VIII deficiency can develop severe haemorrhage (184). CAT evaluated the effect of factor VIII levels in neonates in cord blood PPP, with varying levels of Factor VIII, Antithrombin and TFPI (157).
Factor VIII-depleted neonatal plasma showed a slight prolongation in lag time and time to peak, with no change in peak thrombin. In a neonate with confirmed factor VIII deficiency, the lag time and time to peak were slightly prolonged, however, the peak thrombin was reduced by 25%.

An increase in TFPI levels resulted in a prolonged lag time and time to peak, but had little effect on peak height or ETP. In contrast, an increase in the Antithrombin level, resulted in a large reduction in ETP (64%) and peak height (33%) but no change to the lag time. This study illustrated the mechanisms by which the inhibitory pathways impact on thrombin generation parameters in neonates.

#### *1.11.7.3 Cholestatic liver disease*

Preterm infants are at risk of cholestasis due to prolonged parenteral nutrition use. Cholestasis reduces vitamin K absorption and thus, vitamin K dependent coagulation factors. It was hypothesised that infants with intestinal failure associated liver disease may have impaired thrombin generation (185). CAT was performed in PPP at birth and day 30 in the presence of thrombomodulin. In spite of prolonged standard clotting tests in the liver disease group, there was no difference in ETP between groups at either timepoint.

## 1.12 Research aims

The first aim of the EVENT study (Extracellular Vesicles in Early preterm Neonates and Thrombin generation Study) was to compare thrombin generation in neonatal platelet rich plasma between preterm and term infants, and to evaluate the effect of platelets on neonatal thrombin generation.

# Chapter 2 : Introduction II: Extracellular vesicles in neonates

## 2.1 Introduction to extracellular vesicles

This section pertaining to the introduction to extracellular vesicles (EVs) and the current knowledge of EVs in neonates was published in *Pediatric Research* in 2020 (186) and has been updated to include recently published studies.

EVs are mediators of physiological and pathological processes, biomarkers of disease and therapeutic targets (187-189). EVs are nanoparticles surrounded by a lipid bilayer, which are released from cells but cannot replicate (169). The very first description of EVs was by Wolf in 1967, who noted that there were tiny particles in plasma that displayed features of platelets and described them as “platelet dust” (190). Subsequently, in 1983, two different groups described the process by which extracellular vesicles are released, following the identification of transferrin receptor recycling in reticulocytes (191, 192).

EVs contain proteins, lipids and miRNA (193). The biological roles of EVs vary depending on their cell or membrane of origin (194). Platelet-derived EVs (PDEVs) were initially thought to account for more than 70% of plasma EVs (94) but this has been revised down to less than half of all EVs (195), and play an important role in haemostasis by increasing both the phospholipid surface available for secondary haemostasis and the amount of tissue factor (TF) present in the environment (170). EVs also have a role in pathological processes, including inflammation and tumour metastasis (196, 197).

EV profiles have been used as biomarkers of disease. For example, in tumours such as glioblastoma, a diagnostic EV profile is detectable in the cerebrospinal fluid (198). Moreover, there have been advances in the use of EVs as potential therapeutic targets, particularly in pathologies of prematurity such as bronchopulmonary dysplasia (BPD) (199). Numerous studies have demonstrated potential efficacy of Mesenchymal stem cell EVs in the treatment of necrotising NEC and BPD in pre-clinical models of disease (200-202). In addition, EVs from microglia (rich in the microRNA miR-24-3p) have been shown to attenuate ROP in a mouse model (203).

For the purposes of clarity, the terms small EVs (SEVs) and large EVs (LEVs) are used in this review, although frequently referred to as exosomes and microparticles respectively in the literature. SEVs are isolated by ultracentrifugation at 100,000 xg and are generally less than 100-150nm in size, while LEVs are isolated at 20,000 xg and measure up to 1000 nm (169, 204). SEVs are predominantly derived from inward blebbing of multivesicular bodies, and participate in intercellular communication (205). LEVs typically derive from the plasma membrane and play a role in inflammation and coagulation (206).

The International Society of Extracellular Vesicles (ISEV) has published position statements on the “Minimal Information for Studies of Extracellular Vesicles” (MISEV). These guidelines describe the minimal recommended information for EV studies, with the aim of improving the reliability and reproducibility of the results of EV studies (169, 207, 208). These guidelines recommend detailed reporting of the collection, separation and storage methods used (169, 207). Moreover, MISEV recommends the characterisation of EVs using global EV markers and at least two single EV characterisation techniques (208).

Several techniques are available to characterise EVs. Nanoparticle tracking analysis (NTA) uses Brownian motion to calculate the size and concentration of SEVs (of less than 300 nm, but it can visualise particles up to 1000 nm) (209). The fluorescent mode of NTA utilises fluorophores (Quantum Dots) attached to antibodies to fluorescently label SEVs, allowing determination of the EV phenotype (209). Flow cytometry is used to measure LEVs in the range 300 to 1000 nm (210). Individual EVs pass a laser beam, which produces light scatter used to calculate the size, and fluorescent parameters to detect the cellular origin (211). This is performed using fluorescently conjugated antibodies, e.g. CD144 (endothelial-derived EVs (212)) and CD41/CD42b/CD61( PDEVs (213)). Dynamic light scattering (DLS) is a technique which measures the size of particles (1 nm to 6 µm) in a solution using Brownian motion but does not give information regarding the concentration or the origin of the EVs (214). Transmission Electron Microscopy (TEM) and Immunoblotting are also methods used to confirm the presence of EVs within a sample (208).

Several reviews have discussed the possible therapeutic applications of EVs in neonatology (199, 215-217). However, there is a paucity of information regarding circulating EVs in the neonatal period. In this review, I will describe the current

knowledge of EVs released into the bodily fluids of neonates in the first month of life and the clinical implications of what is known to date.

## *2.2* The role of EVs in neonatal vascular biology

### 2.2.1 Platelet EVs and procoagulant EVs

Ten studies evaluated the role of PDEVs or procoagulant activity of neonatal EVs. The first used flow cytometry to evaluate large PDEVs in umbilical cord blood (UCB) from preterm compared with term infants and adults (173). After activation there was a significant increase in PDEVs (CD42b) in both preterm and term infants.
The procoagulant activity of the EVs was measured using a novel method of flow cytometry-detected binding of Fluorescein isothiocyanate (FITC)-labelled factor V/Va to the surface of PDEVs (GPIb positive). At baseline, there was no significant difference in the procoagulant effect, however after stimulation there was a significant reduction in the procoagulant effect of EVs in the preterm group. The reduction in procoagulant effect was corrected by the addition of adult plasma or factor V (173). This suggested a plasma/factor deficiency as the cause for the reduced procoagulant effect.
Schmugge *et al.* determined the percentage of PDEVs in both UCB and neonatal samples in healthy term infants (174). Using flow cytometry (CD41), a higher proportion of PDEVs was reported in both UCB and neonatal samples compared with adults. It was found that neonatal platelets displayed greater platelet activation than adult platelets at baseline, but it was hypothesised that this may have in part been due to difficulties in sampling neonatal blood.
In 2008, Wasiluk *et al.* described the number of PDEVs in preterm and term UCB samples using flow cytometry (CD61) (175). A significant increase in the number of PDEVs in the preterm infants was demonstrated. Moreover, the number of PDEVs was not dependant on the number of platelets present.
Most recently, O'Reilly *et al.* described the EVs released in preterm infants on Day of life (DOL) one and three (218). While there was an increase in the total number of both SEVs and LEVs between DOL 1 and 3, demonstrated using both NTA and flow cytometry, there was a reduction in the number of PDEVs (CD41) over the same time. This is suggestive of an early platelet activation event following preterm delivery. Unfortunately, these findings were not investigated in term controls. It is

therefore unclear if the changes seen are due to prematurity or normal adaption to extrauterine life.

Hujacova *et al.* demonstrated similar counts of PDEVs in preterm and term cord samples, but reported reduced levels of CD36 and CD62 fluorescence intensity (but not CD41) on preterm EVs, suggestive of a lower concentration of these glycoproteins on the PDEVs (219). The same group also demonstrated no correlation between gestational age and PDEV number (220).

Over the last decade five studies have evaluated the procoagulant activity of neonatal EVs. Schweintzger *et al.* characterised EV procoagulant activity in term UCB samples compared with adults (154). Using flow cytometry, no significant difference was found in the total EV number (Annexin V positive) between the groups. Moreover, this study used two techniques to evaluate the functional EV procoagulant activity. The enzyme-linked immunosorbent assay (ELISA) (XYMUPHEN-MP activity kit) measured the procoagulant EV phospholipid content in plasma, but Computer automated Thrombography (CAT) was also used (154, 164), which is a global assay of coagulation that uses a fluorogenic substrate to measure thrombin generation in plasma (132). Both ELISA and CAT, demonstrated significantly increased procoagulant activity of the neonatal EVs.

An alternative ELISA (ACTICHROME Microparticle activity kit) was used to evaluate the procoagulant activity of EVs in UCB of healthy term infants compared with maternal blood and healthy non-pregnant females (164). Again, the procoagulant effect of EVs was higher in UCB. However, this study only performed a functional analysis of EVs and did not characterise EVs using any other technique, as recommended by MISEV (208).

Karlaftis *et al.* investigated the procoagulant activity of EVs in healthy term infants on DOL 1 and 3, comparing them with older children and adults (221). This study used the “STA- Procoag phospholipid kit”. In this assay, patient plasma is added to phospholipid depleted human plasma and the phospholipid content of the EVs impacts thrombin generation (221). Reduced procoagulant activity were demonstrated in neonatal samples on DOL 1 compared with older children and adults. Similar to the last study (164), only one functional technique was used to evaluate EVs. Unlike the ELISAs previously described, this assay does not pre-select the EVs by Annexin V binding.

In 2015, Campello *et al.* described the EVs released in UCB in infants born to mothers with- and without pre-eclampsia (PET) (176). The procoagulant activity of UCB EVs was assessed using the STA-ProCoag phospholipid kit. There was a higher procoagulant activity in the PET group, compared with healthy controls. Moreover, this study also used flow cytometry to characterise the EVs and found the proportion of large PDEVs (CD 61) was significantly higher in the PET group. Finally, Korbal *et al.* described the number of tissue factor extracellular vesicles (TF-EVs) between preterm and term UCB samples (177). An ELISA (XYMUPHEN-MP TF kit) was used to demonstrate a marginally increased TF-EV content in preterm infants. Again, only one functional technique was used. Moreover, none of the three studies (164, 177, 221) measured the total EV concentration, thus it is not clear whether the number or procoagulant activity of the circulating EVs was altered.

### 2.2.2 Endothelial EVs

Haemolytic disease of the newborn (HDN) is a serious condition whereby an infant's red cells are haemolysed by maternal antibodies, resulting in anaemia, hyperbilirubinemia and kernicterus if left untreated. Awad *et al.* used flow cytometry to detect CD144 positive large endothelial-derived EVs (EEVs) in neonates with ABO HDN compared with infants with Rhesus HDN and controls without HDN (212). It was hypothesised that ABO mediated haemolysis would result in endothelial dysfunction due to the presence of the A and B antigens on endothelial cells. A significant increase in EEVs in HDN compared with term controls was found, and infants with ABO HDN had significantly higher levels of EEVs compared to Rhesus HDN. Although this study highlights a potential pathophysiological mechanism of ABO HDN mediated endothelial injury, only one EV characterisation method was used and there was no clinical evaluation of endothelial dysfunction. However, these findings were replicated by Zhu *et al.* in Chinese neonates with ABO-mediated HDN, which supports these findings (222).

Vítková *et al.* described the release of EEVs as a marker of endothelial injury in infants undergoing extracorporeal membrane oxygenation (ECMO) (223). Flow cytometry was used to detect large EEVs in patients receiving ECMO compared with healthy term infants. A significant increase in the total number of LEVs was found in ECMO patients. There was a trend towards increased EEV markers (CD105, CD 31,

CD 309) and a significant increase in EVs positive for mucosal vascular addressin cell adhesion molecule 1 (MADCAM1) in ECMO patients compared with controls. The heterogeneity of clinical indications for ECMO in this study may have confounded the results. Moreover, in patients receiving ECMO, the identification of EVs released in response to interaction with the ECMO circuit as distinct from disease progression can be challenging, and was discussed as a limitation of this study. An increased production of EVs in response to ECMO circuits has been shown in animal models (224).

Hujacova *et al.* demonstrated no difference in EEVs (CD31/CD105) between preterm and term infants in cord samples, and no correlation with gestational age.

Most recently, Sibikova *et al.* investigated the effect of labour on the release of EEVs in cord and neonatal blood (225). Samples were collected from infants born by pre-labour caesarean section and compared to those born by spontaneous vaginal delivery (SVD). Several EEVs (PECAM [Platelet endothelial cell adhesion molecule], ICAM-1 [Intercellular adhesion molecule 1] and EPCR [Endothelial protein C receptor]) were elevated in the group born by SVD in cord samples, however these differences were no longer present on postnatal samples on DOL 3. This demonstrates the importance of considering the method of delivery, and likely the mechanism of onset of labour, when evaluating EVs in cord samples. Moreover, further studies to evaluate the effect of mode of delivery on neonatal endothelium would be useful to develop our understanding of conditions such as transient tachypnoea of the newborn, which more commonly affect babies born by elective caesarean section.

### 2.3 The role of EVs in neonatal respiratory disease

Chronic lung disease (CLD) (or BPD) is a clinical condition defined as the ongoing requirement for respiratory support at 36 weeks corrected gestational age (CGA). It is a serious complication of prematurity, causing respiratory and neurodevelopmental morbidity (226).

In 2018, Lal *et al.* described the role of EVs in neonates with BPD (227). Firstly, NTA was used to demonstrate that tracheal aspirates (TA) from infants with severe BPD (ventilated at 36 weeks CGA) had a reduced modal EV size (65 v 105nm) and higher particle concentration of SEVs than controls. Using EV-depletion techniques, it was

inferred that 63% of the SEVs were derived from epithelial cells (MUC 4-transmembrane mucin receptor family (human)). Subsequently, in a prospective cohort of extremely preterm infants, TA samples were taken within 6 hours of delivery and the infants were divided into BPD-susceptible and BPD-resistant groups based on the outcome of BPD at 36 weeks. 40 differentially expressed micro Ribonucleic acid (miRNA or miR) were identified and in the validation cohort, low miR 876-3p was identified as the most sensitive predictor of severe BPD in early TA. Following the identification of miR 876-3p, a significant reduction of EV miR 876-3p was confirmed in the 36-week TA of infants with severe BPD compared with controls . In addition, epithelial cell culture experiments and a mouse model of BPD were both used to demonstrate that both hyperoxia and lipopolysaccharide (LPS) exposure reduce EV miR 876-3p. Finally, a gain of miR 876-3p (through the intranasal administration of mimic miR 876-3p) in the mouse models, exposed to both hyperoxia and hyperoxia/LPS, resulted in reduced alveolar hypoplasia compared with mice without the miR gain. Through a robust multi-process methodology, this study successfully identified a possible underlying pathological mechanism of BPD, a biomarker of BPD and a therapeutic target.

Go *et al.* compared serum from preterm infants who developed CLD with preterm infants who did not (228). Samples were collected from UCB and neonatal blood at DOL 28 and 36 weeks CGA. At DOL 28, a significant increase in miR-21 was found compared with miR-21 levels at birth in infants with clinical CLD. Using a mouse model, increased miR-21 was also found in lung tissue after exposure to hyperoxia. Thus, a serum EV biomarker of CLD was described. There was a low rate of prenatal steroid administration (59-74%) in this study, which may limit the generalisation of the findings.

Fabietti *et al.* evaluated EV profiles in fetuses with congenital diaphragmatic hernias, undergoing the FETO procedure (fetoscopic endotracheal obstruction) to identify markers of poor postnatal survival, a common complication despite good lung volume growth, usually ascribed to pulmonary arterial hypertension (229). EVs were evaluated in amniotic fluid collected at the time of balloon insertion, and tracheal aspirates at the time of balloon extraction. Using NTA, a significant increase in the number of EVs was identified in both fluids in non-survivors. There were also increases in white cell and epithelial EVs, and of four differentially expressed miRNA

known to target genes involved in lung development, in tracheal aspirates of infants who did not survive.

### 2.4 The role of EVs in neonatal neurology

In 2014, Tietje *et al.* described the variation of CSF EVs with increasing age (230). Using NTA, no difference in the size of SEVs between age groups was identified. However, the number of SEVs in the CSF of children <2 years was significantly increased compared with teenagers and adults. Several differentially expressed miRNAs between the youngest and oldest groups were also identified. However, the indication for lumbar puncture in the youngest group was not clear. Without this information, confounding factors, such as febrile illness, may be missed (230). Therapeutic hypothermia (TH) is a treatment which reduces the risk of death or disability in infants with moderate to severe neonatal encephalopathy (NE) (231). Goetzl *et al.* described the release of neural SEVs using ELISA in the peripheral blood of term infants undergoing TH at 8, 10 and 14 hours after the initiation of treatment (232). It was shown that a decremental changes in synaptopodin levels, a cytoskeletal protein and mediator of synaptic plasticity, were significantly associated with a longer length of hospital stay (LOS), greater need for anti-epileptics and worse diffusion weighted imaging summary scores. Both NTA and ELISA were used to characterise EVs. Although this study did not describe long-term outcomes, it identifies a potential biomarker of short-term outcomes in infants undergoing TH.
In 2019, Spaull *et al.* analysed the CSF EVs of preterm neonates with post-haemorrhagic hydrocephalus (PHH) (233). PHH is a progressive dilatation of the ventricles which can occur after an intraventricular haemorrhage, and which is associated with a high risk of neuro-disability (22). NTA showed heterogenous size and concentration of SEVs between patients was shown. While two patients displayed a similar modal size of EV, all patients displayed similar concentrations of EVs within the 30-100nm size. One infant had serial CSF analysis and there was a decrease in the particle concentration over time, with a corresponding increase in particle size. Although multiple techniques were used to characterise EVs, only three patients were included and only one had serial samples. Moreover, there was no correlation to the clinical outcomes.

The final study used UCB EVs to evaluate brain health in infants at risk of congenital iron deficiency (234). Contactin 2 (CNTN-2) and Brain derived neurotrophic factor (BDNF) were used as markers of brain health. It was hypothesised that risk factors for congenital iron deficiency would result in lower levels of CNTN-2 and BDNF. ELISA was used to measure EV CNTN-2 and BDNF levels in UCB EVs and the results were compared to cord ferritin levels, the marker of congenital iron deficiency used. It was shown that low levels of EV CNTN-2 and high levels of EV BDNF were associated with low ferritin levels and thus markers of congenital brain iron deficiency. While this is a novel method of assessing possible brain iron deficiency, it was not possible to definitively measure the brain iron stores in this study. As with the last two studies (232, 233), the inclusion of developmental outcomes would have strengthened the case for these two as EV biomarkers.

## 2.5 The role of EVs in prenatal and perinatal disease

### 2.5.1 EVs in pre-eclampsia

PET occurs in 3% of pregnancies and can be life-threatening (235). It is responsible for 20% of preterm deliveries <1500g and infants born to mothers with PET have a higher risk of intra-uterine growth restriction (IUGR) and perinatal mortality (236-238).

As previously discussed, Campello *et al.* described the EVs released in UCB in infants born to mothers with and without PET (176). Using flow cytometry, a significant increase in the total LEV count (Annexin V), PDEVs (CD61), activated platelet-derived (CD62P), leukocyte-derived (CD45), EEVs (CD62E) and TF-EVs (CD142) was shown in the PET group, compared with healthy controls.

Jia *et al.* described the proteomic content of UCB EVs in pregnancies complicated by PET versus healthy controls (239). NTA showed a higher concentration of SEVs in the PET group (statistical significance not described). This supports the findings in the previous study (176), although flow cytometry and NTA measure particles of different sizes. The proteomic analysis identified a differential expression in 29 proteins, and the pathways most associated with the PET group were the complement and coagulation pathways.

In 2020, Xueya *et al.* described the differential EV miRNA expression in UCB between infants born to mothers with PET and without (240). Following EV miRNA

analysis, 25 differentially expressed miRNA were identified, including miR 125a-5p which was increased in the PET group. The relative increased expression of miR 125a-5p was also demonstrated in maternal peripheral blood and placental tissue in the PET group. Using cell culture techniques, the authors demonstrated that miR125a-5p may inhibit angiogenesis by regulating VEGFA (vascular endothelial growth factor A) and may be involved in the progression of PET.
It is important to note that these studies were designed to assess the pathophysiological mechanisms and maternal outcomes of pre-eclampsia and not the neonatal clinical outcomes. In each case, the controls were not gestational-age matched (176, 239, 240). The infants with PET were born at an earlier gestation, thus prematurity may confound the findings.

### 2.5.2 EVs in prematurity

Extremely preterm infants (born less than 28 weeks gestation)(241) are at high risk of neonatal death, and long term physical disability and neurodevelopmental impairment (242). Bruschi *et al.* described the effect of polyunsaturated fatty acid (PUFA) supplementation to mothers with threatened preterm labour (243). The EVs in UCB of the treated group (n=10) were compared with term infants (n=12) and untreated preterm infants (n=10). Using mass spectrometry analysis, glutathione synthetase (GSS) was identified as the most discriminating marker between the groups. An ELISA of GSS showed the levels were highest in the untreated preterm group, followed by treated preterm infants and then term infants. Moreover, higher levels of protein oxidation were demonstrated in the untreated preterm group. Antenatal treatment with PUFA may ameliorate some of the biochemical oxidative changes in preterm blood EVs and reduce inflammation. The clinical outcomes of the infants or safety data for the treatment were not described in this study.

### 2.5.3 EVs in intrauterine growth restriction

IUGR is defined as “a fetus with an estimated fetal weight < 10th percentile that, because of a pathologic process, has not attained its biologically determined growth potential” (244). IUGR is associated with an increased risk of intrauterine death, intrapartum asphyxia, and poorer long term neuro-development (245). Miranda *et al.* investigated the role of UCB EV markers in infants with IUGR compared with healthy

controls (matched for gestational age) (246). Using fluorescence NTA with Quantum dots bound to CD63 (EV marker) and placental-type alkaline phosphatase (PLAP), no difference was found in the total number or placental SEVs in UCB. However, the percentage of placental SEVs in UCB was significantly lower in IUGR babies, and correlated with the severity of the growth restriction. Similar findings were shown in the maternal blood samples at the time of delivery, and thus identified the proportion of placental EVs in maternal blood as a potential diagnostic marker of IUGR. However, these findings would need to be replicated in maternal samples earlier in pregnancy and in larger numbers to become a useful clinical marker.

### 2.6 Other roles of EVs in neonatology

Haematopoietic stem cells (HSCs) are multipotent cells which generate all of the cellular blood components (247, 248). In 2019, Xagorari *et al*. described the presence of HSC-LEVs (CD 34) using flow cytometry in UCB of healthy term infants (249). Moreover, haematopoiesis-specific miRNA were identified in both CD34 positive cells and CD34 positive LEVs.

One study compared EV miRNA between UCB from healthy term infants and adults (250). Using NTA, no difference was found in the size or concentration of SEVs. MiRNA sequencing identified that the 30 most abundant miRNAs were similar between groups. 65 differentially expressed miRNA were identified and following functional analysis using KEGG (Kyoto Encyclopaedia of Genes and Genomes) and GO (Gene Ontology) pathways, the differentially expressed miRNAs were shown to be involved in pregnancy and reproduction, cell mobility, biogenesis of exosomes and nervous system pathways. Moreover, the authors showed that miRNA in UCB exosomes were very similar to the miRNA identified in UCB plasma identified in another study, reinforcing miRNA enrichment of SEVs (251).

Mar *et al.* investigated the role of urinary EVs as biomarkers in necrotising enterocolitis (NEC) (252). Urinary EVs miRNA demonstrated differential expression between preterm infants with NEC, preterm infants with sepsis, and healthy preterm infants. Further work in this area would be useful to identify biomarkers able to distinguish between NEC and sepsis, which can be a challenging neonatal issue.

Wang *et al.* described EV miRNA related to lactogenesis, the process by which breast milk is produced, in UCB in healthy term infants (253). Sixty-nine lactation

related miRNA were identified in UCB LEVs. Moreover, the application of these EVs to epithelial mammary cells increased the production of b-casein, an important component of human breast milk. However, the lactation related miRNA were not investigated in the maternal circulation during the peripartum period. This would have provided further insight into the regulation of human lactogenesis as the placenta is expulsed very shortly after delivery.
Finally, Keller *et al.* demonstrated the presence of urinary SEVs during fetal life (amniotic fluid at 16 weeks gestation) and in neonatal and adult urine samples and the preservation of CD24 urinary EVs across species, detected here in a mouse model (254).

### 2.7 Changes in EVs with age

Two recent studies have explored the variation of EVs with age. Penas-Martinez *et al.* compared EV profiles between full-term neonates and adults (255). Neonatal EVs, measured in cord blood, had 65% less protein, smaller EVs measured by TEM and 131 differentially expressed proteins compared to adults. The upregulated neonatal proteins were predominantly linked to haemostasis (vWF, alpha 2 macroglobulin and Factor VIII) and granule secretion, while the downregulated proteins were immunoglobulins. The differential expression of haemostatic proteins in neonatal EVs is unsurprising, and well supported by the literature as discussed in *Chapter 1: Section 1.9*. Ohta *et al.* evaluated changes in EVs, measured in discarded serum, in twenty preterm infants (>1500g) over the first twelve months of life (256). The concentration of EVs increased from birth to 48 weeks of age, and the concentration of EVs at birth showed a moderate correlation with infant gestational age. However, there were limitations to this study as only one technique was used to evaluate the EVs, an exosome quantitation kit, and the EVs were measured in serum, where the process used to generate serum may sequester EVs.

### 2.8 Research aims

The second aim of the EVENT study was to describe the circulating EVs in very preterm infants, over the first two weeks of age and to compare these to healthy full-term infants.

# Chapter 3 : Materials and Methods (EVENT Study)

## 3.1 Ethical approval

Ethical approval was sought and granted by the Rotunda Research Ethics Committee for recruitment of preterm infants and healthy full-term control groups to the EVENT Study (REC-2019-012).

Subsequent amendments to the ethical approval were sought and granted to allow inclusion of outborn preterm infants, to allow collection of a full blood count in the healthy term controls and to increase the number of full term infants from 50 to 100.

Ethical approval was also sought from, and granted, by the Coombe Women and Infants University Hospital (Study No. 23 – 2020). However, it was ultimately not possible to recruit patients at this site due to several reasons including COVID-19 (Coronavirus Disease 2019) restrictions, a Cyber-attack on the Irish healthcare system, logistical challenges recruiting across two sites and subsequent commencement of another coagulation study in preterm infants at that site.

## 3.2 Study design

### 3.2.1 Sample size

No other studies to date have evaluated platelet-dependent thrombin generation in preterm infants. From my review of the literature on neonatal thrombin generation to date, most studies included between 10 and 30 participants, although some included up to 87 participants, from the population of interest (142).

The EV aspect of this study was novel and there is very limited data evaluating the changes in EVs during the perinatal adaption period in preterm or term infants. A literature review on the current knowledge of EVs in the neonatal population was performed (186). Most studies included between 10 and 50 neonatal participants, with 79 participants in the largest study (173, 175, 234).

The EVENT study aimed to recruit 100 very premature infants and 50 term controls (subsequently increased to 100) to examine thrombin generation and the EV profiles in very premature babies.

Comparable work performed by our group on platelet-poor plasma in a similar cohort recruited 116 infants and used 205 blood samples in order to allow for subgroup

analysis at different gestational ages (121). Based on this data, I estimated that a cohort of 100 very premature infants would provide statistically significant data on platelet-rich plasma thrombin generation and perinatal changes in EVs, and was feasible to recruit over a two-year period at the Rotunda Hospital (approximately 100-120 VLBW infants per year).

### 3.2.2 Inclusion criteria

Preterm infants born between 24+0 weeks and 30+6 weeks gestation were eligible for inclusion in this study. Healthy full term controls, infants born between 37+0 weeks and 42+0 weeks gestation without underlying anomalies, were eligible for inclusion.

### 3.2.3 Exclusion criteria

Infants with a known antenatal brain haemorrhage or with a family history of coagulopathy were excluded.

### 3.2.4 Consent

In the EVENT Study, ethical approval was obtained to collect umbilical cord blood samples at delivery as well as postnatal research samples at the same time as clinical blood sampling on Day 1, Day 3 and at two weeks of age. In addition a point-of-care cranial ultrasound was performed on Day 1 (preterm only) and a review of clinical records (in all cases).
In the preterm cohort, parents were approached -antenatally where possible- to discuss the study, answer any questions they had and provide them with a Patient Information Leaflet. Following a period of time, to allow them to discuss the study themselves, the investigator returned to seek consent. If they wished to participate, the parents and the investigator signed the consent form. If time did not allow for antenatal consent, parents were approached in the postnatal period, allowing for maternal wellbeing, and the same procedure was followed. In the preterm group, where precipitous delivery did not allow antenatal consent, an umbilical cord blood sample could be stored pending informed parental consent after delivery. If parental

consent was not obtained, the sample was discarded. Prior informed parental consent was required for any postnatal neonatal research samples.
In the term controls, a cohort of parents were approached prior to delivery to seek consent to collect an umbilical cord blood sample, and a postnatal research sample only if their infant was having clinically indicated blood tests. A second cohort of term infants were approached prior to a postnatal clinically-indicated blood test being collected, to seek consent to take a postnatal research sample at the same time. Written informed consent was obtained from all parents for their infant to participate in this study. All neonatal research samples were taken at the same time as clinically indicated blood tests. If an infant did not have a clinical peripheral blood test at a particular time point (e.g. Day 3), no research samples were collected for that time point.

### 3.3 Cranial ultrasound

A cranial ultrasound is a non-invasive imaging technique, which uses sound waves to produce an image and obtains images through the anterior fontanelle in neonates. Standard images were taken in the coronal and sagittal planes to identify intraventricular haemorrhages. A cranial ultrasound was performed on infants in the preterm cohort, within the first 24 hours of life, to evaluate for an early intraventricular haemorrhage (Nobulus, Hitachi, Tokyo, Japan). Where an abnormality was detected on cranial ultrasound, it was reported to the treating clinician. Subsequent cranial ultrasounds were performed as per hospital protocol at the following time points: Day 1 – Day 3, Day 7- Day 10, Day 28 and 34- 36 weeks corrected gestational age or before discharge. Cranial ultrasounds were not performed in the term infants enrolled in this study.

### 3.4 Illness severity scores

Illness severity scores were designed to facilitate comparisons between neonatal units, taking into consideration the severity of illness in neonates treated in that unit. They also have limited roles in predicting individual outcomes and the risk of mortality (257).
There are several illness severity scores available. SNAP II (score for neonatal acute physiology) and SNAP-PE II (includes perinatal risk factors) represents the mortality

risk of physiological derangements (258). Only six physiological variables were used to calculate SNAP II and the collection was limited to the first twelve hours of life to minimise treatment bias. The perinatal risk factors included birth weight, small for gestational age and Apgar <7 at five minutes. The SNAP II tool has been used in many neonatal clinical trials and has recently been re-validated for predicting mortality in a modern Canadian cohort of extremely preterm infants (259). The SNAP II and SNAP-PE II scores were calculated for all preterm infants where data was available.

### 3.5 Data collection

Maternal and neonatal data were recorded from the electronic patient record "Maternal and Newborn Clinical Management System" (MN-CMS) (Cerner, Missouri, United States). Maternal demographics, antenatal history, birth history and neonatal outcomes were included. The definitions of pathologies of prematurity were as defined by the Vermont Oxford Network (VON) (260). Pseudoanonymised data was inputted into Microsoft Excel, using a unique study number as an identifier (see below).

### 3.6 Data management

Data management was compliant with General Data Protection Regulations (GDPR). The primary investigator completed GDPR and Good Clinical Practice (GCP) training.

All patients enrolled were given a unique study identifier, in order of recruitment, in order to code the data. The keycode used to identify patients was kept on an encrypted computer in a locked hospital office. Only the study team accessed patient data. Data was kept on a password protected Microsoft Excel file, only accessible to study team members, and it was stored on a password-protected computer in a locked hospital office. The processed data will be retained for the duration of the study and anonymized data for up to 5 years afterwards, in keeping with hospital policy. This retention is to allow the investigators respond to any queries regarding the study.

Coded blood samples were transferred from the Rotunda to the UCD Conway SPHERE laboratory. The code to identify these samples remained in the Rotunda.

## 3.7 Blood sampling

### 3.7.1 Umbilical cord blood

Umbilical cord blood (UCB) was obtained following delayed cord clamping. Samples were generally taken following placental expulsion. However, in a small number of cases, clinical samples were indicated while the placenta was *in situ*, and so research samples were collected at the same time. A 10 ml syringe and a 21-gauge needle were used to sample umbilical cord blood (ideally 6 ml). Cord blood was transferred to a 3 ml sodium citrate coagulation tube (or 0.5 ml or 1.3 ml tube if only small volumes were available) and gently inverted to mix. Both umbilical venous and arterial blood could be collected but venous blood was more commonly used given the larger volumes available.

The Umbifunnel ® (Key plastics, Wicklow, Ireland) was also trialled for UCB samples. However, given the small volumes of cord blood available in preterm infants, it often resulted in underfilled 3 ml coagulation tubes or clotting within the Umbifunnel® itself. In the term group, where larger volumes of cord blood were present, it was difficult to deliver exactly the correct amount of blood into the coagulation bottle, which is necessary for coagulation studies.

For this reason, the standard technique of needle puncture was the chosen method of umbilical cord collection for this study.

### 3.7.2 Neonatal blood samples

Neonatal blood samples were only collected at the same time as clinical samples. Experienced neonatal nurses and doctors performed phlebotomy and the aseptic non-touch technique was used. Blood samples were not taken from heparinised arterial lines and samples were hand-delivered directly to the laboratory.

The research samples were collected in citrate coagulation microsample tubes (sodium citrate 3.2%) of 0.5 ml or 1.3 ml size (Sarstedt, Numbrecht, Germany, Catalogue number 41.1506.002, 41.1506.005) and gently inverted after collection.

In the preterm group, neonatal blood samples were collected on Day 1 (1.3 ml, 0-24 hours), Day 3 (0.5 ml, 48-72 hours) and at two weeks of age (0.5 ml, Day 14 – Day 21). A smaller 0.5 ml sample could be collected instead on Day 1 where phlebotomy was challenging. Samples were not collected after an infant had reached a corrected

gestational age of 31+0 weeks, and irrespective of postnatal age, if an infant was not having a blood test performed for a clinical indication on that day or if an infant had received a blood product (e.g. platelets, red cells, plasma).
In the term control group, neonatal samples (1.3 ml) were collected at any time over the first 96 hour of age if consent had been obtained and an infant was having a blood test performed for a clinical indication e.g. measurement of serum bilirubin. While I initially aimed to recruit term infants on Day 1 (0-24 hours) and Day 3 (48-72 hours), most healthy term infants have bloods performed on Day 2 so a pragmatic decision was made to expand the time range for sampling to 0-96 hours.

### 3.8 Full blood counts

All preterm infants had full blood counts (FBCs) performed as part of their routine clinical care. A cohort of the term controls had FBCs performed as part of this study to evaluate their platelet counts. This was predominantly limited to infants undergoing cord sampling and was halted in May 2021 when a massive Cyber-attack caused significant disruption to work in clinical hospital laboratories across Ireland.

### 3.9 Plasma preparation

Both platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were produced from UCB and Day 1 samples, while only PPP was made from Day 3 and two week samples.
All samples were processed by the lead investigator as soon as possible after collection and within four hours. Firstly, each sample was manually tested for evidence of clot formation. Any samples with evidence of clot formation were discarded.
PRP was generated by centrifugation (Centrifuge 5810 R, Eppendorf, Stevenage, United Kingdom) of the sample at 200 xg for 10 minutes with no brake at 21 ˚C. PPP was generated by double centrifugation at 3000 rpm for 6 minutes with full brake. Where possible, the platelet count in PRP was measured. Where only small volumes of PRP were available, this was done by diluting 20 µl of PRP in 180 µl of Phosphate Buffered Saline (PBS) and performing an optical measurement to evaluate platelet count using the Cell-Dyn Ruby Hematology Analyzer ( Abbott, Illinois, United States).

The value was then multiplied by the dilution factor (10) to obtain the PRP platelet count. This technique was validated in PRP using umbilical cord plasma.

### 3.10 Sample storage and transfer

PPP was aliquoted into vials, typically containing 50 to 100 µl and stored at -80 ˚C in the Rotunda Hospital. Samples were then transferred in batches on dry ice from the Rotunda Hospital to the UCD Conway Institute.

### 3.11 Calibrated Automated Thrombography (CAT)

#### 3.11.1 Thrombin generation in preterm and term infants

This technique measures the ability of plasma to generate thrombin, using a fluorogenic thrombin substrate and is described in more detail in *Chapter 1: Section 1.10.2 and Figure 3.1*. Performing CAT in PRP evaluates the role of platelets in thrombin generation, as the assay is dependent upon the phospholipid content of the PRP. In the EVENT Study, CAT was carried out in PRP from UCB and postnatal neonatal samples (Day 1 only in the preterm group).

PRP was prepared immediately after sampling, as described above, and all CAT run within two hours of initial sample collection. Thrombin generation reagents; “PRP-reagent” (contains 1 pM tissue factor (TF) and “Thrombin Calibrator” (Thrombinoscope BV, Stago, Asnieres sur Seine, France)) were reconstituted by the addition of 1 ml deionised water and allowed to stand for 10 minutes before gently shaking. “Fluo Buffer” was warmed to 37˚C (AccuBlock Mini, Labnet International, USA (D0100-230V).

Samples were run in duplicate where volume allowed (two test wells and two calibration wells per patient) on a 96-well plate (Nunc TM, 96-well transparent U-bottom plates, 500 µl, ThermoFisher Scientific, Waltham, United States), although this was not possible in a small number of samples as insufficient volumes of plasma were obtained. Into each test well, 80 µl of PRP and 20 µl of “PRP reagent” (contains 1 pM TF) were added. Into each calibration well, 80 µl of PRP and 20 µl of “Thrombin Calibrator” were added.

The plate was incubated at 37˚C for 10 minutes. During this time, “Flu-Ca” (fluorogenic Z-Gly-Gly-Arg-AMC.HCl substrate and 100mM $CaCl_2$) was prepared. 40

µl of “Fluo-Substrate” was added to the heated “Fluo-Buffer” (1600 µl). The mixture was immediately vortexed (Vortex-Genie TM 2, Scientific Industries, New York, USA) and protected from light. 20 µl of the combined “Flu-Ca” solution (final concentrations, Z-Gly-Gly-Arg-AMC.HCl, 0.42 mM and $CaCl_2$, 16.67 mM) was added to each well to initiate the coagulation cascade.

Thrombin generation was measured over 60 minutes using the Fluoroskan Ascent Plate Reader (ThermoFisher Scientific, Waltham Massachusetts) and a thrombin generation curve was produced using Thrombinoscope BV software (Version 5.0, Stago, Asnieres sur Seine, France). The parameters measured include the lag time, time to peak thrombin generation, peak thrombin generation and endogenous thrombin potential (ETP). Data was exported to Microsoft Excel.

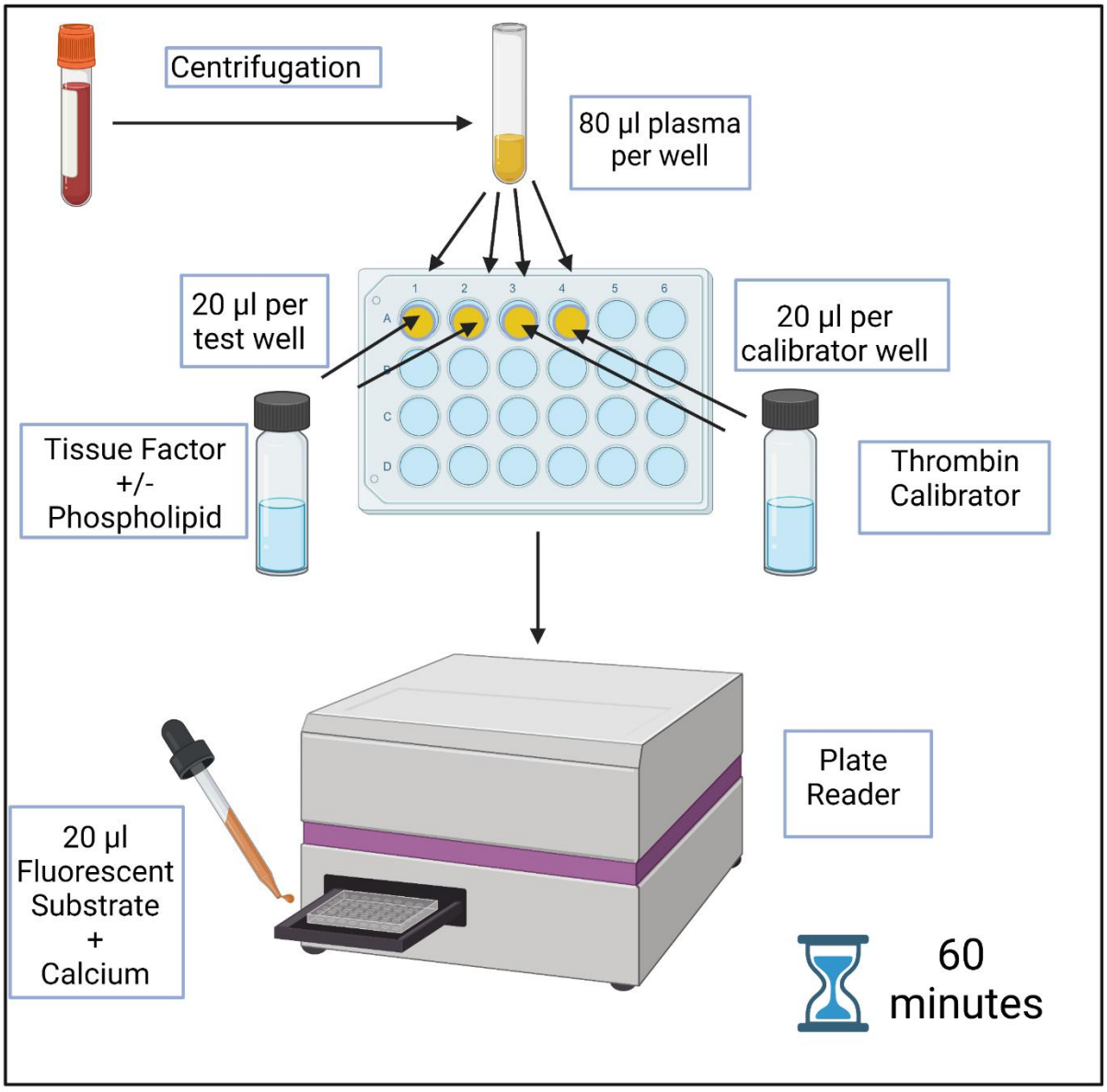


**Figure 3.1: The standard process for performing CAT in plasma**

Figure created with *BioRender.com* and first published in the *European Journal of Paediatrics* (142)

### 3.11.2 Manual injection

Between August 2020 and October 2020, the 1ml glass syringe in the injection apparatus of the Fluoroskan Ascent Plate Reader was out of order. During this time, manual injection of “Flu-Ca” was used to allow CAT to continue using the method described above. Following the 10-minute incubation period of the plate (containing plasma and reagent) at 37 ˚C, the plate was ejected and 20 µl of “Flu-Ca” was added to each well using a multi-channel pipette. The plate was then re-inserted into the Plate Reader and the analysis commenced. Only the peak thrombin and endogenous thrombin potential values were used from this epoch as lag time and time to peak thrombin were distorted by the additional injection time.

### 3.11.3 Contribution of platelets to neonatal thrombin generation

To evaluate the relative contribution of neonatal platelets to the differences seen in thrombin generation between preterm and term infants in PRP, CAT was performed in matched fresh PRP and PPP from UCB (both preterm and term when sufficient volumes were obtained) (*Figure 3.2*).

PRP and PPP were prepared as previously described in *Section 3.9* and the platelet count in PRP and PPP was measured using the Cell-Dyn Ruby Hematology Analyzer.

From each patient, 80 µl of PRP was added to two test wells and two calibrator wells and 80 µl of PPP was added to two test wells and two calibrator wells. 20 µl of “PRP reagent” (contains 1 pM TF) was added to each test well and 20 µl of “Thrombin Calibrator” to each calibrator well. The plate was incubated as before, prior to the automatic injection of 20 µl of “Flu-Ca” to each well.

The key CAT parameters from PRP and PPP were compared to evaluate the relative contribution of neonatal platelets to thrombin generation.

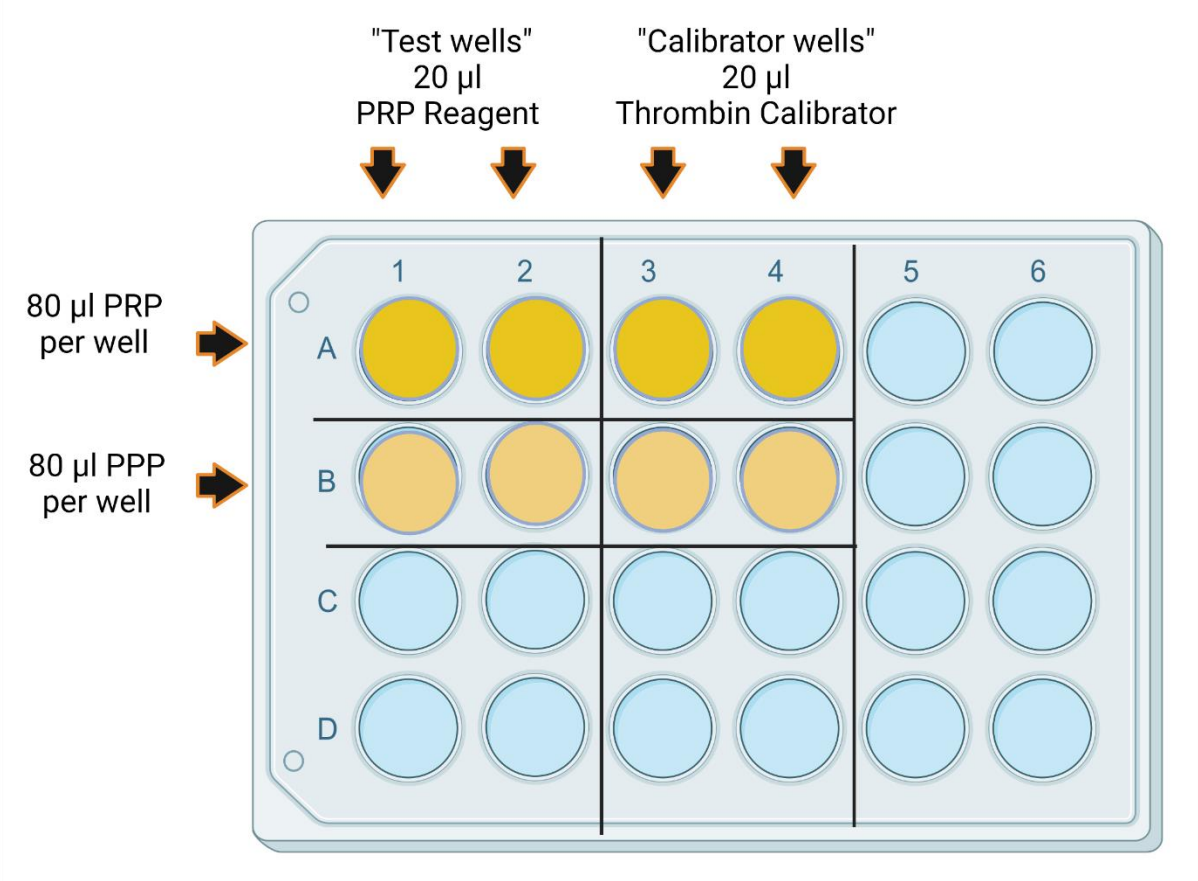


**Figure 3.2: Evaluation of CAT in paired PRP and PPP to evaluate the effects of neonatal platelets on thrombin generation**

Image created with *BioRender.com*

### 3.11.4 Role of Tissue Factor Pathway Inhibitor (TFPI) in neonatal thrombin generation

The differences seen between preterm and term infants in thrombin generation in PRP, were similar to that described by Neary *et al.* in PPP i.e. a shorter lag time and time to peak in preterm infants with similar ETP and peak thrombin (121). I aimed to evaluate the effect of TFPI in neonatal thrombin generation, particularly, whether reduced TFPI activity in preterm infants was responsible for the shorter lag time and time to peak thrombin seen in this group.

Thrombin generation in UCB PPP was evaluated using CAT in the presence and absence of a polyclonal TFPI antibody. This experiment was carried out in a subgroup of preterm and term infants who had CAT in UCB PRP performed and adequate volumes of frozen PPP remaining.

PPP was thawed by incubation at 37 ˚C for 10 minutes. CAT was performed in UCB PPP in the presence of PPP LOW reagent (contains 1 pM TF and 4 µM phospholipid). Eighty µl PPP was placed into each well (3 wells per patient). Into the first well, 20 µl PPP LOW reagent was added, into the second well, 20 µl PPP LOW and anti-TFPI antibody (Anti-human TFPI 100 µg/ml Haematologic Technologies, Vermont, United States, Product code PAHTFPI-S) and into the third well, 20 µl Thrombin Calibrator was added. Following a 10-minute incubation at 37 ˚C, thrombin generation was initiated by the automatic dispensing of 20 µl Flu-Ca and recorded for 60 minutes. Due to the limited volume of plasma remaining, samples were not analysed in duplicate.

The key thrombin generation parameters were compared between preterm and term infants in the presence and absence of TFPI.

### 3.11.5 Role of EVs in thrombin generation

To evaluate the potential role of EVs in neonatal haemostasis, “Microparticle (MP) Reagent” was used in a small cohort of patients in umbilical cord blood. “MP Reagent” (4 µM phospholipid) does not contain TF, and the initiation of thrombin generation is therefore dependent on the TF-EVs present in plasma.

CAT was conducted as previously described in *Section 3.11.1,* but adding 20 µl of “MP Reagent” to 80 µl of PPP in the test wells, and 20 µl of “Thrombin Calibrator” to 80 µl of PPP in the calibrator wells.

The limitation of this experiment is that the intrinsic pathway may eventually initiate thrombin generation, particularly in the absence of corn trypsin inhibitor, an inhibitor of the intrinsic pathway, which was not used in this experiment.

### 3.12 Evaluation of Tissue Factor Pathway Inhibitor (TFPI)

TFPI was characterised in preterm and term infants using two techniques, TFPI antigen levels and TFPI activity levels as described below.

#### 3.12.1 TFPI levels

TFPI levels were measured using R & D Quantikine ELISA (Human TFPI) (Minneapolis, United States, Catalogue number DTFP10). The standard and other reagents were made up as per the manufacturer's instructions.
After thawing at 37 ˚C, UCB PPP was diluted 1:50 with calibrator diluent (4 µl PPP and 196 µl calibrator diluent). A 1:100 dilution was recommended for plasma samples, but given the lower levels of TFPI expected in neonates compared to adults (155, 159, 261), a lower dilution factor was used. Samples were analysed in duplicate and according to the manufacturer's instructions.
The optical density of each well was measured at 450nm, within 30 minutes, on the Plate Reader (Clariostar Plus Microplate Reader, BMG Labtech, Aylesbury, United Kingdom). As wavelength correction was not available, optical density at 570nm was also recorded and subtracted from the 450nm reading to correct for optical imperfections in the plate. Duplicated values were averaged and the blank was subtracted from each value. Data analysis was performed by generating a 4-parametric logistic regression curve fit.

#### 3.12.2 TFPI activity

TFPI activity was measured using the Actichrome TFPI Activity Assay (BioMedica Diagnostics, Windsor, Canada, Reference 848). This assay measures the ability of TFPI to inhibit the catalytic activity of the FVIIa/TF complex to convert FX to FXa. To measure the residual activity of the FVIIa/TF complex, a chromogenic substrate of FXa is cleaved by FXa, releasing a p-nitroaniline chromophore, the absorbance of which is measured.
The reagents were prepared as recommended by the manufacturer's instructions. A solution of ethylenediaminetetraacetic acid (EDTA) was prepared by dissolving 240 mg of EDTA (trisodium dihydrate) in 10 ml deionised water and adjusting the pH to 9.9 with Sodium hydroxide.

PPP was thawed at 37 ˚C and diluted 1:10 with TFPI depleted plasma (10 µl PPP and 90 µl TFPI depleted plasma). None of the aliquots had previously been thawed. 20 µl of standard or sample were added to each well and samples were run in duplicate. 20 µl of TF/VIIa complex was added to each well and incubated at 37 ˚C for 30 minutes. 20 µl of Factor X was then added to each well and incubated for 15 minutes at 37 ˚C, followed by the addition of 20 µl of EDTA to each well.
20 µl of Spectrozyme FXa substrate was added to each well using a Multichannel pipette, with the same time interval between each row. After exactly 5 minutes, the reaction was stopped by the addition of 50 µl of glacial acetic acid, in the same order as the chromogenic substrate was added.
The absorbance was read at 405 nm using the Clariostar microplate reader (BMG Labtech, Aylesbury, United Kingdom). The standard curve was constructed using a polynomial regression fit curve and the TFPI activity was calculated using the PQ formula for solving quadratic equations. The activity values obtained were multiplied by 10 to correct for the dilution factor.

The PQ formula was used to solve the polynomial equation for x

$$x = \left(-\frac{p}{2}\right) \pm \sqrt{\left(\frac{p}{2}\right)^2 - q}$$

Where $x^2 + px + q = 0$.

Equation 3.1: The PQ formula for solving quadratic equations

### 3.13 Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to measure the size and concentration of nanoparticles in the range 0-200 nm in PPP. The Nanosight NS300 (Malvern Panalytical, Malvern, United Kingdom), configured with 488nm laser and a high sensitivity CMOS (complementary metal oxide semiconductor) camera, was used as previously described (262).

Frozen PPP was thawed and diluted in particle free PBS (Gibco, Walthem, United States). For UCB and Day 1 samples, an initial 1:50 dilution of was used, while 1:200 was used for Day 3 and Day 14 samples. Samples were further diluted as required to achieve 10 to 80 particles per frame.

15 x 60 second videos were captured for each sample to optimise the precision (263). Sample analysis was performed at a flow rate of 50 and temperature control was used (25 ˚C). The camera level was set to 13 and the detection threshold was set to 10. Nanosight NTA software Version 3.4 was used to analyse the particle count and concentration. Results were exported to Microsoft Excel.

### 3.14 Flow cytometry

Flow cytometry is an imaging technique which can be used to analyse cells or large EVs. Individual EVs in solution pass through multiple laser beams, producing light scatter (forward and side scatter) and fluorescent parameters. This information can then be used to determine the number, size and granularity of particles, and in conjunction with fluorescently labelled antibodies, the cellular origin of the LEVs (211, 264). Laser beams emitting different wavelengths are used to excite fluorophores which produces a fluorescence emission. This emitted fluorescence passes through a series of filters before being detected by photodetectors and converted to an electrical output. “Gates” are applied around populations of interest. There can be spectral overlap between certain fluorophores, and this needs to be compensated for during the analysis.

I performed flow cytometry at the Department of Pathological Physiology, Faculty of Medicine, Charles University, Prague, Czech Republic with assistance from Prof. Jan Zivny.

#### 3.14.1 Preparation of reagents

All reagents were prepared fresh daily.

Phosphate buffered saline (PBS) (Gibco PBS x 1, Calcium and Magnesium free, Life Technologies, Paisley, UK, Reference number 10010-023) was filtered with a 220nm filter (Spritzen syringe filter 0.22 µm TPP, Trasadingen, Switzerland, Catalogue number 99772), prior to use.

Annexin Binding Buffer (ABB) (10 x Annexin Binding Buffer, Exbio, Prague, Czech Republic) was also prepared daily. Two ml of ABB (10 X) was diluted with 18 ml of ultrapure water type 1 and then filtered through a 220 nm filter.

Two types of calibration beads were prepared and run daily. Apogee Mix Beads (Apogee Flow Systems, Hempstead UK, Catalogue number 1493) included size calibration beads (180, 243, 300, 590, 800, 1300 nm) and two fluorescent beads. The Apogee bead container was vortexed and 1 µl of bead solution was added to 300 µl of filtered ABB and vortexed before use.

Dako calibration beads were also used, which included 8 fluorophores of 3 µm size (Agilent, Santa Clara United States, Catalogue number K0112). The Dako beads

were vortexed and one drop of bead solution was added to 300 µl of filtered ABB and vortexed before use
All antibodies were purchased from BioLegend Europe (Amsterdam, The Netherlands) and stored at 4°C.

### 3.14.2 Isolation and staining of LEVs

Isolation and staining of LEVs was performed as described in *Figure 3.3*. Frozen vials of plasma were incubated at 37 ˚C for ten minutes. 100 µl of plasma from each patient was placed in a 1.5 ml Eppendorf tube. Plasma was centrifuged at 20,800 xg (14,000 rpm) for ten minutes at 10 ˚C to pellet large EVs ( Centrifuge 5810 R, Rotor FA-45-30-11, Eppendorf, Germany).
The supernatant was removed, being careful not to disturb the EV pellet at the bottom of the tube. The EV pellet was resuspended with 1 ml filtered PBS. This was centrifuged at 20,800g (14,000 rpm) for ten minutes at 10 ˚C to wash the EVs. The supernatant was removed, again being careful not to disturb the EV pellet.
450 µl filtered ABB was added to each vial and vortexed to disperse the EVs in the solution. 100 µl of this ABB/EV solution was placed in four Eppendorf tubes. Antibodies were added to each vial as described in *Table 3.1*.
For Tube 3 (Platelet markers) and Tube 4 (Endothelial/other markers), a “Master Mix” of antibodies was prepared immediately prior to addition to the vials to reduce pipetting error (*Table 3.1*). The volume of “Master Mix” prepared depended on the number of samples being tested, and was vortexed and covered in tinfoil. For Tube 2, Annexin V FITC was added directly to the EV solution.
The tubes were incubated in the dark at room temperature for exactly 20 minutes following addition of the antibody. After incubation, 300 µl of filtered ABB was added to each tube and the tubes were centrifuged at 20,800 xg (14,000 rpm) for ten minutes at 10 ˚C to remove unbound stain. The supernatant was removed and the stained EV pellet was resuspended in 200 µl of ABB, vortexed and placed in a polypropylene flow cytometry tube.

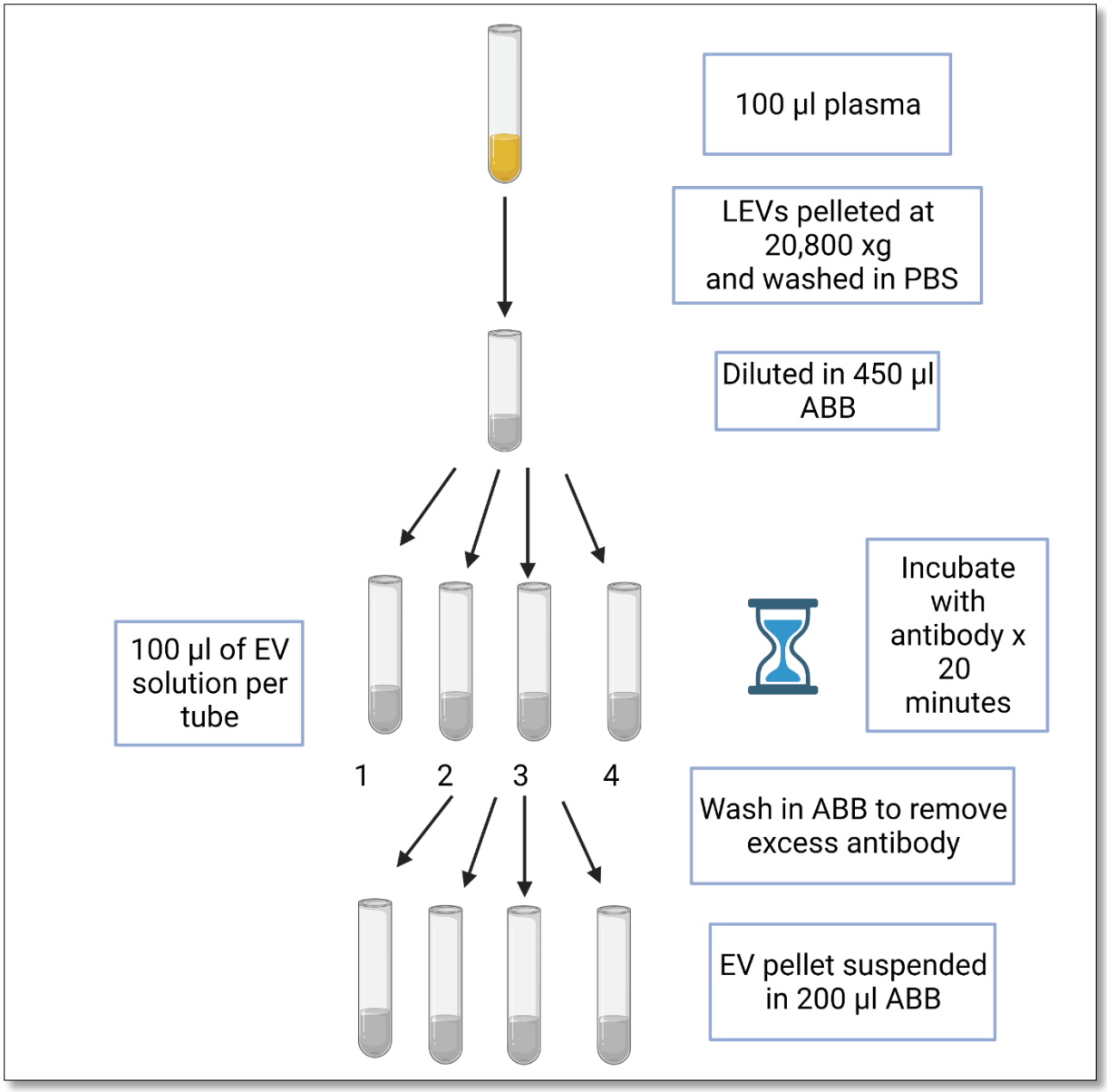


**Figure 3.3: Summary of the isolation and staining procedures of large EVs for flow cytometry.**

Image created with BioRender.com ABB- Annexin Binding Buffer, PBS; Phosphate Buffered Saline, LEV- Large extracellular vesicle

**Table 3.1: Summary of the antibodies added to each tube (1-4) for flow cytometry analysis**

FITC (Fluorescein isothiocyanate), PE (Phycoerythrin), PB (Pacific blue), APC (Allophyocyanin) and AF-700 (Alexa Fluor 700), VEGF (Vascular endothelial growth factor), PECAM (Platelet endothelial cell adhesion molecule), TF (Tissue factor)

| Tube | Label | Volume of EV solution | Antibody | Fluorophore | Cat no | Antibody volume |
|---|---|---|---|---|---|---|
| Tube 1 | Non-stained | 100 µl | None | | | |
| Tube 2 | Annexin V | 100 µl | Annexin V | FITC | 640945 | 1 µl |
| Tube 3 | Platelets | 100 µl | Annexin V | FITC | 640945 | 1 µl |
| | | | CD 62P | PE | 304906 | 5 µl |
| | | | CD 41 | PB | 303714 | 5 µl |
| | | | CD 42b | APC | 303912 | 5 µl |
| Tube 4 | Endothelial/Other | 100 µl | Annexin V | FITC | 640945 | 1 µl |
| | | | CD 309 (VEGF) | PE | 393004 | 5 µl |
| | | | CD 31 (PECAM) | PB | 303114 | 4 µl |
| | | | CD 142 (TF) | APC | 365206 | 5 µl |
| | | | CD 45 | AF-700 | 368514 | 5 µl |

### 3.14.3 Flow cytometry analysis

The BD FACS CANTO II flow cytometer (BD Biosciences, San Jose, California, United States) was used.

Before each use, the machine was cleaned and calibration beads were run followed by patient samples. The flow rate was set to medium flow (1 µl/s) and the analysis was recorded, after a 10 s delay, until 30,000 events or 180 s were recorded (whichever occurred first).

FACS Diva software (Version 6.1.3) (BD Biosciences, San Jose, California, United States) was used to analyse the data. Compensation was applied for the spectral overlap between fluorophores, particularly FITC and PE, and APC and AF-700 fluorophores and applied to all experiments. Gates were applied to include particles less than 1300 nm and the populations of interest and applied to all experiments (*Figure 3.4*).

To calculate the concentration of total LEVs and Annexin V positive events, the average of those gates in Tubes 2 to 4 were calculated.

The concentration of EVs was calculated per gate using the following formula:

$$Concentration\ of\ EVs\ in\ Gate\ 1\ (events/\mu l) = \frac{Gate\ 1\ events\ x\ Dilution\ Factor}{Time\ (s)} \quad (8.9)$$

**Equation 3.2: Calculation of the concentration of extracellular vesicles in individual gates**

Owing to the limitations of the flow cytometer used, it was not possible to determine the size of the EVs, merely the concentration and the fluorescence.

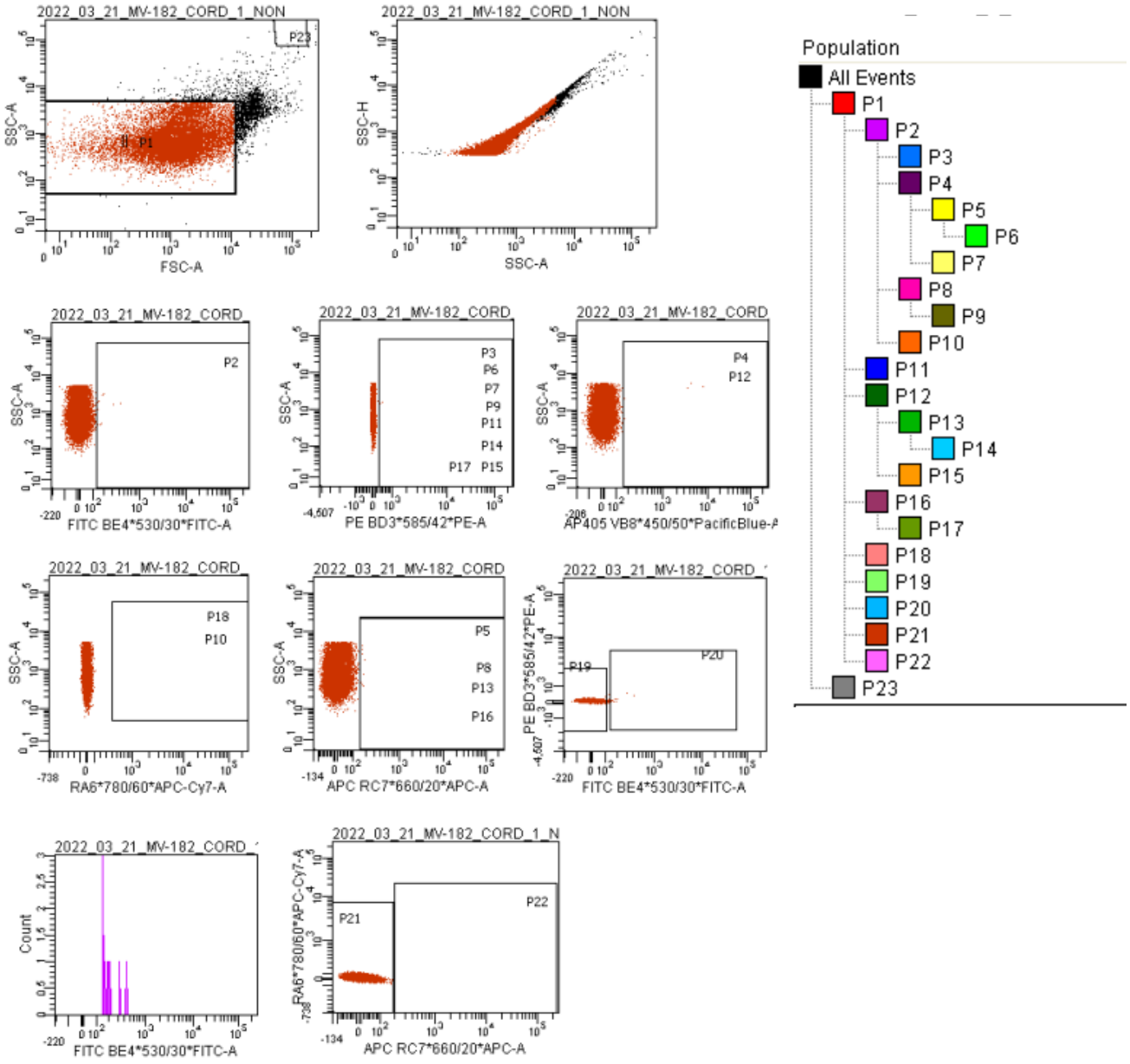


**Figure 3.4: Example of the gates applied for flow cytometry analysis**

### 3.15 Tissue factor extracellular vesicle procoagulant activity

The Zymuphen MP-TF kit (HYPHEN BioMed, Neuville-sur Orsy, France, Reference 521196), a functional assay for the measurement of TF- bearing microparticles procoagulant activity, was used. The principle of this assay is that the absorbance is directly proportional to the amount of TF-EVs in the sample. The plate is coated with a murine monoclonal antibody specific for human TF, to which the TF-EVs bind during an overnight incubation. After washing, Factor VIIa and Factor X are added, Factor VIIa binds TF, and in the presence of Calcium converts Factor X to Factor Xa, where the Tenase complex is dependent on the phospholipid surface of the EVs. Reagents were prepared as per the manufacturer's instructions.

Plasma, frozen at -80 ˚C, was thawed at 37 ˚C for ten minutes for this assay. The plasma was tested undiluted. Samples were run in duplicate.

The optical density of each well was measured at 405 nm on the Plate Reader (Clariostar Plus Microplate Reader, BMG Labtech, Aylesbury, United Kingdom). The blank value was subtracted from each value and duplicates were averaged. A linear calibration curve was generated in Microsoft Excel and the concentration of each sample was calculated from this.

Some samples had an optical density below that of the lowest standard (2.1 pg/ml). These "low" values were arbitrarily assigned a value of "1 pg/ml" and pseudo counts were randomly assigned (using Research Randomizer, www.randomizer.org, free online software) to each "low" value (e.g. 1.0001, 1.0002, 1.003 etc.).

### 3.16 Statistical analysis

Descriptive analysis was performed. To test for normality of continuous data, the Shapiro-Wilk test and histograms were used. Mean and standard deviation are described for normally distributed data, while median and interquartile ranges are used for non-parametric data. Frequency (percentages) are described for categorical data. Comparisons between the preterm and control groups were performed using Chi-squared test for categorical variables (or Fisher's exact test if n<5) and parametric T-test or non-parametric Mann Whitney U-test for continuous variables. Paired T-tests and Wilcoxin Sign Rank tests were used for paired parametric and non-parametric data, respectively. The Spearman Rank Test was used to evaluate correlation between non-parametric variables. Significance was assumed at two-sided $p<0.05$.

Stata (version 17.0) was primarily used for statistical analysis. However, R (version 3.6.3) was used for analysis and graphical representation of the raw NTA data. The analysis of changes in SEVs and LEVs during perinatal adaption, measured by NTA and flow cytometry, was performed on R with assistance from Dr. Stephen Madden, Lecturer in the Data Science Centre in RCSI. Pooled T-tests were used to compare the changes in EVs over time, owing to the mix of paired and unpaired samples at each timepoint (265). To adjust for multiple analysis, the Benjamini-Hochberg method was used (266).

# Chapter 4 : Results I: Neonatal Thrombin Generation

## 4.1 Introduction

Babies born preterm are at risk of haemorrhagic complications such as intraventricular haemorrhage (IVH) (16). The preterm haemostatic system differs from that of a healthy full-term infant or an adult (119, 120). Preterm infants have reduced levels of coagulation factors (119), hyporesponsive platelets (when tested *in vitro*) (105) and prolonged standard clotting tests (PT/APTT) (121). However, evidence is emerging from global coagulation assays to suggest that premature infants may be hypercoagulable compared to full term infants, due to an imbalance of procoagulant factors compared to anti-coagulant factors (126). Moreover, the effect of platelets on neonatal thrombin generation is poorly understood, particularly in preterm infants. The first aim of the EVENT Study was to characterise thrombin generation, using calibrated automated thrombography, in platelet rich plasma in preterm compared to full term infants. Secondly, I aimed to understand the mechanistic pathways which influence neonatal thrombin generation.

## 4.2 Results

### 4.2.1 Clinical demographics

101 preterm infants (born less than 31+ 0 weeks gestation) were recruited to the EVENT Study (*Figure 4.1*). A smaller group of healthy term infants were recruited as controls (n=66).

Among the parents of eligible preterm infants who were approached, 20 (16.5%) declined to participate, often concerned that their infant was too small or too sick to participate in research studies.

Of the preterm infants recruited, there was a high incidence of clotted and insufficient samples. The collection of umbilical cord blood (UCB) samples was challenging, particularly as delayed cord clamping is routine, in keeping with current evidence (267). Of the UCB samples attempted, 27 (48.2%) were successful, while 22 (39.3%) were clotted and 7 (12.5%) were insufficient. A high number of UCB samples (n = 45) were not attempted, owing to the unpredictable and often precipitous nature of preterm birth, highlighted by the proportion of preterm infants (66 [65.4%]), who were

delivered out of hours, and the dependency of the lead investigator on clinical staff to be informed of the delivery.

A high incidence of clotted postnatal samples was also noted in the preterm group, reflecting the challenges of phlebotomy in this cohort, even by experienced clinicians. On Day 1, 70 (83.3%) of attempted samples were successfully obtained, while on Day 3 only 32 (56.1%) were successful, and on Day 14 12 (85.7%) were successful. The use of a 0.5 ml or 1.3 ml sample collection tube did not influence sample success on Day 1 ($p= 1.0$) or Day 3 ($p=0.1$).

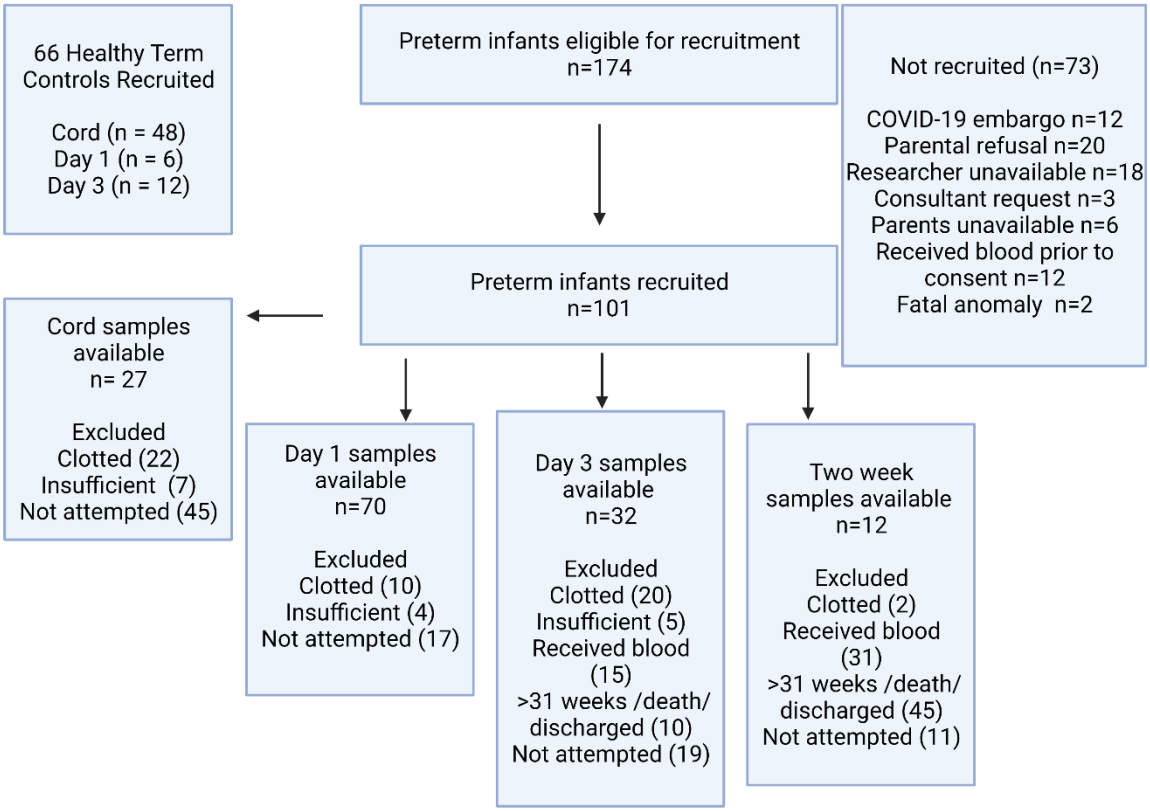


**Figure 4.1: Flow diagram of the infants recruited to the EVENT Study and the plasma samples obtained**

Image created with BioRender.com

The maternal, antenatal, birth and neonatal characteristics are described below (*Tables 4.1, 4.2, 4.3 and 4.4*).

Mothers in the control group were older (33.5 years v 31.6 years, $p$=0.04) and more likely to be Caucasian (92.4% v 77.1%, $p$=0.01) than in the preterm group (*Table 4.1*). However, a higher proportion of mothers in the preterm group smoked during pregnancy (25.3% v 6.1%, $p$<0.01). There was no difference in maternal blood group (ABO or Rhesus group) between the preterm and control groups.

**Table 4.1: Summary of the maternal demographics of infants recruited to the EVENT Study**

For categorical variables, where data was not available for each subject, the denominator is included for clarity

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [b] Unpaired T-test, [c]Fisher's exact test, [d]Chi square test

| Maternal Demographics | Preterm n= 83 | Term N=66 | *p*-value |
|---|---|---|---|
| Maternal Age, years | 31.6 (±6.6) | 33.5 (±4.7) | 0.04[b] |
| BMI, kg/m$^2$ | 27 [22.9 – 30.5] | 25.4 [23.01 – 28.1] | 0.2[a] |
| Gravida | 2 [1 – 3] | 2 [1 – 3] | 0.59[a] |
| Liveborn infants | 0 [0 – 2] | 1 [0 – 2] | 0.5[a] |
| Caucasian | 64 (77.1) | 61 (92.4) | 0.01[d] |
| Smoking | 19/75 (25.3) | 4 (6.1) | <0.01[c] |
| Maternal medication pre-pregnancy | 20 (24.1) | 18 (27.3) | 0.66[d] |
| Maternal Blood Group | | | |
| A | 28 (33.7) | 23 (34.9) | 0.96[c] |
| B | 12 (14.5) | 11 (16.7) | |
| O | 39 (47) | 30 (45.5) | |
| AB | 4 (4.8) | 2 (3) | |
| Maternal Rhesus Group | | | |
| Positive | 70 (84.3) | 56 (84.9) | 0.93 [d] |
| Negative | 13 (15.7) | 10 (15.2) | |

The incidence of assisted reproductive technology (ART) and maternal thrombocytopenia was similar between groups (*Table 4.2*). Unsurprisingly, the incidence of preeclampsia (27.7% v 4.6%, $p<0.01$) and preterm prelabour rupture of membranes (PPROM) (36.1% v 1.5%, $p<0.01$) was higher in the preterm group, as these are common causes of preterm birth.

**Table 4.2: Summary of the antenatal demographics of infants recruited to the EVENT Study.**

For categorical variables, where data was not available for each infant, the denominator is included for clarity

Mean (±SD), Median [IQR], Frequency (%), [c]Fisher's exact test, [d]Chi square test

| Antenatal Demographics | Preterm n=83 | Term n=66 | *p*-value |
|---|---|---|---|
| Assisted reproductive technology | 11/66 (16.7) | 8/59 (13.6) | 0.63[d] |
| PET/PIH requiring treatment | 23 (27.7) | 3 (4.6) | <0.01[c] |
| Gestational Diabetes | 11 (13.3) | 5 (7.6) | 0.27[d] |
| Antenatal steroids administered | 80 (96.4) | 8 (12.1) | <0.01[d] |
| Dose of Steroid: 1<br>2<br>3 | 10 (12.1)<br>69 (83.1)<br>1 (1.2) | | |
| MgSO4 | 77 (92.8) | | |
| PPROM | 30 (36.1) | 1 (1.5) | <0.01 [c] |
| GA at PPROM, weeks | 24.7 (±3.9) | | |
| Maternal thrombocytopenia | 13/81 (16.1) | 7 (10.6) | 0.34 [d] |

For obvious reasons, the gestational age and birth weight were lower, while the incidence of multiple pregnancy, low Apgar score at five minutes and chorioamnionitis was higher in the preterm group (*Table 4.3*).

**Table 4.3: Summary of the birth demographics of infants recruited to the EVENT Study**

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [c]Fisher's exact test, [d]Chi-squared test

| Birth Demographics | Preterm n=101 | Term n=66 | *p*-value |
|---|---|---|---|
| Gestational Age | 28.3 [26.4 – 29.9] | 39.29 [38.9 – 39.9] | <0.01 [a] |
| Birth Weight | 1105 [800 – 1390] | 3445 [3200 – 3830] | <0.01 [a] |
| Male | 56 (55.5) | 32 (48.5) | 0.38 [d] |
| Singleton | 61 (60.4) | 66(100) | <0.01 [c] |
| Multiple pregnancy | | | |
| Twin | 33 (32.7) | | |
| Triplet | 3 (3) | | |
| Quadruplet | 4 (4) | | |
| Duration of delayed cord clamping | 40 [30 – 60] | | |
| Onset of labour | | | |
| Spontaneous | 53 (52.5) | 10 (15.2) | <0.01 [c] |
| Induced | 0 (0) | 15 (22.7) | |
| Pre-labour | 48 (47.5) | 41 (62.1) | |
| Mode of delivery | | | |
| SVD | 25 (24.8) | 13 (19.7) | 0.48 [c] |
| OVD | 4 (4) | 5 (7.6) | |
| Caesarean section | 72 (71.3) | 48 (72.7) | |
| Arterial pH | 7.29 (±0.1) | | |
| APGAR at 5 minutes | 6 [5 – 8] | 9 [9 – 9] | <0.01 [a] |
| | | | |

| Birth Demographics | Preterm<br>n=101 | Term<br>n=66 | *p*-value |
|---|---|---|---|
| Surfactant in delivery suite | 41 (40.6) | | |
| Respiratory status on transfer to NICU | | | |
| Invasive ventilation | 44 (43.6) | | |
| Non-invasive ventilation | 55 (54.5) | | |
| Self-ventilating | 2 (2) | | |
| Clinical chorioamnionitis | 19 (18.8) | 1 (1.5) | <0.01 [c] |

The neonatal outcomes of the preterm group are described in *Table 4.4*. 40 infants (39.6%) had an intraventricular haemorrhage (IVH) and 10 (9.9%) were severe. 6 (5.9%) had a pulmonary haemorrhage and 7 infants (6.9%) died prior to discharge.

**Table 4.4: Summary of the postnatal outcomes of preterm infants recruited to the EVENT Study.**

For categorical variables, where data was not available for each infant, the denominator is included for clarity

Mean (±SD), Median [IQR], Frequency (%). SNAPPE ii; Score for Neonatal Acute Physiology with Perinatal Extension-II, CONS: Coagulase negative staphylococci, IVH: Intraventricular Haemorrhage, PVL: Periventricular leukomalacia, VEGF: Vascular endothelial growth factor, NEC: Necrotising enterocolitis, FIP: Focal intestinal perforation

| Clinical Outcome | Preterm n=101 |
|---|---|
| SNAPPE-ii Score | 23 [5 – 35] |
| Small for gestational age (<10$^{th}$ centile) | 7 (6.9) |
| Congenital anomaly | 13 (12.9) |
| Neonatal Sepsis | |
| Antibiotics administered Day 1 | 80 (79.2) |
| Early onset sepsis | 2 (2) |
| Late onset sepsis | 7/100 (7) |
| CONS infection | 8/100 (8) |
| Respiratory Distress Syndrome | 97 (96) |
| Surfactant | 75 (74.3) |
| Pneumothorax | 11 (10.9) |
| Invasive ventilation | 68 (67.3) |
| Chronic lung disease | 41/96 (42.7) |
| Any IVH | 40 (39.6) |

| Clinical Outcome | Preterm n=101 |
|---|---|
| Severe IVH (Grade 3 or 4) | 10 (9.9) |
| Post-haemorrhagic hydrocephalus | 5 (5) |
| Cystic PVL | 6 (5.9) |
| Pulmonary haemorrhage | 6 (5.9) |
| Retinopathy of prematurity | 26 (25.7) |
| Laser or anti-VEGF therapy | 7 (6.9) |
| Patent Ductus Arteriosus treatment | |
| Paracetamol | 14 (13.9) |
| Ibuprofen | 18 (17.8) |
| Device/surgical closure | 5 (5) |
| Necrotising enterocolitis (≥Bell's stage 2) | 9 (8.9) |
| Abdominal drain for NEC/FIP | 4 (5) |
| Laparotomy for NEC/FIP | 1 (1) |
| Neonatal thrombocytopenia | |
| Any (<150) | 46 (45.5) |
| Severe (<50) | 9 (8.9) |
| Any Red Cell Transfusion | 63 (62.9) |
| Any Platelet Transfusion | 7 (6.9) |
| Any Plasma Transfusion | 5 (5) |
| Neonatal Death | 7 (6.9) |

### 4.2.2 Thrombin generation in platelet rich plasma

Thrombin generation was carried out in PRP from both UCB (n=17 preterm, n=35 term) and postnatal blood (preterm n=16 and term n=13). It was not possible to carry out real-time PRP thrombin generation on all samples, owing to difficult phlebotomy and insufficient volumes of blood obtained in some infants, and availability of the thrombin generation equipment and lead investigator.

The results are presented below. In cord blood, I found a significantly shorter lag time ($p$= 0.02) and time to peak ($p$<0.01) in the preterm infants compared to full-term infants (*Table 4.5*). ETP and peak thrombin remain unchanged.

**Table 4.5: Summary of the thrombin generation parameters in umbilical cord blood platelet rich plasma in preterm and term infants**

Where FluCa was injected manually, only ETP and peak thrombin measurements were used (n=4 preterm, n=7 term)

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [b] T-test

| CAT Parameter | Preterm n=17 | Term n=35 | *p*-value |
|---|---|---|---|
| PRP Platelet count ($x10^9$/L) | 100 [63.6 – 127] | 124 [82.5 – 173] | 0.05[a] |
| Lag time (min) | 3.8 [2.8 - 4] | 4.3 [3.7 – 4.8] | 0.02[a] |
| ETP (nM.min) | 965.6 (±239.2) | 946.6 (±180.9) | 0.76[b] |
| Peak thrombin (nM) | 90.8 (±30.4) | 81 (±24.5) | 0.22[b] |
| Time to peak (min) | 8.8 (±1.5) | 11.9 (±2.7) | <0.01[b] |

In postnatal PRP (*Table 4.6*), the lag time (p=0.04) and time to peak (p=0.01) were significantly shorter, while peak thrombin (p=0.01) was significantly higher and there was a trend towards a higher ETP (p=0.05) in the preterm group, suggestive of a hypercoagulable state.

**Table 4.6: Summary of the thrombin generation parameters in neonatal platelet rich plasma in preterm and term infants**

Where FluCa was injected manually, only ETP and peak thrombin measurements were used (n=2 preterm, n=1 term)

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [b] T-test

| CAT Parameter | Preterm n=16 | Term n=13 | *p*-value |
|---|---|---|---|
| PRP Platelet count ($x10^9$/L) | 29.2 [12.1 – 53.7] | 38.4 [22.9 – 116] | 0.2[a] |
| Lag time (min) | 3.9 [3.17 – 4.17] | 5.2 [3.84 – 5.68] | 0.04[a] |
| ETP (nM.min) | 927.2 (±182.6) | 788.2 (±178.1) | 0.05[b] |
| Peak thrombin (nM) | 87.4 (±20.8) | 67 (±16.6) | 0.01[b] |
| Time to peak (min) | 9.0 [7.7 – 10.3] | 12.2 [9.2 – 14.7] | 0.01[a] |

It was noted that the platelet count was lower in neonatal PRP than in UCB PRP, although similar between preterm and term infants in both sample types. PRP was always prepared at the same centrifugation speed (200 xg x 10 minutes, no brake), however, UCB samples were typically collected in 3 ml sodium citrate containers, while postnatal samples for thrombin generation were collected in smaller 1.3 ml sodium citrate containers, due to the volume limitations in neonatal sampling.
To understand if this may have influenced the PRP platelet count, in a small number of infants (n=4), UCB samples were collected in both 3 ml and 1.3 ml containers, PRP was prepared and the platelet count measured (*Figure 4.2*). All four infants had normal whole blood platelet counts (range 217 – 264 x $10^9$/L). There was a trend towards a higher platelet count in the 3 ml PRP compared to the 1.3 ml PRP, although this did not reach statistical significance (p=0.13) but the samples size was very small.
Umbilical cord blood samples have a slightly lower haemoglobin content and haematocrit than peripheral neonatal blood samples (268). It is possible that the differences in haematocrit may also have influenced the lower PRP platelet count seen in peripheral neonatal samples.

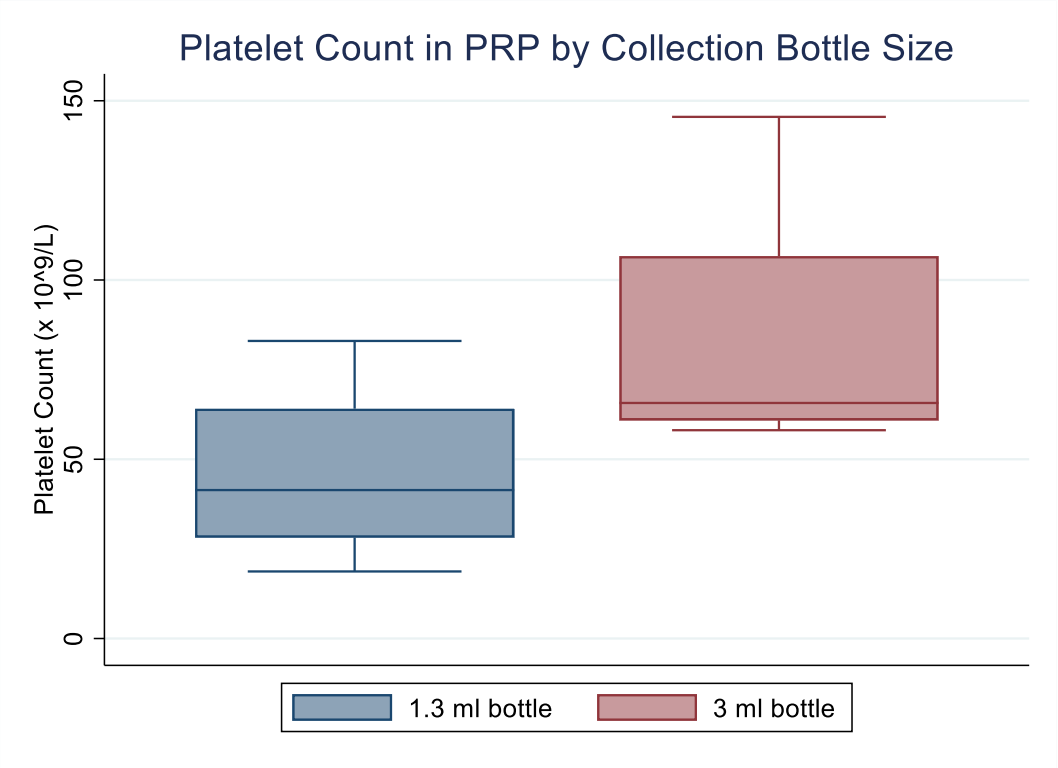


**Figure 4.2: Boxplot of the platelet count in PRP measured in umbilical cord blood, collected in matched 3 ml and 1.3 ml sodium citrate bottles in a small group of infants (n=4)**

### 4.2.3 The contribution of platelets to neonatal thrombin generation

To evaluate the contribution of platelets to neonatal thrombin generation, CAT was performed in UCB PPP and PRP using PRP reagent (containing TF only and no phospholipid). The effect of platelets was evaluated by thrombin generation in a subgroup of infants (n=33, including term and preterm infants), with sufficient fresh UCB PRP and PPP available.

When preterm and term infants were evaluated together, the presence of platelets caused a small but significant increase in peak thrombin ($p < 0.01$) and ETP ($p < 0.01$) but did not affect the lag time or time to peak thrombin (*Table 4.7*). These differences might be considered to be quite modest given the absence of any platelets or any exogenous source of anionic phospholipid in the PPP samples. However, differences in other potential endogenous sources of anionic phospholipid (including platelet-derived micro-vesicles) between groups cannot be excluded.

**Table 4.7: Evaluation of the effect of neonatal platelets on thrombin generation parameters in matched platelet rich and platelet poor plasma from umbilical cord blood in preterm (n= 10) and term (n=23) infants**

Mean (±SD), Median [IQR], Frequency (%),[e]Wilcoxon Signed Rank Test, [f] Paired T-Test

| CAT Parameter | PRP<br>n=33 | PPP<br>n=33 | *p*-value |
|---|---|---|---|
| PRP Platelet count<br>(x10^9/L) | 112<br>[73.4 – 150] | 0<br>[0 – 0.3] | |
| Lag time (min) | 4<br>[3.7 – 4.3] | 4<br>[3.6 – 4.3] | 0.09[e] |
| ETP (nM.min) | 977.2<br>(±202.5) | 892<br>(±209.1) | <0.01[f] |
| Peak thrombin<br>(nM) | 89.7<br>(±22.8) | 75<br>(±21.6) | <0.01[f] |
| Time to peak (min) | 10.4<br>(±2.3) | 10.1<br>(±1.7) | 0.19[f] |

When only preterm infants (n=10) were evaluated, the presence of platelets in plasma (PRP), compared to PPP, caused a reduction in time to peak (p=0.03) and an increase in ETP (p = 0.02) and peak thrombin (p=0.01) (*Table 4.8*).

**Table 4.8: Evaluation of the effect of neonatal platelets on thrombin generation parameters in matched platelet rich and platelet poor plasma from umbilical cord blood in preterm infants only**

Mean (±SD), Median [IQR], Frequency (%),[e] Wilcoxon Signed Rank Test

| CAT Parameter | PRP<br>n=10 | PPP<br>n=10 | *p*-value |
|---|---|---|---|
| PRP Platelet count (x10^9/L) | 102.5<br>[67.9 – 127] | 0.1<br>[0 – 0.5] | |
| Lag time (min) | 3.9<br>[2.8 – 4.3] | 4<br>[3.1 – 4.7] | 0.16[e] |
| ETP (nM.min) | 972.2<br>[806 – 1139.8] | 869.2<br>[704.5 – 1104.4] | 0.02[e] |
| Peak thrombin (nM) | 88.3<br>[80 – 102.3] | 74.4<br>[67.7 – 86.8] | 0.01[e] |
| Time to peak (min) | 8.6<br>[7.1 – 9.5] | 9.8<br>[7.1 – 10.5] | 0.03[e] |

In the full term group (n=23), the presence of platelets also resulted in a small but significant increase in ETP (p<0.01 ) and peak thrombin (p<0.01) but, conversely, caused an increase in lag time (p<0.01) and time to peak thrombin (p=0.01) (*Table 4.9*). While the effect of platelets on ETP and peak thrombin generation was similar in preterm and term plasma, the effect on lag time and time to peak differed.

**Table 4.9: Evaluation of the effect of neonatal platelets on thrombin generation parameters in matched platelet rich and platelet poor plasma from umbilical cord blood in term infants only**

Mean (±SD), Median [IQR], Frequency (%),[e]Wilcoxon Signed Rank Test

| CAT Parameter | PRP<br>n=23 | PPP<br>n=23 | *p*-value |
|---|---|---|---|
| PRP Platelet count (x10^9/L) | 114<br>[73.4 – 158] | 0<br>[0 – 0] | |
| Lag time (min) | 4.3<br>[3.7 – 4.5] | 4<br>[3.7 – 4.3] | <0.01[e] |
| ETP (nM.min) | 1000.1<br>[898.3 – 1076.9] | 929<br>[804.3 – 997.6] | <0.01[e] |
| Peak thrombin (nM) | 85.7<br>[74.2 – 104.9] | 71.1<br>[55.2 – 87.6] | <0.01[e] |
| Time to peak (min) | 11.5<br>[9.3 – 12.5] | 10.7<br>[9.3 – 11.2] | 0.01[e] |

### 4.2.4 Impact of the source of PRP on thrombin generation

In a subgroup of preterm infants who had PRP thrombin generation performed in UCB and neonatal blood PRP (n=7), all parameters measured were similar at both timepoints (*Table 4.10*).

**Table 4.10: Evaluation of the effect of source of PRP on thrombin generation parameters in matched cord and postnatal samples in preterm infants**

Mean (±SD), Median [IQR], Frequency (%),[e] Wilcoxon Signed Rank Test

| CAT Parameter | Cord PRP n=7 | Postnatal PRP n=7 | *p*-value |
|---|---|---|---|
| Lag time (min) | 3.1 [2.8 – 3.8] | 3.4 [3 – 4] | 0.41[e] |
| ETP (nM.min) | 831.5 [724.6 – 1002.9] | 872.6 [831.9 – 946.1] | 0.58[e] |
| Peak thrombin (nM) | 80.8 [72 – 91.2] | 89 [80.3 – 105.6] | 0.94[e] |
| Time to peak (min) | 8 [7.1 – 8.8] | 8.27 [7.7 – 9] | 0.28[e] |

### 4.2.5 Evaluation of the effect of Tissue Factor Pathway Inhibitor (TFPI) on neonatal thrombin generation

Both lag time and time to peak thrombin were shorter in preterm PRP compared with PRP from full-term infants, in both UCB and postnatal neonatal samples (*Table 4.5 and Table 4.6*). This is similar to the findings of the CRISP study, performed in platelet poor plasma, which also demonstrated a shorter lag time and time to peak thrombin in preterm infants, with similar ETP and Peak thrombin to term controls (121).

To evaluate if differential TFPI activity in preterm and term infants was responsible for the differences in lag time and time to peak, thrombin generation was performed in PPP with PPP reagent (contains 1 pM Tissue Factor and 4 μM phospholipid), in the presence and absence of an anti-TFPI antibody. This was performed in a group of infants who had PRP thrombin generation performed and had sufficient frozen PPP remaining (n=11 preterm, n=20 full-term).

Thrombin generation parameters in PPP in preterm and term infants (*Table 4.11*) and in the presence of anti-TFPI (*Table 4.12*) are displayed below (*Figure 4.3*). There was no difference in lag time and time to peak thrombin between preterm and term infants in the subgroup of infants included, both in the presence and absence of anti-TFPI.

**Table 4.11: Thrombin generation in preterm and term umbilical cord PPP with PPP Reagent LOW (contains 1 pm TF and 4 μm phospholipid)**

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [b] T-test

| CAT Parameter | Preterm N=11 | Term N=20 | *p*-value |
|---|---|---|---|
| Lag time (min) | 2.3 [2 – 2.3] | 2.3 [2.3 – 2.6] | 0.44[a] |
| ETP (nM.min) | 864.3 [822.3 – 1116.3] | 779.2 [702.1 – 850.3] | 0.02[a] |
| Peak thrombin (nM) | 115.7 (±20) | 118 (±26.4) | 0.8[b] |
| Time to peak (min) | 5.7 [5.3 – 6] | 5.8 [5.3 – 6.5] | 0.63[a] |

**Table 4.12: Thrombin generation in preterm and term umbilical cord PPP with PPP Reagent LOW (contains 1pm TF and 4µm phospholipid) in the presence of anti-TFPI (100µg/ml)**

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [b] T-test

| CAT Parameter | Preterm N=11 | Term N=20 | *p*-value |
|---|---|---|---|
| Lag time (min) | 1.7 [1.7 – 2] | 1.9 [1.9 – 2] | 0.11[a] |
| ETP (nM.min) | 761.7 [726.3 – 1033.5] | 661.5 [605.3 – 762.4] | 0.01[a] |
| Peak thrombin (nM) | 130.7 (±21.3) | 135.4 (±17.7) | 0.51[b] |
| Time to peak (min) | 4.3 [4 – 4.7] | 4.6 {4.3 – 5] | 0.34[a] |

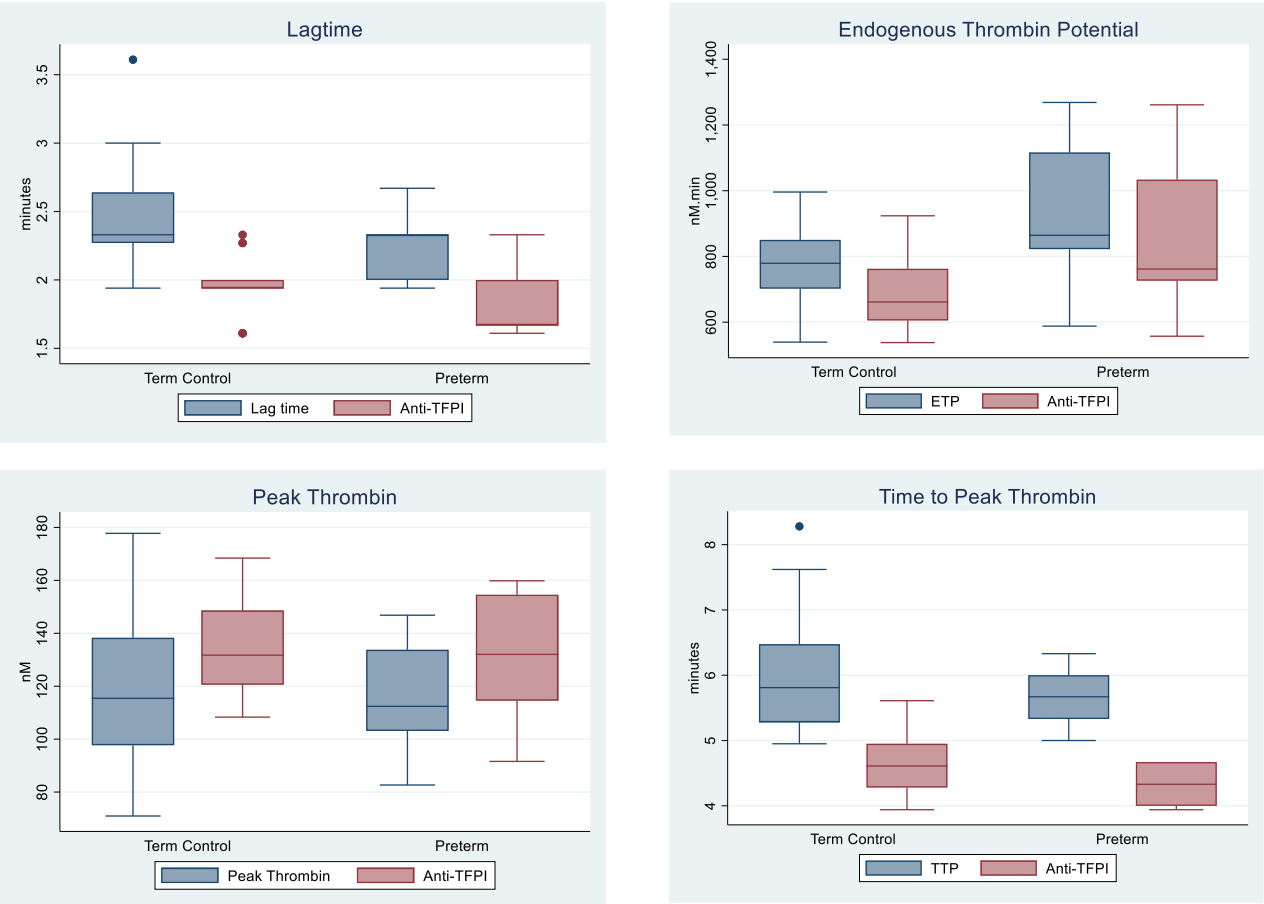


**Figure 4.3: Boxplot of the thrombin generation parameters in preterm and term cord PPP samples in the presence and absence of anti-TFPI (100µg/ml)**

While lag time and time to peak thrombin were similar in preterm and term infants in the presence and absence of anti-TFPI, inhibiting TPFI did influence all thrombin generation parameters (*Table 4.13*). In both preterm and term infants, inhibiting TFPI caused a reduction in lag time and time to peak, a reduction in ETP and a significant increase in peak thrombin ($p<0.01$). Moreover, the magnitude of the effect of inhibiting TFPI, measured by the change in each parameter, was similar in preterm and term infants (*Table 4.14*).

**Table 4.13: Evaluation of the effect of anti-TFPI on thrombin generation in umbilical cord PPP in preterm and term infants**

Mean (±SD), Median [IQR], Frequency (%),[e] Wilcoxon Signed Rank Test, [f] Paired T-Test

| CAT Parameter | Preterm n=11 | | | Term n=20 | | |
|---|---|---|---|---|---|---|
| | -anti TFPI | +anti TFPI | *p*-value | -anti TFPI | +anti TFPI | *p*-value |
| Lag time (min) | 2.33 [2 – 2.33] | 1.67 [1.67 – 2] | <0.01[e] | 2.33 [2.27 – 2.64] | 1.94 [1.94 – 2] | <0.01[e] |
| ETP (nM.min) | 864.31 [822.28 – 1116.32] | 761.74 [726.25 – 1033.54] | <0.01[e] | 779.21 [702.06 – 850.345] | 661.48 [605.29 – 762.41] | <0.01[e] |
| Peak thrombin (nM) | 115.66 (±19.97) | 130.65 (±21.3) | <0.01 [f] | 117.98 (±26.4) | 135.39 (±17.74) | <0.01[f] |
| Time to peak (min) | 5.67 [5.33 – 6] | 4.33 [4 – 4.67] | <0.01[e] | 5.81 [5.28 – 6.48] | 4.61 {4.28 – 4.95] | <0.01[e] |

**Table 4.14: Evaluation of the magnitude of the effect of TFPI on neonatal thrombin generation parameters in preterm and term infants**

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [b] T-test

| CAT Parameter | Preterm<br>n=11 | Term<br>n=20 | *p*-value |
|---|---|---|---|
| Δ Lag time (min) | -0.3<br>[-0.7 - -0.3] | -0.3<br>[-0.7 - -0.3] | 0.5[a] |
| Δ ETP (nM.min) | -79<br>(± - 47) | -90<br>(± - 77) | 0.69[b] |
| Δ Peak thrombin (nM) | 15<br>(± - 6.9) | 17.4<br>(± - 18) | 0.69[b] |
| Δ Time to peak (min) | -1.3<br>[-1.7 - -1.3] | -1.3<br>[-1.7 - -1] | 0.72[a] |

### 4.2.6 Tissue Factor Pathway Inhibitor (TFPI) concentration

TFPI levels and TFPI activity were evaluated in preterm and term infants with sufficient PPP remaining, and inclusive of all infants described in results *Section 4.2.5*. In the TFPI activity assay, results were not available in three infants (n=2 term, n=1 preterm) and these were excluded from the analysis.

Both TFPI levels (*Table 4.15 and Figure 4.4*) and TFPI activity (*Table 4.16 and Figure 4.5*) were similar in preterm and term infants. Collectively, and in conjunction with the findings of *Section 4.2.5*, this suggests that the shortened lag time and time to peak thrombin seen in preterm PRP, are unlikely to be due to reduced TFPI levels in the preterm group.

**Table 4.15: Tissue factor pathway inhibitor levels in preterm and term infants measured in umbilical cord blood plasma**

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test

| | Preterm<br>n=15 | Term<br>n=20 | *p*-value |
|---|---|---|---|
| TFPI Concentration (ng/ml) | 7.1<br>[6.3 – 10.3] | 8.4<br>[7.3 – 9.8] | 0.41 [a] |

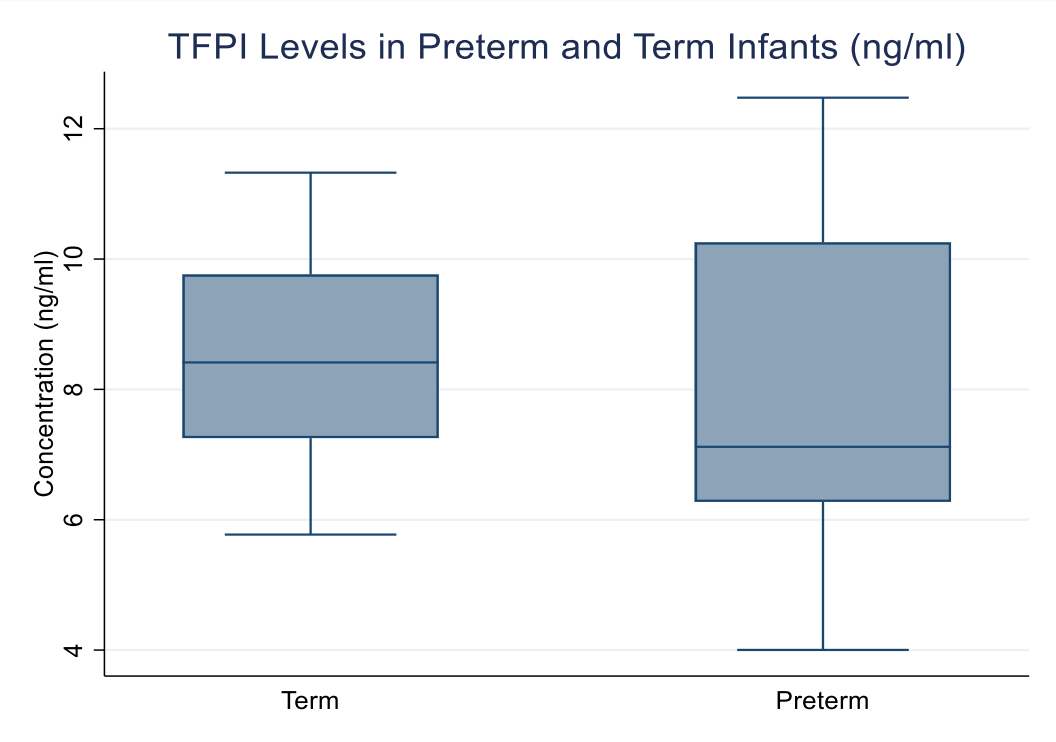


**Figure 4.4: Boxplot of Tissue factor pathway inhibitor levels in preterm and term infants in umbilical cord blood plasma**

**Table 4.16: Tissue factor pathway inhibitor activity in preterm and term infants in umbilical cord blood plasma**

Mean (±SD), Median [IQR], Frequency (%), [b] Unpaired T-test

| | Preterm<br>n=14 | Term<br>n=18 | *p*-value |
|---|---|---|---|
| TFPI Activity (units/ml) | 0.43 (±0.2) | 0.41 (±0.1) | 0.75 [b] |

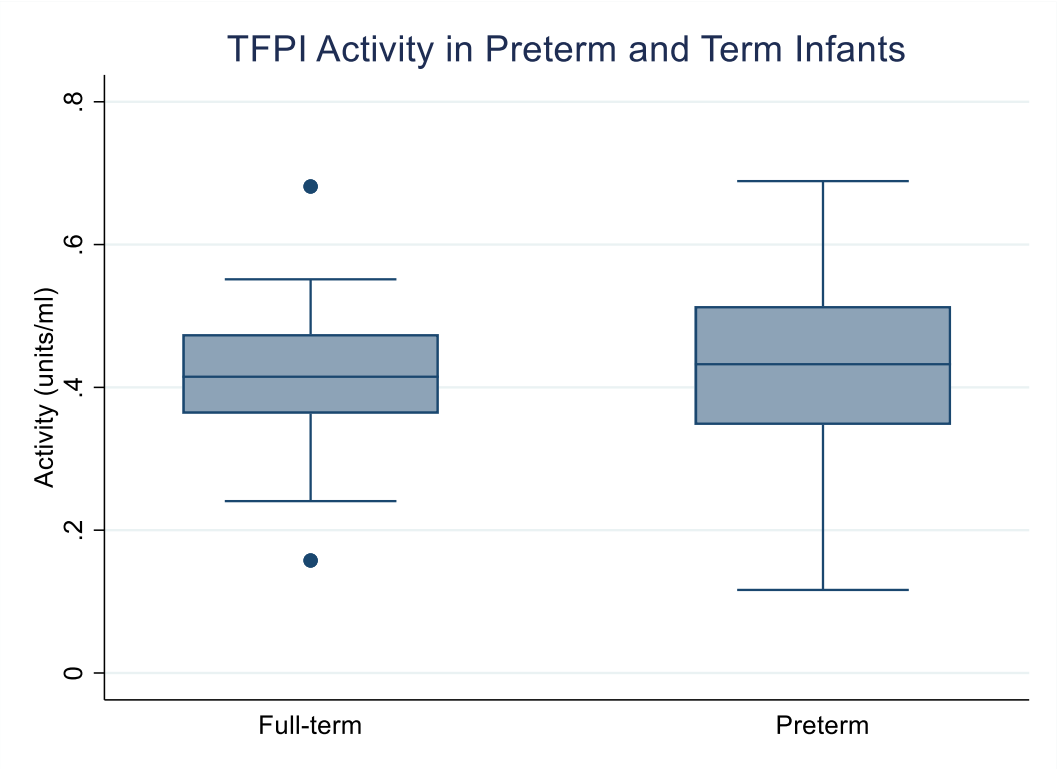


**Figure 4.5: Boxplot of Tissue factor pathway inhibitor (TFPI) activity in preterm and term infants in umbilical cord blood plasma**

### 4.2.7 Thrombin generation and Intraventricular haemorrhage (IVH)

I had planned to evaluate whether there were early differences in thrombin generation between infants who subsequently developed an IVH and those that did not. However, only 4/17 preterm infants who had cord thrombin generation performed had an IVH, of which 1 was a Grade III/IV IVH, and 2/16 infants who had Day 1 thrombin generation had an IVH, none of which were severe. For this reason, thrombin generation was only compared between preterm infants with and without any IVH, in UCB samples. Thrombin generation parameters were similar between infants with and without an IVH in UCB PRP, although the numbers included were very small (*Table 4.17 and Figure 4.6*).

**Table 4.17: Thrombin generation in umbilical cord blood PRP of infants who subsequently developed an intraventricular haemorrhage and those who did not**

IVH: Intraventricular haemorrhage

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test

| | IVH<br>n=4 | No IVH<br>n=13 | *p*-value |
|---|---|---|---|
| Lag time (min) | 3.6<br>[3.4 – 4.1] | 3.8<br>[2.8 – 4] | 0.91[a] |
| ETP (nM.min) | 947.1<br>[881 – 1039.5] | 868.2<br>[806 – 1182.7] | 0.87[a] |
| Peak thrombin (nM) | 71.9<br>[61.5 – 82.7] | 91.2<br>[72 – 121.9] | 0.2[a] |
| Time to peak (min) | 9.8<br>[9.1 – 10.5] | 8.3<br>[7.1 – 9.3] | 0.12[a] |

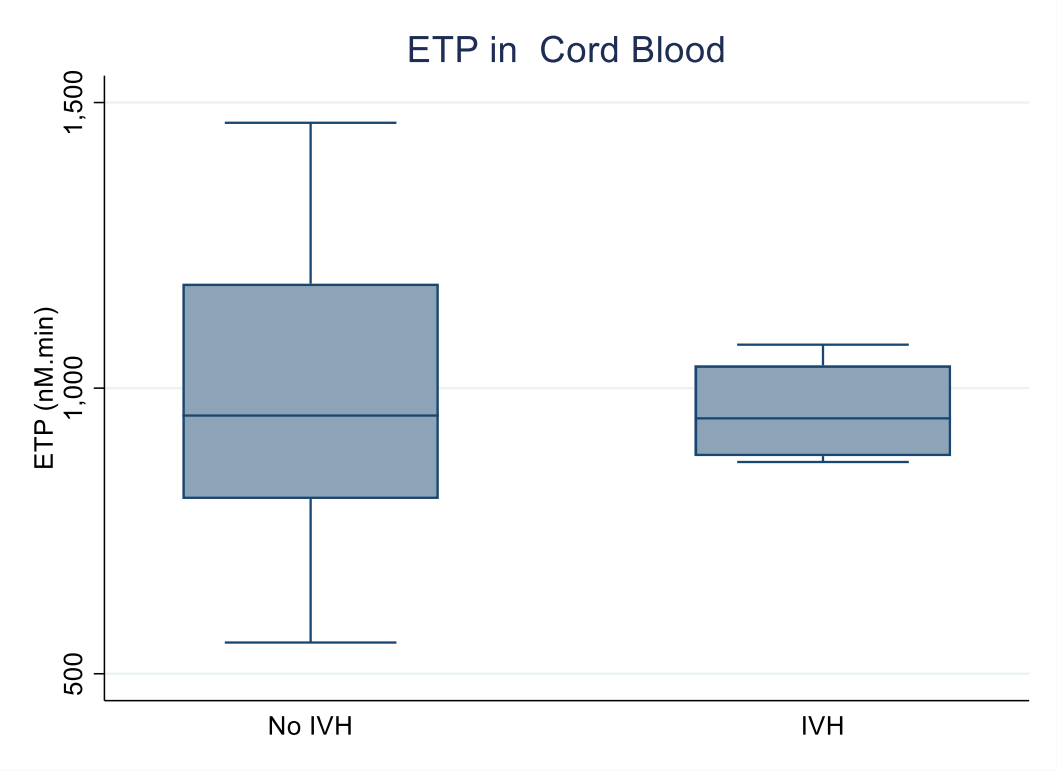


**Figure 4.6: Boxplot of the Endogenous thrombin potential (ETP) in umbilical cord blood PRP of infants who subsequently developed an intraventricular haemorrhage (n=4) and those who did not (n=13)**

IVH: Intraventricular haemorrhage

### 4.3 Discussion

The aim of this chapter of work was to expand our understanding of the role of platelets in secondary neonatal haemostasis and the potential differences between term and preterm infants.

I found that both the lag time and time to peak thrombin generation were significantly shorter in preterm PRP (both umbilical cord PRP and neonatal PRP), when compared to healthy term infants. Moreover, the ETP and peak thrombin were significantly increased in preterm neonatal PRP compared to term neonatal PRP. These findings are suggestive of hypercoagulability in preterm PRP, which has previously been shown in PPP (121, 126).

In this study, I found that the presence of platelets in plasma (PRP), compared to the absence of platelets (PPP), did cause a small but significant increase in peak thrombin and ETP. The effect of platelets on lag time and time to peak differed between preterm and term infants. Interestingly, I found that the presence of platelets in PPP from term infants appeared to prolong the thrombin generation lag time and time to peak but that this effect of platelets was not seen in preterm plasma. These findings differ to those by Haidl *et al.*, who found no difference in thrombin generation parameters between a platelet count of 100 x 10^9/L and 10 x 10^9/L in term infants and are discussed further in *Chapter 6: Section 6.3.*

Our findings in preterm PRP (shortened lag time and time to peak, *Section 4.2.2*) are akin to those described in PPP in the earlier CRISP study (121). Therefore, I wished to explore the plasma factors which may have caused the reduction in these two parameters in the preterm group. Our group has previously demonstrated that an increase in TFPI levels can influence the lag time and time to peak in several pathologies including COVID-19 and pre-eclampsia (137, 269). While blocking TFPI activity caused a reduction in lag time, time to peak thrombin and ETP, and increased peak thrombin in both preterm and term infants, the magnitude of the effect was similar in both groups and did not explain the differences in lag time and time to peak between preterm and term infants. Moreover, I found no difference in TFPI activity levels or TFPI antigen levels between preterm and term infants. Collectively, these results suggest that altered TFPI levels do not account for the reduced lag time and time to peak in the preterm group. For this reason, I evaluated TF-EVs in plasma to evaluate if they may explain the differences in lag time and time

to peak. The results of this are shown in *Chapter 5: Section 5.2.4* and are discussed in *Chapter 6: Section 6.6.2*.

A recent study suggested that blood from the umbilical cord is hypercoagulable compared to neonatal blood (270). Here, I compared CAT parameters in a group of preterm infants with both cord and postnatal thrombin generation available and identified no differences between the two PRP sources to suggest hypercoagulability in cord blood, although the numbers available were small.

Previous neonatal studies have not shown CAT to be a useful test for predicting the risk of haemorrhage, either in preterm infants or post-operatively (126, 166, 179). I attempted to evaluate for differences in thrombin generation parameters between infants who subsequently developed an IVH and those did not. While thrombin generation was similar between infants with and without subsequent IVH in UCB PRP, the numbers included were very small and it is difficult to draw any conclusions from these findings.

There were several limitations to our thrombin generation studies. Firstly, real-time thrombin generation in PRP was not performed in all infants, owing to the challenges of phlebotomy and obtaining adequate volumes of plasma in this cohort, in addition to the availability of the equipment and lead investigator. Secondly, the PRP platelet count in the postnatal samples, was low (29.2 [12.1 – 53.7] v 38.4 [22.9 – 116] x $10^9$/L), although this did not differ significantly between the preterm and term group (p=0.2). The PRP was prepared by centrifugation using the same protocol in both cord and postnatal samples (200g x 10 minutes with no brake) in accordance with ISTH recommendations (167, 168). However, cord samples were typically collected in 3 ml bottles, while postnatal samples were all collected in smaller 1.3 ml bottles and this may explain the difference in PRP platelet counts. While lower centrifugation speeds could have been attempted, the current protocol already resulted in small volumes of PRP, such that postnatal samples were often run in single, instead of in duplicate, and further reductions would likely have made thrombin generation unfeasible. Finally, there was a difference in the age of life at sample collection in the postnatal samples in the preterm and term group. Thrombin generation in preterm infants was only performed on Day 1 samples collected in the first 24 hours of age, while thrombin generation was performed in postnatal samples from the term group, collected at any time in the first 96 hours of age. However, previous work by our group demonstrated similar thrombin generation in preterm infants in PPP on Day 1

and Day 3 of life (179). Therefore, I do not think this discrepancy has affected our findings.

### 4.4 Conclusion

Thrombin generation in platelet rich plasma in premature neonates is similar or enhanced compared to healthy full term infants. Neonatal platelets appear to mediate a small but significant effect on peak thrombin and endogenous thrombin potential. By blocking TFPI, the lag time, time to peak thrombin and endogenous thrombin potential were reduced and peak thrombin was increased in both preterm and term infants. However, the magnitude of the effect was similar in both preterm and term infants, in addition to similar levels of TFPI activity and antigen in preterm and term infants. It is therefore unlikely that differences in TFPI levels shorten lag time and time to peak thrombin in in the preterm group. Unexpectedly, the lag time and time to peak thrombin generation were longer in full term PRP in comparison to PPP when assessed without the addition of any exogenous phospholipid to PPP. No difference was observed between preterm PRP and PPP in the same experiment, potentially suggesting that platelet-derived factors may be influencing this phase of plasma thrombin generation in full term plasma samples *in vitro*.

# Chapter 5 : Results II: Perinatal changes in circulating extracellular vesicles

## 5.1 Introduction

Extracellular vesicles (EVs) are nanovesicles released from cells, surrounded by a lipid bilayer, that cannot replicate (169). Circulating EVs derive from almost all cell types including platelets, megakaryocytes, endothelial cells and leukocytes (271-274). EVs are mediators of physiological and pathological processes, and are increasingly being investigated as biomarkers of disease and as therapeutic targets (198, 217, 275).

After birth, several physiological changes occur in healthy neonates to allow adaption from intrauterine to extrauterine life (1), as previously described in *Chapter 1: Section 1.1*. Evidence is emerging to suggest that the content of circulating EVs change with age (255, 256), although very little information is available regarding the circulating EVs during the perinatal adaption period and the potential role of these EVs (186).

In this study, I aim to characterise the circulating EVs in preterm infants during the first two weeks of age, comparing these results to circulating EVs in a group of healthy full-term infants, to identify whether they have similar responses to perinatal adaption.

## 5.2 Results

### 5.2.1 Changes in circulating small extracellular vesicles (SEVs) during perinatal adaption

SEVs were evaluated by NTA in plasma from preterm and full-term infants (*Table 5.1*).

**Table 5.1: Summary of plasma samples analysed by NTA**

| | Cord | Day 1 | Day 3 | Two weeks |
|---|---|---|---|---|
| Preterm | 24 | 69 | 29 | 12 |
| Term | 48 | 5 | 12 | |

A graphical representation of the distribution of particles in preterm and term infants at each time point (Cord, Day 1, Day 3 and Day 14 [Two weeks of age]) are displayed in *Figure 5.1*.

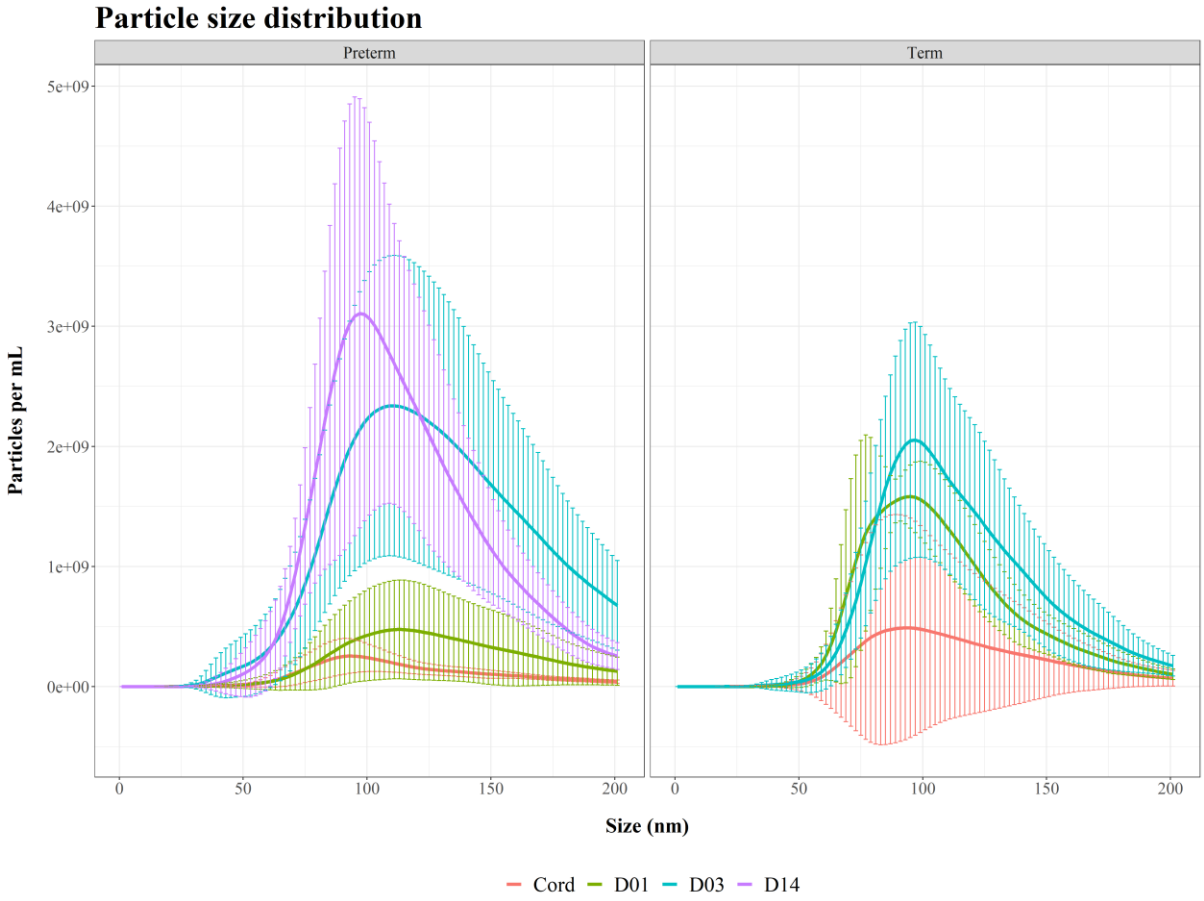


**Figure 5.1: A graphical representation of the particle distribution of SEVs <200 nm in preterm and term infants**

Mean and standard deviation displayed

There was evidence of changes in SEVs during perinatal adaption in both preterm and term infants. In preterm infants, there was an increase in the concentration of SEVs between Cord and Day 1 samples (adjusted $p$<0.01) and between Day 1 and Day 3 (adjusted $p$<0.01) (*Figure 5.2*). No further increase in the concentration of SEVs was seen between Day 3 and Day 14 (adjusted $p$=0.38).

In full-term infants, there was also an increase in the concentration of SEVs between Cord and Day 1 (adjusted $p$<0.01), although no further increase was seen between Day 1 and Day 3 samples (adjusted $p$=0.19).

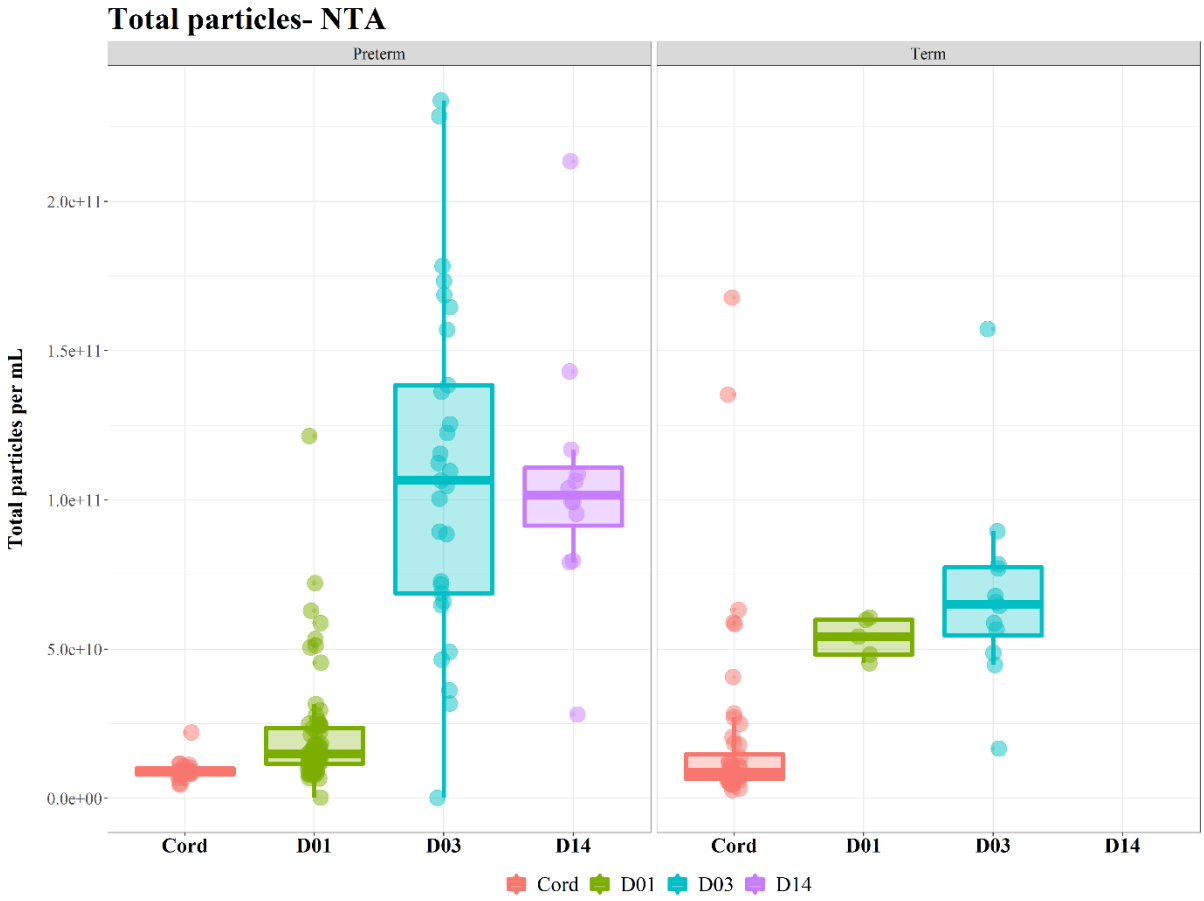


**Figure 5.2: Boxplot of the concentration of SEVs (< 200 nm) during the perinatal adaption period in preterm and term infants**

When the concentration of SEVs was compared between preterm and term infants (*Figure 5.3*), there was no significant difference in cord blood (adjusted *p*=0.05). However, on Day 1, SEV concentrations were higher in the term group (adjusted *p*<0.01), while by Day 3 they were higher in the preterm group (adjusted *p*=0.02).

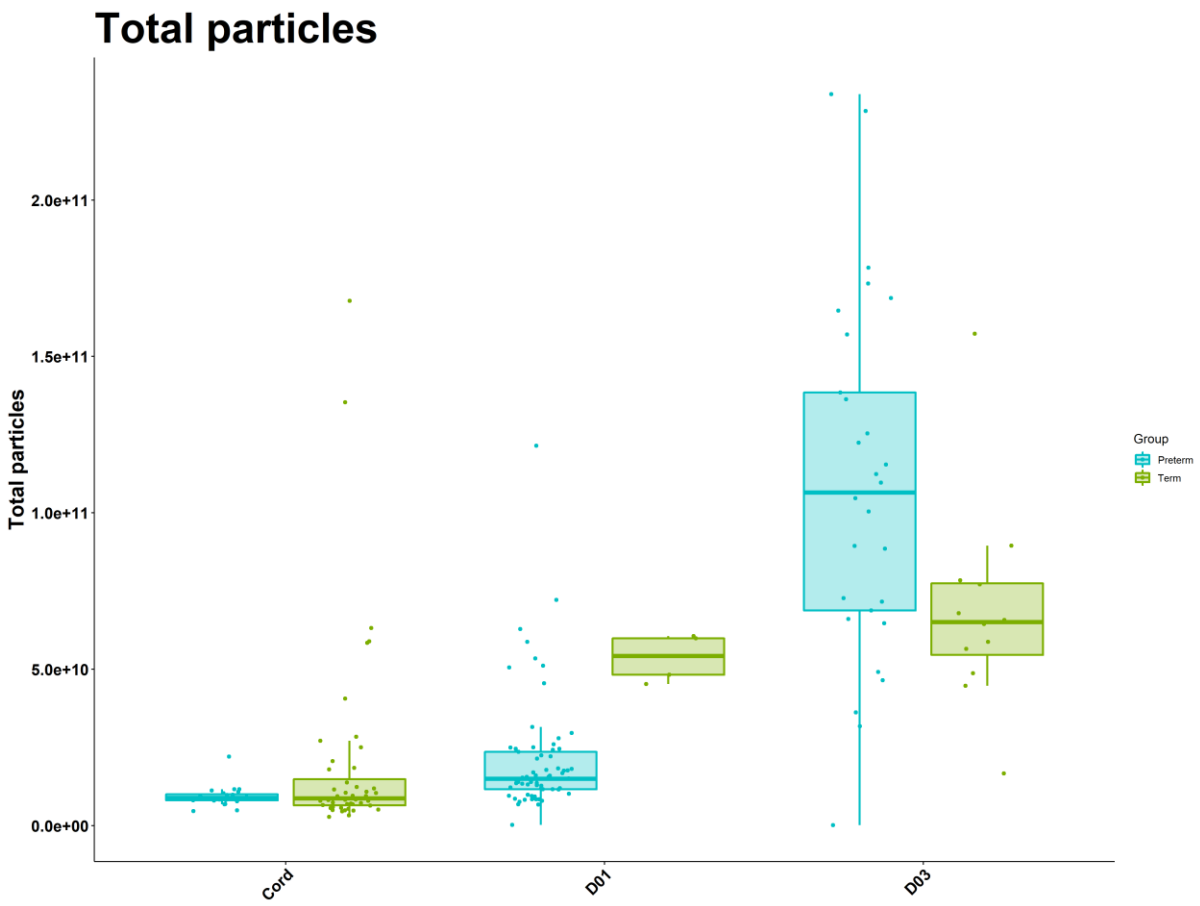


**Figure 5.3: Boxplot of the concentrations of SEVs in cord samples, postnatal Day 1 and Day 3 samples in preterm and term infants**

Day 14 samples were not collected in the term group so no comparison is possible

In the preterm group, there appeared to be two clusters of EV concentrations on Day 1. Samples could be collected at any time in the first 24 hours of life, and in the preterm group, samples were typically collected on admission (at the time of line insertion), or approaching 24 hours of age when the bilirubin and electrolyte levels were typically checked. The SEV concentration on Day 1 correlated with increasing hours of life at the time of sample collection in preterm infants (Spearman's rho 0.33, *p*=0.01) (*Figure 5.4*).

Given the average age (in hours) at the time of sample collection on Day 1 in the term group was 20.5 hours (IQR 19 – 24) hours compared to 1 hour (IQR 1 – 11) in the preterm group (*p*<0.01), this may explain the higher concentration of SEVs in the term group on Day 1. However, there was no relationship between hours at sample collection and Day 3 SEV concentration in preterm (Spearman's rho = 0.1, *p*=0.63) or term infants (Spearman's rho = 0.2, *p*=0.53).

It appears that changes in SEV concentration in preterm infants, begin in the first 24 hours of life.

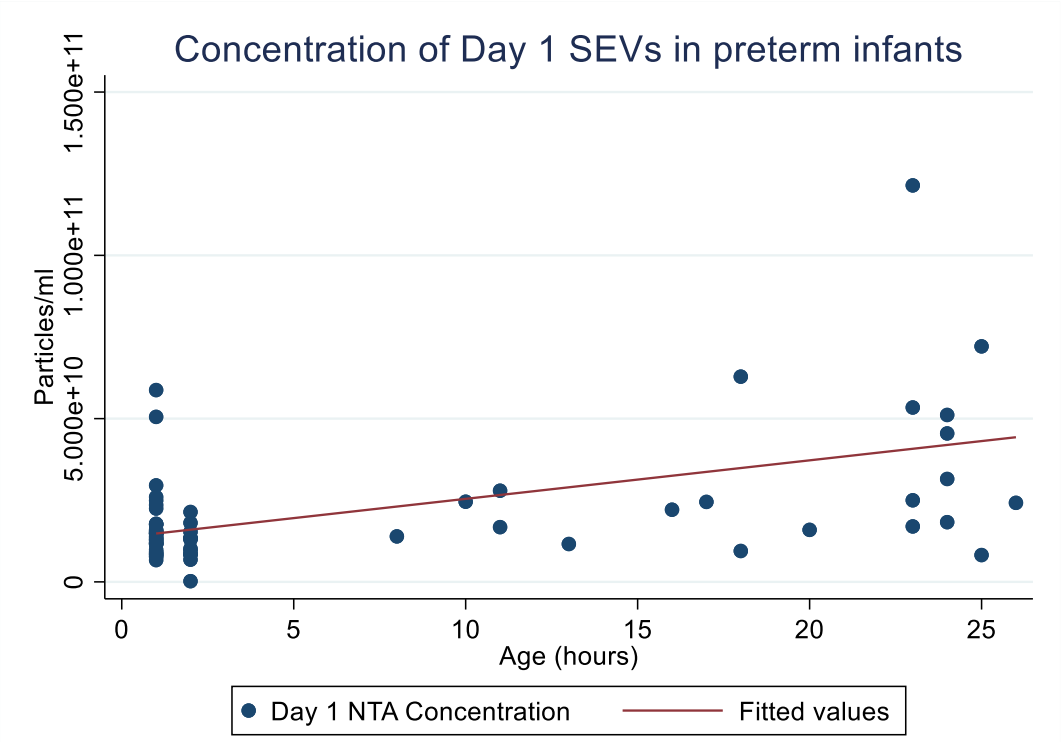


**Figure 5.4: Scatterplot of the concentration of SEVs on Day 1 in preterm infants by the age in hours at sample collection**

Next, the modal size of SEVs were compared over the first two weeks of life (*Figure 5.5*). In preterm infants, the modal size increased between Cord and Day 1 samples (adjusted $p<0.01$). Modal SEV size was similar between Day 1 and Day 3 (adjusted $p=0.54$), but reduced between Day 3 and Day 14 (adjusted $p=0.02$).

No such changes were seen in term infants, where the modal size remained steady between Cord and Day 1 (adjusted $p=0.6$), and Day 1 and Day 3 samples (adjusted $p=0.37$).

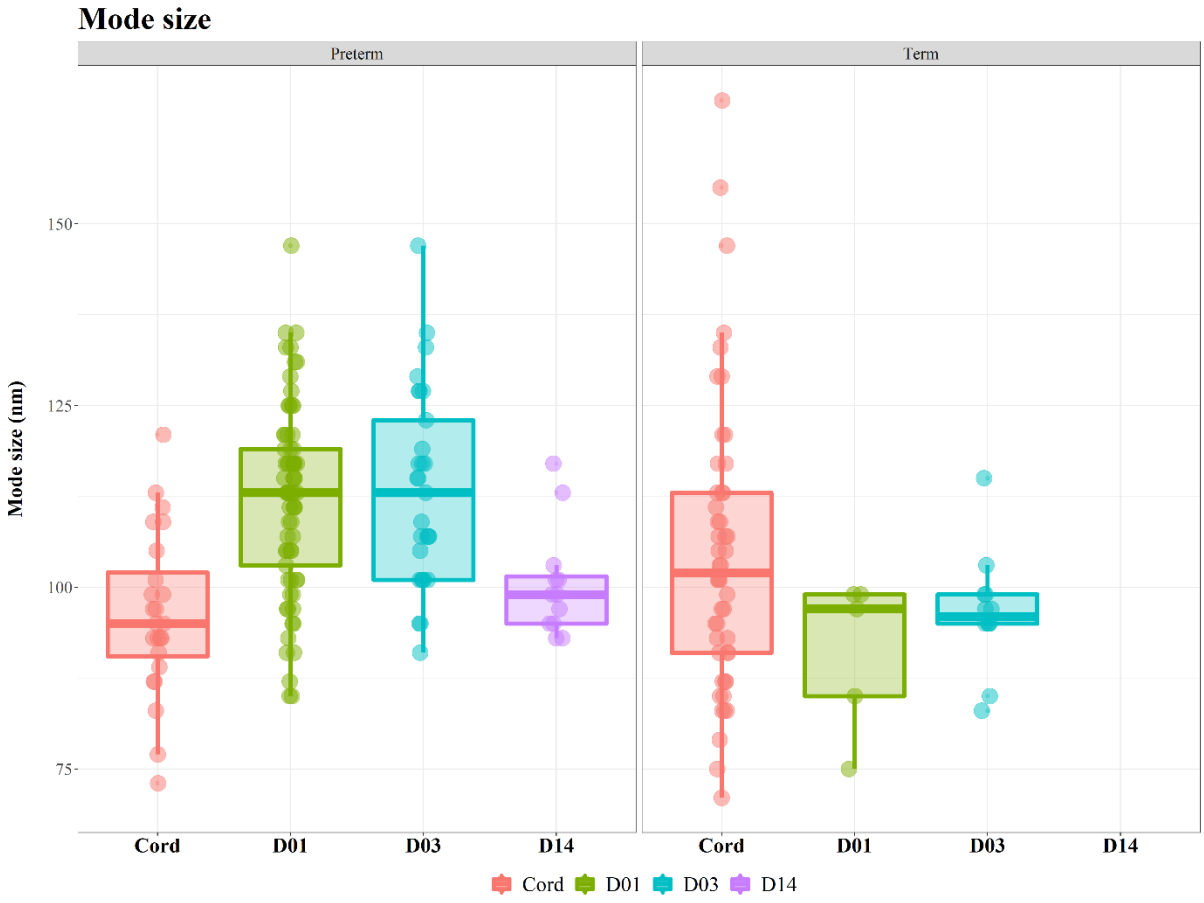


**Figure 5.5: Boxplot of the modal size of SEVs in preterm and term infants during the perinatal adaption period**

When preterm and term SEVs were compared (*Figure 5.6*), there was no significant difference in the modal SEV size in Cord samples (adjusted $p$=0.05), but SEVs were larger on Day 1 (adjusted $p$=0.02) and Day 3 (adjusted $p$<0.01) in the preterm group.

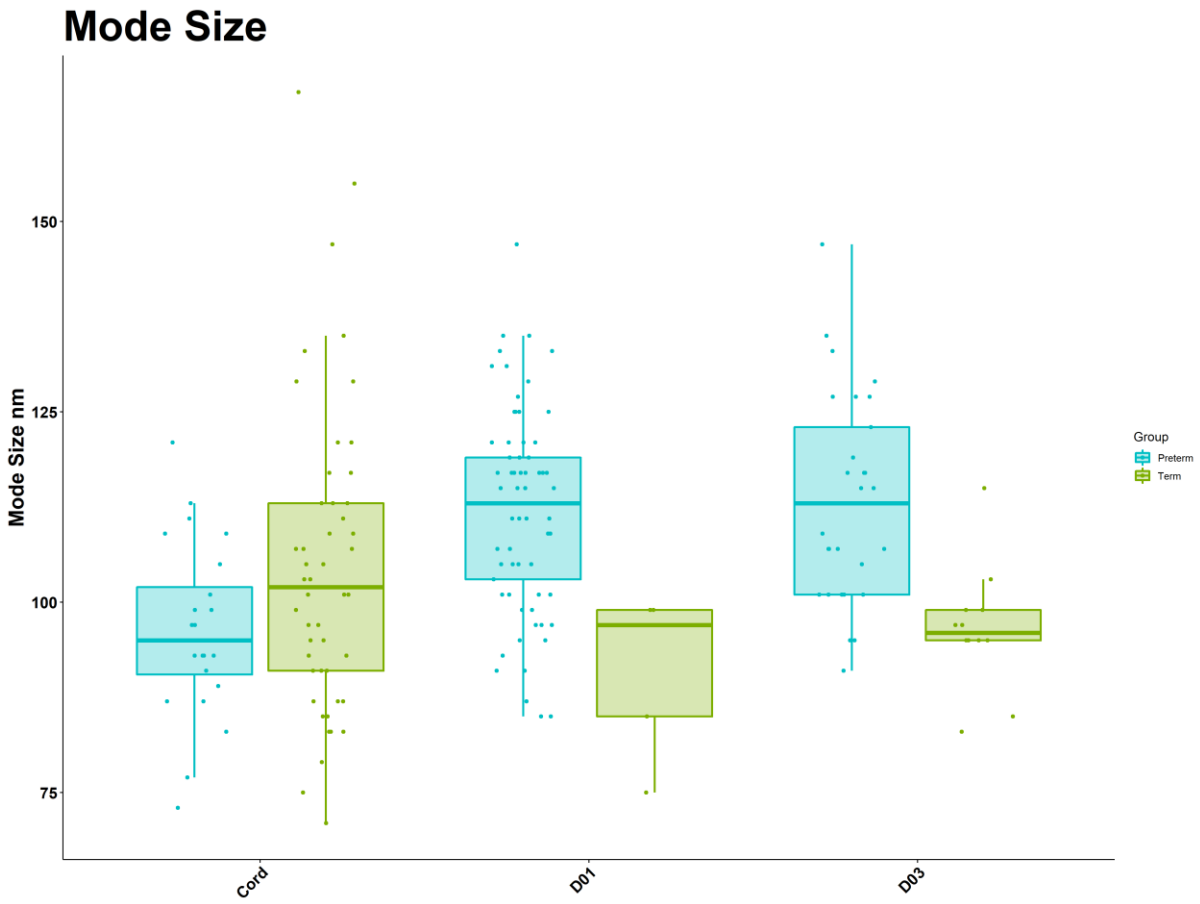


**Figure 5.6: Boxplot of the modal SEV size between preterm and term infants in Cord, Day 1 and Day 3 samples**

### 5.2.2 Changes in circulating large extracellular vesicles (LEVs) during perinatal adaption

Flow cytometry was performed at the Department of Pathological Physiology, Faculty of Medicine, Charles University, Prague, Czech Republic with assistance from Prof. Jan Zivny.

Flow cytometry was used to characterise LEVs isolated from plasma in a subgroup of infants with adequate plasma available, and across all the timepoints as described in *Table 5.2*.

**Table 5.2: Summary of samples analysed by flow cytometry**

| | Cord | Day 1 | Day 3 | Two weeks |
|---|---|---|---|---|
| Preterm | 14 | 23 | 11 | 11 |
| Term | 16 | 5 | 11 | |

Changes were also seen in the concentration of LEVs during perinatal adaption (*Figure 5.7*).

In preterm infants, the concentration of LEVs was higher in Cord than Day 1 samples (adjusted $p$=0.03), and there was a marked increase in the concentration of LEVs from Day 1 to Day 3 (adjusted $p$=0.01), which had reduced again by Day 14 (adjusted $p$<0.01).

When LEVs were evaluated in term infants, Cord samples had higher concentrations than postnatal samples on Day 1 (adjusted $p$<0.01) and which increased between Day 1 and Day 3 (adjusted $p$<0.01).

When the concentration of LEVs were compared between preterm and term infants, Cord and Day 1 LEV concentration was similar (adjusted $p$=0.54 and 0.69, respectively). However, the large increase in LEVs on Day 3 in the preterm group, was greater than in the term group (adjusted $p$<0.01).

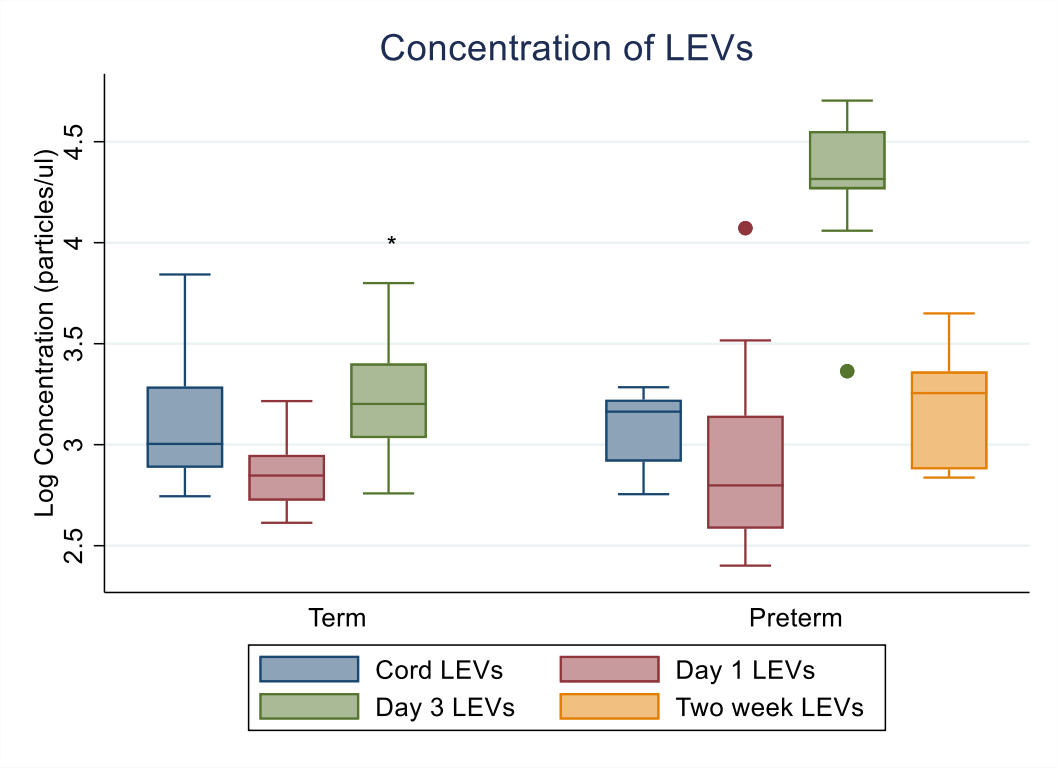


**Figure 5.7: Boxplot of the concentration of LEVs in preterm and term infants during the perinatal adaption period. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

Phosphatidylserine (PS) is expressed on the membrane of EVs and contributes to the their procoagulant activity(170). Annexin V (FITC-labelled) was used to evaluate the concentration of PS + EVs during postnatal adaption.

The concentration of Annexin positive LEVs changed over the first two weeks of life (*Figure 5.8*). In both preterm and term infants, the concentration of LEVs was higher in Cord samples than on Day 1 (adjusted $p<0.01$ in both groups), and increased between Day 1 and Day 3 (adjusted $p=0.01$ and $<0.01$, respectively). There was no change in the concentration of Annexin positive LEVs between Day 3 and Day 14 in preterm infants (adjusted $p=0.27$). The concentration of Annexin positive LEVs was similar between preterm and term infants at each of the three timepoints (Cord, Day 1 and Day 3, adjusted $p=0.54$, 0.61 and 0.79, respectively).

While the concentration of Annexin positive LEVs increased between Day 1 and Day 3, the proportion of Annexin positive EVs of the total LEVs fell significantly during that period in the preterm group (adjusted $p<0.01$), suggesting that the large in increase in LEVs on Day 3 were not Annexin positive (*Figure 5.9*).

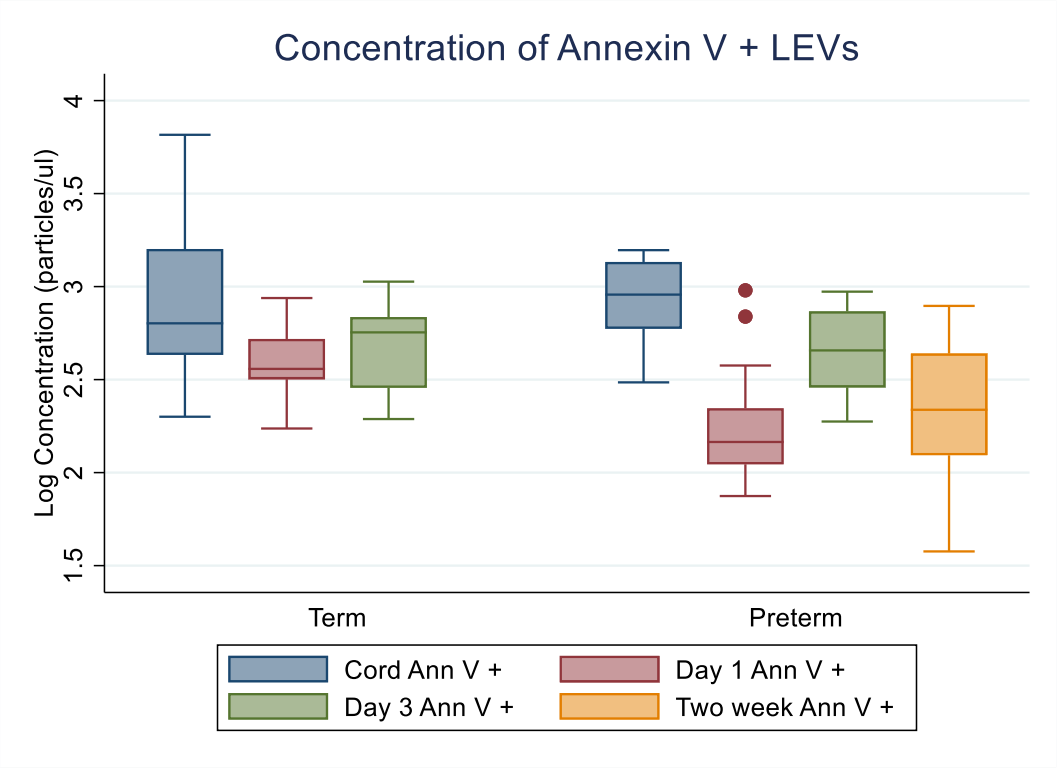


**Figure 5.8: Concentration of Annexin V + LEVs during perinatal adaption in preterm and term infants. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

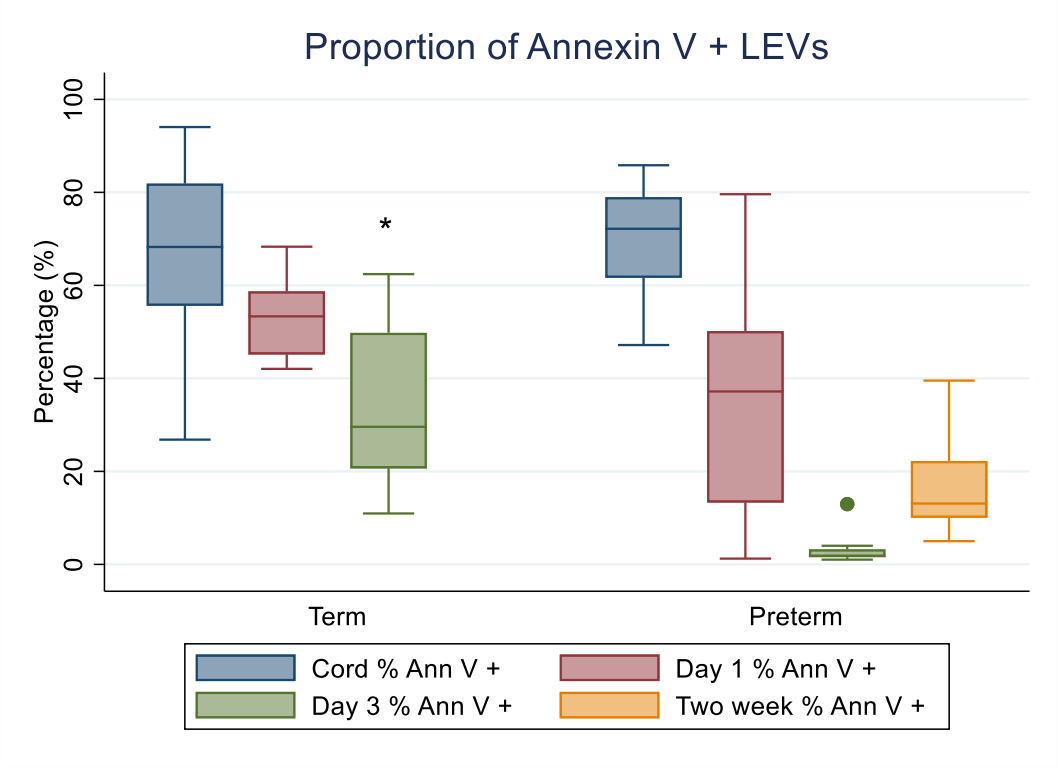


**Figure 5.9: Proportion of Annexin V+ EVs as a percentage of the total number of LEVs. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

### 5.2.3 Cellular origin of circulating extracellular vesicles during perinatal adaption

Several markers were used to evaluate the cellular origin of circulating LEVs, including three platelet markers: CD41 (GPIIb), CD42b (GPIb) and CD62 P (p-selectin). In addition, I evaluated the expression of Vascular endothelial growth factor Receptor 2 (VEGFR2) as an endothelial marker, procoagulant EVs which displayed TF and CD45 positive EVs as a white cell EV marker.

In Cord samples (*Figure 5.10*), there were similar concentrations of CD41/Annexin positive (adjusted *p*=0.54), CD42b/Annexin positive (adjusted *p*=0.54) and CD62P/Annexin positive (adjusted *p*=0.54) LEVs between preterm (n=14) and term (n=16) infants.

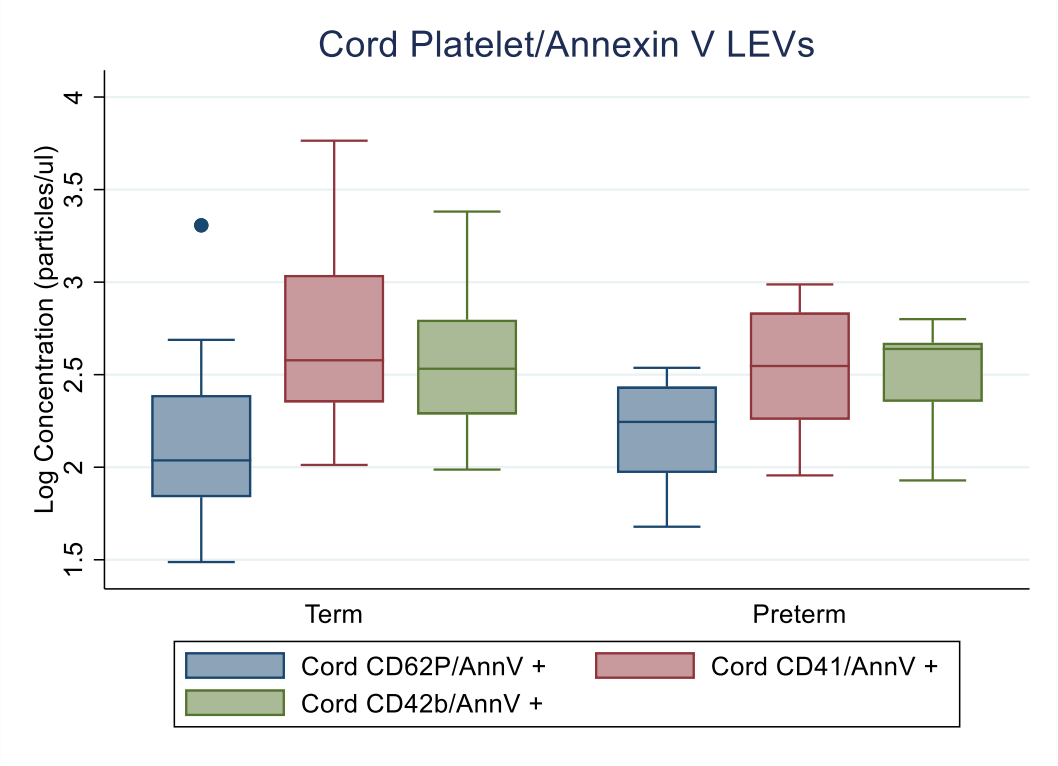


**Figure 5.10: Boxplot of the concentration of CD41, CD42b and CD62P Annexin V + LEVs in Cord samples in preterm and term infants. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

To further evaluate the release of platelet derived LEVs in preterm and term infants, CD41, CD42b and CD62P LEVs were evaluated during the perinatal adaption period (*Figure 5.11*).

In preterm infants, there was a higher concentration of all platelet LEVs in Cord compared to Day 1 samples (CD41 adjusted $p$=0.02, CD42b adjusted $p$=0.04 and CD62P adjusted $p$=0.04) and an increase in the concentration of platelet LEVs between Day 1 and Day 3 (CD41 adjusted $p$=0.01, CD42b adjusted $p$=0.01 and CD62P adjusted $p$=0.01). Similar trends were seen in full-term controls.

However, while the concentration of CD41, CD42b and CD62P were similar between preterm and term infants in Cord samples and on Day 1, the concentration of both CD42b and CD62P was significantly higher on Day 3 in the preterm group (adjusted $p$<0.01 for both) compared to term infants, while CD41 LEVs remained at a similar level to the term samples (adjusted $p$=0.16).

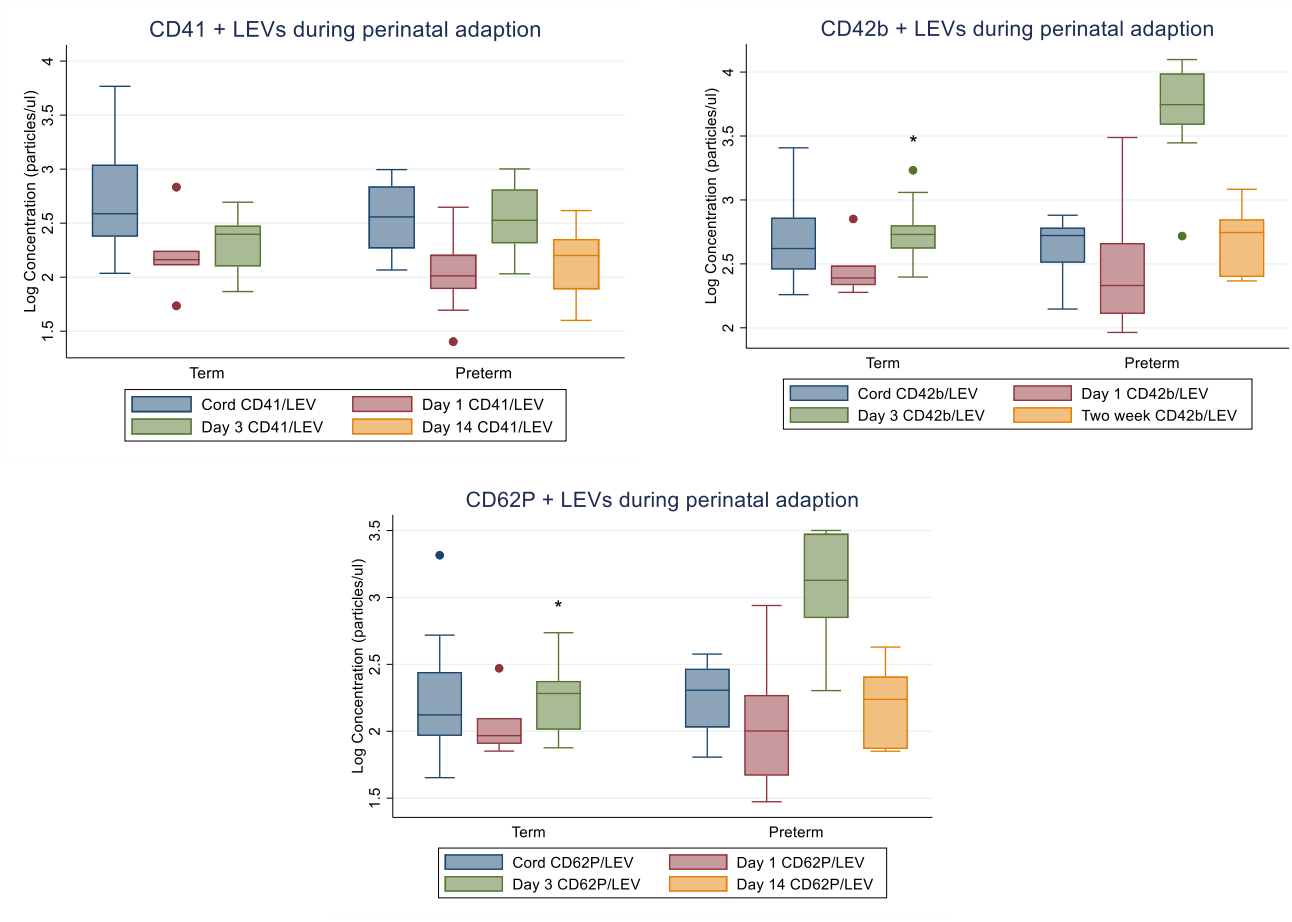


**Figure 5.11: Boxplot of the concentration of platelet LEVs (CD41, CD42b and CD62P) during the perinatal adaption period in preterm and term infants.**

*** Adjusted p < 0.05 between term and preterm infants at the same timepoint**

Similarly, when double positive LEVs were evaluated (*Figure 5.12*), the concentration of CD41/CD42 b, CD41/ CD62P and CD42b/ CD62P increased significantly between Day 1 and Day 3 in both preterm (adjusted $p$=0.01 for each) and term infants (adjusted $p$<0.01 for each). However, CD41/CD42 b and CD41/ CD62P concentrations were similar on Day 1 and Day 3 between preterm and term infants, while the increase in CD42b/ CD62P LEVs on Day 3 was significantly higher in the preterm group (adjusted $p$<0.01).

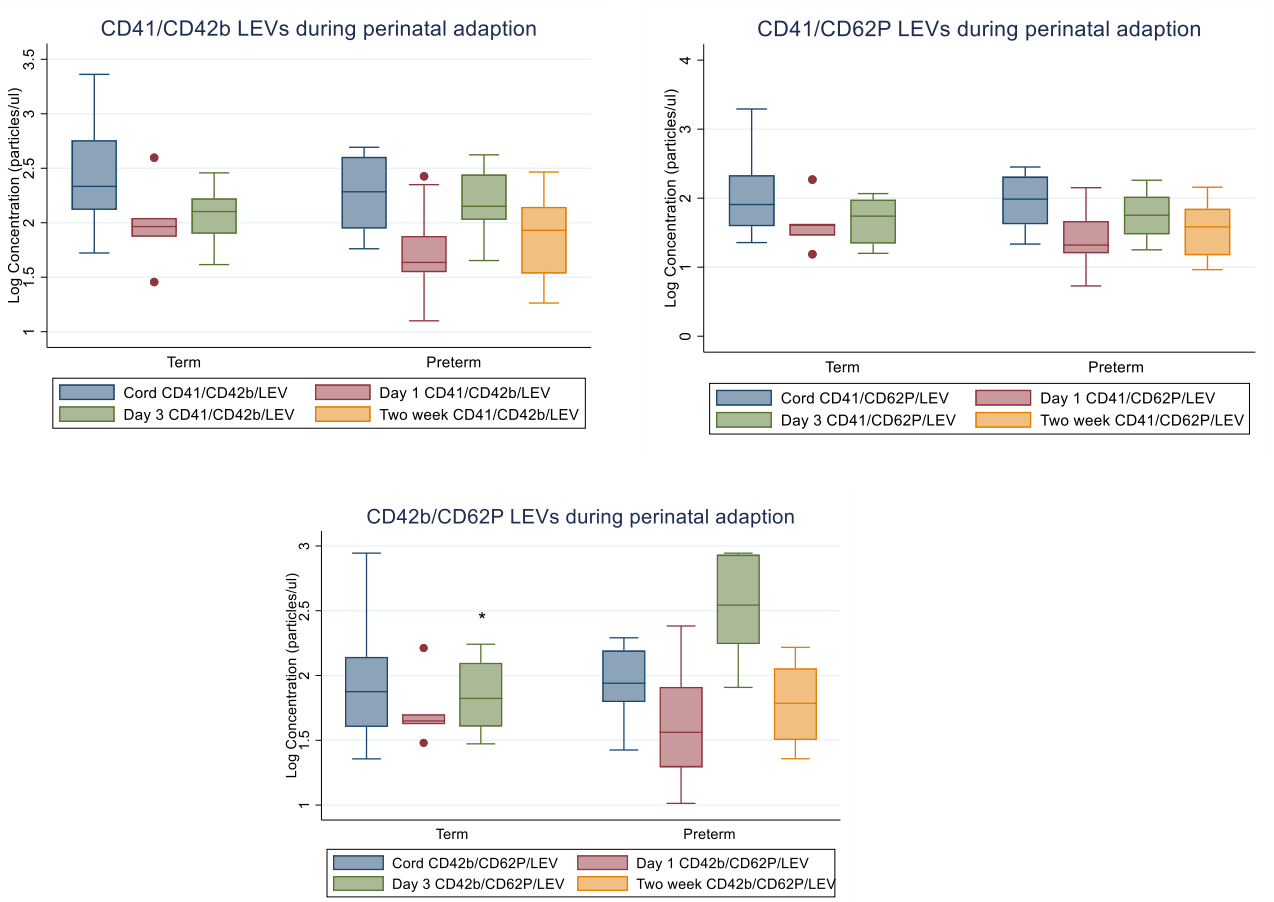


**Figure 5.12: Boxplot of double positive platelet LEVs during postnatal adaption in preterm and term infants. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

In addition to the evaluation of platelet-derived EVs, I evaluated procoagulant TF-EVs. Firstly, TF/Annexin V positive LEVs were evaluated (*Figure 5.13*) and I found higher levels in Cord samples than postnatal samples in preterm and term infants (adjusted $p<0.01$).

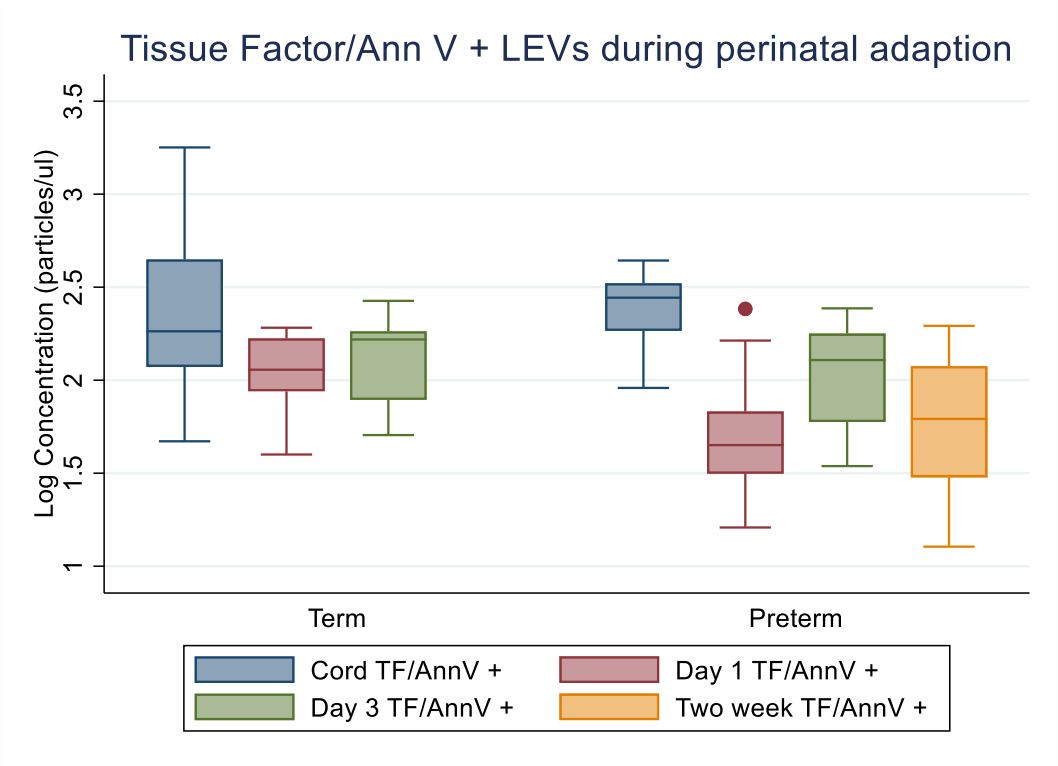


**Figure 5.13: Boxplot of the concentration of Tissue Factor/Annexin V positive LEVs during the perinatal adaption period in preterm and term infants.**

*** Adjusted p < 0.05 between term and preterm infants at the same timepoint**

When I evaluated TF positive LEVs (not Annexin V positive), a significant increase was seen between Day 1 and Day 3 in preterm (adjusted $p$=0.01) and term (adjusted $p$<0.01) infants (*Figure 5.14*). While the Day 1 concentration of TF LEVs was similar between preterm and term infants (adjusted $p$=0.69), they were significantly higher in the preterm group on Day 3 (adjusted $p$<0.01).

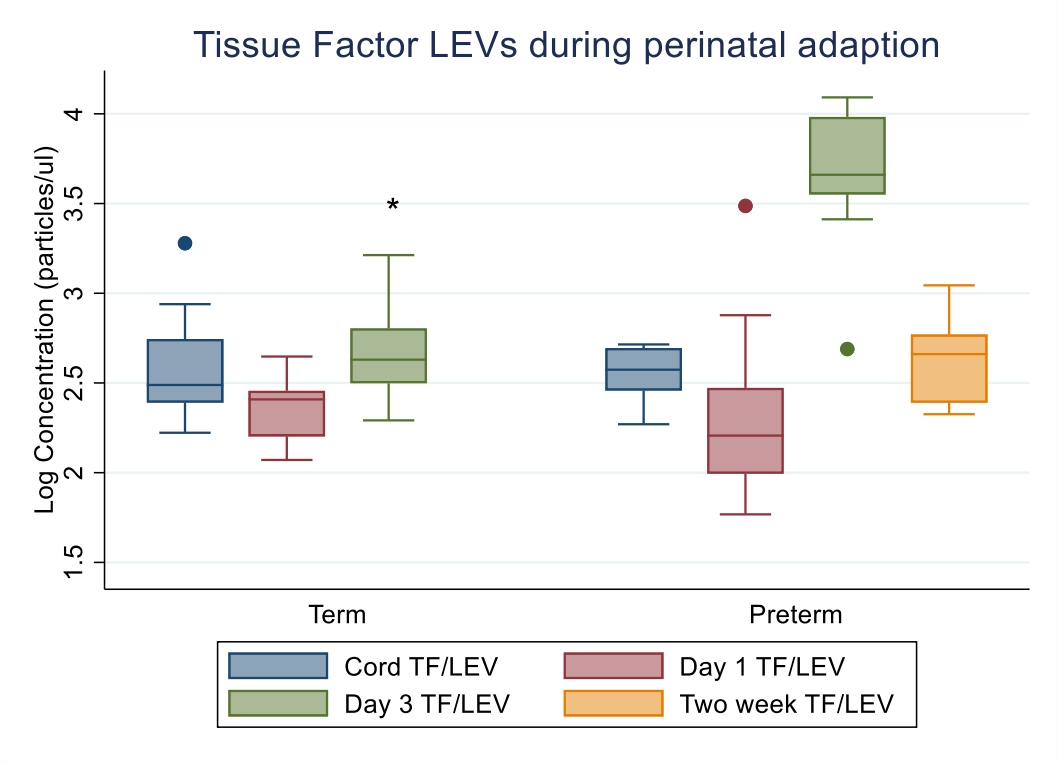


**Figure 5.14: Concentration of Tissue Factor positive LEVs in preterm and term infants during postnatal adaption. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

Endothelial EVs (EEVs) were evaluated by the presence of Vascular Endothelial Growth Factor Receptor 2 (VEGFR2). There was a significant increase in the concentration of VEGFR2 LEVs between Day 1 and Day 3 in preterm and term infants (adjusted $p$=0.01 and $p$<0.01 respectively), although the levels were significantly higher on Day 3 in the preterm group than in the term group (adjusted $p$<0.01) (*Figure 5.15*).

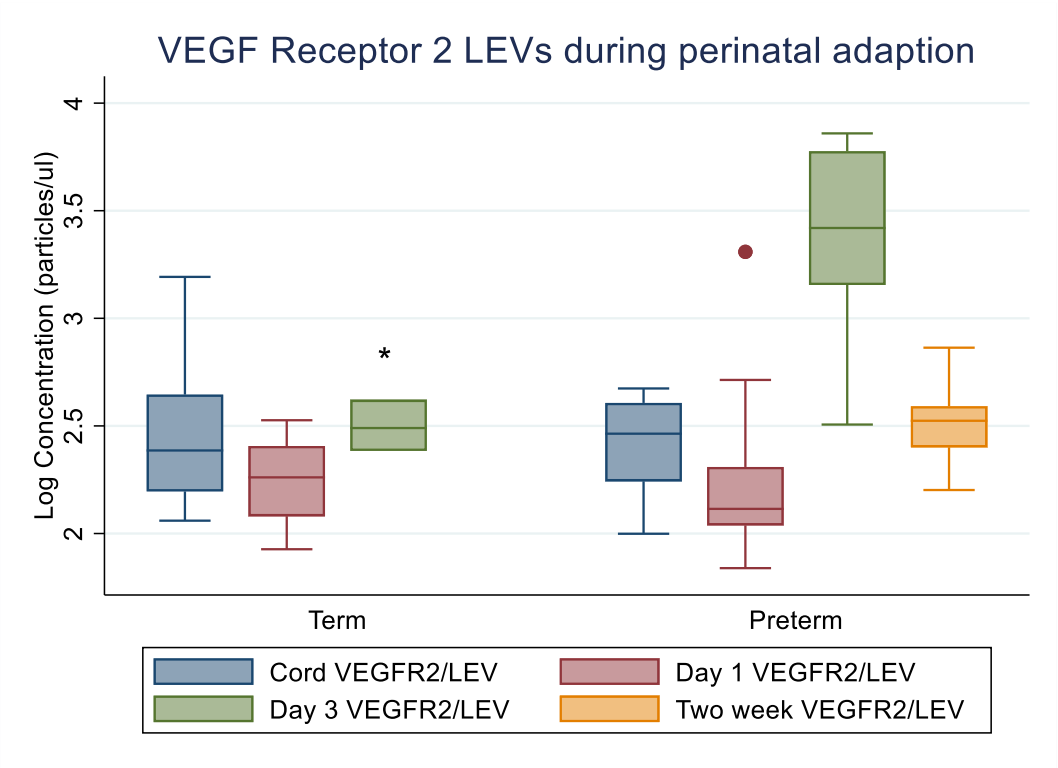


**Figure 5.15: Boxplot of the concentration of VEGF Receptor 2 LEVs during the perinatal adaption period in preterm and term infants. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

Finally, white cell derived LEVs (CD45 positive) were evaluated and a low proportion (<5%) of circulating LEVs were found to be CD45 positive throughout perinatal adaption. The concentration of CD45 positive LEVs did not differ between preterm and term infants at any of the three time points (Cord [adjusted *p*=0.97], Day 1 [adjusted *p*=0.69] and Day 3 [adjusted *p*=0.13]) (*Figure 5.16*).

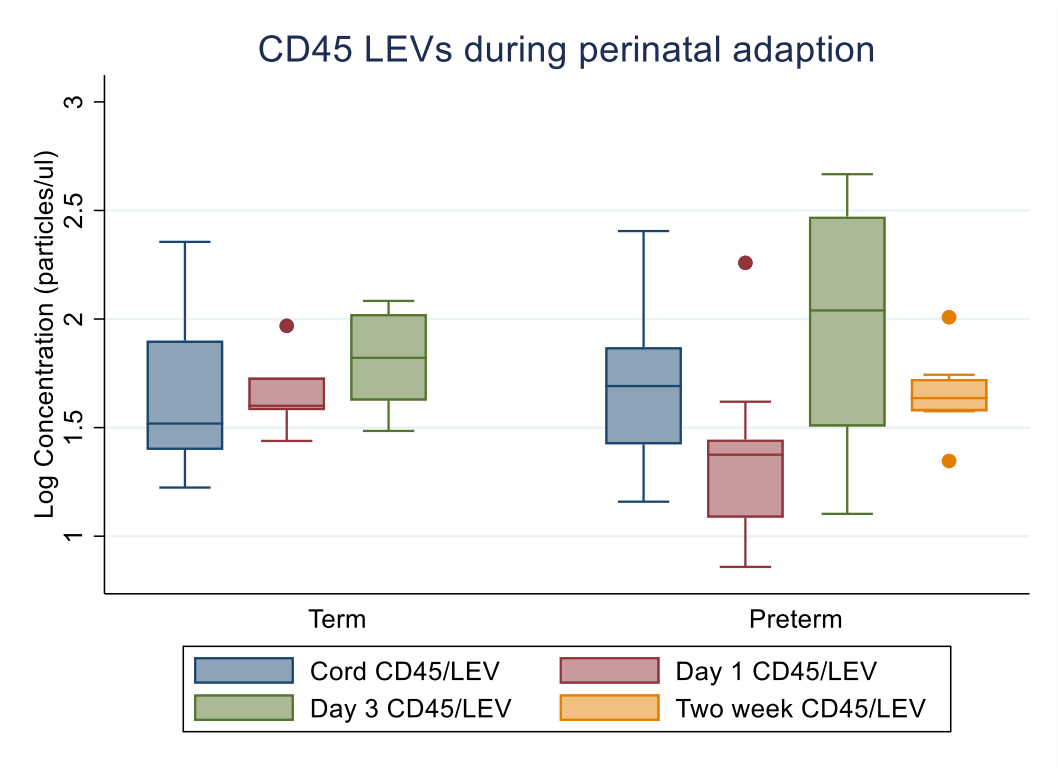


**Figure 5.16: Boxplot of the concentration of circulating CD45 white cell LEVs during perinatal adaption in preterm and term infants. * Adjusted p < 0.05 between term and preterm infants at the same timepoint**

### 5.2.4 Procoagulant activity of circulating extracellular vesicles

Two techniques were used to evaluate the procoagulant activity of EVs

#### *5.2.4.1 EV-TF ELISA*

The procoagulant activity of circulating TF-EVs was evaluated in undiluted plasma from unpaired cord (n=10 preterm, n=10 full-term) and postnatal samples (>24 hours of age) (n=11 preterm, n=10 full-term), using the Zymuphen MP-TF, as previously described in *Chapter 3: Section 3.15*.

Fourteen results were below the absorbance of the lowest standard (2.1 pg/ml, full-term cord n =4, full-term postnatal n = 6 and preterm postnatal n =4). These "low" values were arbitrarily assigned a value of "1 pg/ml" and pseudo counts were randomly assigned (using Research Randomizer, www.randomizer.org, free online software) to each "low" value (e.g. 1.0001, 1.0002, 1.003 etc.). One preterm cord sample duplicate had severely discordant results and was excluded from analysis. There was a higher concentration of TF-EVs in preterm cord samples compared to preterm postnatal samples (*Table 5.3 and Figure 5.17*). While there was a trend towards a higher concentration of TF-EVs in preterm compared to control cord samples, this did not reach statistical significance.

**Table 5.3: Concentration of TF-EVs in cord and postnatal plasma in preterm and term infants**

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test

| Concentration of TF-EVs (pg/ml) | Preterm<br>n=9 cord<br>n=11 postnatal | Term<br>n=10 cord<br>n=10 postnatal | *p*-value |
|---|---|---|---|
| Cord plasma | 5.2<br>[4.8 – 6.2] | 2.9<br>[1 – 6.4] | 0.16[a] |
| Postnatal plasma (>24 hours of age) | 3.0<br>[1 – 4.5] | 1<br>[1 – 3.7] | 0.25[a] |
| *p*-value | 0.03[a] | 0.25[a] | |

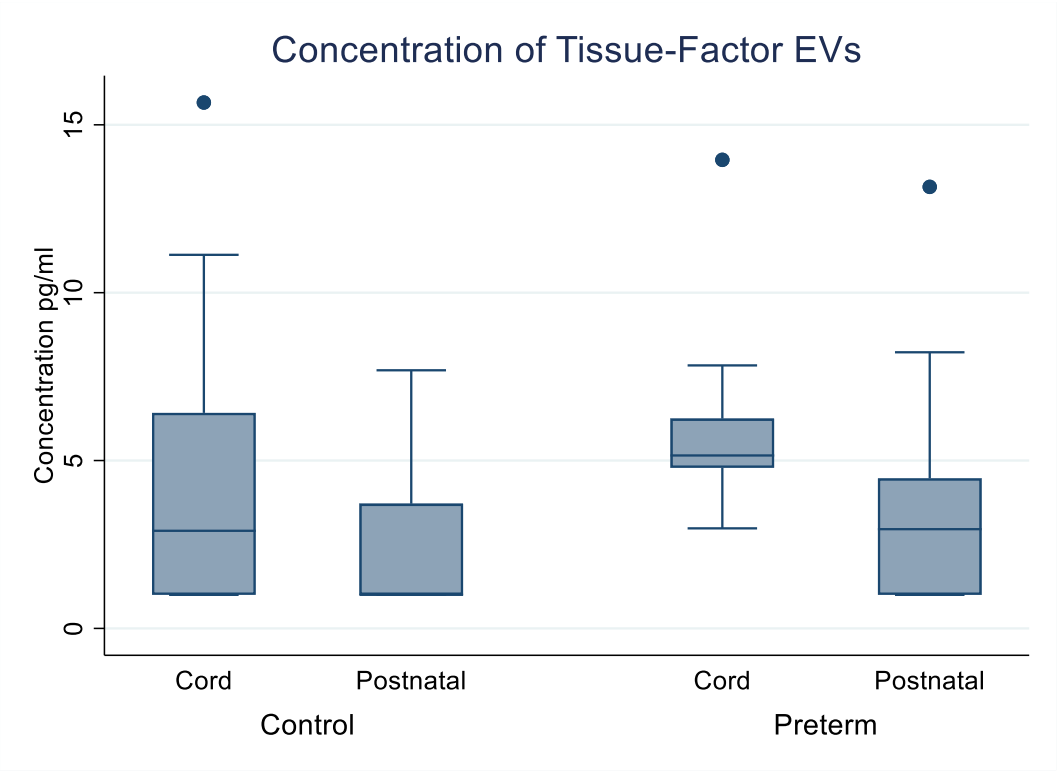


**Figure 5.17: Boxplot of the concentration of TF-EVs in preterm and term infants measured in both cord and postnatal samples**

### *5.2.4.2 Evaluation of TF-EVs using Calibrated Automated Thrombography*

In a subgroup of preterm and term infants, thrombin generation was performed in cord PPP (n=8 preterm, n=22 term) in the presence of Microparticle reagent (contains phospholipid only), which renders the assay dependent on the TF-EVs to initiate coagulation.

In the presence of MP Reagent, thrombin generation was similar in preterm and term PPP, suggesting that TF-EVs initiate thrombin generation in a similar manner in both groups, although a higher peak thrombin was identified in the term control group (*Table 5.4*).

**Table 5.4: CAT parameters measured in PPP in cord samples from a subgroup of preterm and term infants with MP Reagent**

MP reagent contains 4µm phospholipid

Mean (±SD), Median [IQR], Frequency (%), [a] Mann Whitney U Test, [b] T-test

| CAT Parameter | Preterm<br>n=8 | Term<br>n=22 | *p*-value |
|---|---|---|---|
| Lag time (min) | 6.2<br>[4.3 – 7.8] | 6.8<br>[6.1 – 9.2] | 0.22[a] |
| ETP (nM.min) | 798.4<br>[621.9 – 1029.2] | 959.2<br>[835.1 – 1067.4] | 0.26[a] |
| Peak thrombin (nM) | 91<br>[73 – 109.3] | 130.4<br>[100 – 148.8] | <0.01[a] |
| Time to peak (min) | 10.8<br>[9.3 – 12.9] | 10.5<br>[10 – 12.8] | 0.77[a] |

### 5.2.5 Extracellular vesicles and clinical outcomes of prematurity

The primary aim of this chapter was to evaluate the changes in concentration, size and origin of EVs during the perinatal adaption period. However, I also wanted to evaluate if the clinical characteristics of preterm infants, influenced the release of EVs during perinatal adaption. This analysis was limited to SEVs on Day 1 of life as it represented the largest group of preterm infants in the study (n=69).

In preterm infants, the gestational age (Spearman's rho = -0.07, *p*= 0.56, *Figure 5.18*), birth weight (Spearman's rho = -0.2, *p*= 0.08, *Figure 5.19*) and SNAPPE-ii illness severity score (Spearman's rho = -0.2, *p*= 0.14, *Figure 5.20*) did not correlate with the concentration of SEVs on Day 1 of life, measured by NTA.

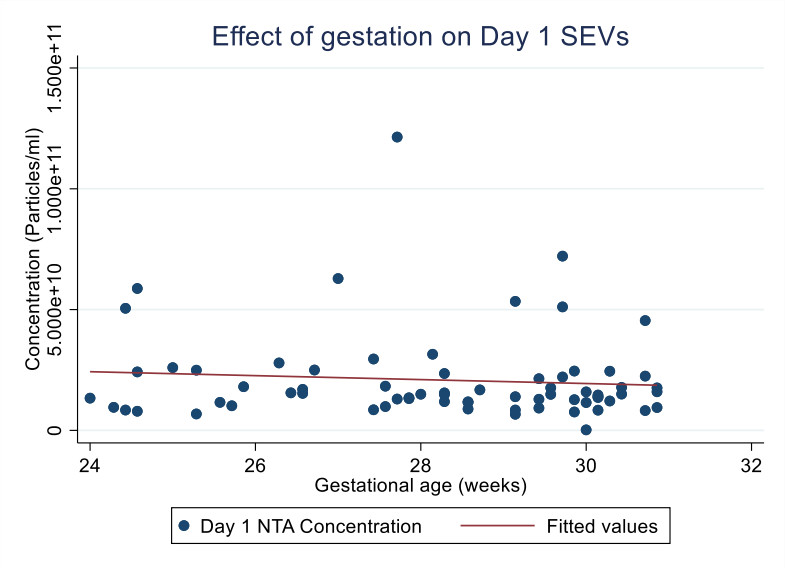


**Figure 5.18: Scatterplot with a fitted linear prediction line of the gestational age and concentration of SEVs on Day 1 measured by NTA in preterm infants**

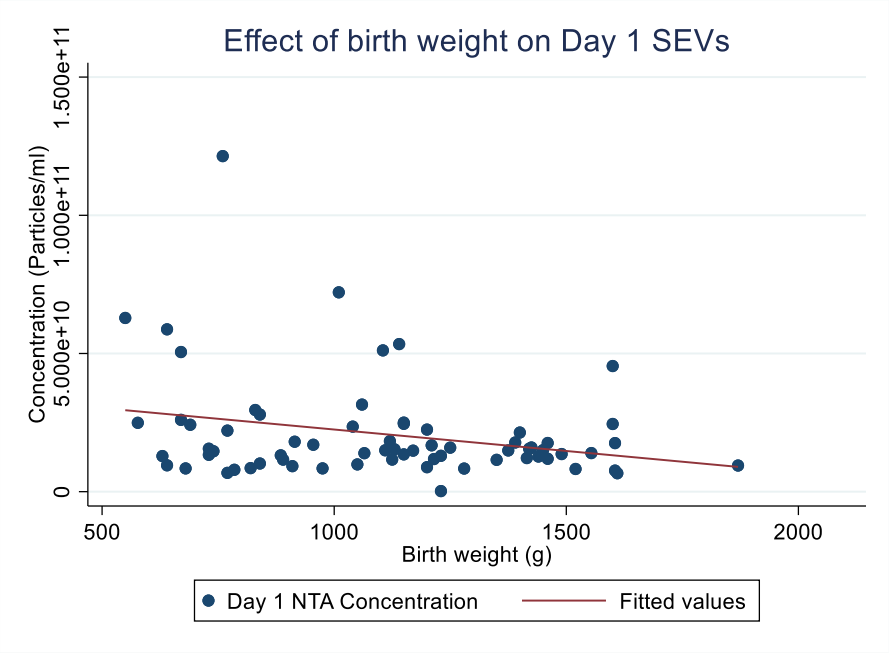


**Figure 5.19: Scatterplot with a fitted linear prediction line of the birth weight and concentration of SEVs on Day 1 measured by NTA in preterm infants**

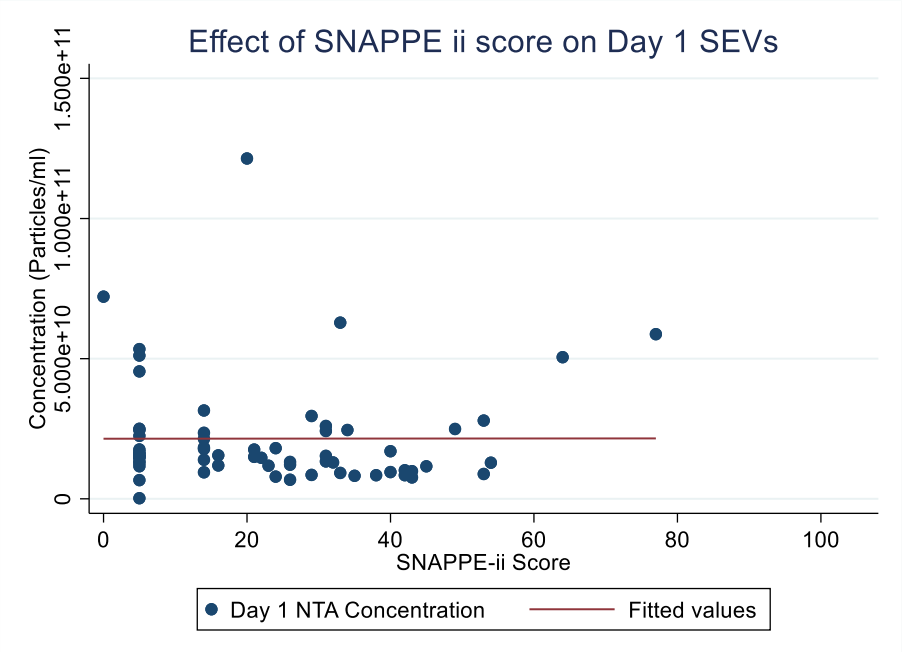


**Figure 5.20: Scatterplot with a fitted linear prediction line of the SNAPPE ii illness severity score and concentration of SEVs on Day 1 measured by NTA in preterm infants**

There was no difference in Day 1 SEV concentrations in preterm infants who developed an IVH (n= 27) compared to those who did not have an IVH (n=42) (*p*= 0.88) (*Figure 5.21*). Similarly, a severe IVH (Grade 3/4) (n=5) was not associated with a higher Day 1 SEV concentration compared to those with no IVH or mild/moderate IVH (n= 64) (*p*= 0.92), although the numbers with severe IVH were small.

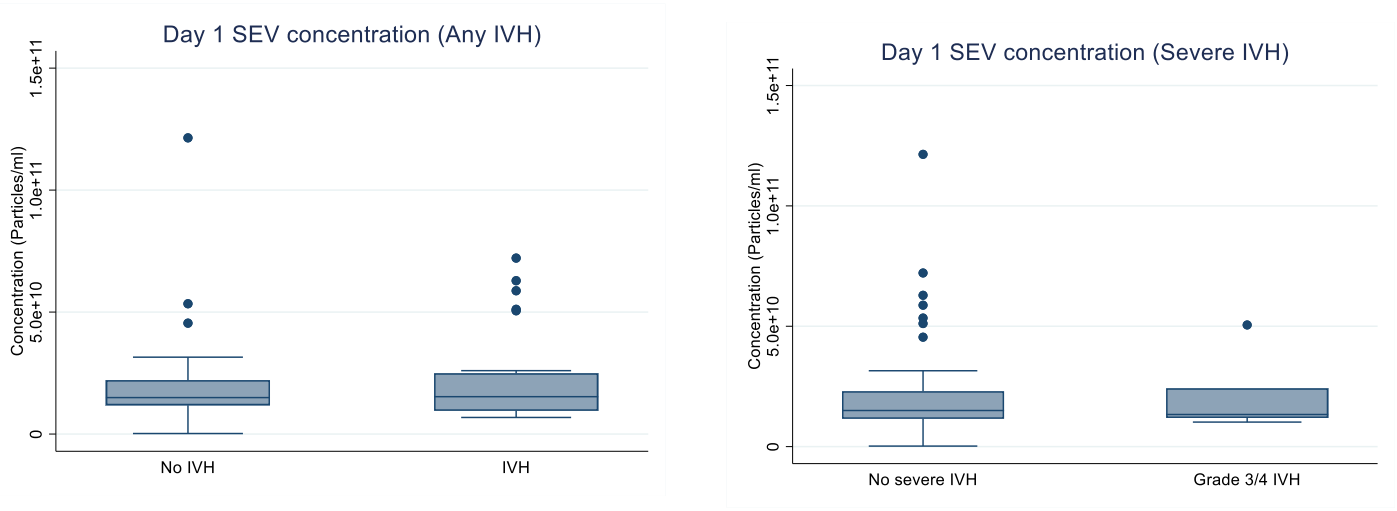


**Figure 5.21: Boxplots of Day 1 SEV concentrations in preterm infants with and without any IVH, and in the presence and absence of severe IVH (Grade 3/4)**

IVH: Intraventricular haemorrhage

As platelets may have a role in closure of the ductus arteriosus, and may be the origin of a majority of circulating EVs, the Day 1 SEV concentrations by presence and absence of a haemodynamically significant duct during hospitalisation was investigated (*Figure 5.22*). There was no difference in Day 1 SEV concentration in the presence (n= 26) and absence (n = 42) of a haemodynamically significant PDA (hsPDA) (p= 0.88).

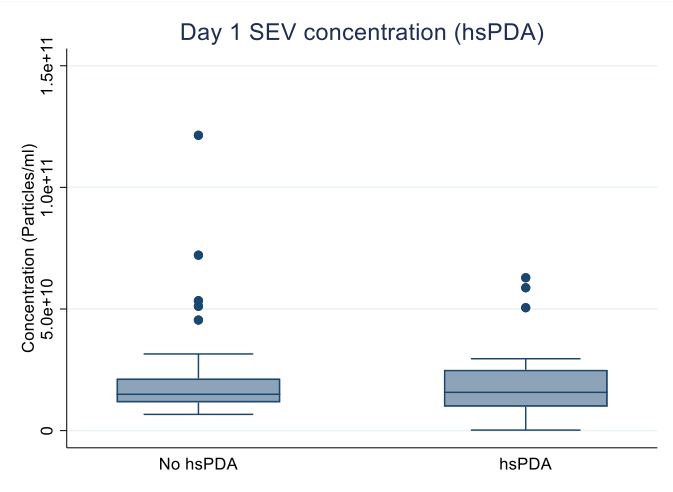


**Figure 5.22: Boxplot of the Day 1 SEV concentration in infants diagnosed with a haemodynamically significant patent ductus arteriosus (hsPDA)**

Finally, to investigate whether inflammatory preterm diseases which develop over the course of time in NICU were associated with an increase in Day 1 SEV concentration, a combined outcome of periventricular leukomalacia (PVL), retinopathy of prematurity (ROP) and chronic lung disease (CLD) was evaluated (*Figure 5.23*). In infants with any of PVL/ROP/CLD (n= 35), there was no difference in the Day 1 SEV count compared to those preterm infants without any of PVL/ROP/CLD (n=34) ($p$= 0.84).

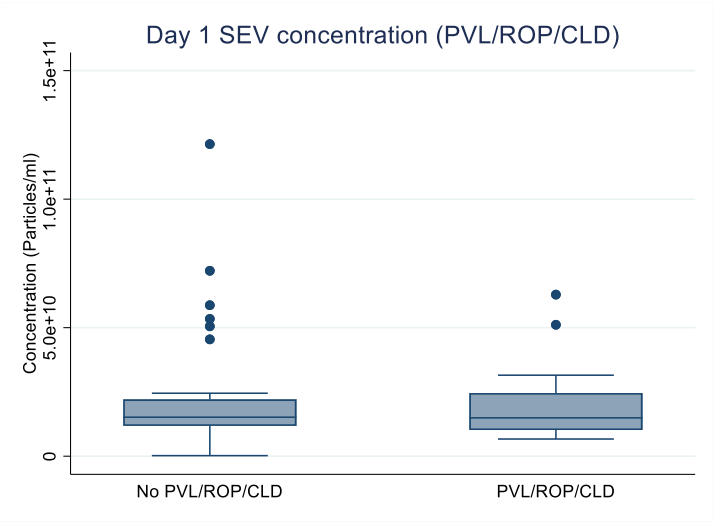


**Figure 5.23: Boxplot of the Day 1 SEV concentrations of infants with an inflammatory outcome (PVL/ROP/CLD) compared to preterm infants with none of these complications**

PVL: Periventricular leukomalacia, ROP: Retinopathy of prematurity, CLD: Chronic lung disease

### 5.3 Discussion

The EVENT study is the largest study to date to describe circulating EVs during perinatal adaptation. I have demonstrated changes in both SEVs and LEVs in preterm infants during perinatal adaption. The concentration of SEVs increased between Cord samples and Day 1 samples, and again between Day 1 and Day 3, and remained elevated at two weeks of age. The concentration of SEVs appears to increase during the first 24 hours of life in preterm infants. Changes in the modal size of SEVs were also detected in the preterm group, with an increase in size of SEVs between cord samples and Day 1 samples.

These changes in concentration of SEVs in preterm infants were mirrored in LEVs with a significant increase between Day 1 and Day 3. However, in contrast to SEVs, the concentration of LEVs was higher in Cord samples than Day 1 samples.

To understand if these changes were related to prematurity, or normal adaption to extrauterine life, I also investigated SEVs and LEVs in a cohort of healthy term infants. Similar to preterm infants, SEV concentration increased between Cord and Day 1 samples, although no significant changes were seen between Day 1 and Day 3 in the term cohort and the modal SEV size remained static over time. Changes in LEVs were similar in the term group to those described above in the preterm group, with a higher concentration of LEVs in cord samples compared to Day 1 samples and a subsequent increase in LEVs between Day 1 and Day 3, although this increase was less substantial than in the preterm group.

In addition, I investigated the effect of the clinical condition of the preterm infant on SEV concentration on Day 1, and found no correlation between birth weight, gestation or SNAPPE ii score and SEV concentration, nor was there any difference in infants with IVH, hsPDA and conditions such as ROP, PVL and CLD and those without.

Together, this data suggests that the changes in EV profiles, seen in premature infants, occur as a function of postnatal age, as opposed to the clinical condition of the infant, although I do acknowledge this study was not designed to evaluate EVs in the context of clinical outcomes. Similar increases in SEV concentration over time were seen in the term group, although the timing of sample collection may have influenced the concentration.

The large increase in LEVs on Day 3 of life was significantly greater in the preterm group, although a smaller increase was also seen in term infants. Interestingly, the

cellular origin of the LEVs in this peak on Day 3 included platelet markers CD42b and CD62P, Tissue factor, VEGFR2 and white-cell marker CD45 LEVs. However, this peak on Day 3 was not associated with an increase in Annexin V positive LEVs or CD41 positive LEVs.

The differential detection of platelet LEVs on Day 3 was unexpected. Both CD42b and CD41 are platelet-specific markers found on the plasma membrane of resting platelets (91, 276, 277), while CD62P is found on activated platelets and is expressed on both endothelium and platelets (278, 279). The functional role of these glycoprotein receptors differ. CD42b (Glycoprotein (GP) Ib) binds von Willebrand factor (vWF) during primary haemostasis, facilitating the adherence of platelets to the endothelium (280). CD41 (GPIIb), as part of the GPIIb/IIIa complex, which is uniquely expressed in platelets, predominantly binds fibrinogen during the aggregation of platelets (281). CD62P (p-selectin) is typically expressed on activated platelets and has a role in leukocyte interactions (282). It has previously been shown that the expression of platelet glycoproteins is influenced by gestation (105).

It is possible that the increase in some PDEVs (CD62P and CD42b) but not others (CD41) in preterm infants may reflect physiological differences in haemostasis in preterm infants. However, this is purely speculative and further studies are required to understand the potential functional implications of the differential expression of glycoproteins on PDEVs.

The procoagulant activity of LEVs was evaluated by flow cytometry, ELISA and thrombin generation. The significantly larger increase in LEVs on Day 3 in preterm infants was not associated with a similar increase in phosphatidylserine expressing LEVs. Both flow cytometry and ELISA demonstrated a significantly higher content of TF-EVs in cord blood compared to postnatal samples. However, the trend towards a higher concentration of TF-EVs in cord samples of preterm infants compared to term infants, detected by ELISA, was not demonstrated by flow cytometry.

There was a significantly greater increase in the release of endothelial LEVs (VEGFR2) between Day 1 and Day 3 in preterm infants, in addition to TF-EVs that could be suggestive of endothelial injury. The implications of this are not known, although there is evidence to suggest that ex-preterm infants are at higher risk of hypertensive disorders in adulthood (283, 284).

The cellular origin of these circulating EVs, and the potential implications of our findings are further discussed in *Chapter 6: Section 6.6.*

Conducting neonatal EV studies is particularly challenging for several reasons, and adherence to the MISEV guidelines is difficult (169). In keeping with the other neonatal studies performed to date, there are several limitations to the EV work described in this study.

MISEV recommends the use of an appropriate control population (285). In this study, healthy term infants (>37 weeks), with no parental history of bleeding disorders were chosen as the controls. In both groups, postnatal samples could only be collected at the same time as clinical venepuncture for ethical reasons. Healthy term infants do not routinely have phlebotomy performed, so postnatal samples were typically obtained in infants undergoing a workup for clinical jaundice. One infant, sampled on Day 1, had a positive Direct Coomb's test (DCT) with confirmed ABO incompatibility jaundice and was excluded from subsequent EV analysis, owing to the known increase in EEVs in this cohort (212, 222). Initially, postnatal control samples were collected on Day 1 of life (0-24 hours) and Day 3 of life (48 – 72 hours) to coincide with the sample timing in the preterm group. However, few healthy term infants undergo phlebotomy in the first 24 hours of life, and most well infants with mild jaundice were sampled on Day 2. A pragmatic decision was taken to broaden enrolment of healthy term controls to allow sampling any time within the first 96 hours of life. Given the changes seen in the first 24 hours of life in preterm infants, the term controls were divided into Day 1 samples (0-24 hours) and Day 3 samples (25 – 96 hours of age).

EV analysis was performed in PPP which was stored at -80˚C after collection, although most samples had not previously undergone a freeze-thaw cycle i.e. it was the first time an individual aliquot was thawed at the time of analysis. While some studies suggest that freezing samples can affect EV number (219), others have provided more reassuring data regarding long term storage at -80˚C (286), although repeated freeze thaw cycles do appear to affect EVs (287). One recent study recommended that EV analysis should be performed on fresh samples (288), however, this would not have been feasible in our study, owing to the 24/7 recruitment of samples and the hospital and laboratory were not co-located.

The 2018 MISEV guidelines sets out recommendations for the characterisation of EVs. The general recommendation includes the identification of three protein markers of EVs and one negative protein marker, in addition to the use of at least two techniques to characterise single EVs, ideally one of which should be electron

microscopy (169). However, these recommendations pose significant challenges to neonatal studies, owing to the small volumes of blood available, challenging venepuncture in neonates and the need to time sample collection with clinical samples for ethical reasons to reduce the risk of pain and infection in this vulnerable group. None of the neonatal EVs studies of vascular biology have met these criteria to date (186).

In this study, NTA was performed in PPP, and not on isolated EVs, owing to the limited sample volume available. It is likely that some of the particles detected within the 200nm range were not EVs, and were lipoproteins such as low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (289). However, in an effort to improve the precision and reproducibility of our findings, NTA was performed over 15 replicates of 60 seconds each, using a protocol previously optimised for plasma and published by our group (262). It was not possible to characterise the cellular origin of SEVs using NTA, as this technique was not available to me.

Flow cytometry was performed in plasma EVs, enriched by centrifugation at 20,800 xg and labelled with platelet, endothelial and leukocyte markers, using a protocol previously published (223, 225). Spectral compensation was applied to fluorophores with overlapping spectra, gates were assigned from size calibration beads and unstained controls and samples were run over 180 seconds or until 30,000 events were recorded. Only a limited number of the assay controls suggested in the MIFlowCyt guidelines were used, including non-stained controls, however, isotype controls and serial dilutions were not performed (290).

Unfortunately, no antibody to detect red cell EV marker was used in these flow cytometry experiments. It is possible that red cell EVs may have accounted for some of the increase in EVs between Day 1 and Day 3. There are changes that occur in red cells during perinatal adaption; changes in haematocrit and haemoglobin concentration over time in preterm and term infants (291), and higher numbers of circulating nucleated red blood cells and reticulocytes in preterm infants (292, 293). However, red cell EVs also may be generated by perinatal haemolysis, either *in vivo* or during sample collection and storage (220). Future studies to evaluate red cell EVs would further characterise the perinatal changes of circulating EVs in preterm infants.

### 5.4 Conclusion

There are changes in size, concentration, procoagulant activity and origin of EVs during perinatal adaption in preterm infants. These seem to be related to the postnatal age, rather than the clinical condition of the infant. These findings have implications for future studies of neonatal EVs, highlighting the importance of the postnatal age at sample collection when comparing groups.

# Chapter 6 : Discussion of Results Chapter I and II

## 6.1 Overview

The first aim of the EVENT Study was to characterise thrombin generation in platelet rich plasma (PRP) in preterm infants and to evaluate the effect of neonatal platelets on thrombin generation. Secondly, I aimed to characterise the circulating extracellular vesicles (EVs) during perinatal adaption in preterm infants, describing their concentration, size and cellular origin, with a particular focus on procoagulant and platelet-derived EVs.

Initially, I performed a comprehensive review of the current literature on thrombin generation in neonates and the role of extracellular vesicles in neonates and these were published in the *European Journal of Paediatrics* (142) and *Pediatric Research (186)* respectively. The findings of these two literature reviews are discussed in *Chapter 1: Introduction I and Chapter 2: Introduction II*, and some of gaps identified in the literature are also discussed here. The key findings of the EVENT Study, which included 101 preterm infants <31 weeks gestation, are discussed below.

### 6.2 Thrombin generation in platelet-rich plasma is enhanced in preterm infants

The lag time and time to peak thrombin were significantly shorter in preterm infants in PRP in both umbilical cord samples and postnatal samples, while the ETP and peak thrombin were also significantly increased in the preterm group in postnatal samples. Collectively, this suggests that preterm PRP is hypercoagulable compared to full-term PRP.

To our knowledge, this is the first study to evaluate thrombin generation in PRP in preterm infants. While preterm infants have prolonged standard clotting times (121), reduced levels of procoagulant factors (119) and hyporeactive platelets when tested *in vitro* (105), this study demonstrates that their PRP is hypercoagulable compared to term PRP. This study further supports the growing body of evidence that preterm infants are hypercoagulable compared to their term counterparts (121, 126).

Tripodi *et al.* hypothesised that preterm infants had a procoagulant imbalance, due to a greater reduction in anticoagulant factors than procoagulant factors (126). In this study, I wanted to evaluate the role of platelets in secondary neonatal haemostasis and this is discussed further in *Section 6.3*. A recent study suggested that blood from the umbilical cord is hypercoagulable compared to neonatal blood measured with both thromboelastography and CAT (270). Here, CAT parameters were compared in a group of preterm infants with both cord and postnatal thrombin generation available. No differences between the two PRP sources to suggest hypercoagulability in cord blood were identified, although the numbers available were small.

Preterm infants are at risk of intraventricular haemorrhage (IVH) (294). Due to the concern about IVH, plasma products are often prophylactically administered to non-bleeding preterm infants with deranged standard clotting tests (295). However, this is not evidence based (296) and the usefulness of standard clotting tests in the preterm population is limited (131). In this study, I found no difference in PRP thrombin generation parameters between infants with and without IVH, although the numbers were small. Similar findings have been described in PPP by Neary *et al*. and Tripodi *et al*. (126, 179). Interestingly, preterm infants with prothrombotic gene mutations (e.g. Factor V Leiden), have been shown to be at increased risk of intraventricular haemorrhage than infants without these mutations (30, 31). It is thought that this is due to hypercoagulability causing venous stasis, thrombosis and subsequent

haemorrhagic infarction. TEG has also been used to assess the prediction of IVH in preterm infants. No significant differences between TEG parameters on admission were shown in infants who subsequently developed an IVH compared with those that did not (163). Moreover, there was a trend towards hypercoagulability in those infants with intracranial haemorrhage. These findings suggests that IVH in preterm infants is not caused by hypocoagulability but may be related to hypercoagulability of preterm plasma.

### 6.3 The potential effect of neonatal platelets on thrombin generation

CAT has been reported to be useful in characterisation of a potential effect of platelets on thrombin generation (135). In order to ascertain whether neonatal platelets contributed to thrombin generation in the EVENT study, thrombin generation was initiated by TF alone (without exogenously added phospholipid; [PRP reagent]) in platelet-rich and platelet poor plasma (PRP and PPP respectively) prepared from UCB samples from a subgroup of preterm and term infants with adequate sample volumes. While the lag time and time to peak were similar in PRP and PPP in the whole group, when evaluated individually, a differential effect of platelets were seen in preterm and term infants. The presence of platelets prolonged the lag time to initiation of thrombin generation and time to peak thrombin generation in the term group, while they had no effect on the lag time in the preterm group but shortened the time to peak thrombin generation. In preterm and term infants, there was a small but significant reduction in ETP and peak thrombin generation in PPP, suggesting that platelets do have a role in thrombin generation during secondary haemostasis.

Platelets, in addition to their role in primary haemostasis, contribute to secondary haemostasis by providing a phospholipid surface for the enzymatic reactions of the coagulation cascade. In neonates, the absolute platelet count is a poor predictor of clinical haemorrhage (102), while the gestation (<34 weeks), timing (<10 days of age) and cause of the thrombocytopenia (NEC) are associated with increased bleeding risk (297).

A small number of adult studies have evaluated the effect of platelets on blood coagulation by characterising thrombin generation in the presence of TF but in the absence of exogenous phospholipid, both in PRP and PPP (134, 135, 139). In PRP, thrombin generation is dependent on the platelet count, function and plasma coagulation factors (298). In adults, thrombin generation is dependent on the platelet count of PRP when this count is below 100 x 10^9/L, while further increases to >100 x 10^9/L have little impact on thrombin generation parameters (134, 135, 139). In adults, the presence of platelets increases ETP and peak thrombin generated in PPP (158).

In the EVENT Study, the increase in ETP and peak thrombin in PRP compared to PPP might be considered to be quite modest given the absence of any platelets or any exogenous source of anionic phospholipid in the PPP samples.

This finding does differ from the data of Haidl et al. in which no difference in thrombin generation parameters were reported in term infant cord plasma samples with a PRP platelet count of 100 x 109/L and 10 x 109/L(158), or in our preliminary results with small numbers of patients (299).

There are several potential underlying reasons. Firstly, Haidl *et al.* investigated these findings in small numbers (n=10) of term infants only, while the EVENT Study evaluated a larger cohort (n=33) and included both preterm and term infants. Secondly, they compared PRP to a low platelet count of 10 x $10^9$/L, while my study compared PRP to no platelets (PPP). In the adult population, PRP thrombin generation, namely ETP and peak thrombin, was reduced below a platelet count of 100 x $10^9$/L (158). It is possible that only a small amount of platelet phospholipid is necessary for thrombin generation in neonates, meaning that a PRP platelet count >10X 10^9/L, may be sufficient to allow normal thrombin generation. Interestingly, recent clinical evidence supports the use of a lower platelet transfusion threshold (i.e. 25 x 10^9/L instead of 50 x 10^9/L) in thrombocytopenic preterm infants (117). Transfusing platelets at the lower threshold was associated with a lower risk of major haemorrhage or death within 28 days. Further work is required to determine the optimal threshold and dose of platelets to administer in thrombocytopenic neonates.

To understand why neonatal thrombin generation may have a low platelet-dependency, Haidl *et al.* performed experiments in the presence of low and high levels of TFPI, to mimic neonatal and adult plasma respectively (158). At lower plasma TFPI levels, thrombin generation demonstrated reduced platelet dependency. However, these experiments were conducted using adult plasma and adult washed platelets, so does not account for the effect of neonatal platelets themselves. Interestingly, their study does appear to show a reduction in peak thrombin in the low TFPI plasma (mimicking neonatal plasma) below a platelet count of 10x 10^9/L, although this is not quantified (*Figure 6.1*).

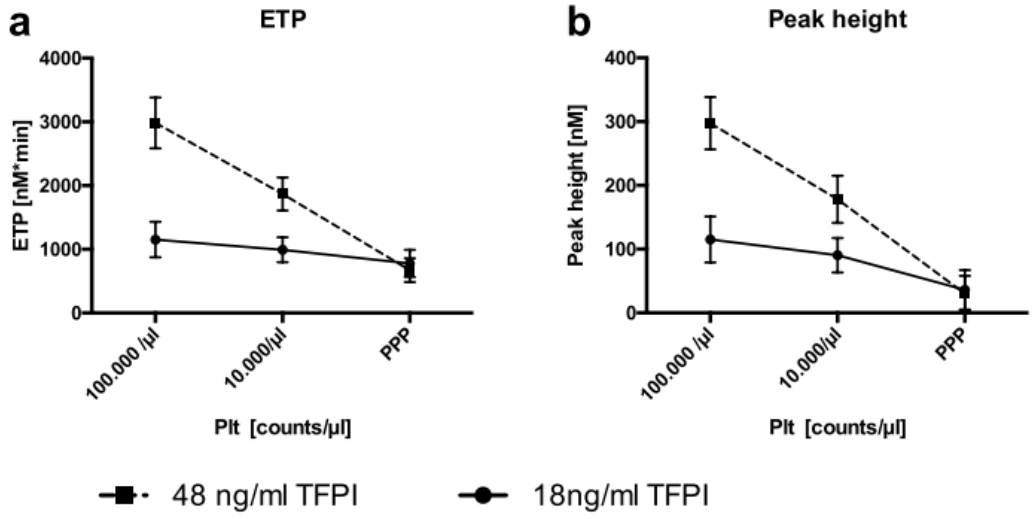


**Figure 4.** Impact of varying TFPI concentrations on platelet count dependency of thrombin generation. CAT measurements were done with TFPI depleted standard plasma reconstituted with recombinant TFPI (18 and 48 ng/ml). ETP (**a**) and peak (**b**) are depicted with varying counts of washed platelets added to the plasma samples (100,000/µl, 10,000/µl, PPP).

**Figure 6.1: Figure published in *Scientific Reports* by Haidl *et al.* "Neonatal thrombocytopenia: Thrombin generation in presence of reduced platelet counts and effects of rFVIIa in cord blood" to investigate the effect of TFPI levels on platelet dependence of thrombin generation"** (158)

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Interestingly, a study by Siegmund *et al.* investigated the effect of platelet count on thrombin generation in patients with Factor VIII and Factor IX deficiency (139). This study showed that with increasing levels of Factor VIII and Factor IX, the platelet dependency of thrombin generation diminished. However, there appears to be a lesser platelet dependency in the neonatal system, which has reduced levels of many factors, although Factor VIII levels are equal to adult levels in preterm and term neonates (119, 120).

The prolongation of the lag time and time to peak thrombin by platelets in the full-term group was unexpected and not seen in the preterm group. I reviewed the effect of platelets on lag time and time to peak thrombin described in adult studies. Haidl *et al.* appears to demonstrate that the presence of platelets causes a prolongation of the time to peak thrombin, compared to PPP in adult samples (*Figure 6.2*), although the statistical significance is not described. Gerotziafas *et al.* did not find that platelets prolonged lag time or time to peak thrombin, however, they used a different concentration of tissue factor in their experiments (134). Siegemund *et al.* and

Hemker *et al.* did not describe the effect of platelets on lag time or time to peak thrombin (139, 298).

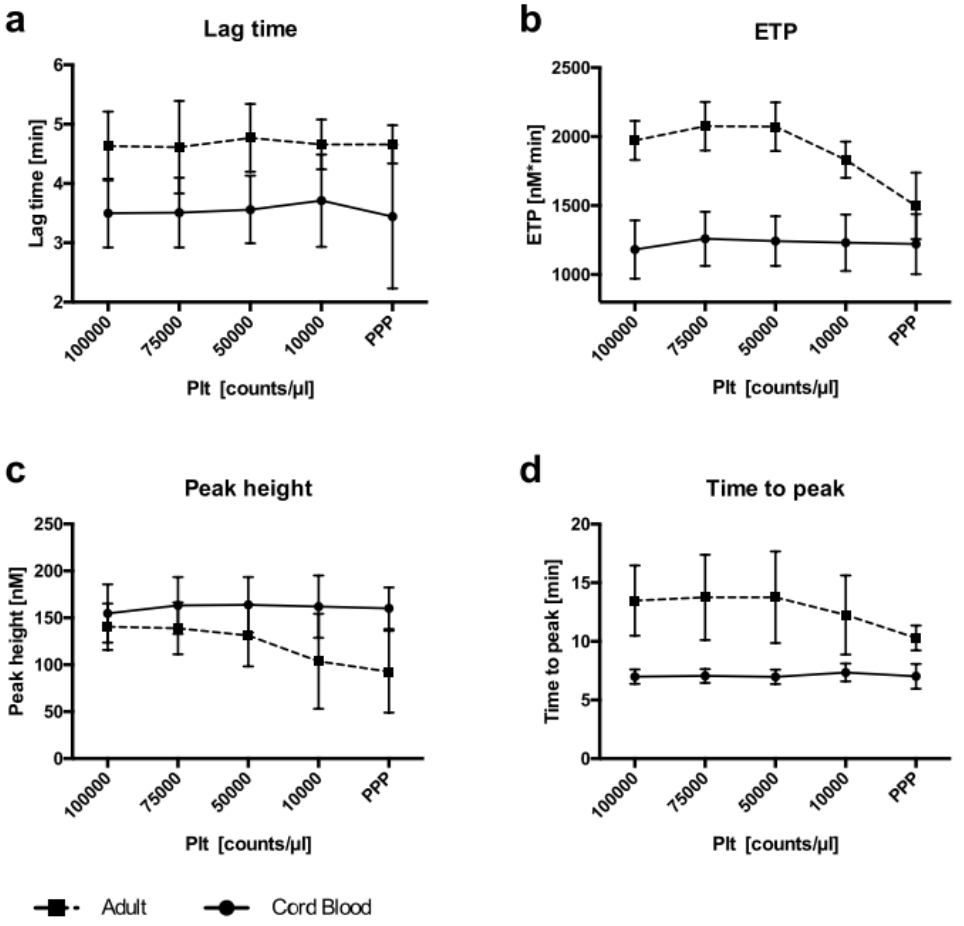


**Figure 2.** Impact of decreasing platelet counts on thrombin generation parameters without rFVIIa. Course of parameters of thrombin generation – lag time (**a**), endogenous thrombin potential (ETP) (**b**), peak height (**c**), time-to-peak (ttpeak) (**d**) – in cord blood derived and adult plasma with platelets at different platelet counts (100,000/µl, 75,000/µl, 50,000/µl, 10,000/µl and PPP; n = 5).

**Figure 6.2: Figure published in *Scientific Reports* by Haidl *et al.* "Neonatal thrombocytopenia: Thrombin generation in presence of reduced platelet counts and effects of rFVIIa in cord blood" to investigate the effect of platelet counts on thrombin generation in neonates and adults** (158)

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A study by Ferrer-Marin *et al.* evaluated the *in vitro* effects of transfusing adult and neonatal platelets on coagulation using TEG (116). The transfusion of neonatal platelets to thrombocytopenic adult blood caused a reduction in reaction time, compared to the transfusion of adult platelets. Similarly, the transfusion of adult platelets to thrombocytopenic cord blood caused a prolongation of reaction time,

compared to the transfusion of neonatal platelets. This suggests that neonatal platelets reduce the time to initiation of coagulation, while adult platelets prolong the initiation of coagulation. However, the authors concluded that, as platelets were not thought to influence reaction time (300) the differences seen were due to contamination of the platelet concentrate with neonatal plasma, which contains lower concentrations of inhibitors such as TFPI.

The findings by Haidl *et al. (158)* would dispute this conclusion by Ferrer-Marin *et al.* in the above study (116). The only difference between PRP and PPP samples in the Haidl *et al.* study was the presence and absence of platelets, the plasma was from the same patient so would be expected to contain similar coagulation factors (158). It is unclear what platelet factor could prolong the lag time and time to peak thrombin. Polyphosphates, present in platelet granules, have been shown to reduce lag time and time to peak thrombin in both adult and cord blood (155). While platelets do contain TFPI, it is not expressed on the surface of resting platelets (301). The experiments discussed below in *Section 6.4* did not identify any differences in TFPI to explain differences in lag time and time to peak in preterm infants, although they only measured plasma TFPI and could not account for TFPI contained within platelets. A previous study has demonstrated the phosphatidylserine exposure on neonatal platelets and phospholipid content of neonatal platelets is similar to adult platelets (302). Few studies have evaluated the proteomic content of neonatal platelets. Stokhuijzen *et al.* compared the platelet proteome of neonatal and adult platelets in a small number of infants (n=5) (303). While there was no difference in the platelet glycoprotein receptor content, proteins associated with platelet degranulation and activation were downregulated in the neonatal group. No studies to date have compared the platelet proteome between preterm and term infants. This may be useful to further elucidate the differential effect of platelets on lag time and time to peak thrombin in the preterm and term group.

Circulating EVs may contribute to phospholipid dependent thrombin generation, and may have a greater haemostatic role in neonates than adults, thus reducing the platelet dependency of thrombin generation (154, 164). Schweintzger *et al.* previously demonstrated that neonatal plasma EVs had a greater impact on thrombin generation, measured with CAT, than adult plasma EVs.

Previous studies have shown that platelet derived EVs (PDEVs) increase thrombin generation in a dose dependent manner and reduce lag time and time to peak thrombin (304). Moreover, the phosphatidylserine component of EVs appears to influence thrombin generation, not the tissue factor component. A study of patients with multiple myeloma demonstrated that the addition of LEVs (isolated at 20,000 xg) to control plasma increased peak thrombin, ETP and reduced lag time and time to peak, while the addition of SEVs (isolated at 100,000 xg) did not (305). A study by Melnichnikova *et al.* of patients with glioma, showed a significant negative correlation between the amount of CD41 EVs and lag time, and a significant positive correlation between the amount of CD41 EVs and ETP (306). Ivanov *et al.* reported that microparticle (MP) rich plasma had a significantly increased peak thrombin compared to platelet free plasma or microparticle depleted plasma, when stimulated with TF only (no phospholipid) (307). When MP rich plasma was added to PRP at varying platelet counts, there was a minimal effect on thrombin generation at a platelet count of 150 x $10^9$/L, but a larger effect on thrombin generation at a platelet count of 1.5 x $10^9$/L, suggesting that EVs may have a more significant role in thrombin generation in thrombocytopenic states.

While evidence suggests that EVs have an effect on thrombin generation, by reducing lag time and time to peak and increasing peak thrombin and ETP (304), it is not clear whether this may have influenced the hypercoagulable changes seen in preterm PRP. My flow cytometry results suggest that the concentration of total LEVs, platelet EVs and TF-EVs are similar in Cord samples and Day 1 samples in preterm and term infants. The large increase seen on Day 3 in the preterm group did not influence the thrombin generation findings as CAT was only performed in Cord and Day 1 samples in the preterm group. The concentration of SEVs was similar in preterm and term infants in Cord samples, and although higher on Day 1 in the term samples, previous studies suggest that SEVs have a less significant impact on thrombin generation (305).

I will further discuss the haemostatic role of EVs below in *Section 6.6.2.*

### 6.4 TFPI levels do not explain the shortened lag time in preterm infants

In the EVENT study, I showed that preterm infants have significantly shorter lag time and time to peak thrombin than full-term infants in PRP, and similar findings have been reported in PPP between preterm and term infants (121).

Cvirn *et al.* previously showed that neonates have lower TFPI levels than adults, and these lower TFPI levels are responsible for the shortened lag time in the full-term neonatal group (159). Moreover, two studies by our group have demonstrated that increased levels of TFPI, in severe pre-eclampsia and in COVID-19, were responsible for longer lag times in these groups, and that the inhibition of TFPI abrogated these findings (137, 269).

I investigated the role of TFPI in neonatal thrombin generation, to establish if TFPI levels were lower in preterm infants than term infants, thus explaining the shorter lag times in this group. However, the addition of anti-TFPI to PPP had a very similar effect on thrombin generation in preterm and term infants, and both TFPI levels and TFPI activity were similar between groups. This suggests that differences in the amount or activity of TFPI does not explain the differences seen in lag time and time to peak between preterm and term infants. The limitation to this is that the endothelial activity of TFPI *in vivo* cannot be accounted for.

Tissue factor also influences the initiation of coagulation, with higher levels associated with shorter lag times (308). TF-EVs are an important source of circulating TF (309), and I aimed to understand whether preterm infants had increased levels of tissue factor EVs compared to term infants. TF-EVs were evaluated in several ways, firstly using thrombin generation in PPP with Microparticle reagent, which renders the assay dependent on the tissue factor content of EVs, secondly by measuring the number of TF-EVs by flow cytometry and finally by quantifying the concentration of TF-EVs in plasma by ELISA.

I identified no difference in thrombin generation between preterm and term infants in the presence of MP reagent. Similarly, TF-EV levels were similar in preterm and term infants in Cord and Day 3 samples measured by ELISA, and by flow cytometry in Cord and Day 1 samples, although the concentration of TF-EVs was higher on Day 3 in preterm infants. This does not confirm or out rule differences in TF-EVs as the cause of the reduced lag time in the preterm group, and larger numbers would be required to it further investigate this.

When CAT is initiated by 1pm TF, in addition to the association between TFPI and lag time, increasing levels of Protein S and fibrinogen are also associated with increased lag time (308), both of which are increased in term infants compared to preterm infants (119, 121, 126). While the cause of the shorter lag time in the preterm group has not been established, it is possible that it is a cumulative effect of several factors including slight reductions in TFPI levels, fibrinogen and Protein S and increases in TF-EVs. Moreover, the differential effect of neonatal platelets on lag time and time to peak discussed in *Section 6.3*, may also suggest that platelet derived factors could influence lag time in preterm infants.

### 6.5 There are changes in the concentration and size of circulating extracellular vesicles during perinatal adaption

The EVENT Study is the largest study to date that evaluates circulating EVs in both preterm (24 – 31 weeks) and term infants. In this study, I found an increase in the concentration of both SEVs and LEVs between Day 1 and Day 3 in preterm infants. There were also changes in the modal size of SEVs during perinatal adaption. While the concentration of SEVs and LEVs increased over time in term infants, the levels of both SEVs and LEVs were significantly higher on Day 3 in the preterm group. Currently, little is known about the circulating EVs during the perinatal adaption period (186). Two recent studies showed that the circulating EVs change over time (255, 256). Ohta *et al.* demonstrated an increase in EV concentration over the first year of life in preterm infants (256). Interestingly, they found that EV concentration at birth correlated to gestational age, while I found no such relationship. However, there are several differences between my study and this study. Firstly, the authors did not investigate the changes during the first two weeks of life, a high risk time for very preterm infants and only included infants >1500g, a group at lower risk of complications of prematurity. EVs were evaluated in serum, while I used plasma EVs, which are of greater relevance for investigating potential haemostatic roles of EVs and are the preferred source of EVs (207). Penas Martinez *et al.* demonstrated that EVs in cord blood of full-term infants had lower protein concentrations, smaller EVs and upregulation of several haemostatic proteins including von Willebrand factor, Factor VIII and alpha-2 macroglobulin, compared to adult EVs (255). These two studies suggest that in both healthy full-term newborns, and preterm infants, the circulating EVs change with increasing age.

I was particularly interested in the change in these EVs over the first days of life, given the physiological and pathological changes that occur during perinatal adaption to extrauterine life in preterm infants. A pilot study in our lab by O'Reilly *et al.,* also demonstrated an increase in size of SEVs and LEVs in a small number of preterm infants (n=21) (218). The pilot study noted a reduction in the proportion of CD41/Annexin V positive LEVs between Day 1 and Day 3 of life in preterm infants, suggestive of an early platelet activation event. However, the pilot study did not include healthy term infants to evaluate if these changes were a physiological or pathological response to preterm birth and the cellular origin of the increase in LEVs on Day 3 was unknown.

## 6.6 Changes in the origin of circulating extracellular vesicles during perinatal adaption in preterm infants

In the EVENT study, for the first time, I have characterised the cellular origin of circulating EVs during perinatal adaption, particularly of the LEV increase on Day 3 in preterm infants, which was previously unknown. I found that while the concentration of most LEVs increased between Day 1 and Day 3 in both preterm and term infants, CD42b, CD62P, TF and VEGFR2 LEV concentrations were significantly higher on Day 3 in preterm infants compared to full-term infants, while Annexin V and CD41 LEVs concentrations remained at similar levels to term infants throughout.

### 6.6.1 Platelet-derived extracellular vesicles

The pilot study by O'Reilly *et al.* suggested that platelets were not responsible for the increase in Day 3 LEVs, due to the reduction in the proportion of CD41/Annexin V LEVs during that time (218). It now appears that platelet derived EVs (PDEVs), namely CD42b and CD62P, may contribute to this increase in LEVs.

In order to hypothesise why there is a differential expression of platelet antigens on LEVs on Day 3 their function must first be considered. CD42b (Glycoprotein(GP) Ib) is uniquely expressed on platelets and binds von Willebrand factor (vWF) during primary haemostasis, facilitating the adherence of platelets to the endothelium (280). CD41 (GPIIb), as part of the GPIIb/IIIa complex, which is uniquely expressed in platelets, predominantly binds fibrinogen during the aggregation of platelets (281). CD62P (p-selectin) is typically expressed on activated platelets and has a role in leukocyte interactions (282).

Platelet glycoprotein expression is influenced by gestation. Sitaru *et al.* demonstrated reduced levels of GPIIb (CD41) and GPIIb/IIIa (CD41/CD61 complex) in platelets in peripheral preterm neonatal samples compared with adults (105). However, platelet expression of GPIb (CD42b) was similar in preterm and term infants compared to adults. Wasiluk *et al.* demonstrated reduced levels of CD62P expression in preterm infants compared to term infants, with the level of expression correlating with gestation in preterm infants (310).

From a functional perspective, the neonatal and adult haemostatic systems are different. Although neonates have significantly reduced levels of procoagulant factors compared to adults, they have several compensatory mechanisms including

increased von Willebrand factor levels and reduced anti-coagulant factors (120, 123). Healthy full term neonates appear to have intact primary haemostasis, demonstrated by shorter bleeding times and PFA-100 closure times compared to adults (110, 112), reportedly due to this increase in von Willebrand factor levels, particularly of larger functionally active polymers (123). Neonatal platelets are hypo-reactive to several agonists *in vitro*, including epinephrine, thrombin, ADP and collagen (105, 107, 108). However, when stimulated with Ristocetin, neonatal platelets were hyperreactive compared to adult platelets (109). In contrast to the increased von Willebrand factor levels in preterm infants, fibrinogen levels are reduced in very preterm infants compared to full-term infants (119).

It is clear that neonatal haemostasis is different to adults, with a highly effective platelet/vWF interaction as a compensatory haemostatic mechanism. It is possible that the relatively increased expression of CD42b LEVs on Day 3, not seen in CD41 LEVs, may be related to an increased functional role of CD42b in preterm haemostasis, although this is purely speculative and this hypothesis will need further testing.

The relative difference in expression of various PDEV antigens seen in the EVENT study, may also explain some of the discrepancies between PDEV findings reported in previous studies. Schmugge *et al*. demonstrated a higher proportion of PDEVs (CD41 positive) in cord blood and postnatal neonatal samples compared to adults (174). Wasiluk *et al.* demonstrated an increased proportion of PDEVs (CD61 positive) in preterm compared to term UCB (175). In contrast Hujacova *et al.* found no difference in PDEVs (CD41/ CD36) or activated PDEVs (CD41/ CD62P) between preterm and term infants in umbilical cord blood (220) and our findings in umbilical cord blood support this. It is likely that the platelet marker chosen to evaluate PDEVs influences the study findings.

### 6.6.2 Procoagulant EV activity

It has been suggested that procoagulant EVs may have a compensatory role in neonatal haemostasis (154). The procoagulant activity of EVs is likely related to the exposure of phosphatidylserine on their outer membrane, providing a phospholipid surface for the enzymatic reactions of the coagulation cascade, in addition to exposure of TF on EVs derived from endothelial cells and monocytes (170). However, the procoagulant activity of TF on EVs is controversial, with some studies suggesting that under physiological conditions, phosphatidylserine is the only part of EVs with procoagulant activity, while under pathological conditions, EVs do seem to express functional TF with procoagulant activity (304). Extreme prematurity likely represents a pathological condition, given the high incidence of haemorrhage and thrombosis associated with this condition.

In the EVENT study, I evaluated the procoagulant activity of LEVs using three techniques. Firstly, I used Annexin V-FITC to evaluate the proportion of LEVs displaying phosphatidylserine by flow cytometry. In preterm infants, the proportion of Annexin V positive LEVs reduced over the first three days of life, although there was a small increase in the concentration of Annexin V positive LEVs between Day 1 and Day 3. Interestingly, the spike in total LEVs on Day 3 in preterm infants was not associated with a similar increase in Annexin V positive LEVs, suggesting that phosphatidylserine is not expressed on all of these LEVs. Previous studies have shown that less than half of EVs may display phosphatidylserine and thus bind to Annexin V (195).

Using flow cytometry, TF/Annexin V positive EVs were increased in cord samples compared to postnatal samples in preterm infants. Using a TF-EV activity ELISA, I also demonstrated an increased concentration of TF-EVs in cord samples compared to postnatal samples on Day three of life in the preterm group. This is interesting, as Raffaeli *et al.* has previously demonstrated that cord blood is hypercoagulable compared to postnatal blood in preterm and term infants (270). Using the ELISA technique, there was also a trend towards an increased concentration of TF-EVs in preterm cord samples compared to term cord samples but this did not reach statistical significance. There was no such difference between TF-EVs measured by flow cytometry between preterm and term infants in umbilical cord blood. Finally, when CAT was performed in the presence of MP reagent, to determine the activity of tissue factor EVs, thrombin generation was similar in preterm and term infants,

although peak thrombin was increased in the term group. This suggests that TF-EVs had a similar impact on thrombin generation in preterm and term infants.

### 6.6.3 Endothelial and leukocyte extracellular vesicles

The large increase in LEVs on Day 3 in the preterm group was associated with a significant increase in TF and VEGF Receptor 2-LEVs, while CD45 LEVs remained similar between preterm and term infants over that period.

Even fewer studies have described the circulating EEVs during perinatal adaption in premature infants. Campello *et al.* demonstrated a significant increase in Annexin V positive EVs, EEVs, TF- EVs and PDEVs in umbilical cord blood of infants born to women with pre-eclampsia, a pathology associated with endothelial dysfunction, compared to healthy controls (176). Hujacova *et al.* demonstrated no difference in EEVs (CD31/CD105) between preterm and term infants in cord samples, and no correlation with gestational age (220). Our findings support the similar levels of EEVs, although with a different EEV marker (VEGFR2), in cord blood of preterm and term infants.

In critically unwell infants undergoing ECMO, Annexin V positive EVs and EEVs (MadCAM) were elevated compared to healthy term controls (223). While this suggests that critically ill infants may sustain vascular endothelial injury, a significant confounder in this study, given what I have shown in the EVENT Study, was the time of sample collection varied between the ECMO group (104 hours) and controls (58 hours).

Sibikova *et al.* recently demonstrated an increase in several EEVs in cord samples of infants born by spontaneous vaginal delivery compared to those born by elective caesarean section (225). However, these differences were no longer present on Day 3 of life. While this work demonstrates that the mode of delivery, or potentially the presence or absence of labour may influence circulating EVs at birth, it does not explain the difference seen in our LEVs on Day 3 of life in the preterm group, as the differences relating to mode of delivery had resolved by that time.

It has previously been shown that endothelial EVs (CD144 positive) are elevated in the presence of ABO incompatibility haemolytic disease of the newborn (212, 222), likely due to endothelial injury mediated by anti-A or anti-B antibodies against ABO antigens on the vascular endothelium. However, as none of the preterm group had

positive Coombe's tests, this is unlikely as a cause of the elevated EEVs on Day 3 of life.

VEGF Receptor 2 was investigated as an endothelial EV marker, given its potential role in the pathogenesis of intraventricular haemorrhage in preterm infants as discussed in *Chapter 1: Section 1.3*. Briefly, VEGF levels, an angiogenic growth factor, are increased within the germinal matrix of premature infants and animals, resulting in increased highly vascularised network of fragile vessels (17). Prenatal inhibition of VEGFR2 in animal models was associated with a reduced incidence of IVH in premature rabbit pups (17, 18).

I identified a significant increase in VEGFR2 on Day 3 of life in preterm infants, in addition to TF-EVs that may be suggestive of endothelial injury. While there is little information regarding endothelial injury in preterm infants, there is evidence to support an increased risk of hypertensive disorders in young adulthood among infants born preterm (283). A study by Bertagnolli *et al.* demonstrated dysfunction of endothelial colony forming cells in adulthood in infants born preterm, in addition to evidence of clinical hypertension (284), while another study demonstrated impaired flow mediated arterial dilatation in ex preterm infants (311). Further longitudinal studies are required to evaluate endothelial function in preterm infants, during the neonatal period, childhood and adulthood, given the known increased risk of cardiovascular disease following preterm birth.

White cell EVs were found in very low concentrations in preterm and term infants. This is not unexpected given low levels are typically seen in healthy adults (312).

### 6.7 Extracellular vesicle content of cord blood differs from postnatal blood

Several studies described neonatal EVs in UCB owing to the larger volumes available and the ethical restrictions to blood sampling in preterm neonates (164, 175, 313). However, UCB may not be the optimal fluid for the evaluation of neonatal EVs, except in conditions such as PET, where placental EVs are relevant (176). However, obtaining adequate UCB samples from extremely preterm infants in the delayed cord clamping era may be a challenge (314).

While platelets are similar, in number, size, structure and function, in cord blood, and peripheral samples taken in the first 24 hours of life (105), it was not clear whether UCB EVs are representative of neonatal EVs in the first Day of life. Our study suggests that there are fundamental differences between cord EVs and peripheral neonatal EVs, particularly in preterm infants. The concentration of SEVs increased from Cord samples to Day 1 samples in both preterm and term infants, while the concentration of Annexin V + LEVs was higher in cord samples than postnatal samples. Similarly, the concentration of platelet EVs (CD41, CD42b and CD62P) and TF-EVs were all higher in cord samples than postnatal Day 1 samples in both preterm and term infants. The modal SEV size was also smaller in cord blood compared to postnatal Day 1 samples in preterm infants.

This data suggests that UCB EVs are not identical to postnatal peripheral EVs. While UCB samples will likely continue to be used, owing to the volume restrictions in preterm infants, it is important to be aware of their limitations.

## 6.8 Study limitations

### 6.8.1 Challenges of conducting haemostasis and coagulation studies in neonates

There are several difficulties associated with conducting research studies in the neonatal population. First, is the issue of consent. For neonatal studies, an infant's guardian must give informed consent for participation on their behalf. Obtaining informed consent can be challenging for neonatal studies, particularly those which occur around the time of birth. Evidence suggests that most parents are supportive of research studies, believing that centres conducting high volumes of research provide better care, in addition to a desire to improve the care of future premature infants (315). Most parents would prefer to discuss research studies in the antenatal period (316), although this is not always possible, owing to the precipitous nature of some preterm births. In this study, 50% of the preterm participants were approached in the antenatal period and while the remainder were approached in the postnatal period. 16.5% of parents approached declined participation, often citing concerns about the size or condition of their infant. However, there was a group of parents who were not available to approach for consent, often due to the clinical condition of the mother, and another group of infants who received blood products before the study could be discussed and were therefore no longer eligible for inclusion.

For obvious reasons, only small volumes of blood can be taken from neonates for research studies, owing to the limited blood volume of these preterm infants and the high risk of iatrogenic anaemia (313). Moreover, phlebotomy in preterm infants is challenging, even by experienced clinicians, and this is reflected in the high incidence of clotted or insufficient samples described in the preterm group, particularly on Day 3 of life (43.9%).

Historically, UCB was used as an alternative to postnatal neonatal samples given the larger volumes available. However, this study highlights the low success (48.2%) of obtaining cord blood in preterm infants in the delayed cord clamping era. Moreover, only 10 (37%) of the preterm cord samples obtained had a volume of at least 6 ml, which by comparison to the volumes available in adult platelet studies, is low. This study and other studies have highlighted the fundamental differences between cord blood and postnatal blood in terms of the EV content and markers of coagulation (270), and these need to be considered when interpreting results.

To perform thrombin generation, at least a 1.3 ml sodium citrate container, filled to the line was required. In several instances on Day 1, the clinician was unable to collect this volume of blood and a 0.5 ml sodium citrate container was selected instead, and thus thrombin generation could not be performed. Moreover, there were several instances where insufficient PRP was obtained from 1.3 ml of citrated whole blood, owing to the high haematocrit in neonates, particularly full-term infants. Thrombin generation in PRP must be run fresh and cannot be freeze-thawed as it influences the thrombin generation parameters (134). Finally, the low platelet count in PRP in postnatal samples, suggests that making PRP from 1.3 ml of citrated blood in the early neonatal period in preterm or term infants may not be feasible.

Standard clotting tests (PT and APTT) were not performed as part of this study, owing to the small volume of plasma available and the limited value of standard clotting tests in preterm neonates (121). Moreover, the management of standard clotting tests which fall outside the expected range in non-bleeding preterm infants is controversial, and inclusion of these tests could have resulted in increased use of prophylactic plasma transfusions which is not evidence based (131).

A large proportion of preterm infants receive blood products, including red cells, platelets and plasma during the neonatal period (317). This presents a challenge for haemostasis and coagulation studies. In this study infants were excluded from further sample collection if they had received any blood product, as we could not ensure that the EVs measured derived from the infant and not from the adult donor. This resulted in a high attrition rate of Day 3 and Two week samples, particularly in the more preterm and unwell infants. Other studies have used a washout period after blood product administration prior to sample collection (126), although given the paucity of information about EVs during perinatal adaption and following blood product administration, including infants who had received a blood product would have made the findings more difficult to interpret.

### 6.8.2 Implications of COVID-19 on research

This research project began in July 2019 and was severely affected by the COVID-19 pandemic. Unfortunately, due to the COVID-19 emergency, all clinical studies at the Rotunda Hospital were stopped for a three-month period between 13th of March 2020 and 12th of June 2020, and RCSI postgraduate research students were required to work from home. No patients were recruited during this time.

In addition, COVID-19 restrictions resulted in reduced access to the laboratory facilities at the Conway Institute in UCD and the calibrated automated thrombography equipment, following this period of complete lockdown, significant delays in sourcing reagents and significant delays in fixing broken parts of essential machines.

However, I took a number of steps in order to mitigate the effects of the COVID- 19 restrictions on this study. Firstly, during the three months period of mandatory “work from home”, I carried out two literature reviews of the role of EVs in neonates and the role of calibrated automated thrombography in neonates, both of which have been published (142, 186). Secondly, I used the preliminary data available on the perinatal extracellular vesicle release in preterm infants and thrombin generation in platelet rich plasma to publicise our study, gain valuable presentation skills and develop my research questions. The work was presented as oral and poster presentations at the Joint European Neonatal Societies Congress, the International Society of Thrombosis and Haemostasis Conference and the American Society of Haematology Annual Meeting during my PhD. Finally, I used the skills I had already developed as part of my PhD to complete a smaller study evaluating the haematological effects of COVID-19 infection during pregnancy and this is discussed in *Chapter 7: Neonatal clinical and haematological outcomes following COVID-19 in pregnancy*.

## 6.9 Future directions

### 6.9.1 Introduction of global coagulation assays into clinical practice

In this study, I have demonstrated that thrombin generation in PRP is hypercoagulable in preterm infants compared to full-term infants, and other studies have shown similar findings in PPP (121, 126). The use of standard clotting tests, which are prolonged in preterm infants, does not reflect this, nor do standard clotting tests predict the risk of clinical bleeding (121). A recent study by Raffaelli *et al.,* showing hypercoagulability in cord blood compared to postnatal neonatal blood, nicely demonstrated the limitations of standard clotting tests (270). Standard clotting tests, which were similar in cord blood and postnatal samples in their study, were not able to detect the hypercoagulable changes identified on both TEG and CAT in cord blood.

While CAT is very useful tool for mechanistic studies of neonatal haemostasis, as described in *Chapter 1: Section 1.11*, there are several reasons why TEG may be more suitable in clinical practice. Firstly, TEG is performed in whole blood, while CAT is performed in plasma, thus requiring the manual preparation of PPP/PRP. CAT reagents can be expensive, particularly in the context of running samples on single patients in real-time, instead of in batches, while TEG is designed for single patient analysis in real-time. Moreover, CAT does not evaluate primary haemostasis or the effect of red or white cells, vascular endothelium or blood flow on secondary haemostasis (135). TEG has the advantage of evaluating the effect of the cellular components of blood on coagulation and the fibrinolytic system, although neither TEG nor CAT can evaluate the effect of vascular endothelium of blood flow on secondary haemostasis. Standard CAT in duplicate requires up to 320 µl of plasma, while TEG requires only 340 µl of whole blood per analysis (146).

Neonatal reference ranges have been reported for TEG/ROTEM (145, 318), the intra-assay reliability is acceptable in TEG, even in VLBW infants (146) and neonatal treatment algorithms have recently been published (151).

### 6.9.2 Implications for the design of future studies of neonatal extracellular vesicles

In this study, I have shown that circulating EVs changes during postnatal adaption in both preterm and term infants. These findings have significant implications for the conduct of future neonatal EV studies. It is essential that the postnatal age of life at the time of sample collection is considered in future studies, to ensure that solely the pathology being investigated, not merely the timing of the sample collection, is influencing the changes in EV profiles under investigation.

Furthermore, while cord plasma may be the preferable source of EVs in neonatal studies from a feasibility perspective, the volume of plasma available, ease of sampling, ethical considerations, it is important to be aware of its limitations and the differences between cord and postnatal EV profiles.

### 6.9.3 The use of more sophisticated techniques to characterise EVs during perinatal adaption.

In this study, NTA, flow cytometry, ELISA and calibrated automated thrombography were used to evaluate the role of EVs in prematurity. Further characterisation of EV profiles using proteomic techniques would yield greater information about the potential role of these EVs during perinatal adaption. While the concentration and source of these EVs is useful, it can only begin to direct us towards the functional implications of these EVs. To understand how these EVs act at a physiological level, a better understanding of the proteins involved would be required.

While EV miRNA have been investigated as biomarkers in several neonatal studies (227, 228), a recent study suggested that miRNA were a minor cargo of EVs which were rarely delivered to target cells (319).

Future studies should evaluate red cell derived EVs as a possible source of the changes in circulating EVs during perinatal adaption. The original discovery of EVs in the 1980's, described the release of vesicles containing transferrin from maturing reticulocytes (191, 192). Preterm infants have higher levels of reticulocytes and nucleated red blood cells than full-term infants (292, 293), and there are changes in both haematocrit and haemoglobin concentration during the first days of life (291). However, caution is required in the evaluation of red cell EVs as they may be generated by perinatal haemolysis, either *in vivo* or during sample collection and storage (220). Future studies could evaluate red cell EVs during perinatal adaption.

### 6.9.4 Further evaluation of the non-haemostatic role of platelets in neonates

To date, most neonatal platelet studies have focused on their role in haemostasis. However, as I have previously discussed in *Chapter 1: Section 1.8*, platelets appear to have a role in closure of the patent ductus arteriosus (98) and in the immune response (95).

Evaluating markers of platelet activation, and particularly the response of platelets to treatment with ibuprofen and paracetamol, may allow us to better understand the mechanisms of PDA closure, and why some infants respond to treatment while others do not. Moreover, little is known about the effect of platelets in neonatal sepsis, and whether they may have a role in the challenging diagnosis of this condition.

### 6.10 Conclusion

The EVENT Study has characterised platelet dependent thrombin generation in preterm infants. Plasma from preterm infants is hypercoagulable compared to full-term infants, in the presence of platelets. Platelets influence coagulation by increasing peak thrombin and endogenous thrombin potential. However, platelets appear to mediate a differential effect in preterm and term infants, by prolonging the lag time and time to peak thrombin in the full-term group. No such effect was seen in preterm infants. The changes seen in lag time and time to peak thrombin between preterm and term infants were not explained by differences in TFPI levels.

The profile of circulating EVs change during perinatal adaption in both preterm and term infants. These changes appear to occur as a function of postnatal age, as opposed to the clinical status of the infant. However, there was an increase in several platelet, procoagulant and endothelial EV markers on Day 3 of life which was significantly greater in the preterm group, and the longer term implications of this need further study. Moreover, the cellular origin of these EVs, particularly the differences seen in platelet glycoprotein receptors, may help us to understand the functional role of these EVs. The timing of sample collection and the source of plasma (cord samples versus postnatal samples) influences the EV profile detected, and is important to bear in mind when designing future neonatal EV studies.

# Chapter 7 : Neonatal clinical and haematological outcomes following COVID-19 in pregnancy

## 7.1 Introduction

### 7.1.1 COVID 19 introduction

Coronavirus disease 2019 (COVID-19) is an infection caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The virus first emerged in Wuhan China in December 2019 (320), spread rapidly across the globe and was declared a pandemic by the World Health Organization (WHO) on 11$^{th}$ March 2020 (321). The first case of SARS-CoV-2 in the Republic of Ireland was identified on 29$^{th}$ February 2020 (322). The clinical features of COVID-19 infection include fever, cough, dyspnoea, fatigue and myalgia (323, 324). In March 2020, the WHO reported that 80% of COVID-19 infections were mild or asymptomatic (325). Irish data (n = 19,789) from the early months of the pandemic (March 2020 to July 2020) described a high incidence of hospitalisation (14.2%), ICU admission (2.2 %) and death (7.5%) (326). This study also described an increase in hospitalisation, ICU admission and mortality among those with chronic heart disease, body mass index (BMI) >40 kg/m$^2$ and male gender. Moreover, chronic renal disease, chronic neurological disease and cancer were associated with an increased risk of mortality. These risk factors are similar to those described in the international literature, which also highlighted increasing age, poverty and race (Black and Asian) as risk factors for adverse outcome (327, 328).

COVID-19 was initially thought to cause mild illness in children (329). However, SARS-CoV-2 can cause severe illness in children, via acute COVID-19 infection or paediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 (PIMS-TS) (330, 331). One US Study reported 6.1% of children with a SARS-CoV-2 diagnosis were hospitalised, and male gender, Black/ African American race and the presence of a complex chronic condition were risk factors for severe disease (331).

### 7.1.2 SARS-CoV-2 variants

Since the first reports of the SARS-CoV-2 virus in China in December 2019, there have been several Variants of Concern (VOC) described, of which several have been identified in this country (332). In Ireland, the first VOC to emerge and become a dominant circulating variant in January 2021 was the Alpha VOC (B.1.1.7.) which was initially identified in the United Kingdom. Subsequently, the Delta Variant (B.1.617.2), first reported in India, became the dominant circulating variant in Ireland in Summer 2021 and had increased transmissibility compared to the Alpha VOC. Most recently, the Omicron variant (B.1.1.529), initially reported in South Africa, has been the dominant circulating variant in Ireland since January 2022. The differential effects of these VOC in pregnancy are discussed further in *Section 7.1.4.1.*

### 7.1.3 Haematological effects of COVID-19

Haematological derangements are a characteristic feature of COVID-19 infection. A high incidence of lymphopenia was noted in patients hospitalised with COVID-19 in the early published reports (323, 324). In addition to lymphopenia, leukopenia and thrombocytopenia have also been described (333).

Patients with COVID-19 are at increased risk of thromboembolic events (334), despite thromboprophylaxis (335, 336). The mechanism of this hypercoagulability appears to be multifactorial and mediated by thromboinflammation; interaction between the endothelium, the immune system and the coagulation system (337). Platelet hyperactivity (338, 339), endothelial injury (340) and activation of the complement system (341) have all been demonstrated in patients with COVID-19. Moreover, extracellular vesicles (EVs), discussed at length in *Chapter 2: Introduction II,* may play a role in this procoagulant state. Several studies have demonstrated an increase in procoagulant Tissue Factor EVs (TF-EVs) in patients with COVID-19 compared to controls, particularly in patient with severe disease (342-345), and one study demonstrated that these TF-EVs remained elevated for 30 days post discharge (343).

The duration of this hypercoagulability is unclear and a recent study demonstrated an increased risk of thrombosis (deep vein thrombosis and pulmonary embolism) for at least two months following an acute infection (346). Some studies have also described persistently increased laboratory markers of hypercoagulability for months

following infection, including patients who did not require hospitalisation for their original illness (347-349).
From a clinical perspective, two randomised controlled trials have recently demonstrated a survival benefit with the use of therapeutic dose heparin in non-critically ill hospitalised patients with COVID-19 (350, 351).

### 7.1.4 COVID-19 in pregnancy

In view of the adverse perinatal outcomes described in Severe Acute Respiratory Syndrome (SARS) and Middle Eastern Respiratory Syndrome (MERS), there was concern from the outset regarding the potential implications of SARS-CoV-2 in pregnant women and their infants (352, 353).

#### *7.1.4.1 Maternal Illness*

Women diagnosed with SARS-CoV-2 during pregnancy usually have mild to moderate symptoms (354). However, there is an increased risk of severe maternal illness among pregnant women, with a greater need for intensive care support, invasive ventilation and even extra-corporeal membrane oxygenation (ECMO) than non-pregnant women (355, 356). The risk factors for severe disease in pregnant women are similar to those in the general population, and include obesity, hypertension, diabetes and increased maternal age (355). Among pregnant women, black and minority ethnic groups were also at increased risk of requiring hospitalisation (357). A majority of hospitalisations for COVID-19 in pregnant women occurred in the second and third trimester (357).
Unfortunately, the emergence of the Alpha and Delta VOCs have been associated with more severe maternal illness, an increased incidence of pneumonia, need for respiratory support and ICU (intensive care unit) admission, particularly during the period of Delta dominance (358). Reports of an increase in the proportion of women who are pregnant being referred for ECMO in the United Kingdom during the Alpha period have also been described (359). These findings are supported by published Irish data (360). No pregnant women were admitted to ICU with COVID-19 before December 2020 in Ireland. However, between December 2020 and August 2021 (periods of Alpha and Delta dominance), 35 pregnant or recently pregnant women were admitted to ICU with COVID-19, 57% required invasive ventilation and 3

required ECMO. Unsurprisingly, in the UK Study the perinatal outcomes were also worse during the Alpha period, with an increased incidence of extremely preterm birth and NICU admission; analysis of the Delta perinatal outcomes has not yet been completed (358).

#### *7.1.4.2 Mother to child transmission of COVID-19*

The transmission of COVID-19 infection in adults is primarily via droplet or airborne transmission (361). There is a paucity of information regarding the mother to child transmission (MTCT) of COVID-19 during pregnancy. Initial reports from the UK and United States described an incidence between 0 - 5%, of infants born to infected mothers, with SARS-CoV-2 polymerase chain reaction (PCR) detected during the neonatal period (357, 362, 363). There are several routes of MTCT of viral infections, including *in utero* haematogenous spread, intrapartum or postnatal transmission (364). Rare reports of the *in utero* vertical transmission of COVID-19 do exist (365). A recent systematic review demonstrated a rate of MTCT of 1.8% (366). In this review, the severity of the maternal illness, maternal ICU admission, maternal death and postnatal maternal diagnosis were associated with the incidence of neonatal SARS-CoV-2 positivity. Moreover rooming in with their mother and breastfeeding were not risk factors for neonatal SARS-CoV-2 diagnosis.

In Ireland, the Royal College of Physicians of Ireland (RCPI) issued “Guidance for Maternity Services” for the management of pregnancies affected by a maternal SARS-CoV-2 diagnosis (367). These guidelines recommended that infants born to women with an active SARS-CoV-2 diagnosis at delivery typically roomed in with their mother on the postnatal ward, albeit with the infant in an incubator in the mother’s room. Infants were only admitted to NICU if there was a medical indication for doing so. Breastfeeding was encouraged, with the necessary infection control precautions (e.g. maternal hand-washing and mask). SARS-CoV-2 testing of well asymptomatic infants was not recommended, and infants only underwent SARS-CoV-2 testing if they had symptoms suggestive of a COVID-19 infection, and ideally only after 72 hours of age to avoid early false negatives.

#### *7.1.4.3 Placental pathology*

There have been reports of placental abnormalities following a maternal SARS-CoV-2 diagnosis during pregnancy.

In the absence of direct placental infection, several studies have reported high rates of placental abnormalities including maternal and fetal vascular malperfusion and thrombi in the larger fetal vessels (368-373). Rebutini *et al.* found that symptomatic women were more likely to demonstrate chronic histiocytic intervillositis (370), while other studies have found no correlation between maternal illness severity and placental findings (369, 372). Some of the early studies were limited by lack of a control group making it difficult to interpret the findings (374). In the absence of direct placental infection, these placental abnormalities may suggest an inflammatory or hypercoagulable maternal state following a SARS-CoV-2 infection in pregnancy (373). However, pathological placental findings must be evaluated in the context of the clinical condition of the infant, many of whom had normal perinatal outcomes and were asymptomatic of COVID-19 infection.

Unfortunately, COVID-19 placentitis, direct infection of the placenta by SARS-CoV-2, emerged as a complication of SARS-CoV-2 infection in pregnancy in late 2020. Schwartz *et al.* described pathological features of COVID-19 placentitis, including chronic histiocytic intervillositis, fibrin deposition, syncitiotrophoblast necrosis and evidence of SARS-CoV-2 in the syncitiotrophoblast in a cohort of liveborn and stillborn infants infected with SARS-CoV-2 via the transplacental route in December 2020 (375). In early 2021 in Ireland, the RCPI reported six cases of stillbirth and one case of second trimester miscarriage due to COVID-19 placentitis (376) and another Irish report described COVID-19 placentitis resulting in fetal distress (377). A more detailed evaluation of the six fetal deaths described a rapidly progressive placental disease, without evidence of fetal growth restriction and all cases caused by the Alpha VOC (378). The time interval from maternal SARS-CoV-2 diagnosis to diagnosis of fetal death ranged from 0 to 19 days. A majority of the mothers had mild symptoms, three had thrombocytopenia and three had attended with reduced fetal movements prior to diagnosis of intrauterine death.

A multicentre review subsequently described 68 cases of COVID-19 placentitis resulting in stillbirth or neonatal death, including the Irish cases (379). The placental findings were similar to those described above and the authors found that severe

placental destruction caused placental insufficiency and fetal death. In addition to placentitis, the next most common placental finding was intervillous thrombosis or haemorrhage (37%). Post-mortem examinations were carried out in 44% of the infants, typically demonstrating normal anatomy, with evidence of intrauterine hypoxia and asphyxia in infants but no evidence that SARS-CoV-2 infection of the fetus had directly caused fetal death.

Preeclampsia, another pathological process associated with endothelial dysfunction (236) and fetal growth restriction are also associated with placental dysfunction (383, 384). Studies have shown that the degree of placental infarction in pre-eclampsia correlates with fetal birth weight (380). Moreover, placental insufficiency and intrauterine growth restriction can cause haematological abnormalities in the fetus including polycythaemia, neutropenia and thrombocytopenia (381, 382).

In our study, Placental Growth Factor (PlGF) was used as a marker of placental function. PlGF is a member of the vascular endothelial growth factor family (VEGF), is pro-angiogenic (383, 384). It is predominantly found in the placenta but is also found in other organs. Maternal levels of PlGF increase during pregnancy, peaking in the second trimester and reducing towards the end of pregnancy (385). PlGF levels are reduced in pregnancies affected by pre-eclampsia (386, 387).

Mestan *et al.* previously showed that PlGF levels, measured in umbilical cord blood, were lower in cases of severe maternal vascular malperfusion of the placenta (388). Broere-Brown *et al.* reported that lower cord PlGF levels were associated with lower birth weight and fetal growth restriction (389).

#### *7.1.4.4 Neonatal Outcomes*

The neonatal outcomes of infants born to women with a SARS-CoV-2 diagnosis during pregnancy can broadly be divided into two categories: well asymptomatic/SARS-CoV-2 negative infants and symptomatic/SARS-CoV-2 positive infants; I will discuss each group separately below.

##### *7.1.4.4.1 Asymptomatic/SARS-CoV-2 negative infants*

As previously discussed in *Section 7.1.4.2,* there is a low incidence of SARS-CoV-2 positivity (1.8%) in infants born to women with SARS-CoV-2 during pregnancy (366). Therefore, this asymptomatic group represents the larger cohort of infants.

While most infants in this group are well, adverse perinatal outcomes have been reported following COVID-19 infection during pregnancy, even in the absence of placental or fetal infection. Several studies have reported a higher incidence of preterm birth (11.6 – 27%) among women with SARS-CoV-2 in pregnancy (390-393). Elevated rates of NICU admission and low birth weight infants have also been described following a maternal COVID-19 infection during pregnancy (393, 394). Unfortunately, there is very little information available about the risk of congenital anomalies following COVID-19 infection during pregnancy, particularly during the first trimester (395, 396).

There is growing concern about the possibility of Multisystem inflammatory syndrome in children (MIS-C) also affecting neonates (MIS-N) following exposure to a maternal SARS-CoV-2 infection during pregnancy (397, 398). Paediatric Inflammatory Multisystem Syndrome Temporally Associate with SARS-CoV-2 (PIMS-TS)/ MIS-C is a post-infectious immune mediated condition, first identified in 2020, which can cause critical illness in children (330). Pawar *et al.* reported a case series of 20 infants with a history of maternal SARS-CoV-2 infection during pregnancy, who presented with multisystem involvement, hyperinflammatory syndrome and IgG antibodies to SARS-CoV-2 in the first week of life (397). 85% of the infants were preterm. There was a high incidence of cardiac involvement (90%) including rhythm abnormalities, coronary abnormalities, cardiac dysfunction and intracardiac thrombi. All infants were treated with steroids and intravenous immunoglobulin (IVIG), 70% received low molecular weight heparin and 60% required inotropes and 10% of the infants died.

##### *7.1.4.4.2 Neonatal COVID-19 infection*

Early evidence suggested that children with COVID-19 experienced milder disease than adults (329). Neonates may acquire SARS-CoV-2 from their mother, either vertically or horizontally, or by postnatal exposure to family members and caregivers. Gale *et al.* described a prospective cohort (n=66) of neonates hospitalised with SARS-CoV-2 during the early stages of the pandemic (399). There was a low incidence of neonatal infections requiring hospitalisation (5.6/10,000 livebirths), 26% were born to women with a SARS-CoV-2 diagnosis and 76% were full-term. Neonatal symptoms included fever, coryza and poor feeding, although 11% were

asymptomatic. 33% required respiratory support, a majority of which was non-invasive and many of whom were preterm. The median duration of admission for infants admitted to a paediatric ward was 2 days. There was one neonatal death, which was unrelated to COVID-19.

There have been rare case reports of severe complications of neonatal SARS-CoV-2 infections, including neonatal seizures and white matter changes (400) and persistent pulmonary hypertension (PPHN) (401, 402). However, neonatal SARS-CoV-2 diagnosis, resulting from either a maternal infection or a postnatal exposure from a family member or caregiver, appear to be rare and usually mild in nature (399). Unfortunately, more recent reports suggest that since the Omicron VOC became dominant the incidence of hospitalisation of children 0-4 years has increased five-fold, and ongoing caution regarding neonatal COVID-19 outcomes is required.

### 7.1.5 Haematological effects of viral infections

Hypercoagulability and thrombotic complications have been described in several viral infections, including Human Immunodeficiency Virus and Cytomegalovirus (403-407). Moreover, a maternal infection during pregnancy with Cytomegalovirus, Parvovirus B-19 or Rubella may cause haematological abnormalities in the fetus including thrombocytopenia and anaemia (408-410).

The haematological abnormalities seen in COVID-19 (lymphopenia, leukopenia and thrombocytopenia), have all been described in pregnant women with SARS-CoV-2 infection (394, 411). While pregnancy itself results in a hypercoagulable state (412), Servante *et al.* described an increased incidence of bleeding and thrombosis in pregnant women affected by COVID-19, although the absolute risk remained low (413). The Royal College of Obstetricians and Gynaecologists issued guidance recommending thromboprophylaxis for all hospitalised pregnant women with COVID-19, to continue for ten days post-discharge, and performance of a VTE assessment on pregnant patients managed in the community (414).

The haematological effects of COVID-19 infection in neonates, or exposure to maternal SARS-CoV-2 during pregnancy, are less clear. Some studies have reported haematological abnormalities such as lymphopenia, neutropenia and thrombocytopenia in these groups, although the numbers in these studies are very

small (75, 391, 399). Children infected with SARS-CoV-2 have demonstrable hypercoagulability (415, 416), albeit with a very low incidence of clinical thromboembolic complications (417). No reports have been identified to date, on the coagulation status of the fetus exposed to maternal COVID-19 or neonates with COVID-19.

### 7.1.6 Research aims

The aims of this study were to evaluate whether a maternal SARS-CoV-2 diagnosis during pregnancy resulted in adverse neonatal clinical outcomes (retrospective cohort study), and to evaluate fetal haematological derangement or hypercoagulability measured in UCB at birth (prospective study).

## 7.2 Materials and Methods

### 7.2.1 Retrospective study

Two retrospective studies were performed to determine neonatal clinical outcomes of liveborn infants delivered to women who had had SARS-CoV-2 during pregnancy. An initial 4-month study was performed to inform acute clinical practice and prospective research design in the initial phase of the pandemic. Subsequently, an extended retrospective review was performed to further investigate rates of preterm birth and other non-infectious outcomes (growth restriction, congenital anomalies, NICU admission) in this cohort. The results of the 4-month study were published in the *Irish Medical Journal (418)* and the results of the 12-month study were published in *The Pediatric Infectious Disease Journal (419)*.

#### *7.2.1.1 Ethical approval*

Ethical approval was also sought and granted by the Research Advisory Group in the Rotunda Hospital for two retrospective chart reviews of the neonatal clinical outcomes of liveborn infants, born to women with SARS-CoV-2 during pregnancy: the first over a 4-month period (RAG-2020-023) and the second over a 12-month period (RAG-2021-001).

#### *7.2.1.2 Study design*

##### *7.2.1.2.1 First study: Four-month review*

A retrospective observational study was performed to describe the neonatal outcomes of liveborn infants, delivered between 1st March 2020 and 1st July 2020, to women with a SARS-CoV-2 diagnosis at any time during pregnancy, at a single tertiary maternity and neonatal unit.

##### *7.2.1.2.2 Second study: Twelve-month review*

In view of the high incidence of preterm birth identified in the initial review, a subsequent 12-month study was performed to interrogate the incidence and cause of preterm birth in a larger cohort and to understand possible non-infectious implications of a maternal COVID-19 infection in their infants (e.g. growth restriction,

NICU admissions, congenital anomalies). Liveborn infants delivered during the 12-month period from 1st March 2020 to 1st March 2021 were included if their mother had a confirmed diagnosis of SARS-CoV-2 at any time during pregnancy.
Symptomatic women were screened for SARS-CoV-2 according to the Irish Health Service Executive recommendations, which were updated over time in keeping with emerging evidence (420). In addition, pre-admission SARS-CoV-2 screening was introduced in our hospital for asymptomatic women undergoing elective caesarean section or induction of labour in June 2020, and for all non-elective admissions in December 2020. All in-house SARS-CoV-2 diagnosis were reported to a central hospital COVID-19 notification system, in addition to community diagnosis which were self-reported by patients via a COVID-19 helpline or their healthcare provider. In this study, cases were identified from this central hospital COVID-19 notification system and from the NICU.
Miscarriages and stillbirths in women with a SARS-CoV-2 diagnosis were excluded as this was outside the scope of our review. Clinical data were collected from the maternal and neonatal electronic patient records and anonymised data were collated in Microsoft Excel (maternal history, antenatal history, SARS-CoV-2 diagnosis, birth history and postnatal outcome). Where a precise date of SARS-CoV-2 diagnosis was not available, the 15$^{th}$ of the month concerned was arbitrarily used as the date of diagnosis (n=1). If two SARS-CoV-2 diagnosis were recorded for a patient, the first was chosen, as the second test likely represented asymptomatic shedding. There were multiple births within the cohort (n=3) and in these cases infant data were considered independently.
Historical hospital demographic data, describing annual pregnancy outcomes for all patients attending the same centre, was available for many variables (maternal age, parity, mode of delivery, birth weight gestational age, NICU admission and preterm birth). For available variables, the year from which data was derived is described in the relevant Tables in *Section 7.3.2*.

#### *7.2.1.3 Statistical analysis*

For both studies, descriptive statistics were generated using Stata SE (version 17.0). For categorical variables, the frequency and percentage were described and for continuous variables means (standard deviations) and medians (interquartile ranges)

were described for normal and non-normative data, respectively. As discussed in *Section 7.2.1.2.2*, historical hospital data was available for certain variables and the Chi square test was used for comparison of categorical variables with historical hospital data.

To further interrogate the findings, analysis was performed comparing neonatal outcomes by date of infection before (1st March 2020 to 31st December 2020) and after the outbreak of the B.1.1.7. (Alpha) variant (1st January 2021 to 1st March 2021), between symptomatic and asymptomatic maternal infections and by interval from infection to delivery (greater than or less than 10 days). Univariate comparisons between these groups were performed using the Chi square test for categorical variables and the Mann-Whitney U test (nonparametric) or independent samples t-test (parametric) for continuous variables. Significance was assumed at 2-sided $p<0.05$.

### 7.2.2 COVID Cord Blood Study (Prospective Study)

Early in the pandemic it became apparent that COVID-19 infection in adults was associated with lymphopenia, thrombocytopenia, hypercoagulability and thrombosis, often in spite of thromboprophylaxis (323, 324, 333-336). Very little information was available regarding the fetal effects of a COVID-19 infection during pregnancy. Fetal haematological sequelae of other maternal viral infections during the antenatal period have been described (408-410). A prospective case-control study was designed to investigate if a maternal SARS-CoV-2 diagnosis during pregnancy resulted in haematological derangements or hypercoagulability in the fetus, measured in umbilical cord blood at birth. Some of the results from this prospective study (clinical demographics and thrombin generation results) have been published in the *European Journal of Obstetrics and Gynecology and Reproductive Biology (421).*

#### *7.2.2.1 Ethical approval*

Ethical approval was sought and granted by the Rotunda Research Ethics Committee (REC-2020-022) for the COVID-19 Cord Blood Study. Approval was also obtained for the retrospective use of control samples from the EVENT Study, where parents had consented to future uses in research projects, provided that ethical approval was obtained.

#### *7.2.2.2 Study design*

This was a prospective observational case-control study conducted in a single tertiary university maternity hospital and neonatal intensive care unit (approximately 8,500 deliveries/year) between January 2021 and March 2021.
Women who had SARS-CoV-2 ribonucleic acid (RNA) detected at any time during pregnancy were eligible for recruitment.
Women were initially contacted by phone about this study, prior to presentation to hospital for delivery, and were provided with a Patient Information Leaflet.
A group of healthy full-term infants, with no major congenital anomalies or family history of coagulation disorders, who had been recruited to the EVENT study, were used as controls (n=20). These infants were either born prior to the outbreak of

SARS-CoV-2 in Ireland (n=10), or after the outbreak of SARS-CoV-2 where there was a negative maternal SARS-CoV-2 PCR on admission to hospital at the time of delivery and no history of maternal SARS-CoV-2 infection during pregnancy (n=10). The former group of controls (pre-COVID) were used in the initial parts of this study (thrombin generation and platelet studies). However a decision was made to include the latter group (negative maternal SARS-CoV-2 PCR and no history of COVID-19) as there was limited volumes of plasma available from the first group, and the later control group had the advantage of also having Full Blood Count data available. Umbilical cord blood (UCB) samples were collected at delivery. Maternal and neonatal data were collected from the maternal and neonatal electronic patient record.

##### *7.2.2.2.1 Sample size*

This was a pilot study. The effect of COVID-19 on umbilical cord blood haematological parameters has not been described previously. This was a small descriptive study to identify if there were any differences between the infants exposed to COVID-19 *in utero* and controls. The number selected (n=15) was based on the estimated likely number of deliveries by COVID positive mothers during this time period.

##### *7.2.2.2.2 Inclusion criteria*

Women with a SARS-CoV-2 diagnosis at any time during pregnancy with a live fetus were eligible for recruitment.

##### *7.2.2.2.3 Exclusion criteria*

Women less than 18 years of age, those with a personal history of a coagulation disorder, an antenatal diagnosis of a major fetal abnormality and those who did not speak English were excluded.

##### *7.2.2.2.4 Consent*

Women with a confirmed diagnosis of SARS-CoV-2 at any time during pregnancy were approached. This was done by a member of the study team contacting the

women by phone, after their COVID-19 diagnosis. Their permission was sought to discuss the COVID Umbilical Cord Blood study. If they agreed, the study background, voluntary nature of the study, interventions (cord blood sampling for research sample and FBC and chart review), benefits and risks were discussed. A patient information leaflet (PIL) and consent form were provided to them (either by post/email, or if they preferred, on arrival to hospital).
After reading the PIL, any further questions they had were answered, and if they wished to consent, the consent form was signed and returned by post or signed when they come to hospital for delivery.
If the delivery was imminent, the PIL and consent form could be given to the woman on arrival to hospital and consent sought at that time, providing the woman was in a clinical condition to be able to give consent. Informed written consent was obtained from all parents prior to inclusion in this study.

#### *7.2.2.3 Data collection*

Maternal and neonatal data were recorded from the electronic patient record "Maternal and Newborn Clinical Management System" (MN-CMS) (Cerner, Missouri, United States) on the Case Report Form (*Figure 7.1*) Maternal demographics, antenatal history, details of the SARS-CoV-2 diagnosis and neonatal outcomes were recorded. Data was input into Microsoft Excel, using the unique study number.

#### *7.2.2.4 Data management*

Data management was compliant with GDPR regulations. The primary investigator completed GDPR and GCP training with the RCSI.
All enrolled patients were provided with a unique study identifier in order of their recruitment in order to code the data. The code to identify patients was kept on an encrypted computer in a locked hospital office. Patient data was accessed by the study team only. Data was kept on a password protected Microsoft Excel file, only accessible to study team members, and it was stored on a password protected computer in a locked hospital office. The processed data will be retained for the duration of the study and anonymized data for up to 5 years afterwards in keeping with hospital policy. This retention is to allow the investigators respond to any queries regarding the study.

Coded blood samples were transferred from the Rotunda to the UCD Conway SPHERE laboratory. The code to identify these samples remained in the Rotunda.

**COVID_UCB_Case Report Form**

Study No:_____ GA:_____ BW:_____

Gender:_____ Single/Multiple:_____

**Antenatal History**

**Maternal History**

| | |
|---|---|
| Age (years) | |
| BMI | |
| Gravity | |
| Liveborn | |
| Mis/Still/TOP | |
| Ethnicity | |
| Smoking (Yes/No) | |
| Conception (spont/ART) | |
| Rh status | |
| Blood group | |
| Maternal Medication | |
| Family hx bleeding | |
| Other maternal hx | |
| Family hx of note | |

| | |
|---|---|
| GA at booking (weeks) | |
| Anatomy Scan: Yes/No<br>Normal/Abnormal | |
| APH requiring admission | |
| GDM: yes/no<br>If so: insulin yes/no | |
| PET requiring ttt | |
| PPROM<br>If so GA at PPROM | |
| Abnormal Dopplers recorded (increased resistance/ AEDF/REDF) | |
| Liquor volume: poly/oligo/anhydramnios | |
| Any Antenatal Steroids | |
| Maternal Aspirin use | |
| Maternal Thrombocytopenia in pregnancy | |

**Birth History**

| | |
|---|---|
| Presentation: Cephalic/ breech/ transverse | |
| Duration delayed cord clamping (secs) | |
| Onset of labour: SOL/IOL/pre-labour | |
| Mode of delivery: Vaginal/ kiwi/ forceps/ Em LSCS/ El LSCS | |
| Delivery indication | |
| Liquor: clear/ blood/mec | |
| Apgar 1 | |
| Apgar 5 | |
| Arterial pH | |
| DS Resuscitation: | |
| None | |
| CPAP/ free flow O2 | |
| IPPV | |
| Intubated | |
| Need for further resuscitation (Chest Compressions, adrenaline, saline bolus, emergency blood) | |
| Vitamin K: Yes/ No | |
| GBS at delivery: yes/no/unknown | |
| Chorioamnionitis: yes/no | |
| Intrapartum antibiotics | |

**Birth Measurements**

| | |
|---|---|
| Weight centile | |
| Weight <3rd centile (yes/no) | |
| OFC (cm) | |
| OFC centile | |
| OFC <3rd centile (yes/no) | |
| | |
| **Control patient only** | |
| Pre March 2020 | |
| Negative PCR on admission | |

**COVID cases only**

| | |
|---|---|
| Mother Symptomatic (yes/no) | |
| Indication for test | |
| Date of test | |
| Community/Rotunda | |
| GA at diagnosis | |
| Days from diagnosis to delivery | |
| Diagnosis <10 days before delivery | |

**Inpatient Course**

| | |
|---|---|
| Congenital Anomaly (yes/no)<br>If so, what? | |
| Ever required admission to NICU (yes/no)<br>If so, why? | |
| Duration of admission (days) | |
| Ever required resp support after DS( yes/no) | |
| Infant ever tested for SARS-CoV 2 PCR | |
| Indication for test | |
| SARS-CoV2 result (detected/ not detected) | |
| Jaundice requiring phototherapy (yes/no) | |
| Weight loss >10% (yes/no) | |
| Hypoglycemia <2.6 (yes/no) | |
| **Infection** | |
| Ever received antibiotics (yes/no) | |
| Neonatal sepsis (EOS or LOS) (yes/no) | |
| **Imaging** | |
| Any performed (yes/no)<br>Indication | |
| **Haematology** | |
| Any blood product received (yes/no) | |
| DCT status: pos/neg/unknown | |
| Documented clinical haemorrhage (yes/no) | |
| Documented clinical thrombosis (yes/no) | |
| **Discharge** | |
| Death before discharge (yes/no) | |
| Day of death | |
| Transfer to another hospital (yes/no) | |
| Medications on discharge | |
| DOL at discharge | |
| Any maternal breast milk during admission (breast fed or EBM) | |
| Exclusive breast feeding at discharge | |
| Exclusive formula feeding at discharge | |
| Combined feeding at discharge | |
| Followed up in POPD (yes/no) | |
| Indication for same | |

**Figure 7.1: Example of the Case Report Form used in the prospective COVID Cord Blood Study**

#### *7.2.2.5 Blood sampling*

Following delayed cord clamping (where applicable), a sample of UCB was collected using a 10 ml syringe and a 21 gauge needle. Samples were typically collected following placental expulsion. However, if clinical samples were indicated while the placenta was in situ, research samples were also collected at this time. Blood was collected in sodium citrate 3.2% (3 x 3 ml) and EDTA (1 x 2.5 ml). All samples were manually checked for clot and excluded if detected. Samples were transferred directly to the laboratory within one hour of collection.

#### *7.2.2.6 Full blood count*

A full blood count (FBC) was performed on the EDTA sample using the "CELL-DYN Sapphire Hematology Analyzer" (Abbott). Results were compared to local neonatal reference ranges.

#### *7.2.2.7 Plasma preparation*

Platelet poor plasma (PPP) was prepared from umbilical cord blood samples collected in sodium citrate 3.2%. PPP was made by double centrifugation of whole blood at 3000m rpm for 6 minutes with full brake.

#### *7.2.2.8 Sample storage*

PPP was aliquoted into vials containing 100 to 400 µl and stored at -80 ˚C in the Rotunda Hospital.

#### *7.2.2.9 Sample transfer*

Samples were transferred in batches on dry ice from the Rotunda Hospital to the UCD Conway Institute.

#### *7.2.2.10 Computer Automated Thrombography (CAT)*

This technique uses a fluorogenic thrombin substrate to measure the ability of plasma to generate thrombin and is described in more detail in *Chapter 1: Section 1.10.2*. In the COVID-19 Cord Blood Study, CAT was carried out in PPP from UCB.

Frozen PPP was thawed by incubating aliquots at 37 ˚C for 10 minutes in a dry bath (AccuBlock Mini, Labnet International, USA (D0100-230V)).
Thrombin generation reagents (“PPP-reagent LOW” (contains 1 pM tissue factor and 4 µM phospholipid) and “Thrombin Calibrator” (Thrombinoscope BV, Stago, Asnieres sur Seine, France) were reconstituted by the addition of 1 ml deionised water and allowed to stand for 10 minutes before gently shaking. The “Fluo Buffer” was also warmed to 37˚C.
Samples were run in duplicate (two test wells and two calibration wells per patient) on a 96-well plate (Nunc TM, 96-well transparent U-bottom plates, 500 µl, ThermoFisher Scientific, Waltham, United States). 80 µl PPP and 20 µl “PPP reagent-LOW” were added into each test well. 80 µl PPP and 20 µl “Thrombin Calibrator” were added into each calibration well.
The plate was incubated at 37˚C for 10 minutes. During this time, “Flu-Ca” (fluorogenic Z-Gly-Gly-Arg-AMC.HCl substrate and 100mM $CaCl_2$) was prepared. 40 µl of “Fluo-Substrate” was added to the warmed “Fluo-Buffer” (1600 µl). The mixture was immediately vortexed (Vortex-Genie TM 2, Scientific Industries, New York, USA) and protected from light. 20 µl of the combined “Flu-Ca” solution (final concentrations, Z-Gly-Gly-Arg-AMC.HCl, 0.42 mM and CaCl2, 16.67 mM) was added to each well to initiate the coagulation cascade.
Thrombin generation was measured over 60 minutes using the Fluoroskan Ascent Plate Reader (ThermoFisher Scientific, Waltham Massachusetts) and a thrombin generation curve was produced using Thrombinoscope BV software (Stago, Asnieres sur Seine, France). The parameters measured include the lag time, time to peak thrombin generation, peak thrombin generation and endogenous thrombin potential (ETP). Data was exported to Microsoft Excel.

#### *7.2.2.11 Platelet Factor 4 (PF4)*

PF4 Quantikine ELISAs (Human CXCL4/PF4) were purchased from R & D Systems (Minneapolis, United States, Catalog no DPF40). The standard and other reagents were made up as per the manufacturer’s instructions.
After thawing, UCB PPP from cases and controls was diluted 1:40 with calibrator diluent (the assay having previously been optimised for neonatal plasma). Samples were analysed in duplicate and according to the manufacturer’s instructions.

The optical density of each well was measured at 450nm, within 30 minutes, on the Plate Reader (Clariostar Plus Microplate Reader, BMG Labtech, Aylesbury, United Kingdom). As wavelength correction was not available, optical density at 570nm was also recorded and subtracted from the 450nm reading to correct for optical imperfections in the plate. Duplicated values were averaged and the blank was subtracted from each value. Data analysis was performed by generating a 4-parametric logistic regression curve fit.

#### *7.2.2.12 Placental Growth Factor (PlGF)*

The Human PlGF Quantikine ELISA was purchased form R and D Systems (Minneapolis, United States, Catalog no DPG00). The kit was brought to room temperature before use and all reagents were made up as per the manufacturer's instructions.

UCB PPP from cases and controls was thawed at 37 ˚C and was tested undiluted. The standard concentrations used were 1000, 500, 250, 125, 62.5, 31.3 and 15.6 pg/ml and an additional dilution of 7.8 pg/ml was also included for completeness as previous neonatal studies have described relatively low levels of PlGF (388). Samples were analysed in duplicate and according to the manufacturer's instructions.

The optical density of each well was measured at 450nm within 30 minutes on the Plate Reader (Clariostar Plus Microplate Reader, BMG Labtech, Aylesbury, United Kingdom). As wavelength correction was not available, optical density at 570nm was also recorded and subtracted from the 450nm reading to correct for optical imperfections in the plate. The blank was subtracted from each value and duplicated values were averaged. Data analysis was performed by generating a 4-parametric logistic regression curve fit.

All of the samples measured had an absorbance below the lowest standard measured. As undiluted plasma was used it was not possible to re-run the assay with a lower dilution factor. For this reason, a Log-Logit regression was performed to extrapolate the curve and provide a concentration value, and is discussed further in the results.

#### *7.2.2.13 Nanoparticle tracking analysis*

NTA was used to measures the size and concentration of EVs in the 0-200 nm size range in PPP and is discussed in greater detail in *Chapter 3: Section 3.13*. The Nanosight NS300 (Malvern Panalytical, Malvern, United Kingdom) was used. Frozen PPP was thawed and diluted in Phosphate Buffered Saline (PBS) (Gibco, Walthem, United States) prior to use. Typically, 800 µl of a 1:50 dilution was prepared for UCB samples.

#### *7.2.2.14 Tissue Factor Extracellular Vesicle (TF-EV) procoagulant activity*

The Zymuphen MP-TF kit (Reference 521196) (HYPHEN BioMed, Neuville-sur Orsy, France), a functional assay for the measurement of TF bearing microparticles' procoagulant activity, was used as previously described in detail in *Chapter 3: Section 3.15*. The reagents were prepared and assay performed as per the manufacturer's instructions. Undiluted UCB PPP from the COVID-19 group and controls was used.

Some samples had an optical density below that of the lowest standard (2.1 pg/ml). These "low" values were arbitrarily assigned a value of "1 pg/ml" and pseudo counts were randomly assigned (using Research Randomizer, www.randomizer.org, free online software) to each "low" value (e.g. 1.0001, 1.0002, 1.003 etc.).

#### *7.2.2.15 Statistical analysis*

Descriptive analysis of continuous data included mean and standard deviation for normally distributed data, while median and interquartile ranges are used for non-parametric data, and frequency (percentages) for categorical data. For some categorical data, the values were combined to ensure sufficient numbers available for analysis. Comparisons between the COVID-19 and control groups were performed using Fisher's exact test for categorical variables and non-parametric Mann Whitney U-test or parametric unpaired T-test for continuous variables. Significance was assumed at two-sided $p<0.05$. Stata SE (version 17.0) was used for statistical analysis and R (version 3.6.3) was used for analysis of the raw NTA data and its graphical representation.

## 7.3 Results

### 7.3.1 Four-month retrospective study

In my initial review of the first four months of the COVID-19 outbreak, 26 liveborn infants were born to women with a SARS-CoV-2 diagnosis during pregnancy in the Rotunda Hospital. The results were published in a “Letter to the Editor” in the *Irish Medical Journal* (418) and are described in *Tables 7.1 and 7.2.*

During this period, the median gestational age at detection of maternal SARS-CoV-2 was 36.9 weeks (IQR 32.1 – 39.1), and 14 (53.9%) were diagnosed within 14 days of delivery, considered the duration of COVID-19 infectivity at that time. Four of the women identified (15%) were from Ireland’s Roma Community, a group disproportionately affected by the COVID-19 pandemic (422).

There was a high incidence of preterm birth in this group, with 6 infants (23%) born less than 37 weeks gestation, although all were moderate to late preterm. It was unclear whether this high incidence of preterm birth was related to maternal SARS-CoV-2 infection.

As advised in the Irish National Guideline “COVID-19 infection: Guidance for Maternity Services”, infants roomed in with their mother on the postnatal ward and breast feeding was encouraged, with the necessary infection control precautions if the mother was deemed to still be within the infectious period of the illness (367). Infants were only admitted to NICU if there was a medical indication e.g. need for respiratory support. Seven infants (27%) required admission to the Neonatal Unit, three due to prematurity and four for non-COVID neonatal problems. Moreover, infants were only tested for SARS-CoV-2 if there was a clinical suspicion of infection and the symptoms were not otherwise explained e.g. by prematurity. Two infants were tested for SARS-CoV-2, (one due to excessive nasal congestion and one following discharge); both were negative, and the infants remained clinically well. Seventeen infants (65.4%) were followed up after discharge from hospital, either in the paediatric outpatient clinic for routine postnatal issues e.g. jaundice, or by a virtual clinic appointment at 4-8 weeks. All 17 infants remained well, and none had developed a symptomatic COVID-19 infection.

**Table 7.1: Maternal demographics of the liveborn infants to women with a diagnosis of SARS-CoV-2 during the four month period, 1st March 2020 to 1st July 2020**

Data first published in the *Irish Medical Journa*l (418)

Mean (±SD), Median [IQR], Frequency (%)

| Demographics | COVID-19<br>n=26 |
|---|---|
| *Maternal* | |
| Maternal Age (years) | 30.8 (±7.3) |
| Caucasian | 14 (53.9) |
| Nulliparous | 18 (69.2) |
| Underlying medical condition | 8 (30.8) |
| *Antenatal* | |
| Pre-eclampsia | 4 (15.4) |
| Gestational Diabetes Mellitus | 3 (11.5) |
| *SARS-CoV-2 Diagnosis* | |
| Gestational age at diagnosis (weeks) | 36.9<br>[32.1 – 39.1] |
| Location of test: | |
| In-hospital | 22 (84.6) |
| Community | 4 (15.4) |
| Diagnosis within 14 days of delivery | 14 (53.9) |
| Time from diagnosis to delivery (days) | 1.5<br>[0.1 – 4.6] |

**Table 7.2: Neonatal demographics of the liveborn infants to women with a diagnosis of SARS-CoV-2 during the four month period, 1st March 2020 to 1st July 2020**

Data first published in the *Irish Medical Journal* (418)

Mean (±SD), Median [IQR], Frequency (%)

| Demographics | COVID-19<br>n=26 |
|---|---|
| Mode of delivery: | |
| Spontaneous vaginal delivery | 14 (53.9) |
| Instrumental vaginal delivery | 4 (15.4) |
| Caesarean Section | 8 (30.8) |
| Male | 16 (61. 5) |
| Gestational age (weeks) | 39.4<br>[37 – 40.1] |
| Birth weight (kg) | 3.3<br>(±0.7) |
| Preterm (<37 weeks) | 6 (23.1) |
| Chorioamnionitis | 1 (3.9) |
| Received breast milk (breast fed or expressed breast milk) | 19 (73.1) |
| Infant SARS-CoV-2 PCR test | 2 (7.7) |
| Infant SARS-CoV-2 detected | 0 (0) |
| NICU Admission | 7 (26.9) |
| Congenital anomaly | 2 (7.7) |
| Jaundice requiring phototherapy | 4 (15.4) |
| Weight loss >10% | 1 (3.9) |
| Day of life at discharge | 4<br>[3 – 6] |
| Discharge location: | |
| Home | 25 (96.2) |
| Isolation facility | 1 (3.9) |
| Out-patient follow up | 17 (65.4) |

### 7.3.2 Twelve-month retrospective study

The results of this study were published in *The Pediatric Infectious Disease Journal (419)*.

#### *7.3.2.1 Maternal demographics*

During the 12-month period, 1st March 2020 to 1st March 2021, I identified 133 infants born to 130 women with a SARS-CoV-2 diagnosis during pregnancy. Patients were identified from the central hospital COVID-19 notification system (n=131) and from the NICU (n=2) (maternal SARS-CoV-2 diagnosis was made prior to booking at this hospital). The maternal demographics are displayed in *Table 7.3*. The mean maternal age was 30.9 years (± 5.8 years). The underlying maternal illnesses included thyroid disease (15 [11.5%]), renal, liver and respiratory disease.
While there was no control group in this study, the Rotunda Hospital publishes data on patient demographics and important clinical outcomes annually as part of corporate governance and transparency. These data from the approximately 8,500 patients attending the hospital each year are in the public domain, and are displayed as a reference value for comparison purposes in the third column of *Table 7.3*. Aggregate data from 2015-2019 inclusive are labelled (a), and individual year 2020 data, where available at time of preparation, are labelled as (b) in this table.

**Table 7.3: Maternal and antenatal demographics of women with SARS-CoV-2 detected during pregnancy (March 2020 – March 2021)**

Data from the Annual Hospital Report, which publishes data on key maternal and neonatal clinical parameters each year, are included in the third column for comparison purposes. Table first published in *The Paediatric Infectious Disease Journal* 2021 (423)

[a] Aggregate Hospital Data 2015 – 2019 [b] Individual Year Hospital Data 2020

| Demographics | COVID-19 group n=130 n(%) | Annual hospital data for reference |
|---|---|---|
| *Maternal* | | |
| Nulliparous | 73 (56.2) | 41-44%[a] |
| Ethnicity | | |
| Caucasian | 88 (67.7) | 81.4%[b] |
| African | 11 (8.5) | 2.1%[b] |
| Asian | 18 (13.9) | 4.9%[b] |
| Members of Ireland's Roma Community and Irish Travellers | 10 (7.7) | 1.8%[b] |
| Other | 3 (2.3) | 9.7%[b] |
| Underlying medical illness | 38 (29.2) | |
| *Antenatal* | | |
| Gestational Diabetes Mellitus | 17 (13.1) | 9.3 – 14.3%[a] |
| Pre-eclampsia/ pregnancy induced hypertension | 7 (5.4) | |
| Smoking during pregnancy | 10 (7.7) | |
| Thrombocytopenia in pregnancy (<150 x 10^9/L) | 24 (18.5) | |

*7.3.2.2 SARS-CoV-2 diagnosis*

Details of the maternal SARS-CoV-2 diagnosis are displayed in *Table 7.4*. In 79 cases (60.8%), the women had at least one symptom of COVID-19 documented. A majority of the diagnosis occurred in the third trimester (116 [89.2%]). While most women experienced mild to moderate symptoms, four women (3.1%) required escalation of care to a high dependency unit or transfer to an acute adult hospital for symptoms related to COVID-19 infection.

**Table 7.4: Details of the SARS-CoV-2 diagnosis detected during pregnancy (March 2020 – March 2021)**

Mean (±SD), Median [IQR], Frequency (%)

| SARS-CoV-2 diagnosis | COVID-19 group<br>n=130 |
|---|---|
| Gestational age at diagnosis (weeks) | 36.4<br>[32.6 – 39] |
| Location of test: | |
| In-hospital | 78 (60) |
| Community | 52 (40) |
| Indication for test | |
| Symptomatic | 61 (46.9) |
| Close Contact | 17 (13.1) |
| Asymptomatic pre-admission screen | 41 (31.5) |
| Unknown | 11 (8.5) |
| Time from diagnosis to delivery (days) | 15<br>[1 – 39] |

#### *7.3.2.3 Neonatal outcomes*

One hundred and thirty-three infants were identified, and neonatal demographics are described in *Table 7.5*. Five infants had SARS-CoV-2 PCR tests performed, two prior to transfer/re-admission and three for symptoms suggestive of COVID-19; however SARS-CoV-2 was not detected in any infant.

The incidence of preterm birth following a maternal SARS-CoV-2 diagnosis over this twelve-month period was 10.5% (n=14). This did not differ significantly from the pre-pandemic hospital incidence (2015–2019) (6.3–7.9%, *p*=0.13). The majority of the infants born prematurely (12 [85.7%]) were late preterm (34 – 36 weeks gestation), a group at low risk of severe morbidity and mortality. The median preterm birth weight was 2.45 kg (IQR 2.24– 2.68 kg) and gestation was 36.6 weeks (IQR 34.3–36.6 weeks). To further understand how a maternal SARS-CoV-2 diagnosis may cause preterm birth, the indications for each preterm delivery were evaluated. Four (28.6%) were due to preterm labour/chorioamnionitis, four (28.6 %) due to intrauterine growth restriction, three (21.4%) were due to pre-eclampsia and three (21.4%) were due to preterm prelabour rupture of the membranes. None of the preterm deliveries were in the maternal interest due to severity of COVID-19 infection during this period.

I also evaluated the indication for NICU admission in the overall study cohort. Twenty-two infants (16.5%) were admitted to NICU; six (27.2%) for complications of prematurity. The NICU admission rate in the COVID-19 exposed infants was similar to the hospital admission rate over the 5-year period, 2015–2019 (16.5% v 13.1%–15.5%, *p*=0.49). Among the full-term infants admitted to NICU, the indications included jaundice requiring phototherapy, infection requiring antibiotics, hypoglycaemia and congenital anomalies. Most remained well, however 4 infants (3%) were severely unwell and required invasive ventilation. There was one neonatal death, unrelated to COVID-19 infection.

12 infants studied (9%) had congenital anomalies, five of which were genitourinary. Five of the 12 anomalies were diagnosed antenatally and the remainder in the postnatal period.

As in *Section 7.3.2.*1, annual hospital data from the approximately 8,500 patients attending the hospital each year are in the public domain, and are displayed as a reference value for comparison purposes in the third column of *Table 7.5*. Aggregate data from 2015-2019 inclusive are labelled (a) in this table.

**Table 7.5: Characteristics and neonatal outcomes of infants born to women with SARS-CoV-2 detected during pregnancy (March 2020 – March 2021)**

Data from the Annual Hospital Report, which publishes data on key maternal and neonatal clinical parameters each year, are included in the third column for comparison purposes. First published in *The Paediatric Infectious Disease Journal* 2021 (423)

Mean (±SD), Median [IQR], Frequency (%), [a] Aggregate Hospital data 2015 – 2019

| Demographics | COVID-19<br>n=133 | Annual hospital<br>data for reference |
|---|---|---|
| Mode of delivery | | |
| Spontaneous vaginal delivery | 63 (47.4) | 49 - 51 %[a] |
| Operative vaginal delivery | 18 (13.5) | 16 – 17%[a] |
| Caesarean section | 52 (39.1) | 29 – 35%[a] |
| Male | 68 (51.1) | 50.6 – 51.7%[a] |
| Gestational age (weeks) | 39.3<br>[38.4 – 40.3] | 39[a] |
| Birth weight (kg) | 3.45<br>[3.01 – 3.84] | 3.39 – 3.41[a] |
| Preterm | 14 (10.5) | 6.3 – 7.9%[a] |
| Small for gestational age (weight <10$^{th}$ centile) | 7 (5.3) | |
| Admission to NICU | 22 (16.5) | 13.1 – 15.5%[a] |
| Congenital anomaly | 12 (9) | |
| Neonatal death | 1 (0.75) | |
| Received breast milk (breast fed or expressed breast milk) | 92 (69.2) | |

| Demographics | COVID-19<br>n=133 | Annual hospital data for reference |
|---|---|---|
| Weight loss >10% | 4 (3) | |
| Hypoglycaemia <2.6 mmol/L | 6 (4.5) | |
| Jaundice requiring phototherapy | 13 (9.8) | |
| Neonate ever tested for Sars-CoV-2 | 5 (3.8) | |
| Sars-CoV-2 detected | 0 (0) | |
| Day of life at discharge | 3<br>(2-4) | |
| Discharge Location | | |
| Home | 129 (97.7) | |
| Other hospital | 1 (0.8) | |
| Isolation facility | 2 (1.5) | |
| Outpatient follow up | 77 (57.9) | 55 – 60%[a] |

#### *7.3.2.4 B.1.1.7. (Alpha) variant*

The B.1.1.7. SARS-CoV-2 variant of concern (VOC) was first identified in the United Kingdom and emerged as the dominant variant in Ireland in January 2021 (424). To evaluate whether the B.1.1.7. VOC was associated with a greater incidence of adverse neonatal outcomes, the outcome data were analysed before and after the emergence of the B.1.1.7. variant (*Table 7.6*). Unfortunately, genotyping of maternal SARS-CoV-2 virus was not available, and so a pragmatic analysis was performed based on the timing of maternal SARS-CoV-2 diagnosis and the predominant variant prevalent at the time. The neonatal outcomes (preterm birth, NICU admission, small for gestational age fetus) were similar before and after the outbreak of VOC B.1.1.7.

**Table 7.6: Neonatal outcomes by maternal SARS-CoV-2 diagnosis before and after the emergence of VOC B.1.1.7**

First published in *The Paediatric Infectious Disease Journal* 2021(423)

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test, [d]Chi-squared test

| Neonatal Outcome | Pre-B.1.1.7 emergence<br>n=67 | Post B.1.1.7 emergence<br>n=66 | *p*-value |
|---|---|---|---|
| Time of maternal SARS-CoV-2 diagnosis | 1st March 2020 – 31st December 2020 | 1st January 2021 – 1st March 2021 | |
| Gestational age at diagnosis (weeks) | 34.9<br>[30 – 38.9] | 37.7<br>[35 – 39] | <0.01[a] |
| Gestational age at birth (weeks) | 39.6<br>[38.6 – 40.3] | 39.3<br>[38.3 – 40.4] | 0.57[a] |
| Birth weight (kg) | 3.46<br>[3.05 – 3.84] | 3.39<br>[2.97 – 3.82] | 0.91[a] |
| Preterm birth (<37$^{+0}$ weeks) | 7 (10.5) | 7 (10.6) | 0.98[d] |
| NICU admission | 11 (16.4) | 11 (16.7) | 0.97[d] |
| Small for gestational age (<10th centile) | 4 (6%) | 3 (4.6%) | 0.71[d] |

*7.3.2.5 Maternal symptom status*

As previously mentioned, a majority of women with a SARS-CoV-2 diagnosis during pregnancy were symptomatic (79 [60.8%]), although few had severe symptoms requiring hospitalisation (4 [3.1%]). To evaluate whether symptomatic infection resulted in worse clinical outcomes, I compared the neonatal outcomes in women with and without symptomatic infections (*Table 7.7*). Cases (8) without documentation of symptom status were excluded. Maternal symptom status did not appear to influence neonatal outcome.

**Table 7.7: Neonatal outcomes based on maternal symptom status**

Patients with unknown symptom status were excluded (n=8). First published in *The Paediatric Infectious Disease Journal* 2021(423)

Mean (±SD), Median [IQR], Frequency (%), *p <0.05, [a]Mann Whitney U test, [b]Chi-squared test

| Neonatal Outcome | Symptomatic n=80 | Asymptomatic n=45 | *p*-value |
|---|---|---|---|
| Gestational age at diagnosis (weeks) | 35 [31.8 – 38] | 39 [36.1 – 40] | <0.01[a]* |
| Days from diagnosis to delivery | 22.5 [10 – 48.5] | 2 [1 – 8] | <0.01[a]* |
| Gestational age at birth, weeks | 39.1 [38.4 – 40.2] | 39.6 [38.1 – 40.9] | 0.17[a] |
| Birth weight (kg) | 3.47 [3 – 3.82] | 3.48 [3.05 – 3.91] | 0.51[a] |
| Preterm birth (<37 weeks) | 9 (11.3%) | 5 (11.1%) | 0.98[b] |
| NICU admission | 13 (16.3%) | 7 (15.6%) | 0.91[b] |
| Small for gestational age (<10$^{th}$ centile) | 4 (5%) | 3 (6.7) | 0.7[b] |

#### *7.3.2.6 Timing of SARS-CoV-2 diagnosis in relation to delivery*

At the time this study was completed, the public health advice regarding likely infectious period following a SARS-CoV-2 infection was reduced from 14 to 10 days. To evaluate if an infection closer to the time of delivery influenced neonatal outcomes, analysis was performed as below (*Table 7.8*) and no differences were identified in neonatal outcomes.

**Table 7.8: Neonatal outcomes based on the number of days (greater than or less than 10) from maternal diagnosis of SARS-CoV-2 to delivery**

Includes both symptomatic and asymptomatic women. First published in *The Paediatric Infectious Disease Journal* 2021 (423)

Mean (±SD), Median [IQR], Frequency (%), *p <0.05, [a]Mann Whitney U test, [b]Chi-squared test

| Neonatal Outcome | Diagnosis <10 days before delivery n=58 | Diagnosis >10 days before delivery n=75 | *p*-value |
|---|---|---|---|
| Days from diagnosis to delivery | 1 [0 – 3] | 36 [21 – 58] | |
| Gestational age at birth (weeks) | 39.5 [38.1 – 40.6] | 39.3 [38.4 – 40.3] | 0.46[a] |
| Birth weight (kg) | 3.61 [3.12 – 3.91] | 3.33 [2.99 – 3.72] | 0.07[a] |
| Preterm birth (<37 weeks) | 7 (12.1) | 7 (9.3) | 0.61[b] |
| NICU admission | 9 (15.5) | 13 (17.3) | 0.78[b] |
| Small for gestational age (<10$^{th}$ centile) | 3 (5.2) | 4 (5.33) | 0.97[b] |

### 7.3.3 Prospective study: COVID Cord Blood Study

Some results from this study have been published in the *European Journal of Obstetrics and Gynecology and Reproductive Biology (421).*

Fifteen infants born to women with a confirmed SARS-CoV-2 diagnosis during pregnancy were included (*Figure 7.2*). Twenty healthy term controls were identified from infants previously recruited to the EVENT Study. As discussed in *Section 7.2.2.2*, ten of these controls were born prior to the first case of COVID-19 in Ireland and the remaining ten were born to women with no history of SARS-CoV-2 during pregnancy, and SARS-CoV-2 was not detected on PCR prior to admission to hospital, at the time of delivery.

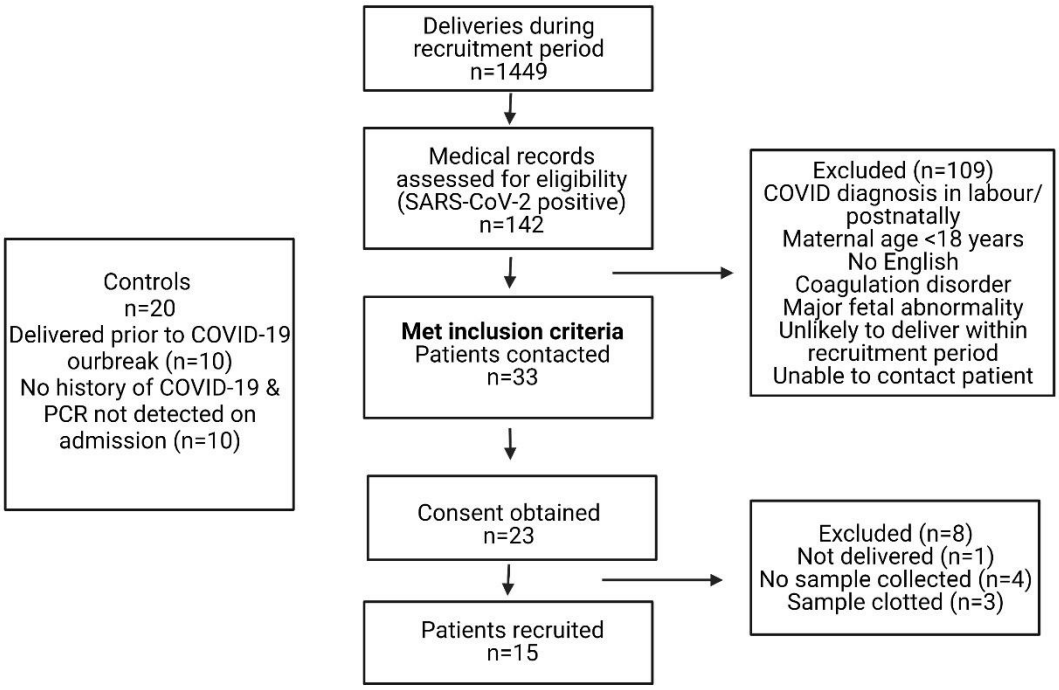


**Figure 7.2: Flow diagram of the infants recruited to the COVID-19 Umbilical Cord Blood Study**

Image created with BioRender.com

*7.3.3.1 Clinical demographics*

The clinical demographics of the mothers and infants in the COVID-19 group, and healthy term controls are described in *Tables 7.9 and 7.10*. The maternal demographics were similar between the COVID-19 group and controls, although mothers in the COVID-19 group were younger (30 [28 – 25] v 34 [32.5 – 36], *p*=0.03). There were differences in the onset of labour and mode of delivery between groups with more infants delivered by caesarean section in the control group (7 [46.7%] v 17 [85%]), *p*=0.03). The remaining maternal characteristics were similar between groups.

**Table 7.9: Maternal and antenatal demographics of infants in the COVID-19 Umbilical Cord Blood Study**

BMI: Body mass index

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test, [c]Fisher's Exact Test

| Clinical outcome | COVID-19 n=15 | Controls n=20 | *p*-value |
|---|---|---|---|
| *Maternal Demographics* | | | |
| Maternal age (years) | 30 [28 – 35] | 34 [32.5 – 36] | 0.03[a] |
| BMI (kg/m2) | 23.8 [22.9 – 26.7] | 26.4 [24.9 – 28.7] | 0.18[a] |
| Caucasian | 14 (93.3) | 19 (95) | 1.0[c] |
| Nulliparous | 10 (66.7) | 7 (35) | 0.09[c] |
| Underlying medical diagnosis | 5 (33.3) | 8 (40) | 0.74[c] |
| Tobacco use in pregnancy | 1 (6.7) | 1 (5) | 1.0[c] |
| *Antenatal History* | | | |
| Gestation at booking (weeks) | 12.9 [12.3 – 13.3] | 12.6 [12 – 13.9] | 0.8[a] |
| Aspirin use in pregnancy | 0 (0) | 4 (20) | 0.12[c] |
| Thrombocytopenia (platelet count ever <150 x 10^9/L) | 1 (6.7) | 1 (5) | 1.0[c] |

| Clinical outcome | COVID-19<br>n=15 | Controls<br>n=20 | *p*-value |
|---|---|---|---|
| Pre-eclampsia | 1 (6.7) | 0 (0) | 0.43[c] |
| Gestational diabetes | 1 (6.7) | 2 (10) | 1.0[c] |
| *SARS-CoV-2 Diagnosis* | | | |
| Gestation at SARS-CoV-2 diagnosis (weeks) | 34.7<br>[31.6 – 36.1] | | |
| Trimester at time of diagnosis | | | |
| 1st trimester | 0 (0) | | |
| 2nd trimester | 2 (13.3) | | |
| 3rd trimester | 13 (86.7) | | |
| Any symptoms of COVID-19 | 14 (93.3) | | |
| Maternal hospitalisation with COVID-19 symptoms | 0 (0) | | |
| Time from diagnosis to delivery (days) | 36<br>[21 – 58] | | |
| Delivery within 10 days of diagnosis | 1 (6.7) | | |

**Table 7.10: Neonatal and postnatal outcomes of infants in the COVID-19 Umbilical Cord Blood Study**

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test, [c]Fisher's Exact Test

| Clinical Outcome | COVID-19 n=15 | Control n=20 | *p*-value |
|---|---|---|---|
| Onset of labour | | | |
| Spontaneous/ Induced labour | 11 (73.3) | 5 (25) | 0.01[c] |
| Pre-labour | 4 (26.7) | 15 (75) | |
| Method of Delivery | | | |
| Spontaneous/ operative vaginal delivery | 8 (53.3) | 3 (15) | 0.03[c] |
| Caesarean section | 7 (46.7) | 17 (85) | |
| Male | 10 (66.7) | 10 (50) | 0.49[c] |
| Gestational age (weeks) | 39.3 [38.9 – 40.3] | 39.3 [38.8 – 40.4] | 1.0[a] |
| Birth weight (g) | 3600 [3270 – 4040] | 3465 [3300 – 4020] | 0.84[a] |
| Chorioamnionitis | 1 (6.7) | 0 (0) | 0.43[c] |
| Need for resuscitation | 2 (13.3) | 1 (5) | 0.57[c] |
| Admission to NICU | 1 (6.7) | 2 (10) | 1.0[c] |
| Congenital anomaly | 2 (13.3) | 2 (10) | 1.0[c] |
| Tested for SARS-CoV-2 | 0 (0) | 0(0) | |

*7.3.3.2 Full blood count*

Full blood counts (FBCs) were performed in 13 COVID-19 patients (n=1 sample not processed, n=1 insufficient). There was no evidence of thrombocytopenia, anaemia or lymphopenia in the COVID-19 group and all results were within our hospital's neonatal reference ranges (*Table 7.11*). FBCs were available in 8 control infants (n=10 were recruited before the project was modified to collect FBCs in controls, n=2 not processed). There was no difference in haematological parameters between the COVID-19 group and controls.

**Table 7.11: Results of the full blood counts performed in umbilical cord blood in the COVID-19 group and healthy term controls**

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test,

| FBC parameter | COVID-19 n=13 | Controls n=8 | *p*-value | Neonatal reference ranges |
|---|---|---|---|---|
| Haemoglobin (g/L) | 15.7 [14.7 – 16.4] | 14.9 [13.4 – 15.7] | 0.20[a] | 13.5 – 19.5 |
| Haematocrit (L/L) | 0.47 [0.44 – 0.51] | 0.46 [0.41 – 0.5] | 0.43[a] | 0.42 – 0.6 |
| Platelet count (x 10^9/L) | 260 [214 – 281] | 262.5 [245.5 – 294] | 0.54[a] | 150 – 450 |
| White cell count (x 10^9/L) | 16.5 [16.2 – 18.9] | 15.3 [10.5 – 19.5] | 0.37[a] | 9 - 30 |
| Lymphocyte count (x 10^9/L) | 5.6 [4.7– 7.2] | 4.9 [3.8 – 5.8] | 0.16[a] | 2-11 |

#### *7.3.3.3 Thrombin generation*

CAT was used to evaluate thrombin generation in PPP, in the presence of PPP-LOW reagent (1 pM TF and 4 µM Phospholipid) as described in *Section 7.2.2.10.* CAT was performed in 14 infants in the COVID-19 group (n=1 excluded as thrombin generation curve suggested a clotted sample) and 10 healthy controls (all 10 were recruited prior to the outbreak of COVID-19 in Ireland). There was no evidence of altered coagulability in UCB PPP in infants born to women with SARS-CoV-2 compared to healthy term infants (*Table 7.12*). These results were published in the *European Journal of Obstetrics and Gynaecology and Reproductive Biology* (421).

**Table 7.12: Thrombin generation parameters measured using Calibrated Automated Thrombography in PPP from UCB in the COVID-19 group and healthy term controls**

Table first printed in the *European Journal of Obstetrics and Gynaecology and Reproductive Biology* (421)

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test

| CAT Parameter | COVID-19<br>n=14 | Control<br>n=10 | *p*-value |
|---|---|---|---|
| Lag time (min) | 2.8<br>[2.7 – 3] | 2.8<br>[2.7 -3.3] | 0.92[a] |
| Peak thrombin (nM) | 136.7<br>[130.7 - 156.1] | 133.9<br>[129.2 - 149.5] | 0.44[a] |
| ETP (nM.min) | 967.8<br>[869 - 1055.6] | 861.1<br>[826.3 – 980] | 0.24[a] |
| Time to peak (min) | 6.4<br>[6.0-7.0] | 6.3<br>[5.7 - 7.3] | 0.94[a] |

#### *7.3.3.4 Platelet Factor 4*

Circulating levels of PF4 were evaluated in PPP to assess platelet activation in infants in the COVID-19 group (n=13) and healthy controls (n=9). Two infants in the COVID-19 group were removed from all further analysis following thrombin generation studies (n=1 clotted sample as previously mentioned in *Section 7.3.3.3*, n=1 mother infectious at the time of delivery which limited transport of sample due to infection control restrictions). Nine controls recruited before the outbreak of COVID-19 were included in the analysis (n=1 excluded as the remaining frozen plasma had aggregated). There was no statistically significant difference detected between PF4 levels in the COVID-19 group and controls, indicating that platelets remained quiescent in both cohorts (*Table 7.13 and Figure 7.3*).

**Table 7.13: Platelet Factor 4 levels were similar in the COVID-19 and control groups in umbilical cord blood PPP**

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test

| | COVID-19<br>n=13 | Control<br>n=9 | *p*-value |
|---|---|---|---|
| PF4 concentration (pg/ml) | 380.2<br>[241.1 – 434.6] | 280.5<br>[208.9 – 304.7] | 0.6[a] |

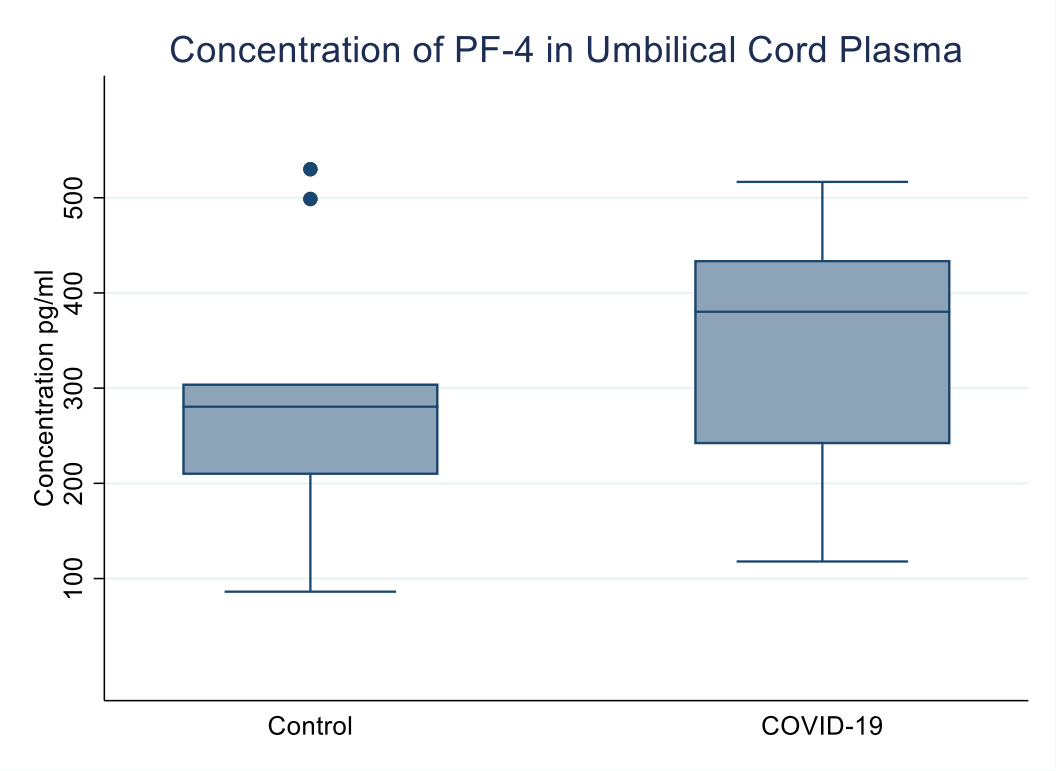


**Figure 7.3: Boxplot of the Platelet Factor 4 levels in UCB PPP in the COVID-19 group (n=13) and controls (n=9)**

*7.3.3.5 Placental growth factor*

In view of the increased risk of low-birth-weight infants and abnormal placental histology reported in the literature following a maternal SARS-CoV-2 infection during pregnancy, I evaluated PlGF levels in UCB PPP. PlGF levels were measured in 13 infants in the COVID-19 group (2 excluded as previously described in *Section 7.3.3.4*) and 18 infants in the control group (excluded: n=1 insufficient plasma, n=1 remaining plasma aggregated). Unfortunately, all PlGF concentrations measured were below the lowest standard used in this ELISA. A Log-Logit regression analysis was performed to obtain concentrations and the results are described in *Table 7.14* below (n=2 controls were unable to obtain a result). However, these results should be interpreted with caution as they are extrapolated from the standard curve and below the detection threshold as per the manufacturer's instructions.

**Table 7.14: Placental growth factor levels in umbilical cord blood PPP in the COVID-19 and control groups.**

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test

| | COVID-19<br>n=13 | Control<br>n=16 | *p*-value |
|---|---|---|---|
| Placental growth factor (pg/ml) | 2.8<br>[1.8 – 3.8] | 2.8<br>[0.9 – 3.4] | 0.69[a] |

*7.3.3.6 Extracellular vesicles*

Nanoparticle tracking analysis was used to evaluate small extracellular vesicles (SEVs) less than 200 nm in UCB plasma in the COVID-19 group (n=13) (2 excluded as previously described in *Section 7.3.3.4*) and healthy controls (n=20). There was a trend towards a higher concentration of SEVs in the healthy controls (*Figure 7.4 & 7.5 and Table 7.15*) but this did not reach statistical significance. The modal size of SEVs was similar between groups (*Table 7.15*).

**Table 7.15: Evaluation of the small extracellular vesicles in UCB plasma measured by nanoparticle tracking analysis (0-200 nm) in the COVID-19 and control groups**

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test

| SEV characteristics | COVID-19 n=13 | Controls n=20 | *p*-value |
|---|---|---|---|
| Concentration (particles/ml) | $1 \times 10^{10}$ [$6.8 \times 10^{9}$ - $1.3 \times 10^{10}$] | $1.4 \times 10^{10}$ [$8.9 \times 10^{9}$ - $5 \times 10^{10}$] | 0.06[a] |
| Modal size (nm) | 101 [87 – 111] | 94 [85 – 107] | 0.54[a] |

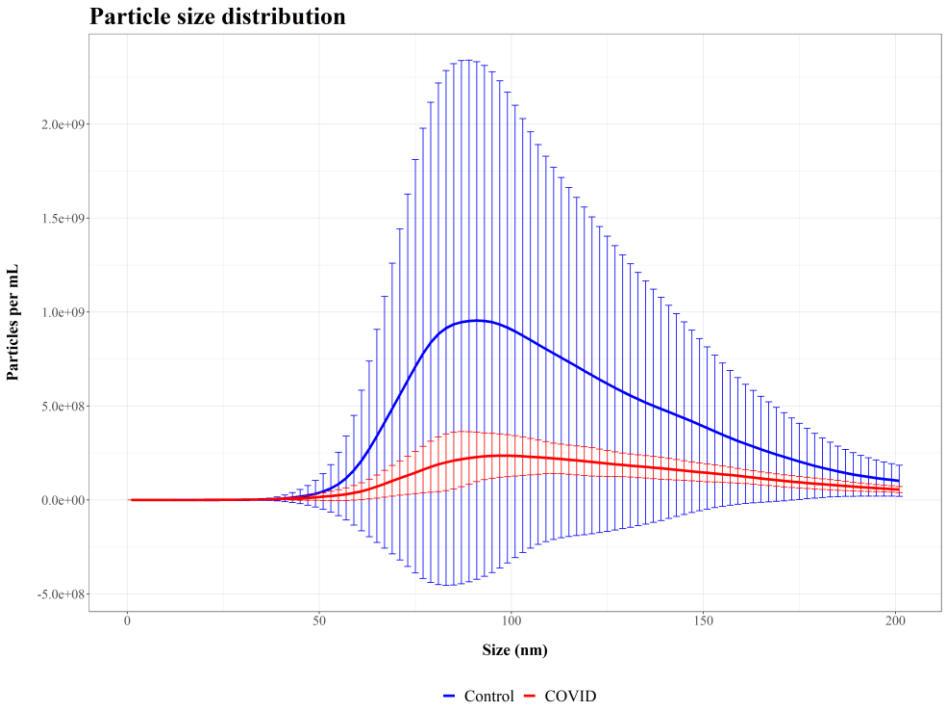


**Figure 7.4: Graphical representation of the distribution of SEVs in the COVID-19 group and controls**

Mean and standard deviation displayed

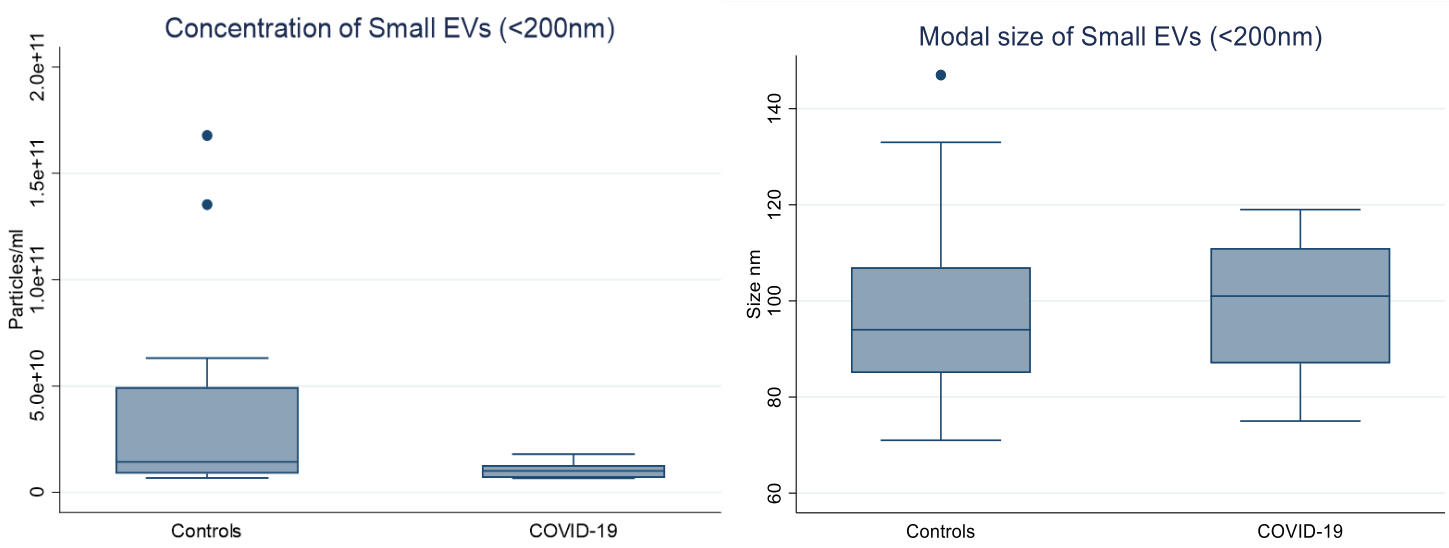


**Figure 7.5: Boxplot of the concentration and modal size of SEVs measured in UCB PPP using nanoparticle tracking analysis in the COVID-19 and control groups**

#### *7.3.3.7 Tissue factor extracellular vesicles*

The procoagulant activity of circulating TF-EVs was evaluated in 13 infants in the COVID-19 group ((2 excluded as previously described in *Section 7.3.3.4*) and 17 in the control group (excluded: n=2 insufficient plasma, n=1 remaining plasma aggregated). Five results (n=1 COVID-19 group, n=4 controls) were below the absorbance of the lowest standard (2.1 pg/ml). These “low” values were arbitrarily assigned a value of “1 pg/ml” and pseudo counts were randomly assigned (using Research Randomizer, www.randomizer.org, free online software) to each “low” value (e.g. 1.0001, 1.0002, 1.003 etc.). There was a trend towards a higher concentration of TF-EVs in the COVID-19 group but this did not reach statistical significance (p=0.07) (*Figure 7.6*).

**Table 7.16: Concentration of extracellular vesicles exposing Tissue Factor in the COVID-19 group and controls**

Mean (±SD), Median [IQR], Frequency (%), [a]Mann Whitney U test

| Tissue Factor Extracellular Vesicles | COVID-19 n=13 | Control n=17 | *p*-value |
|---|---|---|---|
| Concentration (pg/ml) | 5.3 [2.7 – 11.0] | 2.4 [1.9 – 4.9] | 0.07[a] |

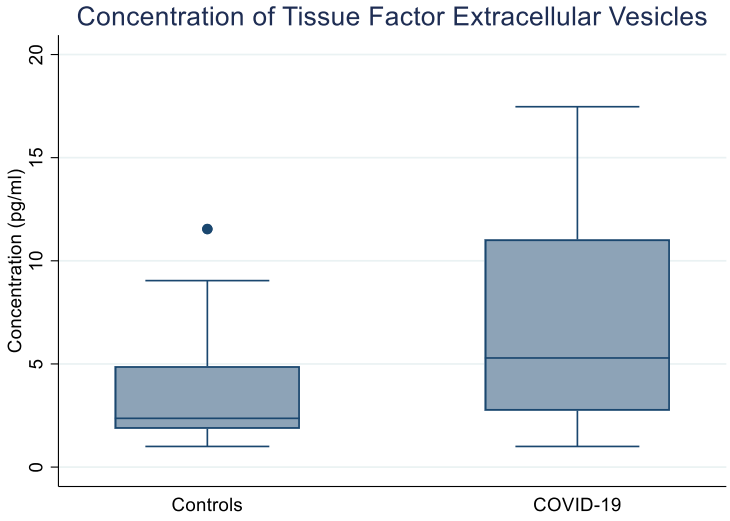


**Figure 7.6: Boxplot of the concentration of Tissue Factor extracellular vesicles in UCB PPP in the COVID-19 group (n=13) and controls (n=17)**

### 7.4 Discussion and future directions

A high incidence of preterm birth following a maternal COVID-19 infection in pregnancy was identified in the first review (*Section 7.3.1)*, although, in a larger cohort over a 12-month period *(Section 7.3.2)*, the rates of preterm birth and NICU admission were similar to 5-year hospital incidence. Maternal symptom status, infection before or after the emergence of the B.1.1.7. VOC and time interval from diagnosis to delivery did not influence neonatal outcomes. In the prospective COVID Cord Blood Study *(Section 7.3.3)*, in infants born to women with SARS-CoV-2 during pregnancy I found similar clinical outcomes between cases and controls and no evidence of haematological abnormalities (thrombocytopenia, leucopoenia or lymphopenia) in infants exposed to COVID-19 *in utero*. Moreover, in umbilical cord blood I did not detect altered coagulability, increased platelet activation or greater procoagulant extracellular vesicle activity in the COVID-19 group compared to controls.

Wide variation in the neonatal outcomes following a maternal SARS-CoV-2 infection during pregnancy has been reported in the literature (394). In the initial four-month review (*Section 7.3.1*), a high incidence of preterm birth was identified (n=6 [23%]) (418), which was similar to that reported in other UK and US studies (17 – 26%) at that time (357, 362). There was significant concern initially regarding the possible risk of mother to child transmission of SARS-CoV-2, either by vertical or horizontal transmission, although it rapidly became clear that the rate of neonatal infection were low (0 - 5%) (357, 362) once recommended precautions were implemented. The Royal College of Physicians in Ireland issued guidelines on “COVID-19 infection: Guidance for Maternity Services” (367), urging that healthy infants would room in with their mother on a postnatal COVID ward and be encouraged to breastfeed, albeit with the use of appropriate infection control precautions (e.g. hand hygiene and a maternal mask for breastfeeding). Our four-month review *(Section 7.3.1)* provided some reassurance to clinical staff regarding the infection control procedures in place in our hospital.

In view of the high incidence of preterm birth identified in our first study (*Section 7.3.1*) (418), a larger review was performed over the twelve-month period following the outbreak of COVID-19 in Ireland *(Section 7.3.2)* (423). The aim of this study was to interrogate the incidence and cause of preterm birth in this cohort and to

understand possible non-infectious implications of a maternal COVID-19 infection in their infants (e.g. growth restriction, NICU admissions, congenital anomalies). In this study *(Section 7.3.2)*, the incidence of preterm birth was 10.5% and did not differ significantly from the 5-year hospital incidence (6.3–7.9%) (p = 0.13). Moreover, there was no unifying cause of preterm birth identified with similar rates of preterm labour, PPROM, intrauterine growth restriction and hypertensive disorders of pregnancy resulting in preterm delivery. It is important to note that during this 12-month period, none of the preterm deliveries were performed in the maternal interest due to severe COVID-19. There are several factors which may explain the differences in the incidence of preterm birth in the first study (23%) *(Section 7.3.1)* versus the 12-month study (10.5%) *(Section 7.3.2)*. Firstly, there was a very small number of cases included during the first (n=26) compared to the second study (n=133). Secondly, particularly during the early stages of the pandemic, vulnerable groups such as The Roma Community of Ireland, Members of the Travelling Community and those living in homeless accommodation or direct provision were disproportionately affected by COVID-19, both in Ireland and abroad (422, 425, 426). These vulnerable groups have poorer pregnancy outcomes at baseline (427-430) and this may have confounded our four-month results. Finally, it has been suggested that the societal lockdowns implemented globally following the outbreak of COVID-19 may have caused a reduction in preterm birth during this time in the general population, although the evidence is conflicting (431-434).

There is large variation in the reported incidence of NICU admission following a maternal COVID-19 infection during pregnancy (10 – 76.9%) (355, 394). This may be partially explained by differing policies regarding the location of care for exposed infants. In our hospital, neonates were only admitted to NICU if there was a clinical indication for doing so, e.g. respiratory distress or prematurity less than 34 weeks (367). The NICU admission rate in the COVID-19 exposed infants (16.5%) was similar to the hospital admission rate over the 5-year period, 2015–2019 (13.1%–15.5%) (p =0.49). However, as mentioned above, four of these infants were critically unwell and PPHN did contribute to this pathology. Interestingly, a three-fold increase in the annual incidence of neonatal PPHN was noted in one centre in Israel following the outbreak of COVID-19 and the authors described 5 infants with unexplained PPHN following a maternal COVID-19 infection in the third trimester (435).

To date, there is very little information about the effect of *in utero* COVID-19 exposure and the incidence of congenital anomalies (395, 396). The incidence of congenital anomalies in the 12-month study was 9% *(Section 7.3.2.3)* which is higher than the European reported incidence of 2.4% major congenital anomalies (436). Our figure consisted of both major and minor anomalies. SARS-CoV-2 was diagnosed after 24 weeks in each case, making it unlikely to be the underlying cause of the anomaly. Moreover, this centre is one of four fetal medicine referral centres for Ireland so may be expected to see additional cases of anomalies. Women with a SARS-CoV-2 diagnosis were offered a scan in the third trimester to monitor fetal growth, in addition to the routine 20 week anomaly scan, offered to all patients. It has previously been shown that third trimester growth scans are associated with an increase in detection of incidental anomalies (437), particularly renal anomalies, which represented the largest cohort of anomalies in this group.

The B.1.1.7. VOC emerged as the dominant variant in Ireland in early 2021 (424). It was associated with more severe maternal illness (438) and COVID-19 placentitis, which resulted in intrauterine death in a number of cases (376). However, no difference in neonatal outcomes was identified (small for gestational age, preterm birth, NICU admission) between mothers infected with SARS-CoV-2 before and after the emergence of B.1.1.7. VOC *(Section 7.3.2.4)*.

In order to evaluate the effect of maternal symptom status, neonatal outcomes were compared between women with and without documented symptoms *(Section 7.3.2.5)* and the reasons for this analysis are explained below. It is known that severe COVID-19 can cause hypoxia, activation of the coagulation system and endothelial dysfunction (439-441), and other causes of pneumonia during pregnancy have been associated with adverse pregnancy outcomes (442). Numerous studies have documented placental abnormalities following a COVID-19 infection during pregnancy, such as maternal and fetal vascular malperfusion and thrombosis of the fetal vessels (368, 371, 373). These findings were seen in the absence of direct placental infection (COVID-19 placentitis) and may suggest a hypercoagulable or hyperinflammatory state within the mother. I hypothesised that a more symptomatic maternal infection would result in worse outcomes than an asymptomatic infection. However, no difference in neonatal outcomes between women with and without symptoms of COVID-19 was found *(Section 7.3.2.5)*. However, it is worth nothing that a very small proportion of women (4 [3.1%]) had severe disease requiring

hospitalisation, most had mild to moderate symptoms. Similar findings were described in an American report; maternal symptom status did not impact the incidence of preterm birth (443).

A trend towards a reduced birth weight in the group with a SARS-CoV-2 diagnosis remote from delivery was noted (p=0.07) *(Section 7.3.2.6)*. It is plausible that given the pathological placental findings discussed above (368, 371, 373), an infection remote from delivery could result in placental dysfunction over a longer period of time, thus influencing birth weight. However, when patients with asymptomatic infections diagnosed on pre-admission PCR screening were removed (many had high cycle thresholds, suggestive of previous infection and therefore the true timing of their infection was unclear), the median birth weight in those diagnosed less than 10 days from delivery was 3.54kg (IQR 3.23–3.86kg) and 3.35 kg (IQR 2.99–3.72kg) in those diagnosed more than 10 days before delivery and the findings were not significant (p = 0.22).

In the prospective COVID Cord Blood Study *(Section 7.3.3)*, careful consideration was given to the selection of a suitable control population. A historical cohort of healthy term infants recruited as controls to the EVENT Study were initially chosen (n=10), all of whom had been recruited prior to the first reported case of SARS-CoV-2 in Ireland. These are the controls initially reported in the *European Journal of Obstetrics and Gynecology and Reproductive Biology* publication (421) and included in thrombin generation and PF4 analysis. Due to the limited plasma remaining in this group of controls, a pragmatic decision was taken to also include healthy term controls (n=10) born to women with SARS-CoV-2 PCR not detected on admission to hospital for delivery and no documented history of COVID-19 infection during pregnancy. This group had the advantage of also having UCB full blood counts available and were included in the PlGF and EV components of this study.

I found no haematological derangements (anaemia, thrombocytopenia, leucopenia or lymphopenia) on FBCs in UCB in the infants born to women with SARS-CoV-2 during pregnancy *(Section 7.3.3.2)*. All values were within our neonatal reference ranges and similar to healthy neonatal controls. Data is limited on the incidence of neonatal haematological derangements following a maternal COVID-19 infection, particularly in asymptomatic infants. Two studies reported FBC results in SARS-CoV-2 negative infants born to women with SARS-CoV-2 during pregnancy (n=30 and n=9), and several infants had haematological derangements (444, 445). Unlike

our study, there was a high incidence of preterm infants and symptomatic infants in their studies. Our findings are similar to those reported in two smaller studies with normal FBC parameters in infants born to women with SARS-CoV-2 (n=5, n=3) (446, 447).

I did not identify evidence of hypercoagulability in UCB measured by CAT in infants born to women with SARS-CoV-2 during pregnancy *(Section 7.3.3.3)*. Although adults with COVID-19 infections may be hypercoagulable and at increased risk of thrombosis (337), these infants did not appear to be hypercoagulable compared to healthy term controls. Given that these infants were well and asymptomatic of COVID-19, it is perhaps not surprising that they had normal coagulation, as measured by CAT. However, there is evidence of thrombosis in fetal and maternal surfaces of the placental (373) in the absence of direct placental infection, and I wished to evaluate whether the hypercoagulable COVID-19 phenotype was transmitted in the absence of fetal SARS-CoV-2 infection, however this does not appear to be the case.

Previous work in our group has demonstrated a hyperactive phenotype in circulating platelets in hospitalised COVID-19 patients compared to both hospitalised and healthy controls (338). In adults, circulating PF4 levels were significantly increased in COVID-19 compared to controls, although the levels did not differ significantly between those with severe and non-severe disease. In UCB, no difference in PF4 levels were identified, suggesting that platelets were quiescent in the COVID-19 group *(Section 7.3.3.4),* again providing reassurance that the procoagulant phenotype is not transmitted to infants *in utero*.

Several studies have shown an increase in the procoagulant activity of circulating extracellular vesicles in adults with COVID-19 compared to healthy non-infected adults (342, 344). Campello *et al.* demonstrated an increase in both Annexin positive EVs and TF-EVs in COVID-19 patients compared to controls at baseline, measured by flow cytometry, and the TF-EVs remained persistently elevated 30 days post discharge (343). Other placental pathologies such as pre-eclampsia, have been associated with an increase in procoagulant extracellular vesicle activity measured in UCB (176). However, a trend towards a lower concentration of SEVs was found in the COVID-19 group, and the size of the SEVs was similar between groups *(Section 7.3.3.6)*. Moreover, when the procoagulant activity of the TF-EVs was evaluated, no significant increase in the Tissue-Factor EV activity in the COVID-19 group was

detected *(Section 7.3.3.7)* and both groups had similar levels to that described in another neonatal study of TF-EVs (177).

Overall, our results are quite reassuring. They suggest that well, asymptomatic infants born to women with SARS-CoV-2 during pregnancy did not display evidence of haematological abnormalities or hypercoagulability in umbilical cord blood at birth. It appears that the fetus may be protected from the systemic maternal infection and subsequent inflammatory response. The placenta, the maternal-fetal interface, is a complex regulator of immunological function and both a physical and immunological barrier to infection of the fetus (448). Placental EVs appear to play an important role in the immunosuppressive action of the placenta, facilitating implantation, angiogenesis and transferring miRNA to regulate gene expression (449). Moreover, the EVs released from trophoblast cells in the placenta, mediate an anti-viral effect on recipient cells via the miRNA they contain (450). The effects of COVID-19 on the placenta are poorly understood at present. However, the pathological findings described in the literature (373) should not be interpreted in isolation, but in the context of the neonatal clinical outcomes.

There were several limitations to these studies. All three studies (*Sections 7.3.1, 7.3.2 and 7.3.3)* evaluated the effects of COVID-19 infections during the wildtype and B.1.1.7. VOC dominance. Recruitment was completed prior to the emergence of the Delta variant, which caused more severe maternal illness (358) and, resulted in an increase in the number of pregnant/recently pregnant women with SARS-CoV-2 admitted to ICU in the Republic of Ireland, several of whom were delivered in ICU due to the severity of maternal illness, often at preterm gestations (360).

The 12-month study (*Section 7.3.2)* was limited by its retrospective nature, with inherent confounding and other biases and only small numbers were included. It is likely that some cases of SARS-CoV-2 diagnosed in the community were not reported by the patient or their healthcare provider to the COVID-19 notification system, particularly infections which may have occurred prior to booking the pregnancy. A majority of the SARS-CoV-2 diagnosis occurred in the third trimester and therefore, conclusions cannot be made about the effects of first or second trimester infections. Unfortunately, genotyping was not available on the maternal SARS-CoV-2 variants, therefore infection with B.1.1.7. variant was inferred from the date of diagnosis and available data public health data on the dominant strains (424). This study was not designed to evaluate maternal outcomes, the incidence of

miscarriage or stillbirth or the rate of mother to child transmission following a COVID-19 infection.

The prospective COVID Cord Blood Study (*Section 7.3.3)* was also limited by small numbers. At the time of study design, there was no available literature on fetal hypercoagulability following a maternal COVID-19 infection and it is possible that it was not adequately powered to detect subtle differences between groups. There were demographic differences between the COVID-19 group and controls, with a higher incidence of caesarean section and increased maternal age in the control group, as control infants were frequently recruited prior to elective caesarean section, often performed for a maternal history of previous caesarean section. Unfortunately, it was not possible to perform placental pathology in the COVID-19 group as part of this study and maternal coagulation values were not available.

A majority of the infants (n=14) in the COVID-19 group were born after the acute maternal infectious period and there were numerous reasons to explain this. For ethical reasons, parents had to be given sufficient time to consider the study information and could not be approached for the first time when they presented in labour. I aimed to evaluate whether there was an ongoing risk of increased fetal coagulation and haematological derangements following a COVID-19 infection. As many infections occur remote from delivery, it was essential to evaluate for a possible cause of the adverse perinatal outcomes reported in the literature (394). Finally, a number of the patients with a SARS-CoV-2 detected on PCR on admission were asymptomatic and had high cycle threshold, suggestive of a previous infection, and may not have been truly acute infections either.

Further research is required to improve our understanding of the effects of the SARS-CoV-2 virus on pregnant women, the placenta and the developing fetus. Future studies should investigate the effects of first and second trimester SARS-CoV-2 diagnosis, particularly their effect, if any, on fetal growth and congenital anomalies as there is currently very little evidence in this area (396). One of the challenges of studying neonatal outcomes in this area is the rapid emergence of new SARS-CoV-2 variants which highlights the need for real-time reliable information on their effect in pregnancy. Groups such as UKOSS (United Kingdom Obstetric Surveillance System) have been instrumental in providing this information to date (438) and ongoing large international collaborations are essential to providing up to date accurate information for clinicians.

If future studies are to further evaluate haematological effects of *in utero* COVID-19 exposure of the fetus, the inclusion of women with acute severe symptomatic infections would be important as these likely represent a higher risk group. Moreover, the inclusion of maternal coagulation and inflammatory markers in addition to placental histology would provide a more holistic understanding of the potential effect on the fetus.

### 7.5 Conclusion

These studies provide mostly reassuring data regarding the outcome of a maternal SARS-CoV-2 diagnosis during pregnancy, over the first twelve months of the COVID-19 pandemic in Ireland. Most infants born to women with SARS-CoV-2 were well and asymptomatic of COVID-19 and had similar NICU admission and preterm birth rates compared to the general hospital population. Moreover, I did not identify haematological derangements or hypercoagulability in umbilical cord blood in these infants. Further work is required to evaluate whether the severity of the maternal COVID-19 infection, such as that seen during the Delta wave, alters fetal coagulation. Moreover, evaluation of the clinical and placental pathological outcomes following COVID-19 infections in early pregnancy is essential to understand the longer-term effects of the virus on placental function.

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# Appendices

**Peer-reviewed publications related to this work**

1. Murphy CA, O'Reilly DP, Neary E, El-Khuffash A, NíAinle F, McCallion N, et al. A review of the role of extracellular vesicles in neonatal physiology and pathology. Pediatr Res. 2020.

2. Murphy C. O'Reilly D., McCallion N., Drew R., Ferguson W. Infants Born to Mothers with COVID-19 During Pregnancy: The First Four Months of the Pandemic. Ir Med J. 2020;113(9):193.

3. Murphy CA, Neary E, O'Reilly DP, Cullivan S, El-Khuffash A, NiAinle F, et al. The role of the calibrated automated thrombogram in neonates: describing mechanisms of neonatal haemostasis and evaluating haemostatic drugs. Eur J Pediatr. 2021.

4. Murphy CA, O'Reilly DP, Edebiri O, Weiss L, Cullivan S, El-Khuffash A, et al. Haematological parameters and coagulation in umbilical cord blood following COVID-19 infection in pregnancy. Eur J Obstet Gynecol Reprod Biol. 2021;266:99-105.

5. Murphy CA, O'Reilly DP, Edebiri O, Donnelly JC, McCallion N, Drew RJ, et al. The Effect of COVID-19 Infection During Pregnancy; Evaluating Neonatal Outcomes and the Impact of the B.1.1.7. Variant. Pediatr Infect Dis J. 2021.

**Peer-reviewed publications during this PhD (2019 – 2022) beyond the core themes of this thesis**

1. Murphy C. Loftus E, Malone F., McCallion N. Born into Direct Provision: Outcomes of Infants Born to Asylum Seekers. Irish Medical Journal. 2020;113(10):206.

2. Comer SP, Cullivan S, Szklanna PB, Weiss L, Cullen S, Kelliher S, et al. COVID-19 induces a hyperactive phenotype in circulating platelets. PLoS Biol. 2021;19(2):e3001109.

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4. Finnegan C, Murphy C, Breathnach F. Neonatal polycystic kidney disease: a novel variant. BMJ Case Rep. 2021;14(7).

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10. Murphy C, Bussmann N, Staunton D, McCallion N, Franklin O, El-Khuffash A. The effect of patent ductus arteriosus treatment with paracetamol on pulmonary vascular resistance. J Perinatol. 2022.

**Published abstracts arising from this work**

1. Murphy CA, Neary E, Kevane B, O'Reilly D, O'Loughlin J, El-Khuffash A, et al. The Effect of Platelets on Thrombin Generation in Cord Blood and Peripheral Neonatal Blood in the Premature Infant; The Event Study. Blood. 2021;138:3188.

**Research Awards**

1. Runner Up, Best Oral Presentation at the Irish Neonatal Research Symposium 2021.

**Oral presentations**

1. C Murphy, L Weiss, P Szklanna, S Comer, D O'Reilly, E Neary, A EL-Khuffash, F NíAinle, N McCallion, P Maguire. "Postnatal adaption of extracellular vesicles in neonates" UCD Conway Festival, October 2020 (Virtual)

2. C Murphy, L Weiss, P Szklanna, S Comer, D O'Reilly, E Neary, A EL-Khuffash, F NíAinle, N McCallion, P Maguire. "Postnatal changes in extracellular vesicles following preterm birth" UK Extracellular Vesicle Early Career Research Event, October 2020 (Virtual)

3. C Murphy, E Neary, B Kevane, D O'Reilly, J O'Loughlin, A EL-Khuffash, P Maguire, F NíAinle, N McCallion. "The effect of platelets on thrombin generation in the premature infants; The EVENT Study", C Murphy, ISTH Congress (International Society on Thrombosis and Haemostasis), July 2021 (Virtual)

4. C Murphy, E Neary, B Kevane, D O'Reilly, J O'Loughlin, A EL-Khuffash, P Maguire, F NíAinle, N McCallion, "Thrombin generation in the premature infant; the effect of platelets", JENS (Congress of joint European Neonatal Societies) September 2021 (Virtual)

5. C Murphy, D O'Reilly O Edebiri, L Weiss, S Cullivan, A EL-Khuffash, E Doyle, J Donnelly, F Malone, W Ferguson, R Drew, J O'Loughlin, E Neary, P Maguire, B Kevane, F NíAinle, N McCallion. "COVID-19 infection in pregnancy did not result in haematological abnormalities or hypercoagulability in umbilical cord blood". Irish Neonatal Research Symposium November 2021 (Virtual)