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To characterize standard laboratory coagulation parameters and plasma thrombin generation in very preterm infants and to investigate their relationship to clinical outcomes

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To characterize standard laboratory coagulation parameters and plasma thrombin generation in very preterm infants and to investigate their relationship to clinical outcomes

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

Supervisor (s): Prof. Naomi McCallion
Dr. Fionnuala Ni Ainle
Dr. Melanie Cotter

2016
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of a PhD, 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.

Signed: [Signature]

Student Number: 12188239

Date: 13th October 2016
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<tbody>
<tr>
<td>α2m</td>
<td>Alpha 2-Macroglobulin</td>
</tr>
<tr>
<td>AGA</td>
<td>Appropriate size for gestational Age</td>
</tr>
<tr>
<td>APC</td>
<td>Activated Protein C</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated Partial Thromboplastin Time</td>
</tr>
<tr>
<td>AT</td>
<td>Antithrombin</td>
</tr>
<tr>
<td>BW</td>
<td>Birthweight</td>
</tr>
<tr>
<td>BPD</td>
<td>Bronchopulmonary Dysplasia</td>
</tr>
<tr>
<td>CA²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CAT</td>
<td>Calibrated automated thrombography</td>
</tr>
<tr>
<td>CRUSS</td>
<td>Cranial Ultrasound</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulopathy</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ETP</td>
<td>Endogenous thrombin potential</td>
</tr>
<tr>
<td>FII</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>FV (a)</td>
<td>(activated) factor V</td>
</tr>
<tr>
<td>FVII (a)</td>
<td>(activated) factor VII</td>
</tr>
<tr>
<td>FVIII (a)</td>
<td>(activated) factor VIII</td>
</tr>
<tr>
<td>FIX (a)</td>
<td>(activated) factor IX</td>
</tr>
<tr>
<td>FX (a)</td>
<td>(activated) factor X</td>
</tr>
</tbody>
</table>
FXI (a)  (activated) factor XI
FXII (a)  (activated) factor XII
FXIII (a) (activated) factor XIII
FP       Frozen Plasma
GA       Gestational Age
HDN      Haemorrhagic disease of the newborn
HMWK     High Molecular Weight Kininogen
IVH      Intraventricular Haemorrhage
NEC      Necrotising Enterocolitis
NICU     Neonatal Intensive Care Unit
PBS      Phosphate-buffered saline
PDA      Patent Ductus Arteriosus
PIVKA-II Acarboxy prothrombin
PT       Prothrombin Time
PVL      Periventricular Leucomalacia
RCT (s)  Randomised Controlled Trial (s)
RDS      Respiratory Distress Syndrome
ROP      Retinopathy of Prematurity
SD       Standard Deviation
SEM      Standard Error of the Mean
SGA      Small for gestational age
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>SNAPPE-II</td>
<td>Score for neonatal acute physiology, perinatal extension, version II</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue Factor Pathway Inhibitor</td>
</tr>
<tr>
<td>VKD</td>
<td>Vitamin K Dependent</td>
</tr>
<tr>
<td>VLBW</td>
<td>Very Low Birth Weight</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand Factor</td>
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SUMMARY

Very premature infants are at risk of bleeding and frequently given plasma because of perception that coagulation tests are abnormal, which may simply be due to immaturity. We hypothesized that characterization of coagulation tests alongside assessment of thrombin generation would provide information on preterm haemostasis.

In a prospective observational study, cord and peripheral blood was drawn from neonates <30/40 and on day 1, 3 and fortnightly until 30/40 corrected gestational age (GA). Exclusion criteria included antenatal intraventricular haemorrhage and parental bleeding disorder. Prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, procoagulant and anticoagulant factor activity were measured and thrombin generation was characterized. Cord blood of term neonates provided control plasma.

Between 2013 - 2015, 137 infants <30/40 were recruited. Median (range) GA and birth weight were 27.9 (23.7-29.9) weeks and 1020 (510-1730) g respectively. Median (5th-95th percentile) Day 1 PT, APTT, and fibrinogen were 17.9 (12.8-27.7) s, 79.1 (48.8-134.3) s and 1.3 (0.7-3.9) g/L respectively (n=127). PT and APTT from preterm cord blood (n=42) were prolonged vs. controls (n=27, p<0.001), which corrected with increasing postnatal age; p<0.001.

Preterm cord blood peak thrombin and endogenous thrombin potential were similar to controls (132 (40.6) nM vs. 136.7(35) nM; p=0.66 and 1168(289) nM*min vs. 1303 (190) nM*min; p=0.11) respectively (n=27).
Mean activity of procoagulant factors II, VII, IX and X (0.31 (0.18-0.5) IU/ml vs. 0.44 (0.35-0.6) IU/ml, p=0.003; 0.33 (0.09-0.57) IU/ml vs. 0.42 (0.31-0.59) IU/ml, p=0.29; 0.16 (0.09-0.5) IU/ml vs. 0.29 (0.19-0.37) IU/ml, p=0.004 and 0.28 (0.13-0.52) IU/ml vs. 0.44 (0.32-0.58) IU/ml, p=0.02) respectively, n=12 and anticoagulant factors Protein C (0.11U/ml (0.06-0.24) IU/ml vs. 0.27 (0.18-0.39) IU/ml; p=0.002), free protein S (0.38 (0.28-0.55) IU/ml vs. 0.47 (0.36-0.59) IU/ml; p=0.02), antithrombin (0.22 (0.06-0.36) IU/ml vs. 0.53 (0.38-0.69) IU/ml; p<0.001) and tissue factor pathway inhibitor (6.4 (2.6-16.9) ng/ml vs. 9.2 (4.2-15.6)ng/ml; p=0.1) were reduced in preterm vs. controls.

In conclusion, we describe ranges for coagulation tests, demonstrate differences in both procoagulant and anticoagulant pathways, and show that thrombin generation is similar in very preterm and term infants.
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Finally, I would like to thank my family and friends for their enduring support and encouragement.
CHAPTER 1: INTRODUCTION

1.1 Prematurity

Neonatology encompasses the care of all infants less than a month of age and prematurity refers to all infants born less than 37 weeks gestational age (GA). In the US, the rate of preterm births is 12.5%, rising by 33% over the previous two decades (Horgan, 2015). Initial management is focused on stabilisation of the infant in the delivery room. With reduction in GA, rates of complications of prematurity increase. Late preterm infants may have difficulties with feeding or thermoregulation. Those born less than 30 weeks GA are at highest risk. Infants born less than 28 weeks GA are referred to as extremely preterm infants and those less than 30 weeks as very preterm infants. Survival rates for infants at the limits of viability at 24 weeks GA are approximately 60% and of survivors there is a 30% chance of lifelong severe disability (Dani et al., 2009). Common complications of prematurity include respiratory distress syndrome (RDS), chronic lung disease, necrotising enterocolitis (NEC), and patent ductus arteriosus (PDA). These very premature infants are at high risk of developing bleeding complications including intraventricular haemorrhage (IVH) with significant impact on long-term morbidity and mortality (McCrea and Ment). Care by neonatologists is directed towards minimising these potential complications. Advances in neonatology have led to a higher survival rate for very premature infants (Guillen et al.), but the incidence of complications and rates of long term neurodisability has remained high in these infants (Costeloe et al., 2012, Stoll et al., 2015).
1.2 Intraventricular Haemorrhage

The risks of neurodisability are highest for the smallest and sickest preterm babies, but it is not possible to determine precisely which infants will go on to develop significant neurological motor or cognitive problems. One of the major complications premature infants face is that of IVH with its potential for significant associated morbidity and mortality. IVH is most commonly found in premature infants and occurs in approximately 20-25% of infants <1500g (Volpe JJ 2008). IVH rarely occurs antenatally. The majority of cases occur in the first few days of life. Half of cases occur in the first 48 hours after birth (Brouwer et al., 2012) increasing to eighty per cent within 72 hours and ninety per cent by day seven of life (Whitelaw, 2001).
1.2.1 What are the implications of intraventricular haemorrhage?

It is known that the presence of IVH, particularly severe IVH, increases the risk of adverse neurological sequelae (Volpe JJ 2008). IVH is routinely examined for with cranial ultrasound (CRUSS) imaging; a safe bedside screening tool (Figure 1.1). The severity of IVH is graded in line with Papile’s classification (Papile et al., 1978). In Papile’s classification, grades 1-4 IVH are described as bleeding that is limited to the germinal matrix; bleeding within the ventricles but without ventricular dilation; ventricular bleed with dilation and bleeding that has extended beyond the ventricles into the surrounding parenchyma respectively. Grades III and IV IVH are associated with the worst outcomes, including death from hypovolaemic shock, hydrocephalus and later disability including cerebral palsy and seizures (Klebermass-Schrehof et al., 2012). This has led to interventions to try to prevent or limit the extent of IVH in an effort to improve long term outcome and quality of life of these infants.
Figure 1.1: Preterm infant, GA 27 weeks CRUSS performed on day 1, day 4 and day 5 of life. Coronal views showing a normal CRUSS on admission, with subsequent development of an IVH. Adapted from Gerda van Wezel-Meijler and Linda S de Vries, Cranial ultrasound - optimizing utility in the NICU. *Current Pediatric Reviews*, 2014;10(1):16-27
1.2.2 Aetiology

IVH originate in the subependymal germinal matrix, which is located beside the lateral ventricles. This area is comprised of neural cells which migrate into the cerebral cortex during the second and third trimester (Roland and Hill, 2003). In addition, this area is rich in immature blood vessels poorly supported by connective tissue which are prone to haemorrhage (Ballabh, 2014). As infant matures, the germinal matrix involutes and is absent from 34 weeks GA thus reducing the rate of IVH (Vergani et al., 2000). The pathogenesis of IVH is probably multifactorial, with contributions from fluctuations or acute increases in cerebral blood flow secondary to immature cerebral blood flow autoregulation (Whitelaw, 2001, Ment et al., 1995), poor vascular integrity and extravascular factors around the germinal matrix capillaries (Roland and Hill, 2003, Brouwer et al., 2012). Other contributing factors include ventilation induced changes in intrathoracic and venous pressures conveyed to the germinal matrix (Ballabh, 2014) and potential roles of metabolic disturbances. Irrespective of the aetiology, blood diffuses from the germinal matrix and progresses into the ventricles (Robertson, 2006).
1.2.3 Role of coagulation system in development of IVH

Abnormalities of coagulation have been suggested as part of the pathogenesis of newborn IVH (Beverley et al., 1984, Ballabh, 2014). The majority of studies supporting this hypothesis were performed several decades ago (McDonald et al., 1984, Beverley et al., 1984). Beverly et al and McDonald et al observed a significant association between coagulation values at birth and at 48 hours of life and grade of IVH in preterm infants (Beverley et al., 1984, McDonald et al., 1984). Setzet et al demonstrated that in very low birthweight (VLBW) infants (n=54), those with IVH had a significantly prolonged mean bleeding time in comparison to the infants who did not develop IVH (Setzer et al., 1982). Van de Bor et al observed a difference in factor V (FV) levels in those who had or had not IVH in 49 infants less than 34 weeks GA (Van de Bor et al., 1986).

Several advances in neonatology have occurred since which limits the generalisability of these studies including the improvements in outcomes and increased use of antenatal steroids as well as advances in obstetrics and NICU care since the 1980s. The study by Beverley et al was also limited by the fact that many of the samples taken at 48 hours life were drawn from heparinised umbilical lines, and the fact that cord blood values were compared to values taken at 48 hours (Beverley et al., 1984). McDonald et al also drew samples from heparinised lines (McDonald et al., 1984).

More recently Porella et al demonstrated an association between low factor II (FII), FV, factor VII (FVII), factor VIII (FVIII), factor X (FX) and anti-thrombin levels and IVH although low FVII was the only factor indicated as a significant risk factor in development of IVH (Poralla et al., 2012a). Piotrowski et al observed a lower FVII level in infants with high
grade IVH in a study of 38 VLBW infants (Piotrowski et al., 2010) and Salonvarra et al observed a low prothrombin level in infants with IVH (Salonvaara et al., 2005).

On the other hand Motta et al examined data on 290 infants with a mean GA of 30 weeks (Motta et al., 2014). Upon multivariate analysis, no laboratory tests of haemostasis (prothrombin time, activated partial thromboplasatin time, fibrinogen and platelet count) were associated with haemorrhage.

Christensen et al observed that abnormal coagulation values drawn at delivery did not predict clinical bleeding episodes during the first week (Christensen et al., 2014). However they noted that that a larger sample size would be warranted to make a definitive conclusion. Altuntas el al. performed a retrospective study of FFP use in NICU, and have stated that “There is currently no evidence that a particular coagulation value as measured by the prothrombin time (PT) or activated partial thromboplasatin time (APTT) is associated with an increased risk of bleeding” (Altuntas et al., 2012).

Although controversial, the low risk of intracranial haemorrhage observed in very premature neonates with documented haemophilia also supports the hypothesis that coagulation does not play a major role in the development of intracranial haemorrhage (Fink et al., 2014).

Alternatively pro-thrombotic states have been postulated to act as a mechanism of causation of IVH. A thrombosis occurring in the germinal matrix could obstruct venous drainage (Ramenghi et al., 2011). This obstruction would increase pressure in the tissue and capillaries and result in extravasation of blood (Kuperman et al., 2013). Cerebral sinus thrombosis was noted to account for approximately one third of cases of IVH in term neonates in one study (Wu et al., 2003). Proposed mechanisms for venous thrombosis in neonates include maternal
hypercoagulability, physical effect of labour, high haematocrits, lower levels of coagulant factors and neonatal morbidities including sepsis, birth asphyxia, dehydration, congenital heart disease and thrombophilia (Moharir et al., 2011, deVeber et al., 2001). Genetic factors including those related to genes related to coagulation are been further considered in IVH aetiology (Ryckman et al., 2011).

1.2.4 Therapeutic Options

Interventions which prevent or limit IVH would improve morbidity and mortality for very premature infants. As there is currently no treatment for IVH once it has occurred, most strategies have been aimed at preventing or treatment of potential risk factors (McCrea and Ment, 2008). In order to prevent IVH, one could ideally prevent prematurity. However with the limits of viability decreasing further and preterm births affecting 12.7% of pregnancies this is unlikely to occur (Sayres, 2010).

Therefore neonatologists focus majority of their care on the secondary prevention of IVH be that antenatally, intrapartum or postnatally. Antenatal administration of corticosteroids and postnatal surfactant use are both examples of evidence based therapies that have through reduction of RDS led to reduction of IVH (Whitelaw, 2001). Indomethacin used in the prevention of PDA is a non-steroidal anti-inflammatory agent which reduces the incidence of IVH through its action on stabilising baseline cerebral blood flow (Fowlie et al., 2010). The TIPP trial was a large international multicentre randomised controlled trial which examined the use of empirical intravenous indomethacin compared with saline acting as a placebo in the prevention of IVH in preterm infants less than 37 weeks (Schmidt et al., 2001). 1200 infants
were recruited, none of whom had a known IVH at time of enrolment. Adequate concealment and blinding of the intervention to those caring for the infant was performed. A reduction in IVH and PDA was observed in the group receiving prophylactic indomethacin. However no benefit in the study’s primary outcome (improved survival/neurodevelopmental outcome) was observed following indomethacin administration. A Cochrane meta-analysis has also shown that however despite this finding, neurodevelopment in cases compared to placebo was not improved (Fowlie et al., 2010). Antenatal magnesium sulphate is incorporated into standard practice as a measure to reduce IVH. In a study by Petrova et al, the authors found that in preterm infants with gestational age 23–31 weeks with IVH, antenatal MgSO4 exposure was less likely than in infants with the same clinical characteristics but without IVH (Petrova and Mehta, 2012). Neither phenobaritone, ibuprofen or vitamin E administration play a role in preventing IVH (Robertson, 2006).

### 1.2.5 Coagulation-directed therapy

Concern about coagulopathy arises because VLBW infants are observed to have prolonged PT and APTT times compared with term infants and adults (Christensen et al., 2014). Despite controversy over the role of coagulopathy in the causation of IVH (Volpe JJ 2008), the treatment of coagulopathy or perceived coagulopathy in premature infants has been advocated by some as a means to prevent, or prevent extension of, IVH in VLBW infants. However evidence regarding haemostatic agents is limited by small sample samples, poor study design and data regarding long term effects.
Potential therapeutic strategies in prevention of IVH that have been studied include ethamsylate, a non-steroidal drug that decreases the degree of bleeding in small blood vessels through enhancing capillary endothelial resistance and activating platelet adhesion (Kuperman et al., 2013). Although ethamsylate appears to reduce IVH in preterm infants, a meta-analysis showed that there was not long-term benefit with respect to mortality or neurodevelopmental disability (Schulte et al., 2005). Recombinant activated FVII has been proposed to reduce IVH through augmentation of haemostasis locally at site of damaged blood vessels, but there is insufficient evidence against potential adverse risk of increased thromboembolic events (Robertson, 2006). Prothrombin complex concentrate or antenatal vitamin K was not shown to be efficacious in the prevention of IVH. Prothrombin complex concentrate or antenatal vitamin K was not shown to be efficacious in the prevention of IVH (Waltl et al., 1973).

Frozen plasma (FP) is an example of therapeutic agent that is used in adult, paediatric and neonatal patients to correct coagulopathy in patients who are bleeding or perceived to be at risk of bleeding. Perceived abnormalities in haemostatic system have been postulated to be associated with aetiology of IVH (Beverley et al., 1984). The administration of FP could reduce bleeding tendency in these preterm infants who are known to have prolonged laboratory measurements of coagulation. This could either prevent the occurrence of haemorrhage or the extension of an established low grade IVH into more severe forms of Grade III and IV (Papile et al., 1978).

Within plasma there are also many proteins including albumin, complement, transport molecules, immunoglobulins (gamma-globulins). Derivatives of plasma can be produced from whole blood or through plasmapheresis and then frozen to -18 degrees Celsius within a specified
time period (6-8 hours for fresh FP or 24 hours for F24). FP contains both procoagulant and anticoagulant components of the coagulation cascade including fibrinogen, FVIII, Von Willebrand Factor (VWF), FXIII, and vitamin K dependent (VKD) coagulation factors (FII, FVII, F IX, FX). As FVIII, FV, AND FVII have low half-lives, these factors are lower in F24 compared to FP, although the term FP is often used for both these products. FP is generally administered in doses of 10ml/kg (although can range to 20mls/kg), a volume which may impact on the haemodynamic status and overall fluid balance in preterm infants. As most IVH occur in the first 72 hours of life, FP is also most commonly given early in the postnatal period.

However the effects of FP may be exerted by a mechanism other than the improvements of coagulation factor concentrations. IVH is known to be associated with fluctuating cerebral blood flow and blood pressure instability (Van Bel et al., 1987, Perlman et al., 1983, Ballabh, 2010), and plasma may exert its effect through volume alone - stabilising the circulation and thus preventing rapid changes in blood pressure through a pressure passive cerebral circulation. Through these mechanisms, it might prevent a new haemorrhage, or prevent the extension of a haemorrhage (Fanaroff and Fanaroff, 2006, Julkunen et al., 2008, Salonvaara et al., 2005). If this is the mechanism, other fluids could have a similar effect without the risk of adverse transfusion reaction. This is supported by recent evidence demonstrating that deferred cord clamping following delivery in preterm infants reduces the incidence of hypotension and severe IVH (Mercer et al., 2006).

Despite the perceived benefit of FP in prevention of IVH, the benefit of this practice has not been clearly shown. Administration of FP carries potential risks due to volume overload, infection (including possible infection with as yet unidentified pathogens) and adverse transfusion reactions (Pal et al., 2014). Transfusion reactions following
FP transfusion include transfusion-associated lung injury and transfusion associated circulatory overload with incidence for both at 1/5-10000 in US and 6% respectively (Li et al., 2011, Sanchez and Toy, 2005). Both are likely underreported in the neonatal population (Pal et al., 2014). Neonates are also at a greater risk of transfusion errors (Stainsby et al., 2008).

The potential benefits need to be considered in terms of dosing, route, and duration of administration of this product. To minimize the risk of infection pathogen inactivation technology has been developed. Plasma is pooled usually from 300-5000 donations and exposed to solvent detergent treatment, methylene blue treatment (a phenothiazine dye and light) or amotosalen treatment (psoralens are added to plasma and exposed to UVA light). These methods have the advantage of enhanced virus and microbial protection, but are associated with altered or loss of coagulation factor content.

It is unclear whether prophylactic administration of FP prevents neonatal IVH in all high risk patients. It is also not known whether infants identified as having prolonged PT and APTT times benefit from administration of FP to prevent IVH. There is no consensus on what therapeutic modalities and when should be given to very premature neonates. Prophylactic use of FP in response to perceived abnormalities in coagulation test results, without signs of active bleeding accounted for approximately half of cases of FP transfusions in an international audit of practice (Stanworth et al., 2011). The dilemma regarding the correct use of FP arises from the lack of understanding of the physiology of the neonatal haemostatic system and what are normal parameters for this population. By delineating normative values for this cohort, one could provide guidance on when therapeutic modalities may be indicated thus potentially limiting transfusions in this population. However the role of coagulation in bleeding tendencies in this age group has not been well established. The value of coagulation tests as a predictor of bleeding is
limited in all patient populations. In addition the role of FP in modifying coagulation tests when mild and moderated results are documented is questionable (Segal and Dzik, 2005). There appears to be paucity of data supporting the empirical use of FP in neonates to prevent IVH (Group, 1996, Stanworth, 2007, Yang et al., 2012).
1.3 Conclusion

Prevention of IVH remains elusive, with concern that current practices may even be harmful. Evidence for and against therapeutic strategies are limited by studies examining different agents and methods of administration and diagnosis of IVH. Aetiology of IVH must be considered further in order to assess effective therapies. The role of coagulation in relation to IVH and the use of FP in response to perceived coagulation abnormalities will be examined in this thesis.
CHAPTER 2: BLOOD COAGULATION

A broad understanding of physiology of the adult coagulation system is required in order to examine coagulation system in premature neonates. Haemostasis refers to a balanced and regulated system that maintains blood in a fluid state. In addition excessive blood loss is prevented by a combination of events that are initiated in the event of injury. Virchow’s triad illustrates these complementary activities including vasoconstriction, sequestration of von-Willebrand factor (VWF) and platelets to site of injury followed by activation of coagulation cascade. The physiological response to vessel injury involves formation of a temporary platelet plug which is subsequently stabilised by generation of cross linked fibrin, whilst repair occurs (Furie and Furie, 2008).
2.1 Primary Haemostasis

Primary haemostasis results in the formation of a platelet plug at a site of vascular injury and involves four sequential overlapping phases: vasoconstriction, platelet adhesion, platelet activation and platelet aggregation (Israels et al., 2011). VWF, a large multimeric glycoprotein stored in endothelial cells and platelet alpha granules, interacts with exposed collagen and platelet receptors (GPIβ/IX/V and GPVI) to facilitate the process of platelet aggregation (Reininger, 2008). Binding of VWF to platelet receptors results in conformational changes in platelet integrin’s α2β2 and α2β1 which stabilises the platelet plug (Rivera et al., 2009). Activated platelets release adenosine di-phosphate, serotonin and thromboxane A2, amplifying platelet activation. Activated platelets express negatively charged phospholipids to their surfaces which act as a surface for the enzymatic processes of the coagulation cascade i.e. secondary haemostasis.
2.2 Secondary Haemostasis

Secondary haemostasis is the process whereby the haemostatic system is instigated, propagated and continued by a cascade effect of coagulation protein activation and is tightly regulated. The pathway is complex and involves many different proteins; zymogens (inactive precursors) of serine proteases (FII, FVII, Factor IX (FIX), FX, Factor XI (FXI), Factor XII (FXII)); cofactors (Tissue Factor (TF), FVIII, FV); a transglutaminase zymogen (Factor XIII (FXIII)); and fibrinogen (Figure 2.1). A serine protease is an enzyme containing the amino acid serine in its active site that hydrolyses specific peptide bonds in proteins, and a transglutaminase is an enzyme that forms peptide bonds between the side chains of specific glutamine and lysine amino acid residues.

When injury occurs to a blood vessel, sub-endothelial TF, a 47kDa transmembrane glycoprotein on the surface of extravascular cells is exposed (Drake et al., 1989, Bouchard et al., 1997, Schecter et al., 2000). TF forms a complex with VKD serine protease FVII, resulting in morphological alterations in the serine protease domain that enables activation (Dickinson and Ruf, 1997). Activated FVII is also present in plasma in free form constituting 1-2% of total FVII and can bind to TF directly. TF-FVIIa then binds to and forms ternary complex with FX or FIX, thus activating them to FXa and FIXa respectively (Mackman et al., 2007, Mann et al., 2003).

FXa converts a small amount of prothrombin to thrombin, which in turn activates FV and FVIII (Pieters et al., 1989). A feedback loop is created which results in amplification of thrombin generation by the generation of active cofactors for the prothrombinase and intrinsic tenase complexes respectively (Brummel et al., 2002). Sufficient thrombin generation is essential for satisfactory haemostasis. It is postulated that enhanced thrombin generation is associated with prothrombotic
tendencies whereas reduced thrombin generation may be associated with a haemorrhagic phenotype (Kremers et al., 2015).

Thrombin generation can occur in the absence of TF, via the ‘contact pathway’ and is initiated by activation of FXII to FXIIa, which in turn activates FXI to FXIa (Renne, 2012). The result of this pathway is FXIa-mediated FIX activation, which activates FX through the intrinsic tenase complex (FVIIIa + IXa + X + phospholipids).

Irrespective of the mode in which the cascade is initiated, both pathways meet at the level of FX activation. Activated platelets provide a negatively charged phospholipid surface on which FXa in association with FVa in the presence of Ca$^{2+}$ form the prothrombinase complex (FVa + FXa + FII + phospholipids). This complex is 300,000 times more efficient than FXa alone in converting prothrombin to thrombin (Rosing et al., 1980). The negatively charged phospholipid membrane of activated platelets, monocytes and endothelial cells provide a surface for the activation of the serine protease zymogens.

The formation of a blood clot occurs when thrombin cleaves fibrinopeptides from fibrinogen to fibrin, which can then polymerize and form an insoluble fibrous mesh in association with platelets and VWF (Mann et al., 2003). Thrombin also activates FXIII, a transglutaminase which covalently cross links fibrin at glutamine and lysine residues (Spraggon et al., 1997). This increases fibrin clot resistance to proteolytic degradation.

Thrombin is a multifunctional enzyme that catalyses reactions that promote coagulation, particularly the conversion of fibrinogen to fibrin monomer, but also reactions that limit coagulation, including activation of the anticoagulant protein C. Thrombin is regulated by binding irreversibly to inhibitors such as antithrombin (AT) and alpha 2-macroglobulin (α2m) and to a lesser extent by a group of miscellaneous serpins (alpha1antitrypsin, antichymotrypsin, antitrypsin, protease nexin 1, C1inhibitor, and others).
2.2.1 Procoagulant Mechanisms

**Vitamin K dependent proteins**

The VKD factors prothrombin, FVII and FX have varying half lives in circulation. The factors with the longest and shortest half-lives are FII and FVII respectively (Forestier et al., 1986, Reverdiau-Moalic et al., 1996). FVII, FIX, FX, prothrombin, protein C, protein Z and protein S are VKD coagulation proteins characterized by the presence of a negatively charged \(\gamma\)-carboxyglutamic acid domain which mediates binding to phospholipid membranes (Stafford, 2005). FVII, FIX, FX and protein C are further comprised of two epidermal growth factor (EGF) like domains and a C-terminal serine protease domain (McDonald et al., 1997). VKD coagulation proteins require post-translational modification by the enzyme \(\gamma\)-glutamyl carboxylase in order for the N-terminal \(\gamma\)-carboxyglutamic acid domain to adopt a function conformation which enhances the association of these factors with negatively charged phospholipid membrane surfaces.

**Contact Activation System**

Contact Activation System is a protease cascade that consists of four proteins called contact factors, including FXII, FXI, and prekallikrein which are serine pro-enzymes, and high molecular weight kininogen (HMWK), being a cofactor protein. In the “contact” or “intrinsic” pathway of blood coagulation, contact with negatively charged surfaces, autoactivates FXII to FXIIa in the presence of prekallikrein and high molecular weight kininogen (HMWK). According to the waterfall hypothesis, this initiates the intrinsic pathway of coagulation as described previously (Uszynski et al., 2015). *In vivo*, FXI can be activated by
alternative routes and therefore deficiencies of FXII, prekallikrein or HMWK are not associated with abnormal bleeding.

### 2.2.2 Anticoagulant Mechanisms

**Alpha 2-Macroglobulin**

One of main regulators of thrombin is α2m, a broad-spectrum protease binding protein present on the luminal surface of endothelial cells in normal human arteries, veins and lymphatics, which acts by blocking thrombin’s active site from interacting with its substrates (Sottrup-Jensen, 1989, Ling et al., 1995)

**Tissue Factor Pathway Inhibitor**

Another important regulator of thrombin is Tissue Factor Pathway Inhibitor (TFPI) (Bajaj et al., 2001); a 43kDa, 276 amino acid glycoprotein with 3 Kunitz domains (K1, K2, and K3), an acidic N-terminal region and a highly basic C-terminal region. TFPI regulates thrombin generation through the FVIIa/TF pathway by initially binding to FXa before complexing with FVIIa-TF, and preventing further activation of FX (Girard et al., 1989). Some thrombin can continued to be generated because FIXa is, also activated by FVIIa/TF pathway and proceeds to activate FX. FIXa is not inhibited by TFPI (Camerer et al., 1996, Ofosu, 1995).
**Antithrombin**

AT is a 58 kDa, 432 amino acid glycoprotein and exists as two distinct isoforms, native AT or latent AT. In addition AT circulates in two glycoforms; α-AT has four identical sialylated complex oligosaccharides attached to asparagines, which account for about 90-95% of AT and β-AT is only glycosylated on three of the potential four asparagine sites (Picard et al., 1995). AT slowly reacts with and irreversibly inhibits thrombin, FIxa, FXa and FXIa (Rau et al., 2007, Pike et al., 2005). AT inhibits its target proteases in two steps. In the first step, an encounter complex is formed, in which the target protease recognizes the sequence of the AT reactive site loop. Ser-195 of the protease attacks the peptide bond between the P1 and P1’ residues, resulting in formation of a covalent bond between P1 and active site Ser 195. This is followed by protease conformational change and loss of enzymatic activity (Dementiev et al., 2004, Rau et al., 2007). The inhibition of more than one factor in the cascade amplifies its effect.

**Protein C and Protein S**

Protein C is a VKD serine protease which circulates in plasma as a zymogen and is activated to activated protein C (APC) by the complex of thrombin bound to thrombomodulin (Dahlback and Villoutreix, 2005a). Thrombomodulin is expressed in the vascular endothelium, and consists of a C-type lectin-like domain, six EGF-like domains, a serine/threonine-rich region, a transmembrane section and a short cytoplasmic tail. Thrombin binds to EGF 5 and 6, while protein C binds to EGF 4 during activation (Yang and Rezaie, 2003). Thrombomodulin binding effectively switches the function of thrombin from procoagulant to anticoagulant because exosite I (which is required for substrate binding) becomes occupied by TM domains (Dahlback and Villoutreix, 2005b).
Protein C activation is modulated by the interaction of protein C with the endothelial protein C receptor (Stearns-Kurosawa et al., 1996). APC mediates its anticoagulant function by cleaving and inactivating cofactors FVa (Walker et al., 1979) and FVIIIa (Fay et al., 1991), which is dependent upon the presence of the anticoagulant cofactor protein S; a VKD glycoprotein, in plasma. Amplification occurs because one molecule of thrombin can activate many molecules of protein C and each molecule of APC can cleave many molecules of FVIIIa or FVa.

In addition, APC has recently been shown to possess signalling properties, which mediate anti-inflammatory, anti-apoptotic and endothelial barrier protective effects. The half-life of APC in plasma is ~30 minutes (Gruber and Griffin, 1992). The proteolytic function of APC is inhibited by protein C inhibitor, plasminogen activator inhibitor-1, α-1-antitrypsin (Heeb and Griffin, 1988), α2m and α-2 antiplasmin (Heeb et al., 1991).
Figure 2.1: The coagulation cascade
2.3 Fibrinolysis

The final components of the haemostatic system involves fibrinolysis or break down of the blood clot once the blood vessel has repaired the defect and allows blood flow to resume as normal in the vessel. Plasminogen, the principal zymogen in the fibrinolytic pathway (92 kD), is activated by proteolytic cleavage at Arg 560 to generate active serine protease plasmin (Holvoet et al., 1985, Cesarman-Maus and Hajjar, 2005) which in turn degrades fibrin. The two major plasminogen activators are tissue plasminogen activator and urokinase plasminogen activator (Cesarman-Maus and Hajjar, 2005). Serine protease inhibitors (serpins) plasminogen activator inhibitor-1 and 2 and α2-antiplasmin inhibit fibrinolysis. Plasminogen activator inhibitor-1 regulates both tissue plasminogen activator and urokinase plasminogen activator and is the principal inhibitor of plasminogen activation (Cesarman-Maus and Hajjar, 2005). Release of plasminogen activator inhibitor-1 from platelets at the site of injury protects the developing thrombus from fibrinolysis (Rau et al., 2007). In contrast, tissue plasminogen activator, plasminogen and plasmin are bound to fibrin later during clot development (Cesarman-Maus and Hajjar, 2005).
2.4 Assessment of Haemostatic System

Blood haemostasis represents equilibrium between pro- and anticoagulant proteins to maintain thrombin at a steady state (Dahlback, 2005). In routine practice, alterations in this balance is assessed for by use of tests for isolated components of the coagulation cascade with assays such as PT and APTT or by measuring levels of individual proteins.

2.4.1 PT, APTT, Fibrinogen

PT is a reflection of time taken for coagulation to occur following supplementation of decalcified, platelet poor plasma with TF, phospholipid and calcium. The PT is a measurement of the activity of the extrinsic and common pathways of coagulation and therefore, is reflective of FVII, FX, FV, FII (prothrombin) and fibrinogen. In contrast the APTT measures the activity of the intrinsic and common pathways of coagulation.

These assays have inherent limitations in that they are not very sensitive or specific to detection of some clinical bleeding and thrombotic disorders. The results of these tests can imply that the patient is normal when they are at risk or abnormal when there is no risk. They were primarily designed for monitoring of anticoagulant therapy and for the care of patients with inherited bleeding disorders. These tests provide information about blood chemistry but do not provide information about vessel wall and flow which as described in Virchow’s triad contribute to thrombosis and haemostasis.
In addition to the lack of sensitivity and specificity of these tests, these tests only reflect the initiation phase of blood coagulation. Ninety-five per cent of thrombin generation is known to occur following clot initiation and is therefore not reflected by these assays. Similarly, the naturally occurring anticoagulant systems are not reflected by these assays as insufficient thrombin is formed for their activation. *In vivo*, the anticoagulant functions of AT and protein C is activated by glycosaminoglycans (Tripodi et al., 2008) and thrombomodulin (Dahlback, 2005) respectively on endothelial cells. The routine tests of blood coagulation therefore do not accurately reflect *in vivo* haemostatic systems as these assays measure isolated components of the coagulation cascade without consideration of other interacting components. Consequently PT and APTT can indicate whether a patient is deficient in pro-coagulants but not as to whether that deficiency is balanced by a deficiency in anticoagulants. Despite these limitations, these assays have some use in routine hospital practice. An aspirational aim would be to supplement these tests with clinically validated assays that more accurately reflect the entire haemostatic pathway.

### 2.4.2 Calibrated Automated Thrombography Assay

Prediction of the overall bleeding risk in patients with prolonged coagulation times can be challenging, as there may be underlying abnormalities in both procoagulant and anticoagulant mechanisms. Consequently, further detailed characterization of haemostatic mechanisms can be of potential clinical significance and may be further defined using global assays of haemostasis including thromboelastography and calibrated automated thrombography (CAT) which describe the function of the haemostatic system.
Thromboelastography examines blood coagulation followed by clot lysis in whole blood through analysis of viscoelastic changes in fibrin polymerisation (Matsumoto et al., 2013). Disadvantages of this method are that distinguishing the separate components of coagulation and fibrinolysis can be challenging and that analysis must be performed immediately after sampling given that whole blood samples are required (Matsumoto et al., 2013).

Analysis of thrombin generation via CAT assay was first described by Hemker (Hemker et al., 2003). Thrombin is a coagulation enzyme which cleaves fibrinogen to generate the fibrin blood clot, activates the anticoagulant protein C pathway and “feeds back” to activate other coagulation factors. The concept behind this assay was to quantify the evolving properties of thrombin generation in the presence of fibrinogen after a TF stimulus. This assay is suitable to account for both the action of pro- and anticoagulants. Contrary to the clotting times, thrombin generation is very sensitive to variations in prothrombotic and antithrombotic around the normal mean as well as to the activity of the APC system (when a modification is incorporated).

Although this assay has been in use for many years it is only recently that automated adaptations have facilitated its use in a semi-high-throughput manner. In this assay, a trigger is used to replicate in vivo vessel wall damage. In platelet poor plasma, procoagulant phospholipids (4uM) amplify the effects of TF. Thrombin generated is automatically measured by determining the rate of cleavage of a fluorogenic thrombin substrate. Using specialized software, curves representing thrombin generation in real time are generated (Figure 2.2). These “thrombin generation curves” allow parameters to be measured which are not available with standard PT and APTT assays, including lag time to initiation of thrombin generation, time to peak thrombin, peak thrombin generated and endogenous thrombin potential (ETP; area
under the thrombin generation curve), with the ETP considered most reliable of all these.

The time taken for thrombin generation to occur is known as the lag time. The clot appears at start of the thrombin burst when thrombin is at its lowest concentration. Therefore, the clotting time as measured by PT and APTT is comparative to the lag time of thrombin formation. Thrombin generation increases steadily towards a peak. The time taken to reach peak thrombin generation corresponds to amplification phase of coagulation.

This assay provides a greater representation of the complete haemostatic capacity as the generation of thrombin continues to be measured after the initial fibrin clot has been generated. The majority of thrombin formation occurs within the clot. This is important to measure because thrombin acts through feedback mechanisms to promote further coagulation and also on other tissues in its other functions. ETP reflects the quantity of free thrombin found in the sample from commencement to completion of the coagulation cascade (Wolberg, 2007). The quantity of thrombin generated is dependent on levels of all clotting factors and inhibitors. For this reason the ETP is considered to be the parameter that is most reflective of overall haemostasis, representing both pro- and anticoagulant functions (Al Dieri et al., 2002). It has been suggested that the higher the ETP, the greater the risk of thrombosis and the lower the ETP, the greater the risk of haemorrhage as demonstrated by the flattened thrombin generation profile seen in haemophilic patients compared to healthy adults (Young et al., 2013). ETP approximately less than 20% of the mean is indicative of a haemorrhagic risk.

Subsequently, inhibitors are activated which reduce the level of thrombin and the curve decreases to baseline. Other parameters that can be measured include slope of the ascending and descending curves.
Control of pre-analytical variables is required given the greater degree of sensitivity of the assay. However the conditions of a thrombin generation experiment can be obtained reproducibly at almost any concentration of TF.
Figure 2.2: Thrombin generation assay parameters
2.5 Developmental Haemostasis

The neonatal coagulation system differs from the adult in all phases, including primary and secondary haemostasis, anticoagulant and fibrinolytic mechanisms. These differences are observed in rates of production and turnover and in both concentration and functionality of plasma proteins (Uszynski et al., 2015). Developmental haemostasis refers to the concept that the neonatal coagulation system is a dynamic evolving system which progresses from a fetus to an adolescent (Jaffray and Young, 2013).

The coagulation system forms early in utero. All factors are present at birth; however most proteins are present at a lower level than in adults. Therefore “screening” coagulation tests including PT and APTT are prolonged compared with adults. Fetal forms of some proteins exist which generate and regulate thrombin differently or are synthesized at a different rate. Maturation of the coagulation system is generally complete by six months of age although some proteins continue to evolve towards adult levels until adolescence. The evolving changes in the functional level of the coagulation proteins lead to several challenges for the clinician (Jaffray and Young, 2013).

The components of the coagulation system with particular reference to secondary haemostasis and their role in neonatal coagulation are the focus of this thesis, as described below.
2.5.1 Primary Haemostasis in the Neonate

Platelets in the fetus have reached adult values by 22 weeks GA (Forestier et al., 1991) and increase further in the first 9 weeks postnatally (Wiedmeier et al., 2009). Although platelet quantity is broadly equal to that of adults (Revel-Vilk, 2012, Wiedmeier et al., 2009, Strauss et al., 2011), qualitative changes are observed. For example, platelet surface glycoproteins exhibit altered expression and pattern of response to agonists (Wiedmeier et al., 2009, Strauss et al., 2011). Neonatal platelets have a decreased response to agonists, decreased granule secretion, and decreased expression of fibrinogen-binding sites. The decreased platelet response persists for the first 2-4 weeks after delivery (Israels, 2009). Unlike adults, neonates are unable to increase megakaryocyte ploidy in times of stress and are limited to increasing the number of bone marrow megakaryocytes (Sola-Visner et al., 2007).

Despite platelet hyporeactivity in neonates (Strauss et al., 2011), in vivo global assays of platelet function, such as bleeding time and the platelet function analyser (PFA-100), do not show platelet dysfunction, instead are shortened in newborns and normalise before the end of the first month of life (Israels, 2009). Furthermore global in vitro testing for haemostasis using thromboelastography and rotating thromboelastometry show accelerated coagulation and strong clot firmness (Strauss et al., 2010).

This inconsistency may be explained by the role of VWF in neonatal haemostasis (Revel-Vilk, 2012). VWF also plays a critical role in platelet function. VWF levels are increased in neonates and there are a greater number of hyper-functional large VWF multimers. These findings may counteract platelet hyporeactivity in neonates (Andrew et al., 1987, Katz et al., 1989, Chalmers, 2004). These levels then decrease reaching adult levels after 1 year of life. The higher haematocrit levels in neonates
may also explain this inconsistency, because a higher haematocrit level is associated with a shorter bleeding time (Del Vecchio et al., 2008).

2.5.2 Secondary Haemostasis in the Neonate

Procoagulants

At birth the only procoagulant factors that are within the adult range are fibrinogen, FV, and FVIII. Despite quantifiably similar levels of fibrinogen, qualitative changes are described in fibrinogen term neonates (Monagle et al., 2003). This “fetal” fibrinogen has a higher proportion of sialic acid and phosphorous levels are four times that of adult fibrinogen, giving it an increased molecular weight. Sialic acid binds to Ca$^{2+}$ reducing the intermolecular repulsion between fibrinogen chains which facilitates fibrin polymerisation and reduced susceptibility to thrombin as compared to adults (Uszynski et al., 2015, Kuhle et al., 2003, Schmaier, 2014). Other examples of qualitative differences in the neonate include fetal forms of TF (Cvirn et al., 2003).

Vitamin K dependent proteins

Coagulation factors are synthesised by the fetal liver from approximately 10 weeks GA and do not cross the placenta (Monagle and Massicotte, 2011). Physiologically low levels of FII, FVII, FIX, FX are observed in neonates and are approximately 50% of adult values at birth (Andrew et al., 1987). These factors rapidly increase in the first few weeks of life and overlap substantially with the adult range by 6 months of age, although
the average values of most remain 20% lower until the teenage years (Andrew et al., 1987, Andrew et al., 1988).

Vitamin K deficiency is associated with increased bleeding risk in newborns and older children and consequently vitamin k is routinely administered at birth. Despite vitamin k prophylaxis at birth, neonatal coagulation factor levels remain low compared to adults, including VKD factors such as acarboxy prothrombin (PIVKA-II factor) (Cornelissen et al., 1993), secondary to fetal liver immaturity where vitamin K is synthesised. PIVKA-type factors are observed in adults in cases of liver failure or secondary to vitamin K deficiency when coumarin or phenyloindandione derivatives are administered. These factors lack COOH groups in the gamma position. As a result they are unable to form complexes with calcium and phospholipids during thrombin generation (Uszynski et al., 2015).

The Contact System

As described in section 2.2.1 the contact system is an alternative route to thrombin generation in the absence of TF. In this system factor XII is activated by combination of phospholipids, HMWK, and PK. FXIIa thus activates FXI which in turn activates FIX to FIXa, and the cascade of factor activation continues with thrombin generation. Traditional thinking is that levels of the contact system are lower in term neonates compared to adults (Andrew et al., 1981) although this has recently been challenged (Uszynski et al., 2015). Further consideration of this topic will be discussed later in thesis.
Regulators of Coagulation

Alpha 2-Macroglobulin

Infants and children have higher levels of α2m in comparison with adults (Andrew et al., 1987, Andrew et al., 1992, Ignjatovic et al., 2007, Monagle et al., 2006). Therefore α2m is a more important inhibitor of thrombin in neonatal plasma than in adult plasma (Levine et al., 1987, Schmidt et al., 1989). In addition α2m also plays a role as regulator of APC activity (Cvirm et al., 2002).

Tissue Factor Pathway Inhibitor

TFPI is observed from 10 weeks gestation and is found in syncytiotrophoblasts, cytotrophoblasts, vascular endothelium, and extravillus trophoblasts. TFPI appears to be required for fetal survival as gene deletion studies in animals lead to fetal death (Huang et al., 1997). One hypothesis is that TFPI maintains an anticoagulant surface at the maternal-fetal interface. TFPI is synthesized by endothelial cells of which 50-80% remains bound to the cell surface. The remainder circulates in plasma in a free active state, or inactive bound to lipoproteins (80%) or platelets (5-10%) (Bridey et al., 1998).

Antithrombin (AT)

AT functions initially by forming a reversible complex with the target protease resulting in disruption of its reactive site loop. This leads to the formation of a covalent bond between AT and target protease rendering the target protease inactive and the complex is cleared from the
circulation (Rau et al., 2007). A glycosaminoglycan such as heparin sulphate (negatively charged linear polysaccharide molecules secreted by mast cells) on the endothelial surface bind to AT and act as cofactors that accelerate its inhibitory effect (approximately 1,000-fold in the case of thrombin inhibition and 10,000 fold with respect to FXa) (Rau et al., 2007, Rezaie, 1998). AT and heparin cofactor II levels are 50% of adult values at birth and increase to adult levels by 3 months of age (Andrew et al., 1992, Monagle et al., 2006).

**Protein C and Protein S**

Protein C glycosylation modulates is functional effects (Ni Ainle et al., 2011). Animal data suggest that fetal protein C is glycosylated to a greater degree than adult protein C (Manco-Johnson et al., 1995). Protein C is a VKD clotting protein. Similarly to other VKD proteins, plasma concentrations of protein C are greatly reduced at birth compared with adult level. Protein C levels remains decreased during the first six months of life, at which point they are only 60-70% of adult plasma concentrations (Monagle and Massicotte, 2011, Salonvaara et al., 2004, Andrew et al., 1988, Andrew et al., 1992). Interestingly despite these data, there is no evidence to suggest that protein C is functionally different in neonates compared with adults (Monagle and Massicotte, 2011).

The anticoagulant function of APC in plasma is dependent upon interaction with its VKD cofactor, protein S. Mean total protein S concentration in the neonate is approximately 35% of adult; however, the mean concentration of free or functional protein S is approximately twice that. In adults, 40% of protein S circulates bound to C4b-binding protein, and this complex functions in the complement system but has no anticoagulant function. In the neonate, plasma levels of C4b-binding protein are very low and essentially all plasma protein S functions in the
protein C anticoagulant system (Manco-Johnson, 2005, Thornburg and Pipe, 2006, Schwarz et al., 1988). Moreover, the increased levels of α2m in the newborn may facilitate interaction of protein S with APC (Monagle and Massicotte, 2011).
2.5.3 Fibrinolysis

Neonatal plasminogen quantities are 50% that of adult concentration. Despite the lower levels of plasminogen in neonates (50% that of adult concentration), overall fibrinolysis is adequate, given that there are lower levels of histidine rich glycoprotein (a physiologic inhibitor of plasminogen binding) and delayed inactivation of neonatal plasmin (Corrigan and Jeter, 1990, Ries, 1997, Saxonhouse and Manco-Johnson, 2009). Neonates also exhibit elevated tissue plasminogen activator and plasminogen activator inhibitor activity concentrations.
2.6 Haemostatic Assessment in Neonates

Levels of procoagulant proteins and contact factors are reduced in neonates compared with adults and result in prolonged PT and APTT in neonates compared with adults. However, these assays are not sensitive to the known reductions in anticoagulant pathways that also affect neonates compared with adults. Despite “prolonged” clotting times; neonates appear in general to have balanced haemostatic activity. Analysis of thrombin generation, as a global assay of haemostasis may provide clarity on the *in vivo* balance between pro and anti-coagulant pathways. Interestingly, term neonates have been reported to have normal plasma TF-initiated thrombin generation curves compared to adults despite having evidence of prolonged PT and APTT (Tripodi et al., 2008).
2.7 Coagulation System in the Premature Neonate

Given the differences in the coagulation system of the term neonate and adult, infants born at an earlier GA are also likely to have alterations in the coagulation system. Andrew et al have produced comprehensive reference ranges for a range of haemostatic parameters for infants greater than 30 weeks GA (Andrew et al., 1988) which demonstrate differences in APTT and haemostatic factors compared with older infants and adults. The differences in procoagulants are even more marked in preterm infants than in term infants. However, in preterm infant, haemostatic development is accelerated and by 6 months of postnatal age the preterm factor assay results are equivalent to those seen in term neonates and are nearly equivalent to adult levels (Andrew et al., 1988). An important difference between term and preterm infants is in α2m activity. Term infants have values of α2m activity that is 40% higher than those of adults. Levels of α2m are reduced in moderately preterm neonates compared to term values, although still 20% higher than that of adults (Andrew et al., 1988).

It can only be assumed that these differences described also apply to a more premature population as there have been limited studies in this area. Progress in the area of understanding the coagulation system in extremely premature infants has been limited. This is for a number of reasons, given that these infants are usually sick, it is difficult to obtain an adequate sample size, and blood sampling is difficult. To date fetal studies have been used to extrapolate data to extreme premature infants (Reverdiau-Moalic et al., 1996). Haemostatic proteins are first synthesised in the fetus from five-ten weeks GA (Hassan et al., 1990). Because coagulation factors cannot cross the placenta, the fetus starts to produce its own procoagulants and anticoagulants in the liver at about 5-10 week’s GA. At 20 weeks GA the coagulation factors can be
measured in plasma yet they are still at very low levels. By the time the infant is viable (circa 24 weeks GA) all components of the haemostatic system are present. Fetal studies provide evidence that the haemostatic system is evolving. There are large differences between earlier gestation fetuses and term infants (Forestier et al., 1986, Reverdiau-Moalic et al., 1996). This suggests that much of the maturation occurs in early gestation, given that it is known that the maturation between 30-40 weeks GA is of slower nature (Andrew et al., 1988). Fetal studies at increasing GA reveal inverse relationships between GA and standard measurements of PT and APTT (Reverdiau-Moalic et al., 1996). As a result of this maturation, it may be assumed that premature infants have even lower levels of procoagulant and anticoagulant factors than term infants. Data obtained from fetal studies cannot be extrapolated to a premature neonate of corresponding GA, given the observed discrepancies between fetal and infant values at term (Reverdiau-Moalic et al., 1996, Hassan et al., 1990).

Despite the fact that very preterm infants are at greatest risk of bleeding, definitive normal ranges for coagulation parameters have not conclusively been determined in this patient group to date. Previous studies which have attempted to characterize reference ranges for coagulation parameters in extremely premature infants have been limited by very small sample sizes, particularly in extremely low birth weight infants of less than 26 weeks GA (Seguin and Topper, 1994, Barnard et al., 1979, Salonvaara et al., 2003). Schmidt examined 64 infants less than 29 weeks GA and obtained coagulation values on day 1 of life. These data was pooled with those of 106 infants over 29 weeks GA to examine whether abnormalities of coagulation with respect to term reference values could be determined (Schmidt et al., 1992). Plasma levels of FII, FVII, FIX and FX in 15 VLBW infants at birth have been shown to be significantly decreased in concentration compared to infants
at 34-36 weeks GA (Salonvaara et al., 2004). As advances in neonatal care promote the survival rate of extremely premature neonates, it is increasingly important to study the coagulation system in these infants, given their risk of bleeding complications. Current understanding of mechanisms of these morbidities and thus treatment of same is challenged by the limited understanding of the neonatal haemostasis for this high risk premature population.

Thrombin generation assays can potentially provide further information on the neonatal haemostatic system (Muntean et al., 2004). Shah et al. examined 20 premature infants less than 30 weeks GA divided into four plasma pools of 5 each and compared them to infants 30-38 weeks GA and infants at term. Lower prothrombin levels in the more premature infants were balanced against lower levels of AT and α2m and all infants had similar thrombin generation profiles (Shah et al., 1992). This study was limited by the nature of the subsampling technique by which the thrombin generation assay was performed and the use of defibrinated plasma which introduces potential error by introducing a level of complexity to the assay. In addition this study was based on cord plasma pooled from a small cohort of neonates. Tripodi overcame these limitations and found that in preterm infants greater than 30 weeks GA generated significantly more thrombin than their full term counterpart (Tripodi et al., 2008).
2.8 **Implications of Developmental Haemostasis**

The physiological role and basis of developmental haemostasis has not been clearly elucidated. Potential molecular mechanisms underlying this concept include increased clearance, reduced synthesis secondary to hepatic immaturity or altered functionality or fetal forms of coagulation proteins. Post-translational modifications have also been known to occur and may have significant impact on the function (Monagle et al., 2003).

The role of the unique physiology of the fetal and neonatal haemostatic system is not quite understood but may be as a consequence of the roles these proteins play in other systems within the body. The evolving maturation of the haemostatic system may play a role in angiogenesis, inflammation, and wound repair (Schedin-Weiss et al., 2008). Reduced AT concentrations may be beneficial for the healthy development of fetus and early neonate, given the well-recognized potent anti-angiogenic properties of AT (Niessen et al., 1996). The higher level of α2m observed in term neonates could potentially be protective against thromboembolism compensating for the low level of AT described to permit angiogenesis at a time of rapid cell proliferation whilst simultaneously preserving an effective anticoagulant pathway (Monagle and Massicotte, 2011, Monagle et al., 2006, Mitchell et al., 1991). In children with AT deficiency, thrombotic systems typically do not appear until adolescence when the levels of α2m normalise to adult levels (Jaffray and Young, 2013).

Furthermore low intra-uterine levels of vitamin K may also be beneficial to the developing embryo. Transfer of vitamin K across the placenta is tightly regulated. Fetal vitamin K levels are approximately 10% of maternal levels (Shearer et al., 1982, Mandelbrot et al., 1988). Vitamin K is necessary for the synthesis of many proteins including
osteocalcin, an enzyme which enhances mineralisation of cartilage. Low levels of vitamin K, may therefore prevent premature maturation of fetal cartilage by reducing synthesis of osteocalcin, though not at levels low enough to produce punctate dyschondroplasia (Booth, 1997). Vitamin K is also important for regulation of other proteins including growth arrest-specific protein 6, which is involved in cell adhesion, cell proliferation and protection against apoptosis. Reduced synthesis of this protein may also be important to normal fetal development (Manfioletti et al., 1993). High levels of vitamin K are postulated to play a role in DNA mutagenesis in vitro, and as a result reduced vitamin K levels could be protective against this risk at a time of rapid cell proliferation in fetal life (Israel and Israel, 1995).

The contact activation system and kallikrein-kinin system interactions may also be implicated in developmental haemostasis (Schmaier, 2014, Schmaier, 2003). The contact activation system being localised to the uteroplacental unit, may have regulatory roles with respect to placental blood flow and transport of substances across the placenta (Hermann et al., 1996). Kinins such as bradykinin are activated following action of prekallikrein on HMWK, and act on myometrium and uteroplacental unit during labour (Valdes et al., 2001). It is likely that this process consumes coagulation factors but the exact mechanism modulating the levels of the respective contact activation system components in the fetus and mother is still, to date, poorly understood (Uszynski et al., 2015).

Although infants have levels of coagulation proteins that are lower than those of adults, the typical healthy infant has a much lower incidence of thrombosis without an increased risk of bleeding. Healthy neonates do not show easy bruising, do not demonstrate excessive bleeding with surgery, and have normal wound healing. Prediction of the overall
bleeding risk in patients with “prolonged” coagulation times can be challenging, as there may be underlying abnormalities in both procoagulant and anticoagulant mechanisms. The main reason for the lack of excessive bleeding and thrombosis in neonates may be “balanced haemostasis” with physiologically lower levels of both procoagulant and anticoagulant proteins. Consequently, further detailed characterization of haemostatic mechanisms can be of potential clinical significance. To date, plasma thrombin generation in the very preterm infant remains poorly characterized, and no study thus far has attempted to correlate thrombin generation with plasma coagulation times and coagulation factor levels in this patient population.

As discussed in Chapter 1, infants delivered prematurely are a vulnerable population at risk of bleeding complications, including severe IVH and disseminated intravascular coagulation (DIC). Low plasma reserves in pro- and anticoagulant coagulation factors, intravascular volume contraction after birth, and a high incidence of hypoxia and sepsis in critically ill newborns can lead to a decompensation of the coagulation system in this population. DIC can be defined as a systemic thrombo-haemorrhagic disorder seen in association with well-defined clinical situations and laboratory evidence of (1) procoagulant activation, (2) fibrinolytic activation, (3) inhibitor consumption, and (4) biochemical and clinical evidence of end-organ damage or failure (Veldman et al., 2010).

Prospectively characterized reference ranges for the most vulnerable cohort of preterm infants, those born <30 weeks’ GA are lacking. Therefore, care providers face major challenges in interpreting “prolonged” coagulation times in these vulnerable neonates who are the group most at risk of devastating IVH. Improved neonatal ICU care, consultant-led care, prevention of hypoxia/blood pressure fluctuations and prevention of prematurity have been shown to prevent IVH (McCrea and Ment, 2008, Synnes et al., 2006). However, a role for immaturity of
the premature coagulation system as a mechanism underlying IVH has not been proven (Christensen et al., 2014). Despite this, results of plasma coagulation times (PT and APTT) are often measured in very preterm infants and are interpreted in light of reference ranges established for adults or term neonates (Andrew et al., 1987).
2.9 Aims of Thesis

To date, the management of very premature infant has been complicated by the lack of definitive gestation-specific ranges for coagulation parameters. Whether a particular result predicts a risk of serious complications such as IVH remains poorly understood. Consequently, clinicians have a low threshold to transfuse these infants with blood products in order to achieve a coagulation test result which is in the normal reference range for an entirely different patient population: older preterm infants, term neonates or even adults. This practice may be inappropriate and potentially dangerous, given the inherent risk of transfusions in an already compromised population, such as circulatory overload and transfusion-transmitted infection. Moreover, it remains unclear whether corrections for advancing gestational or postnatal age should be made. In addition, mechanisms underlying prolongations in plasma coagulation times remain poorly understood. Normative values, based on gestation and postnatal age are required in the extreme premature population cohort to further our understanding of developmental homeostasis and aid clinical management.
This project aims to:

- Define normal ranges for plasma coagulation parameters in very premature infants.
- Characterize plasma thrombin generation in very premature infants.
- Prospectively assess bleeding complications including IVH and correlate these with thrombin generation and plasma coagulation parameters.
- Assess the effect of increasing gestational and postnatal age on plasma coagulation parameters.
- Evaluate efficacy of FP use in neonates in prevention of IVH.
- Investigate the influence of perinatal factors on coagulation.
- Investigate the relationship between coagulation and other neonatal morbidities.
CHAPTER 3: METHODOLOGY

3.1 Approval by the Hospital Research and Ethics Committee

The data described in the subsequent chapters were obtained from infants admitted to the Neonatal Intensive Care Unit (NICU), Rotunda Hospital, Dublin between April 2013 and April 2015. The Rotunda Hospital is a tertiary centre with approximately 9,000 deliveries a year. A single centre was chosen for this study to standardise operating procedures and facilitate a high quality data study. The Research and Ethics Committees of the Rotunda Hospital agreed that this study could be undertaken as a prospective observational study. The study was also approved by the Research and Ethics Committee of the Children’s University Hospital during funding application for this study. The lead investigator undertook research ethics training including informed consent module and good clinical practice training. Informed parental consent was obtained for each case. As clotting samples are routinely taken in infants born before 30 weeks GA as part of clinical practice in the Rotunda, the Research and Ethics committee determined that parents could be approached for informed consent on day 1 of their baby’s life if antenatal consent had not been obtained. Only infants whose parents gave consent were included in the subsequent study and analysis.
3.2 Infant Eligibility

A pilot study was initially performed undertaking a retrospective review of coagulation profiles on day 1 of life in infants less than 27 weeks GA over a six year period between 2004 to 2010 (Neary et al., 2013). This database was subsequently extended to include all infants less 30 weeks GA from 2008 onwards.

In this subsequent prospective observational study, infants were recruited if less than 30 weeks GA and admitted to the NICU. Exclusion criteria included those with an antenatal brain haemorrhage and parental history of blood coagulation disorder. Infants delivered elsewhere were excluded as initial blood samples were not available for these infants. With respect to serial blood analysis, infants were not studied if clinically unstable, if poor access limited venepuncture sites, if no blood sampling was required for clinical reasons or if plasma products had been administered.

A control group of infants was also recruited into the study. These infants were healthy term infants who were delivered either by spontaneous vaginal delivery or by elective caesarean section. Cord blood was collected from these infants with parental consent to act as a control for laboratory analysis.
3.3 Study Strategy

All preterm infants delivered at or before 29 weeks and six days GA were assessed for eligibility in this prospective observational study. Baseline coagulation times and coagulation factor levels were measured on plasma prepared from cord blood. Following the local protocol, coagulation profiles were performed on admission to NICU from non-heparinised lines and reported to clinical team. Parents were approached for consent antenatally or after delivery. When consent was obtained, infants were included in the study. Plasma was prepared from blood collected from study neonates fulfilling inclusion and exclusion criteria. Serial coagulation times were measured on day three and at week two of life. Haemostatic mechanisms were characterised by measuring of plasma thrombin generation in real time. Demographic and clinical data were recorded. Standardised cranial ultrasound views were performed on day 1 of life by the lead investigator and between days 3 and 7 of life by one of two consultant radiologists as per local protocol.
3.4 Blood collection

3.4.1 Cord Blood Collection

Cord blood was collected immediately after delivery from all eligible preterm infants (less than 30 weeks GA), patterned by the method described by Baer et al (Baer et al., 2013). Briefly, at delivery the obstetrician or midwife attending the delivery clamped the umbilical cord at both ends. If there were multiples, one clamp was placed on the placental end of the umbilical cord for baby A, two for baby B etc. Cord milking occurred as per standard practice. The obstetrician placed the placenta in a sterile basin and then given to the lead investigator for phlebotomy. The umbilical vessels were identified (Figure 3.1) and blood was drawn by needle puncture (18-gauge needle on a 10ml syringe) and transferred to an appropriate blood bottle (3ml tube) containing sodium citrate in 1:9 ratio to blood volume. Massaging the umbilical cord during collection allowed for better blood flow. The needle was inserted bevel down to prevent collapse of the vessel wall as the blood was drawn into the syringe. In the case of a caesarean labour the collection procedure was carried out in the same way as with a natural/vaginal delivery. The blood samples were inverted multiple times to mix the anticoagulant with the whole blood. The blood sample was labelled with patient details (neonate’s medical record number, last name, and date of birth) on each tube. A unique participant-identifier number was allocated to each aliquot for long term storage. After the blood studies were drawn the placenta was managed as per standard practice by the midwifery staff.
Figure 3.1: Placenta

Figure 3.1: Typical size and appearance of placenta/ umbilical cord obtained. Adapted from Baer VL, Lambert DK, Carroll PD, Gerday E. & Christensen RD. Using umbilical cord blood for the initial blood tests of VLBW neonates’ results in higher haemoglobin and fewer red blood cell transfusions. *J Perinatol*, 2013 May;33(5),363-5.
Analysis of pre-analytical factors

A recognised difficulty of using cord blood for coagulation studies is the fact that cord blood coagulates easily (Oleko et al., 2011). Success rates for non-coagulated blood samples vary in studies from 0-100% (Oleko et al., 2011). Factors that influence the rate of coagulation include the haematocrit level of cord blood, the method of blood sampling (needle extraction or collection of dripping blood), the size of the blood collection tube, the volume of blood available for collection, the time between cord blood collection and centrifugation, and workload of clinical staff (Oleko et al., 2011). The birth process itself may also lead to coagulation activation (Kulkarni et al., 2013) and time taken for placenta to deliver can also affect coagulation of cord blood. Gestation of infant and any associated placental pathology also affect blood sampling. Short, thin or snapped cords will have very little, if any blood left and are often associated with premature neonates. Associated maternal events including antepartum haemorrhage or placental position can also influence blood volume remaining.

To address this issue of coagulated blood samples, procedures to minimise haemostatic activation during cord blood collection were employed. A standard operating protocol was prepared concerning sample collection, time to centrifugation, aliquoting and long term storage of multiple aliquots at -80 degrees. Cord blood was primarily collected by the lead investigator. Additionally staff education of midwifery and neonatal staff regarding cord sampling was performed.
3.4.2 Neonatal Blood Collection

Neonates received 0.04mls/kg vitamin K intramuscularly or intravenously immediately after birth. None had received heparin/antithrombotic drugs or plasma products. Venous or arterial blood was collected from each neonate by sterile venepuncture directly into blood bottles containing 0.109 M sodium citrate; (blood/citrate; 9:1). Sampling was coordinated with that of clinical venepuncture. Given the small circulating volume in premature infants, blood sampling was limited to analysis of coagulation profile and thrombin generation if sufficient plasma remained. The practical difficulties of blood sampling have been well documented (Williams et al., 2002, Chalmers, 2004). Methods taken to reduce coagulated samples included minimising time from collection to centrifugation and mixing samples well post collection. Blood sampling in neonates for coagulation testing has been shown to be accurate to 1-2 seconds for PT and APTT respectively with or without a discard blood sample (DePalma et al., 1992). All samples were taken from non-heparinised lines to avoid heparin contamination.

Ethical considerations in neonatal blood collection

An important ethical issue to be considered in this study was the volume of blood to be drawn from very premature infants. Laboratory testing is performed on admission to NICU on premature infants in the belief that these tests will guide management. Given the small circulating blood volume in very premature infants, phlebotomy losses on admission can contribute greatly to iatrogenic blood loss (Carroll et al., 2012). In one study of 14 infants between 24-28 weeks GA, phlebotomy losses on day 1 accounted to 10mls/kg which is over 10% of extremely low birth weight
neonates circulating blood volume (Freise et al., 2010). This reduced to a mean daily rate of 1.72mls/kg over one month study period. Anaemia of prematurity is a common complication of prematurity which is exacerbated by iatrogenic blood loss, and managed by the administration of red blood cell transfusions, a strategy not without potential complications (Freise et al., 2010).

In the NICU where this study recruited, coagulation studies were routinely drawn from very premature infants. One of the driving forces for this thesis was to address the value of routine coagulation studies in this population. Rationalising of investigations is an important consideration in this population. The use of umbilical cord blood offers an opportunity to obtain information about the neonatal condition while minimising neonatal phlebotomy, with the potential to improve neonatal outcomes. As cord blood is otherwise discarded and drawing of samples occurred following clinical care e.g. milking the cord, the cord blood sampling was not deleterious to the infants recruited and offered a potential alternative for use for baseline investigations, the feasibility of which we examined in this study. Factor analysis was limited to cord blood analysis to given the volume of blood sampling required. Additionally phlebotomy occurred in recruited patients from cord blood, on day three and week two of life. Blood sampling on day three and week two of life occurred at times of other clinical blood sampling and at the discretion of the attending clinician. In this way attempts were made to minimise any discomfort to the infant during phlebotomy and impact on clinical state (Howie, 2011).

With respect to blood volume drawn, a review of 10 guidelines revealed that recommendations vary from not exceeding between 1-5% on a single draw (or over 24 hours) and between 3-10% of total blood volume over a period of 4-8 weeks (Union., 2008, Howie, 2011) reflecting that these volumes are likely to below what represents minimal risk (Howie, 2011). The total volume of blood in a neonate is estimated at 80 to 90 ml/kg body weight and rises to a peak of 105ml/kg by the end of the
first month and then drops progressively over ensuing months to 75-80mls/kg (Howie, 2011). Attempts were made during this study to reduce the volume required to analyse routine coagulation parameters in preterm infants to 0.5ml although this was insufficient. In order to limit the volume of blood drawn from preterm infants, factor analysis was limited to cord blood analysis. In term infants, ethical approval was not granted for peripheral blood draw, therefore comparisons with cord blood samples were made. Ideally peripheral blood samples from term infants would have allowed for direct comparison to preterm peripheral samples. Term infants would need to be identified as healthy with no evidence of other comorbidities such as jaundice or at risk of sepsis.
3.5 Blood Analysis

3.5.1 Plasma Preparation

Samples were hand-delivered to the laboratory after collection, with manual mixing of citrate and blood. Plasma was prepared in the clinical laboratory on site. This laboratory is accredited to ISO15189 international standards. Accurate laboratory analysis of blood samples is critical to the diagnosis and therapeutic monitoring of neonatal haemostasis. In no other age group is attention to pre-analytical factors more important (Will, 2015). Review of preanalytical variables was assessed prior to sample processing, including volume of sample to ensure optimal blood-citrate ratio. Samples were examined prior to centrifugation and post centrifugation as centrifugation can initiate the coagulation cascade. If the sample was coagulated, the sample was not processed and this was documented. The resulting coagulation profile curves were examined to ensure validity of values observed and samples where the curve was not standardised were not included. Normal platelet poor plasma was prepared within 3-4 hours of collection by centrifugation of citrated blood at 3000rpm for 10 minutes at room temperature. Plasma was periodically checked to ensure that the platelet count was <10 x 10^9/L. Aliquots with red cells or haemolysis were discarded.
3.5.2 Prothrombin time (PT), Activated partial thromboplastin time (APTT) and Fibrinogen measurement

All samples collected in this study were immediately analysed for a standard coagulation profile PT, APTT, and Fibrinogen in platelet poor plasma on the fully automated ACL TOP 500 coagulometer (Beckman Coulter Inc., Galway, Ireland) using HemosIL APTT lyophilised silica reagent, IL Clauss Fibrinogen and IL RecombiPlasTin reagent respectively (Vendor Brennans and Co., Dublin: Manufacturer Instrumentation Laboratory, Lexington, MA (IL)) with the analysers optical method of measurement. All testing was performed in accordance with manufacturer’s instructions. Plasma was divided into 150µl labelled aliquots and samples were stored at -80 degrees Celsius within 4 hours of collection. Transfer of samples from clinical site to research laboratory was performed with the use of dry ice with temperature maintained and validated at -80 degrees Celsius.

3.5.3 Calibrated Automated Thrombography

To date, plasma thrombin generation in the very preterm infant remains poorly characterised, and no study to date has attempted to correlate thrombin generation with plasma coagulation times and coagulation factor levels in this patient population. Thrombin generation in plasma prepared from cord blood and peripheral venous blood was assessed using a Fluoroskan Ascent Plate Reader (Thermo Lab System, Helsinki, Finland) in combination with Thrombinoscope software (Thrombinoscope BV, Maastricht, the Netherlands). Optimisation and training in this
technique was performed in National Children Research Centre Laboratory and in the Conway Institute, University College Dublin. Thrombin generation was assessed using a human recombinant TF trigger in the presence of phospholipids. 80μl plasma was incubated with 20μl platelet-poor plasma (PPP) reagent containing 1pM soluble TF and phospholipid vesicles (4μM; 60% phosphatidylcholine, 20% phosphatidylserine and 20% phosphatidylethanolamine). Thrombin generation was initiated with automatic dispensation of fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC.HCl) and 100mM CaCl₂ into each well (final concentrations, Z-Gly-Gly-Arg-AMC.HCl, 0.42mM and CaCl₂, 16.67mM). Thrombin generated was determined automatically by comparing the rate of fluorogenic substrate hydrolysis to that of a thrombin calibration standard. The area under the thrombin generation curve, which is the ETP, was then measured. Thrombin generation was determined in duplicate wells for reliability of experimental procedure.

Analysis of pre-analytical variables also needs to be considered with respect to the thrombin generation assay. Centrifugation and concentration of TF can impact thrombin generation results and therefore should be standardised throughout study (Rodgers et al., 2014). Optimisation of the assay was performed at the outset of the study, and commercial PPP-reagent LOW (trigger 1 pM TF) was determined to be the optimal TF concentration for use in this study.

3.5.4 Alpha-2-Macroglobulin

α2m-bound thrombin cleaves the fluorogenic thrombin substrate utilised in the thrombin generation assay and continues to do so following physiological thrombin inhibition. Initial α2M-mediated thrombin inhibition plays an important physiological role, particularly in neonates (Schmidt et
al., 1989). The α2m-bound thrombin is still amidolytically active towards the substrate which is corrected for by the software. α2m levels in the blood samples was estimated simultaneously at time of thrombin generation analysis using the thrombin generation assay incorporating software that estimates individual sample α2M contribution to thrombin generation.

3.5.5 Activated Protein C Function

CAT assay was performed in the presence and absence of the naturally occurring anticoagulant APC 1 and 2.5nM respectively to determine APC anticoagulant function in preterm neonatal plasma.

3.5.6 Factor Analysis

Where sufficient plasma was available from cord blood samples within ethical limitations, procoagulant and anticoagulant factors were measured. Cord blood samples were used to determine FII, FVII, FIX, protein C, free protein S, and AT levels. VKD factors were measured using HemosIL factor-specific deficient plasma on an IL ACL TOP 500 analyzer. Free protein S was measured with an automated latex ligand immunoassay. Protein C and AT were measured by automatic methods with chromogenic endpoints. All reagents were sourced from Brennan and Co., Dublin, Ireland.
3.5.7 To assess the mechanism contributing to prolonged coagulation screening tests

In order to examine the contribution of individual factors to PT and APTT prolongation in neonatal samples, pooled cohorts of cord blood from preterm cases and term controls were prepared. PT, APTT, FII, FVII, FIX, FX and FXII were measured. Preterm factor levels were individually increased to that of term control level in aliquots of 210µl, following which PT and APTT were re-measured.

3.5.8 Determination of the TFPI plasma concentrations

TFPI levels were determined by means of the human TFPI Quantikine ELISA Kit. Briefly 100µl of assay diluent was added to each well of a 48 well plate. 50µl of Standard, control or sample was added to each well. The plate was then aspirated and washed four times using 400µl of wash buffer. 200µl of TFPI Conjugate was then added to each well. The plate was covered with a sealer and incubated at room temperature for 1 hour on a horizontal orbital microplate shaker. The plate was then aspirated and washed four times using 400µl of wash buffer. 200µl of substrate solution was added to each well and then the plate was incubated for a further 30 minutes on the benchtop and protected from light. 50µl of stop solution was added to each well. The colour in the well changes from blue to yellow and the plate was then read in a plate reader at 450nm within 30 minutes to determine the
optical density. As wavelength correction was not available optical density was also set to 540nm or 570nm and difference between the values at 450nm corrected for optical imperfections in the plate.
A standard operating protocol was created for clinical data collection including a case report form assigned to each infant on admission which allowed baseline information to be recorded including demographics, perinatal factors and clinical morbidities. Demographic data collected on infants recruited included maternal age, smoking in pregnancy, maternal medication, gestational diabetes, rates of preeclampsia/HELLP syndrome, clinical chorioamnionitis, maternal bleeding (placental abruption, placenta praevia), twins/triplets (twin-twin-transfusion syndrome), occurrence of fetal growth restriction, prevalence of small-for gestational age infants, mode of delivery, sex-distribution, rates of asphyxia or low Apgar scores, neonatal septicaemia, DIC, NEC, and transfusions (thrombocyte and plasma). Clinical morbidities are described below. The attending clinician was responsible for decisions regarding plasma product transfusion and this data was recorded. A database was created and all data was anonymised on entry. Demographic data were also obtained on term infants including GA, BW, and gender.
3.6.1 Definitions

**Antepartum Haemorrhage:** Antepartum haemorrhage (APH) refers to haemorrhage from or into the genital tract which occurs antenatally. Frequent aetiologies of APH include placenta praevia and placental abruption (RCOG, 2011).

**Birth Asphyxia:** Apgar scores and acid base status are used to identify occurrence of asphyxia at time of birth (Levene et al., 1986).

**Chorioamnionitis:** Chorioamnionitis represents a state of intrauterine inflammation commonly secondary to a bacterial infection. Chorioamnionitis can be confirmed histologically but may be suspected by clinical signs such as maternal fever, leucocytosis, tachycardia, uterine tenderness, and preterm rupture of membranes (Galinsky et al., 2013).

**Gestational Diabetes:** American Diabetes Association defines gestational diabetes as “diabetes diagnosed during pregnancy that is not clearly overt diabetes” (Association., 2012).

**Periventricular Leucomalacia (PVL):** Abnormalities in the white matter of the brain which can impact neurocognitive development (Volpe, 2001).
**Preeclampsia:** Preeclampsia is defined as *de-novo* hypertension presenting after 20 weeks of gestation combined with proteinuria (>300 mg/day), other maternal organ dysfunction, such as renal insufficiency, liver involvement, neurological or haematological complications, uteroplacental dysfunction, or fetal growth restriction (Tranquilli et al., 2014).

**Necrotising Enterocolitis:** Disorder of multifactorial aetiology whereby the intestine becomes necrotic, predisposed to by gut immaturity with associated morbidity and mortality (Neu and Walker, 2011, Bell, 1978)

**Respiratory Distress Syndrome:** Infants with RDS have increased oxygen requirements and work of breathing as a result of impaired surfactant production secondary to lung immaturity (Hallman et al., 2002).

**Retinopathy of prematurity (ROP):** ROP occurs as retina blood vessels mature and develop in an abnormal manner (Casteels et al., 2012).

**Small for gestational age (SGA):** SGA infants are those who have a BW below the 10th centile (Pihkala et al., 1989).
3.6.2 Ultrasound Protocol Scans

CRUSS was performed by lead investigator or consultant radiologist using Hitachi HiVision Noblus, and C42 4 to 8 Mhz convex array transducer of 20mm radius. The lead investigator undertook clinical and educational training in CRUSS to ensure competency and subsequently coordinated and instructed training in professional courses in same. The anterior fontanelle was used as the sonographic window. Ultrasound studies included the 6 standard coronal views and 5 sagittal views. Scans were performed on admission and between day 3 and day 7 of life. Images with aspects that were difficult to interpret were discussed with clinical staff. The images were stored on electronic images software and were available to the clinical team. Primary outcome reported was presence or absence of IVH present in both coronal and sagittal views.

3.6.3 Score for neonatal acute physiology, perinatal extension, version II (SNAPPE-II Score)

All infants had a SNAPPE-II score assigned to them in the first 12 hours of life to stratify infants according to clinical condition. The mean blood pressure, lowest temperature, lowest pH, PaO2/FiO2 ratio, low urine output and presence of seizures over the first 12 hours were documented and used to determine the SNAPPE-II score. In addition to these, SNAPPE-II includes points for low BW, low 5- minute Apgar score and if the infant is SGA. This score has been validated in large cohorts of neonates (n=14,610) (Richardson et al., 2001) and further validated in VLBW infants (Zupancic et al., 2007).
3.7 Data Analysis

The lead investigator undertook a diploma in postgraduate statistics with distinction from Trinity College Dublin and consulted with statistician in review of data from this work. For prospectively collected data, means and standard deviations (SD) were used to express values of continuous variables in groups that were normally distributed, and median (10th-90th) percentile range when they were not. Reference intervals were described as 5th – 95th percentiles as described below. Data were assessed for normal distribution using the Kolmogorov-Smirnov test. Normally distributed data was analysed using the Student’s t-test (paired and unpaired as indicated) and non-parametric data were analysed using the Mann-Whitney U test. “Analysis of variance (ANOVA)” was used to compare more than two groups. Linear regression analysis was used to correlate coagulation values with IVH. Differences between categorical variables were analysed using chi-square. A p value of less than or equal to 0.05 is taken as statistically significant in all cases.

3.7.1 Reference Range Determination

Reference ranges are utilised throughout medicine to aid clinical decision making. The process of reference range determination was examined as per Figure 3.2. The reference individuals were represented by the individual cases. Reference range determination is ideally performed by recruiting a healthy population. Although extremely premature neonates cannot be considered to be healthy, they do represent the typical ‘normal’ population for this gestation encountered in neonatal practice. Consecutive infants were prospectively included to make up the sample
size. Efforts to standardise pre-analytical variables (as described above) were employed.

Blood sample analysis from at least 120 individuals is recommended for determination of reference ranges for coagulation tests (Solberg 1987, NCCLS 2000). Common protocols for determining population-based reference intervals cite 2.5th and 97.5th percentiles as the lower and upper reference limits. A sample size of 40 is considered to be the minimal sample size to enable demarcation of 2.5th to 97.5th percentiles (Speer et al., 2013). Non-parametric analysis of data is advised by the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute. It is recognised that sample sizes of 120 individuals may not be feasible in all circumstances and statistical analyses for smaller samples have been assessed. Although numbers as low as 20 have been used to determine percentiles, 80 or more cases are advisable to produce robust data (Geffre et al., 2009).

Reference ranges in adults have been established by recruiting large cohorts of healthy controls and comparing their blood values to that of the individual patient. In certain populations (for example, neonatal medicine) ethical limitations prevent phlebotomy of healthy neonates for establishing normative values (Henry and Christensen, 2015). In this scenario, reference intervals can be used instead, using values representing 5th to 95th percentile developed from laboratory databases of infants who had particular tests drawn as part of clinical care and were thought to have minimal comorbidities that would affect the result. The 5th to 95th percentile is used rather than the standard 2.5th to 97.5th percentile to take into account that values are obtained from clinical patients.

As PT and APTT are determined using citrated platelet poor plasma to which exogenous reagents are added, coagulation reference ranges are specific to the analyser and reagent used in sample analysis,
but trends across laboratories should be comparable (Monagle P and JN., 2011). Validation of reference ranges can be performed by other laboratories using same reagents with 20 cases and allows generalisability of results to other laboratories.
4.1 Background

Previous studies aiming to characterise reference ranges for coagulation parameters in extremely premature infants have been limited by small sample sizes overall; \( n = 10 \) (Seguin and Topper, 1994), \( n = 21 \) (Barnard et al., 1979), \( n = 52 \) (Salonvaara et al., 2003) and inclusion of very few infants <26 weeks’ GA (Monagle and Massicotte, 2011, Stanworth et al., 2011, Segal and Dzik, 2005); or have examined a limited number of coagulation factors (Poralla et al., 2012b). The Subcommittee on Neonatal Haemostasis and Standardization Committee cautioned that the results of older studies may not be accurate given methodological errors (heterogeneity of the material and methods, other inadvertences, extrapolation of results) (Hathaway and Corrigan, 1991).
4.2 Aims

It was hypothesised that standard coagulation test results in extremely preterm infants differ from those in more mature preterm infants and term infants. The first aim of this thesis was to provide reference ranges for very premature infants’ coagulation parameters on day 1 of life.
4.3 Methods

Study 1 – Retrospective Collection

A retrospective review of coagulation screens measured on day 1 of life was performed. All infants born <27 weeks’ GA admitted to the NICU at the Rotunda Maternity Hospital, Dublin, Ireland, were included. Coagulation screens were obtained via non-heparinised peripheral venous cannulae or umbilical arterial or venous catheters. During the timeframe examined, coagulation samples were routinely performed on all extremely preterm infants on day 1 of life. Blood samples were usually taken during the initial stabilisation of infant and within hours of birth. All infants born <27 weeks’ GA from January 1, 2004 to December 31, 2010 were included. Ethical approval for this study was granted by the hospital’s ethics committee.

A total of 190 infants born at <27 weeks’ GA were identified using the NICU database. Coagulation test results were obtained from the laboratory system. Infants who were excluded included those whose coagulation test results were greater than upper limit of instrument range (n = 7). These patients were excluded in view of concerns that preanalytical variables or heparin contamination may have impaired sample quality. Patients were not excluded based on clinical status because one of the aims of the study was to accurately represent the true clinical phenotype of the extremely premature population, so that results obtained would be of maximum practical relevance to clinicians. Infants underwent standard care, which includes treatment with intramuscular vitamin K to prevent haemorrhagic disease of the newborn. The majority of the infants were intubated and received one dose of
surfactant. Other supportive measures included standard nutritional and antibiotic therapy.

Blood samples were obtained before heparinised fluid infusion and were placed in 1.3-ml standard citrated blood tubes, validated to ensure correct blood to citrate ratio. Plasma PT, APTT and fibrinogen results were obtained from the laboratory information system. From 2004 to May 2008, coagulation testing was carried out on the semi-automated coagulation analyser ACL 9000 (IL) using PT-S reagent and APTT-SP reagents (Instrumentation Laboratory). After May 2008, coagulation tests were carried out on the ACL TOP (IL) analyser using PT Recombinant PlasTin 2G and the lupus-sensitive silica-based APTT reagent SynthASil (Instrumentation Laboratory; Brennan & Co.). The ACL 200 instrument was used during the entire time period of study to determine the plasma fibrinogen level by Clauss method, using the reagent Fibrinogen C (Instrumentation Laboratory; Brennan & Co.). Laboratory protocols are in place to evaluate the potential impact of new reagents on patient test results. Comparison of data obtained prior to and after May 2008 was performed to address the potential impact of changes in instrumentation and reagent type on coagulation test results. Reference range determination is ideally performed by recruiting a healthy population. Although extremely premature neonates cannot be considered to be healthy, they do represent the typical 'normal' population for this gestation encountered in neonatal practice. Laboratory reference ranges in current use are based upon published results for more mature infants which are now quite dated (Andrew et al., 1987). In order to verify that coagulation profiles in this institution was consistent with published data, a cohort of healthy infants born at term was identified who had undergone coagulation screening to investigate for presence of inherited coagulation factor deficiencies. Newborns found not to have inherited the coagulation abnormality were considered to represent a healthy term population that could be compared to published reference ranges. A
minimum sample size of 50 infants was determined to be required for adequate power (Geffré et al., 2011).

**Statistical Analysis**

Data were analysed via PASW Statistics Version 18.0.3 and Excel Reference Value Advisor (Leslie and Greenberg, 1991). Results are expressed as mean and SD or median and range as appropriate. Two-tailed t tests of unequal variance and non-parametric tests were used to compare data before and after 2008 and from different subsets of population as described below. Laboratory reference ranges in use during this period are based on data reported by Andrew et al. (Andrew et al., 1987), which provides reference ranges for infants born at >30 weeks GA using the 2.5th–97.5th centile values. For comparison with these previously published ranges, reference ranges in this study were calculated similarly.

**Study 2 – Prospective Collection**

PT, APTT and fibrinogen were prospectively examined in cord samples collected from preterm infant’s less than 30 weeks gestation and from that of term infants. Coagulation profiles were also performed on admission to NICU. Details of methodology are described in detail in Chapter 3. Briefly blood was drawn from cord blood at delivery and from neonate on admission to NICU into citrated tubes (9:1 Blood: Citrate). Samples were hand delivered to lab, where sample was checked for clots. Platelet poor plasma was obtained by centrifugation of whole blood and PT, APTT and fibrinogen were measured on the sample. The same analyser was used for the duration of study period. Coagulation test results from neonatal samples were available to the clinical team as part
of routine care of the infant. Infants received vitamin K as per routine practice and timing and route of administration was recorded.

**Study 3 – Overall cohort**

This was an observational study including data comprised of a retrospective review of anonymised clinical data, approved by The Rotunda Hospital ethics committee (2008-2013) and data from a prospective cohort recruited with parental consent (2013-2015). All infants born less than 30 weeks gestation admitted to NICU at the Rotunda Maternity Hospital, Dublin, Ireland were reviewed in this study. The database employed in study 1 was extended to include infants less than 30 weeks gestation that had coagulation tests performed on day 1 of life and continued up to April 2015 as part of a larger prospective ‘CRISP’ study. Inclusion criteria included infants admitted to NICU less than 30 weeks gestation who had a coagulation test performed on day 1 of life. Infants who were excluded included those whose coagulation test results were not accurately measured by automated analysis (n=4). These infants were excluded in view of concerns that pre-analytical variables may have impaired sample quality.

Demographic data collected on infants recruited included GA, BW, prevalence of small-for-gestational-age infants, antenatal steroid use, mode of delivery, Apgar scores, and plasma transfusions. Infants received 0.04mls/kg intramuscular vitamin K as per standard practice. Coagulation screens were obtained via non-heparinised peripheral venous cannulae or umbilical arterial or venous catheters and were taken as part of admission bloods performed in this unit during the time period June 2008- April 2015. Blood was collected into citrated tubes with a final 9:1 ratio of blood to anticoagulant, followed by mixing and transferring to the hospital laboratory. Coagulation test results were obtained from the
laboratory system. PT, APTT, and fibrinogen were measured in platelet poor plasma on the fully automated ACL TOP coagulometer (Beckman Coulter Inc., Galway, Ireland) using RecombiPlasTin, HemosIL APTT lyophilised silica reagent, and ILClauss Fibrinogen reagent respectively (Instrumentation Laboratory, Lexington, MA). All testing was performed in accordance with manufacturers’ instructions.

Statistical Analysis

Continuous data were checked for normality using the Shapiro-Wilk Test and a histogram and presented as means (SD) or medians [inter-quartile range] as appropriate. Two group analyses (based on the presence or absence of severe IVH) were conducted using an independent student t-test for normally distributed data or the Mann-Whitney U test for skewed data. Multiple group analysis (across different gestation groups) was conducted using one way ANOVA and values were compared with baseline gestation (23 weeks) if ANOVA was significant. Categorical variables were presented as count (%) and compared using Chi square or Fisher exact test as appropriate. Multivariable linear regression was used to assess the independent effect of perinatal characteristics (gestation, 5 minute Apgar score, gender, mode of delivery and antenatal steroid administration) on coagulation parameters. In addition, multivariable logistic regression was used to assess the independent associations between perinatal characteristics and the coagulation parameters on severe IVH evolution. A p value of < 0.05 was considered significant. SPSS (IBM, version 22) was used to conduct the analysis.
4.4 Results

Study 1– Retrospective Collection

190 patients were identified (84 female and 106 male). Median GA and BW were 25.4 (23–26.9) weeks and 803.9 (490–1,300) g, respectively. Demographic data according to GA are presented in Table 4.1. Upon review of the laboratory database, complete coagulation test results were not available in all infants (n = 45, 47 and 109 for PT, APTT and fibrinogen assays, respectively). Potential explanations may include omission of blood sampling or obtaining a sample which was haemolysed but not repeated. Seven infants were excluded as coagulation test results were greater than upper limit of instrument range. Preanalytical errors could not be excluded in this group given the retrospective nature of the study. Mean GA and BW of these infants were 25.6 weeks and 800.3 g, respectively, similar to infants whose complete results were available. In total, day 1 PT, APTT and fibrinogen results were available for 144/190 (76%), 136/190 (72%) and 80/190 (42%), respectively. No significant differences were noted between APTT results determined prior to and after May 2008 (following instrument and reagent change; p > 0.05). Mean PT values determined prior to and after May 2008 were significantly different (22.4 and 20 s, respectively; p < 0.05). This finding did not translate into differences in reference ranges which would be considered to be clinically significant (13–39 and 13–36 s, respectively). Thus, for the purposes of determining a reference range, the entire cohort was analysed as a single group. Results obtained for PT, APTT, and fibrinogen are presented in Table 4.2. Mean PT, APTT and fibrinogen were 21.5 and 75.2 s and 1.9 g/l, respectively. Reference ranges were defined as the 2.5th–97.5th centile of the population sample (Griffiths et al., 2004). Using these reference ranges, PT, APTT and
fibrinogen reference ranges were determined to be 14.4–36.7s, 40.5–158.5 s and 0.7–4.8 g/l, respectively (Neary et al., 2013). In contrast, current laboratory reference ranges used for PT, APTT and fibrinogen, respectively, are 13.0 ± 1.4s, 42.9 ± 5.8 s and 2.8 ± 0.58 g/l. These reference ranges have previously been determined for infants born at a much later GA (Andrew et al., 1987). Moreover, significant NICU practice changes have been implemented since publication of these data. Describing the values as reference intervals, median (range 5-95th percentile) PT and APTT values were 20.2 (14.8-32.6) and 67.4 (43.3-130.2) s respectively (Neary et al., 2014).

Results were then analysed on basis of GA: group 1 incorporated infants born at 23–24 +6 weeks and group 2 incorporated infants born at 25-26 +6 weeks. Mean PT, APTT and fibrinogen in group 1 infants were 22.6s, 82.6 s and 1.99 g/l, respectively. Mean PT, APTT and fibrinogen in group 2 infants were 21.0s, 71.6 s and 1.79 g/l, respectively (Table 4.3). Trends towards shortening of coagulation times were noted upon comparison of the two groups, although these differences were not significant.

Elevated haematocrit reduces plasma volume and in extreme cases may influence coagulation test results. To address this, comparison of day 1 coagulation screens and day 1 haematocrit revealed minimal correlation between haematocrit and PT/APTT (R² 0.04 and –0.2 for PT and APTT, respectively).

Current laboratory reference ranges are based upon published results for more mature infants which are now quite dated (Andrew et al., 1987). In order to verify that data published by Andrew et al. (Andrew et al., 1987) was consistent with coagulation profiles in this institution, term infants
were identified and coagulation test results compared with published laboratory reference ranges as described. Their coagulation test results corresponded with reference ranges currently in use which are based on published data. In comparison, the majority of coagulation test results in extremely premature neonates were outside this reference range (Table 4.2).
Table 4.1: Demographic data of the study population according to GA

| GA
| 23 weeks (n=9) | 24 weeks (n=50) | 25 weeks (n=53) | 26 weeks (n=71) |
|---|---|---|---|---|
| Male/Female | 7/2 | 27/23 | 34/18 | 40/31 |
| Median BW (range), g | 704 (570-950) | 730 (490-1080) | 821 (550-1170) | 851 (490-1300) |

Table 4.2: Day 1 standard coagulation tests in extremely premature infants

<table>
<thead>
<tr>
<th></th>
<th>PT (s) (n=144)</th>
<th>APTT (s) (n=136)</th>
<th>Fibrinogen (g/l) (n=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean +/- SD</td>
<td>21.5 ± 5.3</td>
<td>75.2 ± 27.8</td>
<td>1.86 ± 1.1</td>
</tr>
<tr>
<td>Median (range)</td>
<td>20.2 (13.3-39)</td>
<td>67.4 (34.9 – 191.6)</td>
<td>1.4 (0.5 - 4.8)</td>
</tr>
<tr>
<td>Reference value advisor range (2.5th-97.5th centile)</td>
<td>14.4 – 36.7</td>
<td>40.5 – 158.5</td>
<td>0.7 – 4.8</td>
</tr>
<tr>
<td>90% CI lower limit</td>
<td>13.3 – 14.9</td>
<td>34.9 – 45.4</td>
<td>0.5 – 07</td>
</tr>
<tr>
<td>90% CI upper limit</td>
<td>31.4 – 39.0</td>
<td>130 – 191.6</td>
<td>4.2 - 4.8</td>
</tr>
<tr>
<td>'Abnormal values', according to existing ranges for more mature preterm infants</td>
<td>140 (97.2%)</td>
<td>124 (91.2%)</td>
<td>66 (82.5%)</td>
</tr>
</tbody>
</table>
## Table 4.3: Coagulation status in each GA group on day 1

<table>
<thead>
<tr>
<th>GA</th>
<th>PT, s</th>
<th>APTT, s</th>
<th>Fibrinogen, g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>23-24+6</td>
<td>n = 49</td>
<td>n = 45</td>
<td>n = 25</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>22.63 ± 7.0</td>
<td>82.62 ± 37.2</td>
<td>1.99 ± 1.4</td>
</tr>
<tr>
<td>Median</td>
<td>22.25</td>
<td>79.45</td>
<td>1.3</td>
</tr>
<tr>
<td>(range)</td>
<td>(13.9 – 39)</td>
<td>(34.9 -191.6)</td>
<td>(0.5-4.8)</td>
</tr>
<tr>
<td>25–26 +6</td>
<td>n = 95</td>
<td>n = 91</td>
<td>n = 55</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>20.96 ± 0.42</td>
<td>71.59 ± 21.0</td>
<td>1.79 ±1.02</td>
</tr>
<tr>
<td>Median</td>
<td>22.3</td>
<td>79.7</td>
<td>1.37</td>
</tr>
<tr>
<td>(range)</td>
<td>(13.3 – 31.4)</td>
<td>(40.2-131.3)</td>
<td>(0.7-4.8)</td>
</tr>
</tbody>
</table>


Study 2 – Prospective Collection

One hundred and eighty term and preterm infants identified for potential inclusion for this study. Of these, 153 preterm infants less than 30 weeks met GA criteria for recruitment in this study. After applying exclusion criteria, 137 infants were enrolled in this study. Forty-two non-coagulated samples of sufficient volume were obtained from cord blood at delivery. Of 137 infants less than 30 weeks gestation recruited, non-clotted coagulation samples on day 1 of life were obtained in 127 infants. Patient recruitment is described in Figure 4.1 and clinical demographic data are displayed in Table 4.4.

Median (5th-95th percentile) day 1 PT, APTT, and fibrinogen were 17.9 (12.8-27.7) seconds (s), 79.1 (48.8-134.3) s and 1.3 (0.7-3.9) g/L respectively (n=127). Preterm PT and APTT from cord blood were prolonged compared with results obtained using cord blood from term infants (preterm; n= 42, term; n=27 p<0.001) however no difference in plasma fibrinogen between the two groups was observed (Figure 4.2 A-C).
Figure 4.1: Infant recruitment

180 term and preterm Infants

Cord samples from 27 term infants recruited

153 premature infants delivered less than 30 weeks gestation

Cord samples available (n=42)
- Excluded
  - Clotted (28)
  - Insufficient (42)
  - Not processed (3)
  - Maternal Indication (3)
  - Failed laboratory analysis (12)
  - No sampling (7)

16 Excluded
- Declined or lack of consent; n=7
- Attending physician decision; n=6
- Excluded due to exclusion criteria; n=3

137 premature infants recruited

Day 1 peripheral blood coagulation profile (n=127; 93%)
- Excluded
  - Clotted; n=5
  - Failed laboratory analysis; n=5

Day 3 peripheral blood coagulation profile (n=50/67; 75%)
- Excluded
  1) No longer meets inclusion criteria
     - Gestational age >30/40; n=10
     - Plasma administration; n=27
     - No blood sampling, n=19
     - Withdrawn due to parental request, n=13
     - RIP, n=1
  2) Other
     - Clotted (9)
     - Insufficient (3)
     - Not processed (1)

Week 2 peripheral blood coagulation profile (n=27/35; 77%)
- Excluded
  1) No longer meets inclusion criteria
     - Gestational age >30/40; n=50
     - Plasma administration; n=20
     - No blood sampling, n=23
     - Withdrawn due to parental request, n=7
     - RIP, n=2
  2) Other
     - Clotted (8)
Table 4.4: Demographics of preterm infants recruited

*HELLP syndrome: Haemolysis, Elevated Liver enzymes, Low Platelet count

<table>
<thead>
<tr>
<th>Total recruited = 137</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal age, median (range)</strong></td>
</tr>
<tr>
<td><strong>Smoking during pregnancy</strong></td>
</tr>
<tr>
<td><strong>Maternal antenatal steroid use</strong></td>
</tr>
<tr>
<td>- Partial</td>
</tr>
<tr>
<td>- Complete</td>
</tr>
<tr>
<td><strong>Gestational diabetes</strong></td>
</tr>
<tr>
<td><strong>Preeclampsia/HELLP syndrome</strong></td>
</tr>
<tr>
<td><strong>Clinical chorioamnionitis</strong></td>
</tr>
<tr>
<td><strong>Antepartum haemorrhage</strong></td>
</tr>
<tr>
<td><strong>Multiple Pregnancy</strong></td>
</tr>
<tr>
<td>- Twin</td>
</tr>
<tr>
<td>- Triplet</td>
</tr>
<tr>
<td>- Quadruplet</td>
</tr>
<tr>
<td>- Twin-twin transfusion syndrome</td>
</tr>
<tr>
<td><strong>Intrauterine growth restriction</strong></td>
</tr>
<tr>
<td><strong>Small for gestational age (&lt;10th percentile)</strong></td>
</tr>
<tr>
<td><strong>Gestational age, median (range), weeks</strong></td>
</tr>
<tr>
<td><strong>Birthweight: median (range), g</strong></td>
</tr>
<tr>
<td><strong>Mode of delivery</strong></td>
</tr>
<tr>
<td>- Spontaneous vaginal delivery</td>
</tr>
<tr>
<td>- Caesarean section with labour</td>
</tr>
<tr>
<td>- Caesarean section without labour</td>
</tr>
<tr>
<td><strong>Infant Gender</strong></td>
</tr>
<tr>
<td>- Male</td>
</tr>
<tr>
<td>- Female</td>
</tr>
<tr>
<td><strong>Cord pH: median (range)</strong></td>
</tr>
<tr>
<td><strong>Low Apgar scores</strong></td>
</tr>
<tr>
<td>- &lt; 7 at 5 minutes</td>
</tr>
<tr>
<td><strong>Neonatal septicaemia (early sepsis)</strong></td>
</tr>
<tr>
<td><strong>Day 1 Platelet count &lt; 50 x 10^9/L</strong></td>
</tr>
</tbody>
</table>
Table 4.5: Outcome data for preterm infants recruited

<table>
<thead>
<tr>
<th>Condition</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNAPPE-II</strong></td>
<td>10</td>
<td>(0-60)</td>
</tr>
<tr>
<td><strong>RDS</strong></td>
<td>123/137</td>
<td>(90%)</td>
</tr>
<tr>
<td><strong>Grade 3-4 IVH</strong></td>
<td>16/137</td>
<td>(12%)</td>
</tr>
<tr>
<td><strong>Ventilated for longer than 12 hours</strong></td>
<td>51/137</td>
<td>(37%)</td>
</tr>
<tr>
<td><strong>Red cell transfusions, Median (range)</strong></td>
<td>1</td>
<td>(0-9)</td>
</tr>
<tr>
<td><strong>ROP (Not documented in all cases)</strong></td>
<td>17/119</td>
<td>(14%)</td>
</tr>
<tr>
<td><strong>RIP</strong></td>
<td>14/137</td>
<td>(10%)</td>
</tr>
<tr>
<td><strong>Chronic Lung Disease</strong></td>
<td>25/137</td>
<td>(18%)</td>
</tr>
<tr>
<td><strong>Patent Ductus Arteriosus</strong></td>
<td>48/137</td>
<td>(35%)</td>
</tr>
<tr>
<td><strong>Pulmonary Haemorrhage</strong></td>
<td>2/137</td>
<td>(1%)</td>
</tr>
<tr>
<td><strong>Pneumothorax</strong></td>
<td>14/137</td>
<td>(10%)</td>
</tr>
<tr>
<td><strong>Periventricular Leucomalacia</strong></td>
<td>6/137</td>
<td>(4%)</td>
</tr>
<tr>
<td><strong>Necrotising enterocolitis</strong></td>
<td>14/137</td>
<td>(10%)</td>
</tr>
<tr>
<td><strong>Transfusions Day 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• <strong>Platelets</strong></td>
<td>3/137</td>
<td>(2%)</td>
</tr>
<tr>
<td>• <strong>Plasma</strong></td>
<td>47/137</td>
<td>(34%)</td>
</tr>
</tbody>
</table>
Figure 4.2: Coagulation values in cord blood

Figure 4.2: Coagulation values PT (A), APTT (B) and fibrinogen (C) in preterm (n=42) and term (n=27) infants from cord blood. Values for PT (A), and APTT (B) were significantly higher in preterm infants compared to that of term infants, p=0.001. The median value is shown by the solid line. * This work was presented in poster format at American Paediatric Society, San Francisco, December 2014, and published in Journal of Thrombosis and Haemostasis (Neary et al., 2015).
Study 3 – Overall cohort

Three hundred and twenty four infants with a mean ± SD GA and BW of 26.6 ± 1.7 weeks and 969 ± 277 grams were included during the study period. One hundred and eighty eight (58%) were male, 290 (90%) were in receipt of at least one dose of antenatal steroids and 226 (70%) delivered via caesarean section. Fifty one infants (16%) developed severe IVH (Grades 3 & 4).

Table 4.6 illustrates the median [IQR] and the 5th/95th centile values for the coagulation parameters across the different GA. There was no difference in PT across the different GA (Table 4.6, Figure 4.3). However, infants in receipt of antenatal steroids had a shorter PT (17.6 [15.6 – 20.2] s vs. 19.6 [18.1 – 22.6] s, p=0.002). This association remained significant when adjusting for other perinatal characteristics (β -2.3, p=0.006). APTT was shorter in infants with higher GA (28 and 29 weeks) when compared with 23 weeks (Table 4.6, Figure 4.3). This association however was no longer significant in the multivariable linear regression model. Finally, infants 27 weeks GA and over had lower fibrinogen values (Table 4.6, Figure 4.3). Fibrinogen was also lower in those delivered via caesarean section (1.2 [0.9 – 2.2] g/L vs. 1.6 [1.1 – 2.8] g/L, p=0.001), and in males (1.2 [0.9 – 2.0] g/L vs. 1.4 [1.0 – 2.9] g/L p=0.007). Although fibrinogen was lower in infants delivered by caesarean section, these results were not clinically significant. The finding that fibrinogen was lower in infants delivered by caesarean section could be due to lack of stress response associated with mothers who do not undergo active labour. This is consistent with data from Franzoni et al who also found that fibrinogen was lower in infants undergoing caesarean section versus vaginal delivery but the difference was not statistically significant (Franzoi et al., 2002). Those associations remained significant in the multivariable linear regression model of all perinatal characteristics. Five minute Apgar score was not associated
with alterations in the coagulation parameters (Data not shown). Table 4.7 and table 4.8 give reference ranges for coagulation parameters in subgroups of infants with and without IVH respectively.
Figure 4.3: Coagulation values according to GA

* indicates a p value < 0.05 compared with baseline gestation (23 weeks).
Table 4.6: Reference Ranges of Coagulation Parameters

One-way ANOVA was used to compare values between the different gestation groups. * indicates p value < 0.05 compared with baseline gestation (23 weeks) if the one-way ANOVA was significant. No.: Number of infants in each gestation bracket. IQR: Inter-quartile range

<table>
<thead>
<tr>
<th>Gestation (weeks)</th>
<th>No.</th>
<th>Prothrombin Time (s)</th>
<th>Activated Partial Thromboplastin Time (s)</th>
<th>Fibrinogen (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median [IQR]</td>
<td>5th &amp; 95th centile</td>
<td>Median [IQR]</td>
</tr>
<tr>
<td>23</td>
<td>10</td>
<td>16.5 [15.9–17.9]</td>
<td>15.2 – 21.4</td>
<td>87 [75–115]</td>
</tr>
<tr>
<td>25</td>
<td>41</td>
<td>17.2 [15.4–20.4]</td>
<td>13.1 – 26.2</td>
<td>77 [63–95]</td>
</tr>
<tr>
<td>26</td>
<td>67</td>
<td>17.5 [15.8–19.9]</td>
<td>13.1 – 23.5</td>
<td>78 [63–98]</td>
</tr>
<tr>
<td>27</td>
<td>57</td>
<td>18.5 [16.7–22.0]</td>
<td>12.8 – 30.5</td>
<td>85 [71–102]</td>
</tr>
<tr>
<td>28</td>
<td>62</td>
<td>18.0 [15.8–20.1]</td>
<td>13.2 – 26.5</td>
<td>74 [63–89]*</td>
</tr>
<tr>
<td>29</td>
<td>41</td>
<td>18.3 [15.3–21.1]</td>
<td>12.1 – 29.5</td>
<td>77 [61–84]*</td>
</tr>
</tbody>
</table>
Table 4.7: Reference Ranges of Coagulation Parameters in infants with no IVH

No.: Number of infants in each gestation bracket. IQR: Inter-quartile range

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>7</td>
<td>17.1 [16.3–18.0]</td>
<td>15.4 – 21.4</td>
<td>75.5 [73.8–115.9]</td>
<td>65.3 – 143.4</td>
<td>2.3 [0.8–3.8]</td>
<td>0.8 – 4.7</td>
</tr>
<tr>
<td>24</td>
<td>31</td>
<td>17.5 [15.8–19.8]</td>
<td>13.4 – 35.7</td>
<td>80 [64.4–95.5]</td>
<td>60.1 – 124.8</td>
<td>1.5 [0.9–2.7]</td>
<td>0.6 – 4.2</td>
</tr>
<tr>
<td>25</td>
<td>33</td>
<td>17.9 [15.4–20.2]</td>
<td>13.9 – 25.6</td>
<td>75.9 [62.4–90.5]</td>
<td>46.7 – 132.0</td>
<td>1.9 [1.3–2.6]</td>
<td>0.7 – 3.5</td>
</tr>
<tr>
<td>26</td>
<td>57</td>
<td>17.3 [15.6–19.2]</td>
<td>12.9 – 22.9</td>
<td>73.3 [60.5–89.4]</td>
<td>53.5 – 129.5</td>
<td>1.2 [0.9–1.8]</td>
<td>0.7 – 4.6</td>
</tr>
<tr>
<td>27</td>
<td>51</td>
<td>18.5 [16.6–20.6]</td>
<td>13.2 – 31.4</td>
<td>81.7 [70.4–100.3]</td>
<td>47.7 – 130.4</td>
<td>1.3 [0.9–1.9]</td>
<td>0.6 – 3.5</td>
</tr>
<tr>
<td>28</td>
<td>50</td>
<td>18.0 [15.7–20.0]</td>
<td>12.9 – 27.4</td>
<td>74.7 [62.4–89.4]</td>
<td>48.4 – 112.4</td>
<td>1.3 [0.9–1.9]</td>
<td>0.7 – 3.6</td>
</tr>
<tr>
<td>29</td>
<td>35</td>
<td>17.5 [14.7–19.6]</td>
<td>11.7 – 25.4</td>
<td>73.4 [59.8–84]</td>
<td>48.4 – 112.4</td>
<td>1.2 [1.0–1.5]</td>
<td>0.5 – 3.7</td>
</tr>
</tbody>
</table>
Table 4.8: Reference Ranges of Coagulation Parameters in infants with IVH

No.: Number of infants in each gestation bracket. IQR: Inter-quartile range

<table>
<thead>
<tr>
<th>Gestation (weeks)</th>
<th>No</th>
<th>Prothrombin Time (s)</th>
<th>Activated Partial Thromboplastin Time (s)</th>
<th>Fibrinogen (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median [IQR]</td>
<td>5th &amp; 95th centile</td>
<td>Median [IQR]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5th &amp; 95th centile</td>
<td>Median [IQR]</td>
<td>5th &amp; 95th centile</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5th &amp; 95th centile</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>15.8 [15.2–16.3]</td>
<td>15.2 – 16.3</td>
<td>101.4 [88–114.8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>88 – 114.8</td>
<td>1.9 [1.1–2.7]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.1–2.7</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>13</td>
<td>20.3 [16.1–23.9]</td>
<td>12.1 – 16.7</td>
<td>73.6 [69.8–95.8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65.2 – 191.6</td>
<td>2.7 [1.4–3.5]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8 – 4.2</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>21.4 [18.1–25.2]</td>
<td>16.4 – 26.4</td>
<td>88.9 [51.9–110.8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48.7 – 110.9</td>
<td>0.9 [0.9–3.8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8 – 4.7</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>9</td>
<td>22.3 [18.4–25.3]</td>
<td>15.8 – 48.1</td>
<td>91.7 [68.8–117.1]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58.4 – 125</td>
<td>1.1 [1.0–2.8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.7 – 3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>59.4 – 140</td>
<td>1.4 [1.0–3.4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0 – 4.0</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>4</td>
<td>18.9 [16.6–21.5]</td>
<td>16.3 – 21.5</td>
<td>73.1 [56.2–79.0]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>52.3 – 79.2</td>
<td>1.3 [0.6–2.8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 – 3.2</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>5</td>
<td>21.9 [16.5–27.0]</td>
<td>15.7 – 32.0</td>
<td>81.5 [71.8–110.3]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65.5 – 119.2</td>
<td>1.3 [1.0–1.5]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.8 – 1.6</td>
<td></td>
</tr>
</tbody>
</table>
4.5 Discussion

Coagulation tests are frequently performed following delivery of extremely premature infants (Andrew et al., 1988, Vasudevan et al., 2010). Interpretation of results is challenging. Preterm infants have lower plasma levels of VKD clotting factors (Andrew et al., 1988) and have longer plasma clotting times than those born at term. However, the range of clotting times which should be considered ‘physiological’ in very preterm infants is poorly defined. These extremely preterm infants represent a relatively small NICU cohort. Moreover, they are rarely clinically ‘well’. Both issues have complicated previous attempts to define ‘normal’ reference ranges. Unfortunately, these are the very babies for whom a clear understanding of what constitutes ‘normality’ is vital. The potential for serious morbidity associated with bleeding in these infants may translate into administration of blood products based upon coagulation test results which may or may not be physiological.

Reference ranges relevant to term infants and subsequently premature infants born greater than 30 weeks gestation were published in a large study by Andrews and colleagues in the 1980s (Andrew et al., 1988, Andrew et al., 1987). Unfortunately, this dataset is of limited relevance to very premature infants, as it is based on a much more mature population and there have been seismic changes in perinatal and NICU practice since publication of these data. Monagle et al later confirmed the concept of developmental haemostasis in term infants (Monagle et al., 2006). Most studies aimed at determining reference ranges for standard coagulation tests in very preterm infants (born <30 weeks GA) or fetus have been hampered by very small numbers (Salonvaara et al., 2003, Reverdiau-Moalic et al., 1996, Seguin and Topper, 1994, Barnard et al., 1979). Subsequently, Christensen and colleagues described reference
ranges in a large prospective study of 144 preterm infants born at ≤34 weeks GA which included 28 infants born at less than 28 weeks GA (Christensen et al., 2014). In the latter group, reference intervals for PT and APTT of 14.5-20.9s and 27-64s were reported. Of particular note, abnormal cord coagulation values at preterm birth were not found to be associated with bleeding during the first week of life.

We recently published a large retrospective cohort of premature infants born less than 27 weeks gestation (Study 1) describing values for PT, APTT and fibrinogen when performed on admission to NICU over a six year period as described above (Neary et al., 2013). In this study, which was adequately powered to determine preliminary ranges for PT, APTT and fibrinogen in extremely premature infants, retrospective data were collated from 183 infants born <27 weeks’ GA. Reference ranges were determined to be 14.4–36.7 and 40.5–158.5 s and 0.7–4.8 g/l for plasma PT, APTT and fibrinogen, respectively. The principal limitations of this study include its retrospective nature, the fact that data were not available for all identified patients, and exclusion of seven infants whose clotting times were prolonged beyond the instrument range. These patients were excluded in view of significant probability that results obtained reflected errors in the preanalytical phase, including heparin contamination and under filling of blood bottles, resulting in an inappropriate blood: citrate ratio.

To overcome the limitations described in Study 1, a prospective observational study (Study 2) was performed. In this, the largest study to date characterising reference ranges in a consecutive series of very preterm infants born <30 weeks gestation, we have determined that median (5th-95th percentile) Day 1 PT, APTT, and fibrinogen were 17.9 (12.8-27.7) seconds, 79.1 (48.8-134.3) seconds and 1.3 (0.7-3.9) g/L
respectively (n=127). High levels of multiple deliveries in this cohort reflect the tertiary nature of the maternity centre.

Cord blood sampling was attempted on all patients (almost always by the lead investigator) however as many very premature infants are delivered because of placental pathology, sufficient samples of blood was not always obtained. This is a reflection of the reality of prospective data collection in this preterm cohort. Consequently the number of selections made in analysis of data is also a possible source of bias. The results obtained are in line with previously published data in much smaller/retrospective case series and I believe them to be valid. Where cord blood samples are used caution should be applied in interpretation as neonatal values. Reference ranges are specific to the analyser and reagent used in sample analysis, but trends across laboratories should be comparable (Monagle and Massicotte, 2011). Validation of reference ranges may be performed by other laboratories using similar reagents but including fewer cases.

In order to provide reference ranges for gestation specific weeks less than 30 weeks gestation, the prospective data was combined with extended dataset of infants less than 30 weeks GA from 2008 onwards (Study 3). Median (range) for PT in retrospective data compared to prospective data is 18 (11-48) vs. 18 (12-37), p=0.57. Median (range) for APPT in retrospective data compared to prospective data is 75 (41-192) vs. 79 (36-148), p=0.02. Of infants with APTT >100s, there were (29/187) 16% in retrospective data and (33/138) 24% in prospective data. However as the median range of APTT in both cohorts is not clinically different, data was combined.
This study has provided gestation specific reference ranges in the largest group to date of infants less than 30 weeks life. Antenatal steroids were provided in the majority of infants (90%) and were associated with shorter PT and APTT. Apgar scores had no effect on coagulation profile. Of note resuscitation is not automatically performed at less than 24 weeks gestation in our institution which is why numbers are small for this gestation. These infants are also a sub selected cohort to have received resuscitation at all.

To our knowledge, this is the first study to provide gestation-specific ranges for infants less than 30 weeks GA. The data show that coagulation follows a characteristic pattern of development with values for APTT and fibrinogen lower for infants born at more mature GA. Absolute values are reagent and analyser-specific (Monagle et al., 2006) however; the trend in changes observed is consistent with data from more mature infants. These can be verified in other laboratories using similar reagents with a smaller number of samples.

Limitations of the study include that this is a mixed method study. Values outside of the accurate recording ability of the analyser were excluded as the presence of pre-analytical variables could not be excluded. A further limitation is that not all infants had coagulation testing performed during this period. This reflects the practical challenges of phlebotomy in the VLBW infant but can increase bias. The results obtained are clinically similar to previously published data of infants less than 27 weeks GA. Despite these limitations this study contributes to knowledge of developmental changes in premature infants with respect to coagulation values. The reference ranges provided here confirm that premature neonates have prolonged coagulation times at birth.
4.6 Conclusion

In conclusion, in the largest cross-sectional study to date of very preterm infants, we describe typical ranges for widely available coagulation tests. Very premature infants have coagulation values which are significantly prolonged compared to infants born at term (Neary et al., 2013, Neary et al., 2014). It should be noted that ranges are laboratory and assay-specific. Interestingly, these values reduce with increasing GA (Reverdiau-Moalic et al., 1996). However, it is unclear whether these values confer an increased bleeding risk. Whether these infants should be administered plasma products to correct a perceived coagulopathy is debated given the potential for introducing another set of risks. This is partly due to paucity of information regarding reference ranges for coagulation values in very premature infants which we have now addressed. In practice, these reference ranges should be interpreted in conjunction with clinical parameters (Revel-Vilk, 2012).
CHAPTER 5: THROMBIN GENERATION

5.1. Background

The precise molecular mechanisms underlying the observed prolongation of coagulation tests in extremely premature infants remains poorly understood (Tripodi et al., 2008, Poralla et al., 2012b). Ex vivo studies are required to elucidate the overall balance of procoagulant and anticoagulant pathways in this population, as plasma clotting time prolongation may not directly correlate with in vivo bleeding tendency. Consequently, the current widespread practice of administering plasma purely in response to an abnormal plasma clotting time may not be justified.
5.2 Aims

We recently published data from a large retrospective cohort of premature infants born less than 27 weeks GA, describing values for PT, APTT and fibrinogen when performed on admission to NICU over a six year period (Neary et al., 2013). We hypothesized that characterization of coagulation tests in a large cohort of very preterm infants alongside assessment of pro- and anticoagulant pathways and measurement of thrombin generation in cord blood samples of very preterm infants would provide information on overall haemostatic balance in this patient cohort, who is known to be at risk of major haemorrhagic morbidity.
5.3 Methods

Preterm infants delivered at less than 30 weeks GA and admitted to the NICU at the Rotunda Hospital were recruited as part of a prospective observational trial, as previously described. Samples were taken from the umbilical cord following delivery, and on day 1, 3 and 14 of life in infants who were under 30 weeks corrected GA who did not meet exclusion criteria (e.g. receipt of plasma products, q.v.). Samples were processed as described in Chapter 3 and standard laboratory measurements of PT, APTT and fibrinogen performed. Samples were then processed to assay thrombin generation in 150µl aliquots. Demographic and clinical outcome data was collected as previously described (Chapter 3).

Calibrated automated thrombography assay

Thrombin generation was assessed using a human recombinant TF trigger in the presence of phospholipids. Plasma was incubated with phospholipid vesicles (4µM; 60% phosphatidylcholine, 20% phosphatidylserine and 20% phosphatidylethanolamine). Thrombin generation was then initiated with 1pM TF and 100mM CaCl₂ and measured automatically by comparing the rate of fluorogenic substrate hydrolysis to that of a thrombin standard. Thrombin generation was assessed using a Fluoroskan Ascent™ plate reader in combination with Thrombinscope™ software.
5.4 Results

*Thrombin generation is similar in very preterm compared with term infants.*

Despite differences in standard laboratory clotting times, we found that thrombin generation was similar in very preterm compared with term infants (n=27) (Figure 5.1). The CAT assay was performed in cord samples to provide direct comparisons between preterm and term samples. Baseline PT and APTT were performed on these cord samples. Median (10-90th percentile) PT and APTT measurements in preterm cord samples compared with term infants were 15 (12.1-19.8) s and 64.2 (48.8-90.7) s respectively (Figure 4.2 A and B). Mean (SD) preterm peak thrombin generated was similar to that measured in term infants (132 (40.6) vs. 136.7 (35) nM; p=0.66 (Figure 5.1 B)). Minor, non-significant differences in the area under the thrombin generation curve (ETP) were observed (1168 (289) vs. 1303 (190) nM*min; p=0.11 (Figure 5.1 A)). Interestingly, lagtime to thrombin generation was shorter in preterm compared with term cord samples (Figure 5.1 C and D).
Figure 5.1: TF-stimulated, phospholipid-dependent thrombin generation (n=27 preterm infants; n=25 term infants; A-D) in platelet poor plasma prepared from cord blood. A-D: Coagulation was initiated in plasma with 1pM TF, 4µM phospholipid vesicles Phosphatidylcholine:Phosphatidylserine:Phosphatidylethanolamine 60%:20%:20%) and 6.67mM CaCl₂. ETP, peak thrombin generation, lagtime and time to peak thrombin generation were measured (A-D respectively). Results are expressed as mean ± standard error of the mean (SEM). *This work was presented in poster format at American Paediatric Society, San Francisco, December 2014, and published in Journal of Thrombosis and Haemostasis (Neary et al., 2015).
To address the hypothesis that infants with extreme APTT prolongation might exhibit reduced thrombin generation, ETP and peak thrombin were also measured using aliquots of peripheral blood in preterm infants only (therefore numbers are larger). In infants with a day 1 APTT greater than 100 seconds, ETP was significantly reduced compared with infants with a day 1 APTT less than 100 seconds (mean (SD) 963 (288) nM*min v 1169 (299) nM*min respectively; (Figure 5.2 A). As opposed to ETP, the peak thrombin generated was mildly increased in the cohort with APTT values greater than 100 (mean (SD) 73(14) nM v 83 (34) nM. Parameters of thrombin generation assay were compared with coagulation indices obtained from infants on day 1 of life (Figure 5.3).
Figure 5.2: Thrombin generation in very premature neonates with corresponding APTT values on day 1 of life

Figure 5.2: In neonatal samples with a day 1 APTT greater than 100 seconds (n=28), ETP was significantly reduced compared with infants with a day 1 APTT less than 100 seconds (n=73) (A) but peak thrombin was increased (B). Results are expressed as mean ±SEM.
Figure 5.3: Correlation of thrombin generation with coagulation indices on day 1 of life in very premature neonates

Figure 5.3: In neonatal samples drawn on day 1 of life, PT (A–D) and APTT (E–H) were correlated with parameters of thrombin generation assay.
5.5 Discussion

Thrombin plays many roles in coagulation cascade including acting as a procoagulant through FV and FVIII activation, and cleaving fibrinogen to form the fibrin clot. On the other hand, it can act in anticoagulant manner by activating protein C when bound to thrombomodulin. Therefore thrombin generation is a key component of the coagulation system. Prolonged clotting times might be expected to predict reduced thrombin generation. However, I observed no reduction in plasma thrombin generation in cord samples of preterm infants (where cord blood was available, n=27) born <30 weeks GA and term infants, using an assay incorporating software that estimates individual sample α2M contribution to thrombin generation. No differences in peak thrombin generation and the area under the thrombin generation curve (ETP) were observed. The precise role of thrombin generation as a predictor of bleeding risk in premature neonates is not known. However, peak thrombin generation has been reported to predict bleeding risk in studies performed in adult patients. At the low TF concentration used in the assay described (1pM), sensitivity to reduced plasma concentration of procoagulant coagulation factors would be expected.

Alpha-2-macroglobulin (α2M)-bound thrombin cleaves the fluorogenic thrombin substrate utilized in this assay and continues to do so following physiological thrombin inhibition. Initial α2M-mediated thrombin inhibition plays an important physiological role, particularly in neonates (Schmidt et al., 1989). The α2m-bound thrombin is still amidolytically active towards the substrate which is corrected for by the software. Previous studies have also reported that thrombin generation is similar in older preterm
infants and term infants and in very small numbers of preterm infants (Streif et al., 2000).

Interestingly, reductions in lagtime to onset of thrombin generation were observed in preterm infants. Primitive guidelines have been established in terms of treatment of abnormal coagulation screens (Christensen et al., 2014). Infants who had an APTT greater than 100 seconds were examined as a subgroup, as it is routine practice to administer FP to infants with APTT greater than 100 seconds in the unit where patients were enrolled. This is a local institution practice only and not based on objective guidelines. While I did observe a modest reduction in ETP in a cohort of infants with extreme APTT prolongations (i.e. Those APTT greater than 100 seconds), the clinical relevance of this mild attenuation is uncertain, with the difference observed less than 20% between cohorts. However it seems reasonable given the fact that infants whose APTT values of greater than 100s also had reduced ETP to treat this cohort as separate and treatment of these infants with FP may be warranted.
5.6 Conclusion

This study has demonstrated that thrombin generation is similar in very preterm and term infants. There is growing international concerns that routinely transfusing non-bleeding very preterm infants with plasma could be associated with potential harm and is not evidence-based. The findings reported here highlight the limitations of using basic laboratory coagulation testing, which are more sensitive to attenuated procoagulant pathways, when making decisions on blood product transfusion.
CHAPTER 6: POSTNATAL MATURATION OF COAGULATION

6.1 Background

Developmental haemostasis refers to the evolving nature of the coagulation system with age. This is reflected in fetal studies as illustrated in Table 6.1 below (Reverdiau-Moalic et al., 1996) and studies in term and moderately preterm neonates with both gestational and postnatal age impacting on coagulation values (Andrew et al., 1987, Andrew et al., 1988, Salonvaara et al., 2004, Gibson, 1989).

Whether or not cord blood coagulation test results are a true reflection of neonatal coagulation is not determined. Validation studies have been performed using cord blood for a variety of neonatal admission investigations including full blood count and leukocyte differentials, blood cultures, neonatal blood type and metabolic screening (Christensen, 2015). An initial pilot study showed a decrease in erythrocyte transfusions and IVH in study patients where cord blood was used as a substitute for postnatal screening for baseline neonatal investigations (Christensen et al., 2011). In a larger multicentre trial there was an increase in the haemoglobin in the first 12-24 hours, fewer transfusions per patient, and low vasopressor use observed in the study patients. The rate of IVH appeared lower although not statistically different (Baer et al., 2013). To date no randomised controlled trials (RCTs) have been reported.
Table 6.1: Coagulation screening tests and coagulation factor levels in fetuses, full term newborns and adults


<table>
<thead>
<tr>
<th>Parameter</th>
<th>19-23 (n = 20)</th>
<th>24-29 (n = 22)</th>
<th>30-38 (n = 22)</th>
<th>Newborns (n = 40)</th>
<th>Adults (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>32.5 (19-45)</td>
<td>32.2 (15-44)†</td>
<td>22.6 (16-30)†</td>
<td>16.7 (12.0-23.5)†</td>
<td>13.5 (11.4-14.0)</td>
</tr>
<tr>
<td>PT (INR)</td>
<td>6.4 (1.7-11.1)</td>
<td>6.2 (2.1-10.6)†</td>
<td>3.0 (1.5-5.0)†</td>
<td>1.7 (0.9-2.7)†</td>
<td>1.1 (0.8-1.2)</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>188.8 (83-250)</td>
<td>154.0 (87-210)†</td>
<td>104.8 (76-128)†</td>
<td>44.3 (35-52)†</td>
<td>33.0 (25-39)</td>
</tr>
<tr>
<td>TCT (s)</td>
<td>34.2 (24-44)*</td>
<td>26.2 (24-28)</td>
<td>21.4 (17.0-23.3)</td>
<td>20.4 (15.2-25.0)†</td>
<td>14.0 (12-16)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Factor</th>
<th>19-23 (n = 20)</th>
<th>24-29 (n = 22)</th>
<th>30-38 (n = 22)</th>
<th>Newborns (n = 40)</th>
<th>Adults (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (g/L, Von Clauss)</td>
<td>0.85 (0.57-1.50)</td>
<td>1.12 (0.65-1.65)</td>
<td>1.35 (1.25-1.65)</td>
<td>1.68 (0.95-2.45)†</td>
<td>3.0 (1.78-4.50)</td>
</tr>
<tr>
<td>I Ag (g/L)</td>
<td>1.08 (0.75-1.50)</td>
<td>1.93 (1.56-2.40)</td>
<td>1.94 (1.30-2.40)</td>
<td>2.65 (1.68-3.60)†</td>
<td>3.5 (2.50-5.20)</td>
</tr>
<tr>
<td>IIc (%)</td>
<td>16.9 (10-24)</td>
<td>19.9 (11-30)*</td>
<td>27.9 (15-50)†</td>
<td>43.5 (27-64)†</td>
<td>98.7 (70-125)</td>
</tr>
<tr>
<td>Vllc (%)</td>
<td>27.4 (17-37)</td>
<td>33.8 (16-54)*</td>
<td>45.9 (31-62)†</td>
<td>52.5 (28-78)†</td>
<td>101.3 (68-130)</td>
</tr>
<tr>
<td>IXc (%)</td>
<td>10.1 (6-14)</td>
<td>9.9 (5-15)</td>
<td>12.3 (5-24)†</td>
<td>31.8 (15-50)†</td>
<td>104.8 (70-142)</td>
</tr>
<tr>
<td>Xc (%)</td>
<td>20.5 (14-29)</td>
<td>24.9 (16-35)</td>
<td>28.0 (16-38)†</td>
<td>38.6 (21-65)†</td>
<td>99.2 (75-125)</td>
</tr>
<tr>
<td>Vc (%)</td>
<td>32.1 (21-44)</td>
<td>36.8 (25-50)</td>
<td>48.9 (23-70)†</td>
<td>89.9 (50-140)</td>
<td>99.8 (65-140)</td>
</tr>
<tr>
<td>VIIIc (%)</td>
<td>34.5 (18-50)</td>
<td>35.5 (20-52)</td>
<td>50.1 (27-78)†</td>
<td>94.3 (38-150)</td>
<td>101.8 (65-170)</td>
</tr>
<tr>
<td>Xlc (%)</td>
<td>13.2 (8-19)</td>
<td>12.1 (8-22)</td>
<td>14.8 (6-26)†</td>
<td>37.2 (13-62)†</td>
<td>100.2 (70-135)</td>
</tr>
<tr>
<td>XIlec (%)</td>
<td>14.9 (6-25)</td>
<td>22.7 (6-40)</td>
<td>25.8 (11-50)†</td>
<td>68.8 (25-105)†</td>
<td>101.4 (65-144)</td>
</tr>
<tr>
<td>PK (%)</td>
<td>12.8 (8-19)</td>
<td>15.4 (8-26)</td>
<td>18.1 (8-28)†</td>
<td>35.4 (21-53)†</td>
<td>99.8 (65-135)</td>
</tr>
<tr>
<td>HMWK (%)</td>
<td>15.4 (10-22)</td>
<td>19.3 (10-26)</td>
<td>23.6 (12-34)†</td>
<td>38.9 (28-53)†</td>
<td>98.8 (68-135)</td>
</tr>
</tbody>
</table>

Values are the mean, followed in parentheses by the lower and upper boundaries including 95% of the population.

* Abbreviations: Ag, antigenic value; c, coagulant activity.

* P < .05.
† P < .01.
6.2 Aims

In this study, we have shown that thrombin generation is similar in preterm and term infants in cord samples and on day 1 of life (data presented in Chapter 5 and that we have recently published (Neary et al., 2015)). This chapter investigates what happens to these values following delivery and matures ex-utero. This thesis aimed to prospectively characterise the relationship between coagulation tests in cord blood and neonatal blood samples to assess the feasibility of cord blood use for coagulation analysis in the preterm infant. Additionally, we hypothesized that prospective characterization of coagulation tests in neonatal blood samples would provide information on the development of coagulation indices (prothrombin, activated partial thromboplastin time, and fibrinogen) in relationship to both gestational and postnatal age. We also aimed to identify the effect of FP administration on coagulation parameters in extremely premature infants compared to infants who did not receive FP.
6.3 Methods

Prospective Collection

In a prospective observational study of infants up to 30 weeks corrected GA, blood was drawn into citrated tubes from cord blood of neonates on day 1 of life from non-heparinised lines and on day 3 and day 14 from peripheral phlebotomy if no plasma product had been administered. Vitamin K was administered at birth. Exclusion criteria included antenatal IVH or parental bleeding disorder. Preanalytical variables were controlled for by ensuring samples were correctly filled, not clotted, and majority drawn by lead investigator. Platelet poor plasma was obtained by centrifugation of whole blood at 3000rpm for 10 min. All samples were analysed for PT, APTT and fibrinogen using ACL TOP 500 coagulometer (Instrumentation Laboratory Lexington, MA; IL) using HemosIL APTT lyophilized reagent, IL Clauss Fibrinogen and IL RecombiPlasTin reagent with analyser optical method of measurement.

Retrospective Collection

Retrospective review of coagulation profiles and solvent detergent FP administration over the first 48-72 hours of life in neonates less than 27 weeks GA between 2004 - 2013. Day 1 and day 3 samples from infants who received plasma were compared with samples taken at the same postnatal age in infants who did not receive plasma products. This was not randomised given the retrospective nature of the study.
6.4 Results

Coagulation analysis in very premature neonates

Between April 2013 - April 2015, 137 patients <30/40 were admitted. Median (25th-75th) GA and BW was 27.9(26.4-28.7) weeks and 1020(815-1215) g respectively. Attempts to collect cord blood (3ml sample) were made in 95% of deliveries. In cases where no sample was feasible (n=31), infants were comparable in terms of GA and BW but had higher incidence of emergency deliveries with placenta morbidity observed (abruption, antepartum haemorrhage, acreta, broken cord). The laboratory received blood samples in 72% of cases (n=99). Samples were checked for pre-analytical variables before processing. 40% of samples were discarded at this point due to either initiation of coagulation (66%) or insufficient volume (44%), resulting in 60 samples eligible for processing. Of these complete coagulation profiles PT, APTT and fibrinogen were available on 42 samples, indicating an error on analysis in the remaining samples. Reasons for potential errors on analysing include potential pre-activation of sample or contamination with Warton’s jelly. Successful cord blood collection was associated with increased GA and BW as shown in Table 6.2. Of infants with successful coagulation sampling from cord blood (n=42), 4 infants had grade III/IV IVH and 14 had any grade IVH. Of all remaining infants recruited (n=95), 10 infants had grade III/IV IVH and 31 had any grade IVH.
Table 6.2: Demographics of infants where cord blood analysis of coagulation profile was feasible compared to those which were discarded due to pre-analytical errors.

<table>
<thead>
<tr>
<th></th>
<th>Infants with cord blood (n=42)</th>
<th>Infants without cord blood (n=58)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BW (Mean, SD)</strong></td>
<td>1124 (251)</td>
<td>985 (267)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>GA (Median, Range)</strong></td>
<td>28.7 (23.7-29.9)</td>
<td>27.1 (23.7-29.9)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>29/42 (69%)</td>
<td>35/58 (60%)</td>
<td></td>
</tr>
<tr>
<td><strong>Caesarean Section</strong></td>
<td>32/42 (76%)</td>
<td>44/58 (76%)</td>
<td></td>
</tr>
</tbody>
</table>
Paired samples with neonatal blood sample on admission to NICU were available on 39 of these infants. Correlation of cord blood results to neonatal blood samples for PT, APTT and Fibrinogen was \( r = 0.8, 0.7, 0.7 \) respectively (Figure 6.1). Median (5th-95th percentile) cord PT of 15 (12 -24.4) seconds was observed vs. 15.8 (12.4-32.2) seconds from neonatal samples. Mean cord APTT was 68.5 (16.5) seconds vs. 74.5 (19.5) seconds in neonatal samples and median cord fibrinogen was 1.2 (0.6-4.2) g/L vs. 1.3 (0.8-4.0) g/L.
Figure 6.1: Scatterplot with least squared regression line for PT, APTT and Fibrinogen. Individual points represent the paired data of each individual patient (n=39). Correlation, Spearman R= 0.8 (A), Pearson R= 0.7 (B), Spearman R= 0.7 (C).
**Demonstration of postnatal reduction in clotting times following delivery and vitamin K administration.**

From a total of 137 neonates that were studied on one to 3 occasions, 127 neonatal samples were obtained on admission to NICU, 50 on day 3 of life and 26 at 2 weeks postnatal age. Attenuation reflected plasma product use and infants passing 30 weeks' corrected age. Reference intervals for day 1 of life were described in Chapter 4. We have determined that median (5th-95th percentile) day 3 PT, APTT, and fibrinogen were 13.7 (11.3-21.3) seconds (s), 50.1 (29.9-74.9) s and 2.3 (1-3.5) g/L respectively (n=50). Serial paired samples were available in 47 infants between day 1 and day 3. In total, 18 infants had serial blood samples on day 1, day 3 and week 2 of life.

Serial analysis in infants who did not receive plasma revealed reduction of median (10-90th percentile) PT from 17.9 (13.9-23.6) s on day 1 (n=127) to 13.7 (11.6-16.2) s on day 3 (n=50) and 12 (11.2-16.2) s in week 2 of life (n=26). Serial analysis of this cohort for APTT revealed reduction from median (10-90th percentile) APTT from 79.1 (56.7-113.6) s on day 1 (n=127) to 49.3 (35.3-68.3) s on day 3 (n=50) and 47.4 (31.9-68) s at week 2 (n=26). Comparison of coagulation values in paired sequential samples on day 1, 3 and 14 of life, showed significant reduction in PT and APTT with increasing postnatal age (**Figure 6.2 A-C**; n=18, Repeated Measures ANOVA, p<0.01) Regardless of GA at birth, these infants reached the same levels by the corrected age of 2 weeks (**Figure 6.2 D-F**).
Figure 6.2: Effect of postnatal age on coagulation indices

Figure 6.2: PT (n=18; A), APTT (n=18; B) and fibrinogen (n=18; C) in platelet poor plasma prepared from paired preterm infants on day 1, day 3 and week 2 of life. These infants did not receive plasma products. Values for PT (D), APTT (E) and Fibrinogen (F) were significantly higher (circle) compared to corrected GA matched infant at two weeks of age. These values represent the mean for PT, APTT, and fibrinogen on Day 1 and Day 14 for each gestational week. The number of patients represented by the mean value for infants on day 1 at was 15, 9, 19, 27, 34 and 22 at 24, 25, 26, 27, 28, and 29 weeks gestation respectively. In figures A-C the 90th percentile is the upper reference interval and the 10th percentile is the lower reference interval. Both of these are shown by the whisker lines. The median value is shown by the solid line. *This work was presented in poster format at Paediatric Academic Society Annual Meeting, San Diego, May 2015
Thrombin generation is similar in very preterm infants on day 1 and day 3 of life.

Despite differences in standard laboratory clotting times, we found that thrombin generation was similar in paired samples from infants with paired coagulation indices measured on day 1 and day 3 of life (n=28) (Figure 6.3 C). The CAT assay was performed in paired samples to provide direct comparisons between preterm infants on day 1 and day 3 of life. Baseline PT and APTT were performed on these samples. Median (10-90th percentile) PT and APTT were 17.6 (13.3-23.8) s and 74.9 (47.2-101.5) s respectively on day 1 which reduced significantly to 14 (11.5-16.7) s and 48.6 (34.7-70.9) s respectively on day 3 of life (Figure 6.3 A and B). Mean (SD) ETP on day 1 was similar to that measured on day 3 of life in very preterm infants (1116 (394) vs. 1158 (251) nM*min; p=0.4 (Figure 6.3 D). Significant differences in preterm peak thrombin generated was observed between day 1 and day 3 of life (95.5 (32.5) vs. 124.3 (43.7) nM; p<0.01 (Figure 6.3 E). Lagtime to thrombin generation and time to peak were similar in preterm infants on day 1 and day 3 of life (Figure 6.3 F and G).
Figure 6.3: Thrombin generation in paired neonatal samples on day 1 and day 3 of life

A) PT (n=28; A), B) APTT (n=28; B) and C) TF-stimulated, phospholipid-dependent thrombin generation (n=28 preterm infants C-F) in platelet poor plasma prepared from paired day 1 and day 3 samples. C-F: Coagulation was initiated in plasma with 1pM TF, 4µM phospholipid vesicles (Phosphatidylcholine:Phosphatidylserine:Phosphatidylethanolamine 60%:20%:20%) and 6.67mM CaCl2. ETP, peak thrombin generation, lagtime and time to peak thrombin generation were measured (C-F respectively). Results are expressed as mean ±SEM.
**Postnatal maturation occurs irrespective of FP administration**

A total of 67 infants were identified as having day 1 and day 3 coagulation tests between 2004-October 2013; 40 who received treatment with FP and 27 who did not. The demographics of the two groups are in **Table 6.3**.

**Table 6.3: Demographics of infants with serial coagulation profiles**

<table>
<thead>
<tr>
<th></th>
<th>FP n=40</th>
<th>No FP n=27</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA (weeks)</td>
<td>24.9</td>
<td>26.1</td>
<td>0.02</td>
</tr>
<tr>
<td>BW (g)</td>
<td>775</td>
<td>840</td>
<td>0.52</td>
</tr>
<tr>
<td>Male Gender</td>
<td>22</td>
<td>16</td>
<td>0.8</td>
</tr>
<tr>
<td>Apgar (1, 5 min)</td>
<td>6,8</td>
<td>7,9</td>
<td>0.19</td>
</tr>
<tr>
<td>Mode of delivery: SVD</td>
<td>15</td>
<td>7</td>
<td>0.43</td>
</tr>
</tbody>
</table>
FP resulted in PT and APTT reductions from mean values (ranges) of 23.1 seconds (s) (14.9s-38.4s) and 97.6s (48.7s-191.6s) to 20s (12.3s-40.9s) and 71.9s (41.7s-182s) respectively (n=40). In infants who did not receive FP (n=27), mean PT and APTT reductions of 17.6s (13.3s-22.4s) to 15.8s (12.5s-27.6s) and 74.7s (41.6s-200.4s) to 55.9s (32.7s-77.8s) over the first 72 hours of life were observed without intervention.

Eighty per cent of those treated had values outside the upper limit as per Andrews et al versus 11% of untreated group. This may be due in large part due to arbitrarily administrating FP when APTT is above a certain threshold. 11 infants had an APTT greater than 80s after FP administration. The statistical difference in PT and APTT between day 1 and day 3 values for the cohort treated with FP was p=0.02 and p<0.01 respectively, compared with p=0.01 and p<0.01 respectively in the cohort who were not treated. The differences between those who did get treated compared to those who were did not reach statistical significance for PT or APTT (p=0.084, p=0.53 respectively) (Figure 6.4).
Figure 6.4: Change in PT (A) and APTT (B) between day 1 and day 3 of life in preterm infants who did receive FP (square) and those that did not (circle). Results are expressed as mean. *This work was presented in poster format at Paediatric Academic Society Annual Meeting, Vancouver, May 2014
6.5 Discussion

The evolving nature of the coagulation system with postnatal age as well as GA necessitates reference intervals for multiple time points. However as it can be difficult to obtain samples on this population, reference intervals may be practically difficult to establish at all-time points (Hathaway and Corrigan, 1991). The use of cord blood, which may otherwise be routinely discarded as an alternative to neonatal sampling, could serve to reduce iatrogenic blood loss due to phlebotomy sampling (Christensen et al., 2011).

6.5.1 Use of Cord Blood

Validation studies for any proposed testing are required to determine whether cord blood values are interchangeable with tests analysed from neonatal samples in preterm infants. This study has shown that fibrinogen correlates well from cord blood compared to that of neonatal blood on admission. PT and APTT in neonatal blood were higher in neonatal samples than that of cord blood. Comparing values for cord blood PT and APTT provided p values of 0.04 and 0.15 respectively, providing a statistical difference between groups for PT, however the median for PT is 15s in cord blood compared with 15.8s in neonatal samples which is not clinically significant as both these values fall within laboratory normal value ranges for this age group (Andrew, 1988). In our cohort, cord blood sampling would not have altered practice compared with neonatal values as the number of values with APTT >100 seconds corresponded in both groups of paired samples which as per the local policy in the hospital would of received FP based on these results. As described earlier in the thesis this policy is not a universal policy. These results are consistent with correlation rates of blood culture and full blood
counts evaluated in previous studies (Carroll et al., 2012) with respect to accuracy.

Despite the undisputed role of cord blood as an abundant and readily available alternative blood source, the relatively difficulties in cord blood collection need to be addressed. The major issues in obtaining cord blood irrespective of intended purpose include obtaining required volume of blood, and minimising delays to avoid clotting of the blood sample (Ballen et al., 2008). Feasibility of cord blood collection for coagulation testing is dependent on a number of factors including GA, co-morbidities and staff resources. Baer et al reports a high success rate of 95% in their study on feasibility of use of umbilical cord blood for baseline investigations, although they recognise that training and practice is required (Baer et al., 2013). In addition milking of cord/delayed cord clamping was only employed in a third of patients and study design was based on a convenience sample. Delayed cord clamping is a birth practice where the umbilical cord is not clamped or cut until after pulsations have ceased, or until after the placenta is delivered. An alternative to delayed cord clamping is umbilical cord milking in which the unclamped umbilical cord is grasped and blood is pushed toward the infant several times before it is clamped to autoinfuse blood into the preterm neonate. The hypothesis behind these practices is to stabilise the infant’s circulatory system which improves blood pressure with improved neonatal outcomes. Feasibility of cord blood collection in this study as defined by complete coagulation profile obtained was available in 41% of cases where blood samples where achieved.

Collection of research samples in a busy clinical unit is a challenge. Clinical samples were collected by priority including haemoglobinopathy screening or cord blood gases as required, which may have impacted the volume of blood remaining. Our study had a high rate of clotting (66%), although cord milking was employed in all cases. This is consistent with reports from the ELFE study which reports
variable rates of clotted samples as high as 100% in some centres (Oleko et al., 2011). The ELFE study is a longitudinal study in which cord blood was to be collected from the infants recruited. In a pilot study, to determine feasibility of cord blood collection, the use of syringe was the main factor correlated with coagulation (relative risk: 2.79 [1.47; 5.31], P<0.01). Maternity unit status was also associated with coagulation (RR: 1.48 [1.03; 2.13] in a private maternity unit vs. a public maternity) as well as time between collection and centrifugation (RR 1.03 [1; 1.07] when time between collection and centrifugation increases from 1 h). Carroll et al had a success rate of 33% in infants less than 27/40 and found cord blood collection of the most immature neonates represents a significant challenge (Carroll et al., 2012). The method of blood collection may influence rate of coagulation with coagulation occurring more commonly with needle aspiration compared to pooling of blood (Oleko et al., 2011). The physiological adaptation to delivery induces hypercoagulability in the newborns blood (Andrew et al., 1990). Consequently all mechanical factors such as the syringe shearing force and mechanical pressure through aspiration induce platelet and TF activation and coagulation. This activation is not induced by natural dripping of blood. However blood bottles were narrower than the width of the cord so could not facilitate dripping of blood. Potential solutions are to add citrate in a 9:1 ratio to universal containers into which the blood can pool, but the potential for error is increased here due to variable volume of blood available. Coagulated samples also have implications for other blood tests warranted including full blood counts.

The application of cord blood collection for coagulation testing in a clinical environment would require significant staff education in regards the drawing of correct sample volume to avoid pre-analytical variables. Commercial vacutainers that allow for pooling of blood directly into citrated bottle may be more suitable to clinical settings to reduce rates of coagulation.
6.5.2 Postnatal Maturation

In this study we provide reference intervals for day 1 and day 3 of life in infants less than 30 weeks GA. Of particular clinical relevance, coagulation profiles have been shown to normalise within a few days of birth, irrespective of GA. Thrombin generation was observed to be comparable over all postnatal ages, indicating that the coagulation system is balanced despite the altering coagulation values.

*In vivo* effects of FP in newborn infants remains poorly understood. Coagulation values are not completely corrected in response to FP administration. (Johnson et al., 1982, Hambleton and Appleyard, 1973, Altuntas et al., 2012). Hyytiainen *et al* demonstrated potential coagulation-modulating effects of FP in a proportion of newborns studied, but did not control for the effect of postnatal maturation (Hyytiainen *et al.*, 2003). We present a report on the effects of FP on coagulation in the largest cohort of extremely premature infants less than 27 weeks described to date, along with gestation and age-matched controls who did not receive FP. Limitations of this study include the retrospective design and in that the cohort examined was limited to infants who had serial coagulation values. In addition, infants who received FP had a higher PT and APTT on day 1 due to the policy in the hospital NICU to administer FP if APTT values were greater than 100s.
6.6 Conclusions

In conclusion, this study shows that fetal blood that would otherwise be discarded could be used as an adjuvant to cord milking and delayed cord clamping to preserve haemoglobin levels in very preterm infants by minimising anaemia from phlebotomy. This study suggests that regulation of coagulation indices follows characteristic patterns relative to the GA of the infant. Postnatal development was similar irrespective of initial GA suggesting that coagulation matures to normal adult levels after birth. Delivery or vitamin K administration seems to hasten the acquisition of “normal” values. The clinical importance of this could be noted also for infants who are past the initial perinatal period and require surgery. It is likely that earlier GA ranges are not relevant after day 1 or 3 of life. In addition, infants who received FP and those who did not receive FP all exhibited a reduction in PT and APTT by 72 hours. We have shown that infants who received FP had higher PT and APTT pre and post FP than those who did not receive it and following FP the levels reached the upper limits of normal. As the indication for FP administration was not known in all cases we cannot comment on whether FP was given unnecessarily.
CHAPTER 7: PROCOAGULANT AND ANTICOAGULANT PATHWAYS IN VERY PRETERM INFANTS

7.1 Background

Thrombin generation is comparable in preterm and term infants despite prolonged coagulation times (Chapter 5). Moreover coagulation indices (PT and APTT) decrease postnatally, despite similar thrombin generation at each time point (Chapter 6). In order to understand why thrombin generation is equivalent in the setting of these prolonged coagulation times, a consideration of the components of haemostasis is warranted. Balanced haemostasis requires both procoagulant and anticoagulant clotting pathways. However, the precise mechanism underlying PT and APTT prolongation in these infants remains poorly understood. It was hypothesized that measurement of individual coagulation factor levels could help to address the paradox in which prolonged coagulation values are observed in preterm infants despite similar thrombin generation.

Furthermore, we hypothesised that concurrent assessment of thrombin generation and activity of anticoagulant pathways including α2M and APC would permit further characterisation of neonatal coagulation. Central to this hypothesis is the multifunctional role of thrombin. Thrombin has both procoagulant and anticoagulant functions. Moreover, thrombin has recently been described to play critical roles in fibrinolysis and inflammation (Colucci and Semeraro, 2012). These functions are promoted by the interaction of surface exosites that bind to range of substrates and cofactors (Crawley et al., 2007). CAT assay facilitate a
global assessment of plasma coagulation that incorporates both pro- and anticoagulant pathways. Modification of this assay by incubation of APC permits for assessment of the anticoagulant protein C pathway (Green et al., 2012)


7.2 Aims

We hypothesized that characterization of coagulation tests in a large cohort of very preterm infants alongside assessment of pro- and anticoagulant pathways and measurement of thrombin generation in cord blood samples of very preterm infants in both the presence and absence of APC would provide information on the overall haemostatic balance in this very preterm patient cohort. Moreover, we hypothesized that those differences in both procoagulant and anticoagulant clotting factors in preterm neonates (compared to healthy term neonates) results in an overall haemostatic “balance”. In addition we hypothesised that the primary haemostatic system in premature neonates is adequate.

The second aim of this chapter was to investigate the mechanisms underlying observed prolongations in clotting times in extremely premature neonates and to identify the contribution of FXII to this prolongation (factors not associated with a bleeding tendency)
7.3 Methods

Preterm infants delivered at less than 30 weeks GA and admitted to the NICU at the Rotunda Hospital were recruited as part of a prospective observational trial, as previously described. Samples were taken from the umbilical cord following delivery, and on day 1, 3 and 14 of life in infants who were under 30 weeks corrected GA who did not meet exclusion criteria (e.g. receipt of plasma products, q.v.). Samples were processed as described in Chapter 3 and standard laboratory measurements of PT, APTT and fibrinogen performed. Samples were then processed to assay thrombin generation in 150ul aliquots. Where plasma was remaining in cord samples, pro and anticoagulant factors were measured. Demographic and clinical outcome data were collected as previously described (Chapter 3).

Pro- and anticoagulant factor assays

Where sufficient plasma was available within ethical limitations, procoagulant and anticoagulant factors were measured. Cord blood samples were used to determine FII, FVII, FIX, protein C, free protein S, AT, and TFPI levels. VKD factors were measured using HemosIL factor-specific deficient plasma on an IL ACL TOP 500 analyser. Free protein S was measured with an automated latex ligand immunoassay. Protein C and AT were measured by automatic methods with chromogenic endpoints. All reagents were sourced from Brennans and Co., Dublin, Ireland.
**Estimation of α2m activity**

α2m level was determined by use of thrombin generation assay. α2m-bound thrombin cleaves the fluorogenic thrombin substrate utilized in the thrombin generation assay and continues to do so following physiological thrombin inhibition. The CAT assay has the ability to separate the amidolytic activity into the part due to free thrombin and that due to α2M-thrombin, which allows for assessment of α2M levels (Hemker et al., 2003).

**Suppression of thrombin generation by APC**

TF-stimulated thrombin generation was characterized in a CAT assay with a 1pM TF stimulus using Thrombinscope™ software in the presence and absence of APC to allow for assessment of the function of the protein C pathway. Control plasma was obtained from cord blood of term neonates.

**Evaluation of Primary Haemostatic System**

All infants had a full blood count performed at time of coagulation sampling on admission to NICU. Quantitative analysis of platelets was performed. VWF antigen was measured on a pooled sample of cord blood of preterm infants (n=16) and compared with that of pooled sample of cord blood of term infants (n=16).
To assess the mechanism contributing to prolonged coagulation screening tests

In order to investigate potential mechanisms underlying the discrepancy between “prolonged” coagulation times and the observed normal thrombin generation in preterm infants compared to term infants, the relative contribution of reduced levels of clotting factors whose deficiencies do predict a bleeding phenotype (FIX, FX, FII etc.) and those whose deficiencies do not predict a bleeding phenotype (FXII) were determined.

Cord blood samples from premature infants and normal controls were pooled to provide sufficient plasma volume for the experimental procedures. Baseline PT and APTT, and plasma levels of FII, FVII, FIX, FX, FXI, and FXII were measured in aliquots of each pool. Factors not determined to differ significantly between preterm and term samples (e.g. FV) were not measured given the small available plasma volumes. Immediately afterwards, aliquots of each pool were incubated with one each of a series of commercially prepared purified coagulation factors (FII, FVII, FIX, FX, FXI, FXII). Sufficient volumes of each commercially prepared purified individual coagulation factor were added to the plasma to mimic concentrations of each factor equivalent to that found in cord blood from normal, healthy, term infants. The hypothesis tested here whether the correction of individual factors in preterm plasma to that of a term neonate would result in reduction of PT and APTT in preterm plasma to that seen in term plasma.

Repeat analysis of PT, APTT and the level of that coagulation factor was performed following the addition of each individual coagulation factors to determine the effect of each individual factor correction to the overall
results. Purified coagulation factor concentrates were diluted to prepare stock solutions such that only 1μl of each stock solution was incubated each plasma aliquot to restore the level of that coagulation factor to that of healthy cord blood. Differences in PT and APTT following restoration of each coagulation factor to normal term levels were compared in order to estimate the individual contribution to the “prolonged” observed clotting times due to deficiency of that coagulation factor.
7.4 Results

Plasma activity of both procoagulant and anticoagulant factors are attenuated in very preterm infants.

Analysis of procoagulant and anticoagulant pathways was performed in a subset of infants where sufficient plasma was available from umbilical cord samples (n=12). Depending on the volume of cord blood that was feasible to collect, the quantity of plasma that remained following analysis of cord blood coagulation profiles was variable. Remaining plasma was first tested by CAT. Subsequently, if sufficient plasma remained, coagulation factor activity was determined. Ethical limitations on the volume of blood draw prohibited analysis of coagulation factor activity in neonatal peripheral blood samples and hence all coagulation factor analysis was performed on cord blood. As described, cord blood correlates well with samples drawn from neonate (Chapter 6).

In keeping with published data in a much more mature cohort (Andrew et al., 1988), mean activity of VKD procoagulant FII, FVII, FIX and FX were lower in preterm compared to term infants (n=12) (Table 7.1). Similarly, activity of anticoagulant factors protein C (n=11), free protein S (n=12) and AT (n=13) were also attenuated in preterm compared to term infants (Table 7.2). Levels of TFPI were not significantly attenuated in preterm compared with term plasma (n=13).
Table 7.1: Procoagulants

<table>
<thead>
<tr>
<th>(IU/ml)</th>
<th>Preterm Median (Range)</th>
<th>Term Median (Range)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FII (n=12)</td>
<td>0.31 (0.18-0.5)</td>
<td>0.44 (0.35-0.6)</td>
<td>0.003</td>
</tr>
<tr>
<td>FVII (n=12)</td>
<td>0.33 (0.09-0.57)</td>
<td>0.42 (0.31-0.59)</td>
<td>0.29</td>
</tr>
<tr>
<td>FIX (n=12)</td>
<td>0.16 (0.09-0.5)</td>
<td>0.29 (0.19-0.37)</td>
<td>0.004</td>
</tr>
<tr>
<td>FX (n=12)</td>
<td>0.28 (0.13-0.52)</td>
<td>0.44 (0.32-0.58)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 7.2: Anticoagulants

*This work was presented in poster presentation format at American Society of Haematology, San Francisco, USA, December 2014

<table>
<thead>
<tr>
<th>(IU/ml)</th>
<th>Preterm Median (Range)</th>
<th>Term Median (Range)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein C (n=11)</td>
<td>0.11 (0.06-0.24)</td>
<td>0.27 (0.18-0.39)</td>
<td>0.002</td>
</tr>
<tr>
<td>Free protein S</td>
<td>0.38 (0.28-0.55)</td>
<td>0.46 (0.36-0.59)</td>
<td>0.02</td>
</tr>
<tr>
<td>AT (n=13)</td>
<td>0.22 (0.06-0.36)</td>
<td>0.53 (0.38-0.69)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TFPI (n=13)</td>
<td>6.4 (2.61-16.9)</td>
<td>9.17(4.2-15.57)</td>
<td>0.1</td>
</tr>
</tbody>
</table>
\textit{\(\alpha 2m\) activity is lower in preterm infants compared to term infants}

\(\alpha 2m\) activity was estimated in plasma samples from 101 infants drawn on day 1 of life, using CAT assay with Thrombinscope\textsuperscript{TM} software. Using this software, \(\alpha 2m\) activity was estimated to be significantly lower in cord blood of preterm infants compared to cord blood of term infants (12.2 (7.8-57.8) nM vs. 19.6 (12-25) nM, p<0.001) (\textbf{Figure 7.1 A}). Median (5\textsuperscript{th} - 95\textsuperscript{th}) \(\alpha 2M\) activity in very premature infants on day 1 of life was estimated to be 10.3 (3.3-23.9) nM and was noted to increase with GA (\textbf{Figure 7.1 B}). No difference between sexes was observed.
Figure 7.1: Estimation of α2M activity in cord samples from very preterm neonates

**Figure 7.1**: Estimated α2M activity in cord samples from preterm (n=24) and term infants (n=24) (A). Estimated α2M levels in neonatal samples on day 1 (n=101), day 3 (n=38) and day 14 (n=19) (B). Results are expressed as mean ±SEM.
Suppression of thrombin generation by APC is similar in very preterm compared with term infants.

Thrombin generation was dose-dependently suppressed in the presence of increasing amounts of APC in both cord samples from term and preterm neonates and in preterm neonatal samples (Figure 7.2). Suppression of thrombin generation by APC in preterm and term infants did not differ significantly.
Figure 7.2: The inhibitory effect of APC on ex vivo thrombin generation in plasma

Figure 7.2: Suppression of ETP (A) and peak thrombin generated (B) by APC in preterm (dashed line, n=13) and term (solid line, n=13) infants (n=27) on cord samples. Results are expressed as mean ±SEM. *This work was presented in oral presentation format at Joint European Neonatal Societies Meeting, Budapest, September 2015
Table 7.3: Primary Haemostasis

<table>
<thead>
<tr>
<th></th>
<th>Preterm Samples</th>
<th>Term Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Count Mean (SD)</td>
<td>233 (70)</td>
<td>N/A</td>
</tr>
<tr>
<td>(n=133, day 1 preterm samples)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VWF Antigen IU/ml</td>
<td>0.79</td>
<td>0.84</td>
</tr>
<tr>
<td>(n=16, pooled cord samples)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Coagulation Indices, PT and APTT did not correct with supplementation of preterm factor levels to that of term infants.

The mechanism behind PT and APTT prolongation in preterm infants was determined in pooled samples of cord blood from preterm and term neonates (n=16).

PT and APTT were first measured in aliquots of pooled preterm (n=16) and term (n=16) plasma (210μl). Interestingly, there was no statistical difference in PT measured in pooled preterm and term plasma for this patient cohort (Figure 7.3 A). In contrast, the APTT of pooled preterm and term plasma was 60.7 and 49.7 seconds respectively (Figure 7.3 B), as expected.

Optimisation of experimental method was performed using pooled normal plasma. As a control, phosphate-buffered saline (PBS) was added to an aliquot of each plasma pool of test cases and controls. Addition of 5ul of PBS had no dilutional effect upon coagulation times of pooled normal plasma (Figure 7.4).
Figure 7.3: Clotting times in preterm and term cord blood samples

**Figure 7.3:** PT (n=16; A), and APTT (n=16; B) in platelet poor plasma prepared from pooled cord blood obtained from preterm and term neonates.
Figure 7.4: Effect of addition of PBS on PT and APTT in control and case plasma

A) B) C) D)

Figure 7.4: Addition of PBS did not affect PT (B) of APTT (D) in control or case plasma pools.
In order to investigate potential mechanisms underlying the observed APTT prolongation in preterm compared with term pooled plasma, the relative contribution of individual clotting factors was determined. Baseline plasma FII, FVII, FIX, FX and FXII activity was measured in aliquots of preterm and term pooled plasma (Table 7.4). Factors not determined to differ significantly between preterm and term samples (FV, FVIII, FIX) were not measured (Andrew et al., 1988), given the small available plasma volumes. In keeping with our published findings in individual cord plasma samples (Neary et al., 2014), activities of FII, FVII, FIX and FX in pooled preterm cord blood plasma were reduced compared with pooled term plasma (Table 7.4).

Immediately afterwards, aliquots of each pool were incubated with one each of a series of commercially prepared, highly concentrated purified coagulation factors (FII, FVII, FIX, FX, FXII). Following addition of each individual coagulation factor (to a level sufficient to restore the concentration of that coagulation factor to that of healthy cord plasma), PT, APTT and the level of that coagulation factor was re-measured (Table 7.4). In order to avoid dilutional effects upon APTT and PT, these highly purified coagulation factor concentrates were diluted to prepare stock solutions such that only and exactly 1μl of each stock solution was added to each plasma aliquot to restore the level of that coagulation factor to that of healthy cord blood.
Table 7.4: PT, APTT and activities of FII, FVII, FIX, FX and FXII in pooled aliquots of preterm (case) and term plasma pre- and post-incubation with individual purified coagulation factor concentrate (1µl)

<table>
<thead>
<tr>
<th></th>
<th>Pooled term plasma (n=16)</th>
<th>Pooled case plasma (n=16)</th>
<th>Case + FII (1µl)</th>
<th>Case + FVII (1µl)</th>
<th>Case + FIX (1µl)</th>
<th>Case + FX (1µl)</th>
<th>Case + FXII (1µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>13.4</td>
<td>13.8</td>
<td>13.6</td>
<td>12.7</td>
<td>13.9</td>
<td>13.7</td>
<td>13.6</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>49.7</td>
<td>60.7</td>
<td>59</td>
<td>59.1</td>
<td>58.1</td>
<td>61.9</td>
<td>61.8</td>
</tr>
<tr>
<td>FII (IU/ml)</td>
<td>0.48</td>
<td>0.336</td>
<td>0.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVII (IU/ml)</td>
<td>0.47</td>
<td>0.368</td>
<td>0.51</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIX (IU/ml)</td>
<td>0.32</td>
<td>0.23</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FX (IU/ml)</td>
<td>0.38</td>
<td>0.358</td>
<td></td>
<td></td>
<td>0.378</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FXII (IU/ml)</td>
<td>0.309</td>
<td>0.244</td>
<td></td>
<td></td>
<td></td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>
Interestingly, restoration of individual coagulation factor activity to term plasma activity had no effect upon pooled plasma APTT, suggesting that no individual coagulation factor deficiency is responsible for the observed APTT prolongation in plasma of very preterm infants compared to term infants.

In order to determine the contribution of combined factor deficiencies to the observed APTT prolongation in pooled preterm compared with term plasma, aliquots of preterm plasma (n=14) were incubated with sufficient quantities of FII, FVII, FIX, FX and FXII together (or a similar volume of PBS) to simultaneously restore the activity of each factor in preterm pooled plasma to that of term pooled plasma. PT, APTT and plasma levels of individual coagulation factors were re-measured following this incubation (Table 7.5) to confirm that each target factor level had been achieved.
Table 7.5: PT, APTT and activities of FII, FVII, FIX, FX and FXII in pooled aliquots of preterm and term plasma pre- and post-incubation with combined purified coagulation factor concentrate, PBS or FP

<table>
<thead>
<tr>
<th></th>
<th>Pooled term plasma</th>
<th>Pooled preterm plasma</th>
<th>Preterm plasma + Combined FII, FVII, FIX, FX, FXII (1 µl)</th>
<th>Preterm plasma +PBS (48 µl)</th>
<th>Case +FP (48 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>14</td>
<td>14.5</td>
<td>11.9</td>
<td>14.3</td>
<td>12</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>49</td>
<td>57</td>
<td>42.2</td>
<td>56</td>
<td>40</td>
</tr>
<tr>
<td>FII (IU/ml)</td>
<td>0.448</td>
<td>0.33</td>
<td>0.46</td>
<td>0.342</td>
<td>40.9</td>
</tr>
<tr>
<td>FVII (IU/ml)</td>
<td>0.403</td>
<td>0.303</td>
<td>0.4</td>
<td>0.305</td>
<td>0.5</td>
</tr>
<tr>
<td>FIX (IU/ml)</td>
<td>0.274</td>
<td>0.223</td>
<td>0.27</td>
<td>0.22</td>
<td>0.38</td>
</tr>
<tr>
<td>FX (IU/ml)</td>
<td>0.433</td>
<td>0.462</td>
<td>0.46</td>
<td>0.459</td>
<td>0.49</td>
</tr>
<tr>
<td>FXII (IU/ml)</td>
<td>0.429</td>
<td>0.371</td>
<td>0.433</td>
<td>0.38</td>
<td>0.65</td>
</tr>
</tbody>
</table>
Prior to addition of this combined factor concentrate, APTT was again observed to be prolonged in preterm compared with term pooled plasma aliquots (49 seconds and 57 seconds respectively; Figure 7.5 B). No difference in PT was observed in preterm compared with term plasma (14.5 seconds and 14 seconds respectively). In contrast to findings following restoration of individual coagulation factor levels in preterm plasma to those of term plasma, simultaneous restoration of FII, FVII, FIX, FX and FXII activity shortened APTT from 57 seconds to 42.2 seconds (Figure 7.5 B).

FP is occasionally administered to sick preterm infants who are bleeding, at a dose of 10 ml/kg. Neonatal circulating blood volume is approximately 80mls/kg. Therefore, 48µl FP incubated with 210µl plasma aliquots would approximate a clinically relevant dose of FP. Interestingly; incubation of preterm pooled plasma aliquot with 48µl shortened the APTT of preterm pooled plasma aliquot to a similar degree to that observed following incubation with combined coagulation factor concentrates (Figure 7.5 B). In contrast, incubation with an equivalent volume of PBS used as a control for the experiment did not shorten the APTT of pooled preterm plasma.
Figure 7.5: Changes in coagulation values in preterm and term pooled cord plasma upon incubation with coagulation factor concentrates.

![Graph A) APTT (n=16) in pooled preterm plasma prior to (black bar) and after (white bar) incubation with each individual coagulation factor in a quantity sufficient to restore activity to that of term plasma (A). APTT (n=14) in preterm pooled plasma prior to ("preterm") and after ("preterm + all factors") incubation with a combined preparation of purified FII, FVII, FIX, FX and FXII in a quantity sufficient to restore activity of each factor to that of term plasma or after incubation with either FP or a similar volume (48 µl) of PBS (B).](image-url)
7.5 Discussion

This study is the first to examine the levels of coagulation factors (FII, FV, FVII, FVIII, FIX, FX, FXI, FXII, and FXIII) and anticoagulation proteins (protein C and protein S) in the same population in preterm neonates at birth. α2M is a glycoprotein that cleaves multiple proteases including FXa, thrombin and APC. In this study, CAT assay was used to estimate the activity of α2M in extremely preterm plasma samples compared to term using Thrombinsoscope™ software. The finding that estimated α2M activity is lower in very preterm infants and increases with postnatal age is consistent with data from moderately preterm infants. α2m appears to be an important regulator of the APC activity present in plasma (Cvirn et al., 2002). APC is one of the primary physiological inhibitors of thrombin generation. In this study we showed that suppression of thrombin generation by APC was comparable in preterm and term population, indicating that APC is an important regulator of thrombin generation in both preterm and term infants. This is consistent with examination of APC activity in term infants as compared to adults (Cvirn et al., 1999). Finally primary haemostasis was shown to be quantitatively similar to term infants.

Additionally in this prospective observational study, plasma APTT was determined to be prolonged in pooled preterm plasma compared to pooled term plasma, which was unaffected by restoration of individual coagulation factor levels to those of pooled term plasma. In contrast, simultaneous restoration of FII, FVII, FIX, FX and FXII activity to those of term plasma shortened APTT from 57 seconds to 42.2 seconds, suggesting that the relative contribution of each individual coagulation factor to the overall APTT prolongation is relatively minor. Similarly, incubation with clinically relevant volumes of FP also shortened preterm plasma APTT to that of term plasma.
Mechanisms underlying “prolonged” coagulation times in preterm neonates are poorly understood. Attard et al reported that the activities of coagulation FII, FVII, FIX and FX in term neonate plasma were approximately one-half to one-third of adult values, despite the fact that all neonates received vitamin K prophylaxis at birth (Attard et al., 2013). A further study reported that low levels of VKD factors may contribute to APTT prolongation during the first months of life (Monagle and Massicotte, 2011). The APTT assay is very sensitive to reduced plasma activity of these factors. However, as outlined above, the APTT is also prolonged by low concentrations of FXII which is not associated with a bleeding tendency. The relative contribution of individual coagulation factor to prolonged plasma clotting times has until now been unknown. In particular, it has not been known whether any single coagulation factor contributes to a major extent to this observed APTT prolongation.

Although it is well established that the APTT is prolonged in preterm infants compared with term infants (Andrew et al., 1988, Monagle et al., 2006, Neary et al., 2014), clinical studies have questioned the relevance of this finding. First, APTT may be prolonged due to deficiencies of coagulation factors that predict bleeding (such as FIX) and those that do not (such as FXII) (Green, 2010). Second, the APTT is not sensitive to deficiencies of anticoagulant coagulation factors (such as AT and protein C), which are known to be reduced similarly to procoagulant factors in preterm relative to term infants (Neary et al., 2014). Thirdly, administration of FP to “correct” the prolonged APTT in preterm infants simply because it is prolonged rather than because a bleeding event has occurred is of no proven clinical benefit (Stanworth et al., 2011, Altuntas et al., 2012, Tran et al., 2012). Finally, coagulation times are not a good predictor of IVH in preterm infants, in whom the primary bleeding mechanism appears to be structural brain immaturity rather than “coagulopathy” (Kuperman et al., 2013, Neary et al., 2014).
We have previously reported similar thrombin generation despite “prolonged” APTT and PT in preterm infants relative to term infants (Neary et al., 2014). We observed reductions in both procoagulant and anticoagulant factors and pathways in these very preterm infants compared to term infants and hypothesized that in the preterm infant a system of “haemostatic equilibrium” exists, with attenuated procoagulant and anticoagulant pathways. Studies such as this highlight the limitations of basic laboratory coagulation testing, which are more sensitive to attenuated procoagulant pathways, when making decisions on blood product transfusion.

Finally reduction in contact pathway factors have been well-characterized in older preterm infants, citing that these factors are 50-70% of the values of adults (Andrew et al., 1988). It is unknown whether very preterm infants (<30 weeks GA) also have low levels of these factors, although there is some fetal in utero work that would support this hypothesis (Reverdiau-Moalic et al., 1996). In contrast, a recent study cited that term fetal and maternal levels of contact factors FXII, FXI and PK were similar and only the level of HMWK was lower in the fetus compared with maternal levels (Uszynski et al., 2015).

These apparently conflicting data may be as a result of differences in methodology and measurement of fetal forms of the coagulation factors. Despite this hypothesis, it is not clear whether morphological differences exist in the contact factors between fetus and adult. Some evidence attesting to this include the fact that immunological values of FXII and FXI were observed to be higher than their respective functional levels, leading authors to speculate that functional contact pathway impairment may contribute to the observed elevated infant APTT (Gordon et al., 1980, Andrew et al., 1981).
Similar to other components of the coagulation system, the contact activation system modulates inflammatory and other pathways including the kallikrein–kinin and renin-angiotensin systems (Schmaier, 2014, Stavrou and Schmaier, 2010, Schmaier, 2003). The interplay of these factors in multiple systems heightens the importance of improved understanding of their role in newborn infants.
7.6 Conclusion

In keeping with the observation that thrombin generation was similar in very preterm infants compared to term infants, we also observed reductions in both procoagulant and anticoagulant factors and pathways in very preterm infants compared to term infants. It appears that in the preterm infant a system of “haemostatic equilibrium” exists, with attenuated levels of both procoagulant and anticoagulant pathways. Increasing knowledge of individual components of the haemostatic system of very preterm infants is important in understanding overall haemostatic balance in this population and highlights the limitations of basic laboratory coagulation testing, which are more sensitive to attenuated procoagulant pathways, when making decisions on blood product transfusion.

Improved understanding of the contribution of different pro- and anticoagulant factors in prolonging standard laboratory clotting times (PT and APTT), is useful in interpreting coagulation values in premature infants and need if any for intervention. We have demonstrated that APTT prolongation in very preterm relative to preterm plasma is not predominantly determined by relative deficiency of any single coagulation factor. Rather, combined deficiencies of coagulation factors that do predict bleeding and those that do not predict bleeding (e.g. FXII) contribute to this observed APTT prolongation. Overall, the neonatal haemostatic system is balanced by similarly attenuated anticoagulant pathways (Neary et al., 2014) and no evidence exists to support either routine measurement of coagulation times or administration of FP to correct these “numbers” (Stanworth et al., 2011). Our findings provide additional data towards greater understanding of the unique preterm
neonatal haemostatic system which differs substantially from that of the term neonate and the adult.
CHAPTER 8: COAGULATION VALUES AND IVH

8.1 Background

This thesis has added to our understanding of neonatal haemostatic mechanisms and has demonstrated laboratory evidence of comparable thrombin generation in preterm and term infants despite prolonged coagulation times in preterm infants. One of the clinical questions posed by this thesis was to determine the influence (if any) of these “prolonged” bleeding times on the risk of severe bleeding complications, including IVH.
8.2 Aims

To prospectively assess bleeding complications including IVH and correlate these with thrombin generation and plasma coagulation parameters.
8.3 Methods

Study 1– Prospective Collection

Preterm infants delivered at less than 30 weeks GA and admitted to the NICU at the Rotunda Hospital were recruited as part of a prospective observational trial, as previously described. Samples were taken from the umbilical cord following delivery, and on day 1, 3 and 14 of life in infants who were under 30 weeks corrected GA who did not meet exclusion criteria (e.g. receipt of plasma products, q.v.). Samples were processed as described in Chapter 3 and standard laboratory measurements of PT, APTT and fibrinogen performed. Samples were then processed to assay thrombin generation in 150µl aliquots. Where plasma was remaining in cord samples, pro- and anticoagulant factors were measured. Demographic and clinical outcome data was collected as previously described. CRUSS imaging was performed on day 1, 3 and 7 of life for diagnosis of IVH.

Study 2- Overall Cohort

Preterm infants delivered at less than 30 weeks GA and who had coagulation testing performed on admission to NICU were reviewed as part of observational study including retrospective data from period 2008-2013 and prospective cohort from 2013-2015. Coagulation screens were obtained via non-heparinised peripheral venous cannulae or umbilical arterial or venous catheters and were taken as part of admission bloods performed in this unit during the time period June 2008- April 2015.
Coagulation test results were obtained from the laboratory system. PT, APTT, and fibrinogen were measured.

**Statistical Analysis**

Two group analyses were conducted using an independent student t-test for normally distributed data or the Mann-Whitney U test for skewed data. Fisher’s exact test was used for categorical data. Linear regression was used to examine the association between coagulation parameters and IVH. Multivariable logistic regression was used to assess the independent associations between perinatal characteristics and the coagulation parameters on severe IVH evolution. Relative risk was determined for subgroups with FP and IVH. A p value of < 0.05 was considered significant. Graphpad Prism 5 and SPSS (IBM, version 22) was used to conduct the analysis.
8.4 Results

*Study 1– Prospective Collection*

One hundred and twenty seven infants with a median ± range GA and BW of 27.9 [26.4 – 28.7] weeks and 1020 [820 – 1220] grams with coagulation testing on day 1 of life were included during the study period. Seventy-four (58%) were male, 115 (91%) were in receipt of at least one dose of antenatal steroids and 94 (74%) delivered via caesarean section. Fourteen infants (11%) developed severe IVH (Grades 3 & 4).

Table 8.1 divides the cohort into those with and without a severe IVH. Infants with an IVH were of lower GA and BW. IVH infants had a higher rate of vaginal delivery, lower 5 minute Apgar score and lower antenatal steroid use, and a higher SNAPPE-II score, although only GA and SNAPPE-II score were significantly different to those without IVH (Table 8.1). Of the 14 infants with severe IVH, 6 infants received FP on day 1. In addition, those with a severe IVH had a longer PT and a higher proportion with an APTT > 100 seconds, these differences did not reach statistical significance. Interestingly, those with severe IVH had comparable ETP and peak thrombin generation compared to those who did not have a severe IVH (Table 8.1, Figure 8.1). In the entire cohort, comparing those with IVH (n=84) compared with those without (n=43), there was no statistical difference in PT (17.8s vs. 17.7 s respectively, p=0.74). APTT (78.2s vs. 82.2s respectively, p=0.5) or fibrinogen (1.4 g/l vs. 1.3 g/L, p=0.42).
Table 8.1: Characteristics of infants ± severe IVH and Coagulation Parameters

Data Presented as mean ± SD, median [inter-quartile range 25th -75th], or count (%).

<table>
<thead>
<tr>
<th></th>
<th>Severe IVH (n=14)</th>
<th>No Severe IVH (n=113)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation (weeks)</td>
<td>26.6 ± 1.8</td>
<td>27.9 [26.8 – 28.7]</td>
<td>0.04</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>926 ± 303</td>
<td>1050 [833 – 1225]</td>
<td>0.06</td>
</tr>
<tr>
<td>Male</td>
<td>9 (64%)</td>
<td>64 (57%)</td>
<td>0.8</td>
</tr>
<tr>
<td>Vaginal Delivery</td>
<td>5 (36%)</td>
<td>28 (25%)</td>
<td>0.5</td>
</tr>
<tr>
<td>Five minute Apgar Score</td>
<td>7 ± 2.6</td>
<td>9 [7 – 9]</td>
<td>0.09</td>
</tr>
<tr>
<td>Antenatal Steroids</td>
<td>11 (79%)</td>
<td>104 (92%)</td>
<td>0.13</td>
</tr>
<tr>
<td>SNAPPE-II Score</td>
<td>16 [9-41]</td>
<td>10 [0-19]</td>
<td>0.04</td>
</tr>
<tr>
<td>Coagulation Parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin Time (s)</td>
<td>19.8 ± 4.2</td>
<td>17.7 [15.7 – 20.5]</td>
<td>0.2</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>88.9 ± 24.4</td>
<td>79.1 [66.9 – 95.6]</td>
<td>0.5</td>
</tr>
<tr>
<td>APTT &gt; 100 (s)</td>
<td>5 (36%)</td>
<td>22 (19%)</td>
<td>0.17</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.2 [1.0 – 1.4]</td>
<td>1.4 [0.9 – 2.3]</td>
<td>0.8</td>
</tr>
<tr>
<td>FP Administration</td>
<td>5 (36%)</td>
<td>23 (20%)</td>
<td>0.19</td>
</tr>
<tr>
<td>ETP (n=10)</td>
<td>1178 ± 192</td>
<td>1103 ± 321 (n=90)</td>
<td>0.47</td>
</tr>
<tr>
<td>Peak (n=10)</td>
<td>107 ± 18.8</td>
<td>101 ± 35.3 (n=90)</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Figure 8.1: Infants with severe IVH compared to those with no or mild IVH

Figure 8.1: PT (A), APTT (B) with none or mild IVH (n=113) compared with severe IVH (n=14) during the first week of life, including those infants that did receive plasma. ETP (C), Peak thrombin (D), lagtime to thrombin generated (E) or time to peak thrombin (F) in infants with none or mild IVH (n=90) compared with severe IVH (n=10) during the first week of life, including those infants that did receive plasma.
Baseline coagulation times do not predict IVH in very preterm infants.

In this observational study, individual care providers made decisions on administration of plasma. To ascertain whether coagulation times were different in infants with and without IVH, the cohort of babies who did not receive plasma was first investigated (n=96). Linear regression analysis revealed no correlation between baseline PT or APPT and IVH either on day 1 (Figure 8.2 A and Figure 8.3A) or during the first week of life (Figure 8.2 B, Figure 8.3 B). When infants who received plasma and those that did not were analysed together (n=127), there was also no correlation between PT or APPT and IVH either on day 1 (Figure 8.2 C, Figure 8.3 C) or during the first week of life (Figure 8.2 D, Figure 8.3 D).
**Figure 8.2**: PT does not correlate with IVH development on day 1 (p=0.8; A) or during the first week of life (p=0.3; B) in very preterm infants who did not receive plasma. In the groups as a whole, including those that did receive plasma, PT does not correlate with IVH development on day 1 (p=0.5; C) or during the first week of life (p=0.2; D). *This work was presented in poster presentation format at American Society of Haematology, San Francisco, USA, December 2014 and published in Journal of Thrombosis and Haemostasis.
Figure 8.3: APTT and IVH

Figure 8.3: APTT does not correlate with IVH development on day 1 (p=0.9; A) or during the first week of life (p=0.6; B) in very preterm infants who did not receive plasma. In the group as a whole, including those infants that did receive plasma, APTT does not correlate with IVH development on day 1 (p=0.9; C) or during the first week of life (p=0.2; D).

*This work was presented in poster presentation format at American Society of Haematology, San Francisco, USA, December 2014 and published in Journal of Thrombosis and Haemostasis.
Thrombin generation does not predict IVH in very preterm infants.

In infants who had an IVH compared to those who did not there was no statistical difference in PT or APTT or in the thrombin generation assay parameters (Figure 8.4 A-F). This observation was consistent in the subgroup analyses of infants who had IVH on day 1 of life (Figure 8.5 A-F).
Figure 8.4: PT (A), APTT (B), ETP (C), Peak thrombin (D), lagtime (E) to thrombin generated or time to peak thrombin (F) does not correlate with IVH development during the first week of life in very preterm infants, including those that did receive plasma.
Figure 8.5: Coagulation parameters in infants with or without IVH on day 1 of life.

Figure 8.5: PT (A), APTT (B), ETP (C), Peak thrombin (D), lagtime to thrombin generated (E) or time to peak thrombin (F) in infants with no IVH compared with IVH (n=10) on day 1 of life before any plasma products were administered.
**Factor levels in infants do not predict IVH**

Factor levels in preterm infants measured in cord blood were comparable in infants who did or did not have IVH (Table 8.2).

**Table 8.2: Factor levels in preterm infants in cord blood according to IVH status**

<table>
<thead>
<tr>
<th></th>
<th>IVH (n=7)</th>
<th>No IVH (n=6)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FII</td>
<td>0.35 ± 0.1</td>
<td>0.28 ± 0.1</td>
<td>0.19</td>
</tr>
<tr>
<td>FVII</td>
<td>0.4 ± 0.11</td>
<td>0.3 ± 0.16</td>
<td>0.24</td>
</tr>
<tr>
<td>FIX</td>
<td>0.2 ± 0.14</td>
<td>0.15 ± 0.04</td>
<td>0.47</td>
</tr>
<tr>
<td>FX</td>
<td>0.34 ± 0.1</td>
<td>0.29 ± 0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>Protein C</td>
<td>0.15 ± 0.07</td>
<td>0.11 ± 0.05</td>
<td>0.32</td>
</tr>
<tr>
<td>Free protein S</td>
<td>0.4 ± 0.09</td>
<td>0.36 ± 0.08</td>
<td>0.41</td>
</tr>
<tr>
<td>AT</td>
<td>0.24 ± 0.07</td>
<td>0.2 ± 0.1</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Timing of cranial ultrasound scan

125 infants had a cranial ultrasound scan on day 1 of life with a median (range) time of 3.5 (0.15 – 23) hours. IVH was noted to occur in 14 (11%) infants on day 1 of life. In infants with a cranial ultrasound performed less than 6 hours of age (n=85), 9.4% had evidence of IVH. Of infants recruited, 116 had both coagulation profile and CRUSS on day 1 of life. In infants who had coagulation profile on day 1 of life and evidence of IVH (n=3), values for PT, APTT, and fibrinogen were non-significantly different than those who did not have IVH on CRUSS on day 1 (Table 8.3).

Table 8.3: IVH day 1 vs. those without IVH

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>IVH (n=3)</th>
<th>No IVH (n=113)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>16.5 (15.4-16.8)</td>
<td>17.9 (11.7-36.8)</td>
<td>0.2</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>74.7 (63.2-82.9)</td>
<td>79.1 (35.8-148.2)</td>
<td>0.5</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.4 (1.2-1.5)</td>
<td>1.3 (0.5-4.8)</td>
<td>0.8</td>
</tr>
</tbody>
</table>
**FP administration does not reduce risk of IVH in very premature infants**

Among the infants in this study, 31 received solvent detergent FP on day 1 of life. The primary indication for FP use was an elevated PT or APTT value. At the time of the study, the NICU policy agreed by consultant neonatologists was to administer FP to infants with APTT >100s. In this group who received FP, IVH occurred in 15 cases. Seven cases were characterized as severe IVH. Of those who did not receive FP, 28 patients experienced IVH. Of these, 7 were characterized as severe IVH. FP treatment was not associated with a reduced risk of severe IVH (RR = 3.1, 95% CI= 1.18-8.14) or IVH in general (RR = 1.66, 95% CI=1.03-2.68). The mean PT and APPT in those who got FP were 22.5 and 107.4 s respectively; higher than those who did not receive FP, as FP is arbitrarily given to infants with APTT >100s in our unit (Table 8.4).
Table 8.4: Values of PT, APTT in infants in those who received FP or did not receive FP

Data are reported as mean (SD) and median (range). *p<0.01 group with FP vs. group without FP

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total Screened (n=127)</th>
<th>FP (n=31)</th>
<th>No FP (n=96)</th>
<th>Relative Risk (Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PT (s)</strong></td>
<td>17.9 (11.7-36.8)</td>
<td>22.5 (16.6-28)</td>
<td>17.2 (13.4-21.6)*</td>
<td></td>
</tr>
<tr>
<td><strong>APTT (s)</strong></td>
<td>79.1 (35.8-148.2)</td>
<td>107.4 (69.3-142.7)</td>
<td>74.7 (53.8-95.3)*</td>
<td></td>
</tr>
<tr>
<td><strong>ETP</strong></td>
<td>1111 (310)</td>
<td>970 (291)</td>
<td>1155 (304)*</td>
<td></td>
</tr>
<tr>
<td><strong>SNAPPE-II score</strong></td>
<td>10 (0-60)</td>
<td>18 (0-60)</td>
<td>10 (0-44)*</td>
<td></td>
</tr>
<tr>
<td><strong>IVH</strong></td>
<td>43</td>
<td>15</td>
<td>28</td>
<td>1.7 (1.0-2.7)</td>
</tr>
<tr>
<td><strong>Grade 3-4 IVH</strong></td>
<td>14</td>
<td>7</td>
<td>7</td>
<td>3.1 (1.2-8.1)</td>
</tr>
<tr>
<td><strong>Mortality</strong></td>
<td>12</td>
<td>8</td>
<td>4</td>
<td>6.2 (2.0-19)</td>
</tr>
</tbody>
</table>
Study 2- Overall Cohort

Three hundred and twenty four infants with a mean ± SD GA and BW of 26.6 ± 1.7 weeks and 969 ± 277 grams were included during the study period. One hundred and eighty eight (58%) were male, 290 (90%) were in receipt of at least one dose of antenatal steroids and 226 (70%) delivered via caesarean section and. Fifty one infants (16%) developed severe IVH (Grades 3 & 4).

Table 8.5 divides the cohort into those with and without a severe IVH. Infants with an IVH had lower GA and BW, a higher rate of vaginal delivery, lower 5 minute Apgar score and lower antenatal steroid use (Table 8.5). In addition, those with a severe IVH had a longer PT and a higher proportion with an APTT > 100 seconds (Table 8.5). On multiple logistic regression, only vaginal of delivery, 5 minute Apgar score and longer PT were independently associated with severe IVH (Table 8.6, Figure 8.6).
Table 8.5: Characteristics of infants± severe IVH and Coagulation Parameters

Data Presented as mean ± SD, median [inter-quartile range], or count (%). APTT: activated partial thromboplastin time; FP: frozen plasma.

<table>
<thead>
<tr>
<th></th>
<th>Severe IVH (n=51)</th>
<th>No Severe IVH (n=273)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation (weeks)</td>
<td>26.0 ± 1.9</td>
<td>26.7 ± 1.7</td>
<td>0.008</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>890 [690 – 1100]</td>
<td>965 [775 – 1190]</td>
<td>0.06</td>
</tr>
<tr>
<td>Male</td>
<td>29 (57%)</td>
<td>159 (59%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Vaginal Delivery</td>
<td>25 (49%)</td>
<td>72 (27%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Apgar Score (5 mins)</td>
<td>7 [5 – 8]</td>
<td>9 [7 – 9]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antenatal Steroids</td>
<td>42 (82%)</td>
<td>248 (91%)</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Coagulation Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin Time (s)</td>
<td>20.5 [17.3 – 24.2]</td>
<td>17.5 [15.6 – 19.6]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>79 [69 – 110]</td>
<td>77 [65 – 92]</td>
<td>0.07</td>
</tr>
<tr>
<td>APTT &gt; 100 (s)</td>
<td>15 (29%)</td>
<td>45 (17%)</td>
<td>0.047</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.3 [1.0 – 2.8]</td>
<td>1.3 [0.9 – 2.3]</td>
<td>0.5</td>
</tr>
<tr>
<td>FP Administration</td>
<td>23 (47%)</td>
<td>91 (33%)</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Table 8.6: Independent Association between Parameters and Severe IVH

Significant associations on univariate analysis in table 20 were used to construct the multivariate logistic regression model. Birth weight was not entered into the model due to collinearity with GA. All the variables in the tables were entered together in the logistic multiple regression model to obtain the adjusted OR. OR: Odds ratio; CI: confidence interval. * highlights a significant association.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation (Per week increase)</td>
<td>0.8 (0.6 – 0.9)*</td>
<td>0.9 (0.7 – 1.2)</td>
</tr>
<tr>
<td>Vaginal Delivery</td>
<td>2.7 (1.4 – 4.9)*</td>
<td>2.4 (1.2 – 4.9)*</td>
</tr>
<tr>
<td>5 minute Apgar Score (Per unit increase)</td>
<td>0.7 (0.6 – 0.8)*</td>
<td>0.8 (0.6 – 0.9)*</td>
</tr>
<tr>
<td>Antenatal Steroids</td>
<td>0.5 (0.2 – 1.1)</td>
<td>0.8 (0.6 – 2.4)</td>
</tr>
<tr>
<td>PT (Per second increase)</td>
<td>1.1 (1.1 – 1.2)*</td>
<td>1.1 (1.1 – 1.2)*</td>
</tr>
<tr>
<td>APTT &gt; 100 (s)</td>
<td>2.1 (1.1 – 4.2)*</td>
<td>0.8 (0.3 – 2.2)</td>
</tr>
<tr>
<td>FP Administration</td>
<td>1.8 (0.9 – 3.3)</td>
<td>1.3 (0.6 – 2.8)</td>
</tr>
</tbody>
</table>
Figure 8.6: Difference in prothrombin time in infants with and without severe IVH. Error bars represent 95% confidence interval.
8.5 Discussion

The aetiological role of coagulation disturbance in development of IVH has been debated as outlined in Chapter 1. This is in part due to lack of reference ranges for this population which I have provided in Chapter 4. Due to concerns about haemostatic disturbance leading to IVH, FP is sometimes administered.

The prospective data provided in this thesis show no association between coagulation parameters and IVH. Our study also showed that there was no statistical difference in cord blood coagulation factor levels in babies who experienced IVH compared to those that did not.

We have demonstrated that in very preterm infants whom the attending neonatologist administered FP to correct a perceived coagulopathy, FP does not result in a reduction of IVH. Studies examining the effectiveness of FP in preventing IVH are further examined in detail in the Cochrane review as described in Chapter 11.

Interestingly, using a global assay of coagulation, thrombin generation was characterized for the first time in infants who developed IVH compared to those that did not, and no significant differences were observed, consistent with a study by Radicioni et al who evaluated neonatal haemostasis by thromboelastography and found no difference in IVH rates according to assay parameters (Radicioni et al., 2015). In fact, the authors suggested that thromboelastography parameters in this study predicted an overall hypercoagulable state in infants with IVH compared to those without IVH. Chen et al reported a similar observation (Chen and Lorch, 1996) however in our hands thrombin generation parameters did not suggest an overall hypercoagulable state in infants with IVH. Strengths of this study include the fact that CRUSS were performed.
on admission to NICU and serially on day 3 and day 7 by one of two consultant radiologists. This allowed for accurate timing of IVH.

In the overall cohort including a large number of infants less than 30 weeks GA, as expected, I observed that GA and BW are significantly associated with the presence of IVH. Although this larger cohort revealed that there is a relationship between PT and IVH irrespective of GA and FP use a causal association has not been proven. Moreover, there is no evidence that correction of prolonged clotting times with FP reduces IVH risk. As IVH is an infrequent event, large cohorts of infants are required to see a relationship. There was a relationship between IVH and APTT for values greater than 100 seconds; however this was negated when PT was taken into account. Limitations of the study of the overall cohort include that this is a mixed method study. Between 2008 - 2013 CRUSS imaging was performed after day 1 of life. Therefore the diagnosis of IVH was subsequent to coagulation profile sampling. Individual attending neonatologists made clinical decisions on plasma transfusion, but this was accounted for in the multiple regression model.

Despite these limitations, this study contributes to knowledge of developmental changes in premature infants with respect to coagulation values. Routine testing of coagulation values has not been shown to be effective in reducing rates of IVH (Pal et al., 2015). Assays such as PT and APTT do not reflect the coagulation cascade in entirety. Using a global assay of thrombin generation I have shown that despite prolonged coagulation times in very preterm infants, that thrombin generated is comparable to term infants (Neary et al., 2015) and is not statistically different in infants with or without IVH. I suggest caution against performing coagulation tests on a routine basis and suggest that these tests be considered on an individual basis with senior input.
8.6 Conclusion

Clinicians who routinely measure standard coagulation tests in premature infants are faced with a dilemma if test results are “longer” than expected. This dilemma is whether administration with plasma products is likely to be of overall benefit when the inherent risks of plasma administration are considered. We have shown that in the prospective cohort, there is no association with standard coagulation indices or in a global assay of haemostasis and rates of IVH clinically although on examination of the entire cohort PT was found to be associated with IVH. In addition, administration of FP is not associated with a reduction in rates of IVH. Further ex-vitro laboratory studies are required to elucidate the mechanism contributing to prolonged coagulation screening tests which will be discussed in Chapter 9.
CHAPTER 9: EFFECT OF PERINATAL FACTORS ON THE COAGULATION SYSTEM IN VERY PRETERM INFANTS

9.1 Background

Our data confirm that haemostasis in the newborn period is balanced by concomitant reduction in both pro and anticoagulant pathways (Chapter 7). Prenatal and perinatal risk factors have the potential to exacerbate a haemostatic system which is already altered from the effects of prematurity itself. This has hindered the development of references ranges in very preterm infants as they are considered to be a heterogeneous population with multiple co-morbidities. Many antenatal and perinatal factors are known to influence haemostasis in pregnant women, but their effects on fetal haemostasis are less clear. These include maternal smoking, preeclampsia, sepsis, gestational diabetes, antenatal corticosteroids, method of delivery, multiplicity, BW, vitamin K, SNAPPE-II score, gender and timing of CRUSS. In this chapter we consider both maternal and neonatal factors that may influence coagulation profiles in very premature infants as below.
9.2 Aims

To investigate any associations between pre- and perinatal factors and coagulation status in very premature infants.
9.3 Methods

Demographic data was collected on all infants recruited into the study including maternal and obstetric factors, perinatal information and neonatal outcomes. Coagulation status at birth was correlated with these variables. Definitions are as described as previously in the methods chapter (Chapter 3). As critical illness may influence haemostatic function (Saxonhouse and Manco-Johnson, 2009), the degree of illness in recruited patients was determined using SNAPPE-II score, a composite of points based on nine physiological variables collected over the first 12 hours after admission, as illustrated in Table 9.1 (Richardson et al., 2001). A variety of thresholds have been used in different studies to define levels of illness. A SNAPPE-II score of 40 was used as the level for partitioning patient cohorts by Mydam et al (Mydam et al., 2015), while Dammann 2008 used a score of 45 (Dammann et al., 2010). For the purposes of this study, an arbitrary SNAPPE score on the 25th centile (as used by Bonnard et al) has been used to define the “unwell patient” (Bonnard et al., 2008). Data was analysed with non-parametric tests and median and range are described below. P<0.05 was accepted as significant.
Table 9.1: SNAPPE-II score

<table>
<thead>
<tr>
<th>Variable</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apgar score &lt; 7 at 5 min</td>
<td>18</td>
</tr>
<tr>
<td>Small for gestational age (&lt; 3rd percentile)</td>
<td>12</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td></td>
</tr>
<tr>
<td>• 750-999</td>
<td>10</td>
</tr>
<tr>
<td>• &lt; 750</td>
<td>17</td>
</tr>
<tr>
<td>Multiple seizures</td>
<td>19</td>
</tr>
<tr>
<td>Urine output (ml/kg/h)</td>
<td></td>
</tr>
<tr>
<td>• 0.1-0.9</td>
<td>5</td>
</tr>
<tr>
<td>• &lt; 0.1</td>
<td>18</td>
</tr>
<tr>
<td>Lowest mean blood pressure (mmhg)</td>
<td></td>
</tr>
<tr>
<td>• 20-29</td>
<td>9</td>
</tr>
<tr>
<td>• &lt; 20</td>
<td>19</td>
</tr>
<tr>
<td>Lowest temperature</td>
<td></td>
</tr>
<tr>
<td>• 35-35.6 °C</td>
<td>8</td>
</tr>
<tr>
<td>• &lt; 35°C</td>
<td>15</td>
</tr>
<tr>
<td>Lowest PO2/FiO2 ratio</td>
<td></td>
</tr>
<tr>
<td>• 1.0–2.49</td>
<td>5</td>
</tr>
<tr>
<td>• 0.33–0.99</td>
<td>16</td>
</tr>
<tr>
<td>• &lt; 0.33</td>
<td>28</td>
</tr>
<tr>
<td>Lowest arterial blood gas Ph</td>
<td></td>
</tr>
<tr>
<td>• 7.10–7.19</td>
<td>7</td>
</tr>
<tr>
<td>• &lt; 7.1</td>
<td>16</td>
</tr>
</tbody>
</table>
9.4 Results

Between April 2013 and April 2015, 137 infants were admitted to the NICU, of which 127 had coagulation profile on day 1 of life. The median (range) GA was 27.9 (23.7-29.9) weeks and median (range) BW was 1020 (510-1730) g. Each factor above was examined to view the impact on coagulation parameters on day 1 of life.
Table 9.2: Comparison of medium coagulation parameters in infants with maternal smoking compared to those without history of maternal smoking

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Maternal Smoking (n=20)</th>
<th>No Smoking (n=107)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>17.2 (12.1-30.2)</td>
<td>18 (11.7-36.8)</td>
<td>0.55</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>70.9 (42.4-134.8)</td>
<td>81.8 (35.8-148.2)</td>
<td>0.14</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.3 (0.56-3.8)</td>
<td>1.3 (0.54-4.8)</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Table 9.3: Comparison of medium coagulation parameters in infants with a confirmed or suspicion of early sepsis compared to those without

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Sepsis (n=18)</th>
<th>No Sepsis (n=109)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>19.5 (14.3-32)</td>
<td>17.7 (11.7-36.8)</td>
<td>0.06</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>80.6 (50.8-140.1)</td>
<td>79.1 (35.8-148.2)</td>
<td>0.57</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.5 (0.58-4.76)</td>
<td>1.3 (0.54-4.4)</td>
<td>0.64</td>
</tr>
</tbody>
</table>
Table 9.4: Comparison of medium coagulation parameters in infants with maternal gestational diabetes versus those without maternal gestational diabetes

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Gestational Diabetes (n=5)</th>
<th>No Maternal Diabetes (n=122)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>16.6 (13.4-28.7)</td>
<td>18 (11.7-36.8)</td>
<td>0.75</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>66.9 (46.6-102.4)</td>
<td>80.3 (35.8-148.2)</td>
<td>0.4</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>0.87 (0.66-4.08)</td>
<td>1.3 (0.54-4.76)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 9.5: Comparison of medium coagulation parameters in infants who received antenatal steroids versus those who did not receive steroids

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Antenatal Steroids (n=116)</th>
<th>No Steroids (n=11)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>17.7 (11.7-36.8)</td>
<td>18.5 (15.2-29.3)</td>
<td>0.24</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>78.2 (35.8-148.2)</td>
<td>89.1 (48.5-139.8)</td>
<td>0.22</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.3 (0.54-4.8)</td>
<td>1.3 (0.6-2.4)</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Table 9.6: Comparison of medium coagulation parameters in infants with vaginal delivery versus those with caesarean section

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Vaginal Delivery (n=33)</th>
<th>Caesarean Section (n=94)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>17.4 (11.7-32)</td>
<td>18.3 (12.1-36.8)</td>
<td>0.78</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>78.2 (62.7-133.5)</td>
<td>81.6 (35.8-148.2)</td>
<td>0.77</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.5 (0.6-4.4)</td>
<td>1.3 (0.5-4.8)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 9.7: Comparison of medium coagulation parameters in infants with vaginal delivery vs. those with caesarean section in labour and not in labour

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Vaginal Delivery (n=33)</th>
<th>Caesarean Section In Labour (n=45)</th>
<th>Caesarean Section Not in Labour (n=49)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>17.4 (11.7-32)</td>
<td>16.7 (12.1-36.8)</td>
<td>20.1 (12.9-30.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>78.2 (62.7-133.5)</td>
<td>75.1 (42.4-148.2)</td>
<td>89.1 (35.8-147.6)</td>
<td>0.12</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.5 (0.6-4.4)</td>
<td>1.4 (0.8-4.8)</td>
<td>1.0 (0.5-3.7)</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table 9.8: Comparison of medium coagulation parameters in infants with multiple births versus singleton births

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Multiple Pregnancies (n=41)</th>
<th>Singletons (n=86)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>17.7 (12.1-32)</td>
<td>18 (11.7-36.8)</td>
<td>0.8</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>83.3 (42.4-148.2)</td>
<td>76.7 (35.8-143.5)</td>
<td>0.3</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.1 (0.72-3.85)</td>
<td>1.4 (0.54-4.8)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 9.9: Comparison of medium coagulation parameters in SGA infants compared to AGA infants

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>SGA (n=12)</th>
<th>AGA (n=115)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>21.9 (15.8-30.2)</td>
<td>17.5 (11.7-36.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>95.3 (62.7-147.6)</td>
<td>78.2 (35.8-148.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>0.83 (0.54-3.9)</td>
<td>1.4 (0.57-4.8)</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 9.10: Logistic regression model of coagulation parameters according to birthweight

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>-2</td>
<td>0.001</td>
</tr>
<tr>
<td>BW</td>
<td>.03</td>
<td>0.001</td>
</tr>
<tr>
<td>PT</td>
<td>-0.18</td>
<td>0.249</td>
</tr>
<tr>
<td>APTT</td>
<td>-.024</td>
<td>0.231</td>
</tr>
<tr>
<td>FIB</td>
<td>0.574</td>
<td>0.278</td>
</tr>
</tbody>
</table>

Table 9.11: Comparison of medium coagulation parameters in infants with a maternal history of preeclampsia/HELLP syndrome or hypertension compared to those who did not have preeclampsia/HELLP**

**HELLP syndrome: Haemolysis, Elevated Liver enzymes, Low Platelet count

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Preeclampsia/HELLP (n=10)</th>
<th>No Preeclampsia/HELLP (n=117)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>19 (17.2-28.7)</td>
<td>17.6 (11.7-36.8)</td>
<td>0.07</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>86.6 (62.7-116.2)</td>
<td>78.2 (35.8-148.2)</td>
<td>0.3</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.5 (0.7-3.7)</td>
<td>1.3 (0.57-4.8)</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Table 9.12: Comparison of medium coagulation parameters according to gender

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Male (n=73)</th>
<th>Female (n=54)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>18.1 (11.7-36.8)</td>
<td>17.8 (12.1-32)</td>
<td>0.3</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>79.1 (35.8-147.6)</td>
<td>80.3 (42.4-148.2)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>147.6</td>
<td>148.2</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.2 (0.5-4.8)</td>
<td>1.4 (0.6-4.4)</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Figure 9.1: Correlation of SNAPPE-II scores to coagulation parameters

Figure 9.1: SNAPPE-II scores were correlated with APTT values on day 1 of life ($r^2 = 0.15$) and with ETP ($r^2 = 0.02$) from plasma samples.
Timing of vitamin K administration

Of the 127 infants with coagulation profiles performed on day 1 of life, all had vitamin K administered as routine practice in our unit. Coagulation testing was performed on admission to NICU with a median (range) of 1.5 (0.5-24) hours. Timing of vitamin K was documented with respect to coagulation testing. In infants whom had coagulation test prior to vitamin K administration compared to infants who had coagulation testing after vitamin K administration, values for coagulation testing were comparable (Table 9.13).

Table 9.13: Comparison of medium coagulation parameters according to timing of vitamin K administration

<table>
<thead>
<tr>
<th>Median (range)</th>
<th>Pre vitamin K (n=21)</th>
<th>Post vitamin K (n=106)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT (s)</td>
<td>18.3 (12.4-25.4)</td>
<td>17.8 (11.7-36.8)</td>
<td>0.6</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>87.3 (53.8-147.6)</td>
<td>76.6 (35.8-148.2)</td>
<td>0.09</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.1 (0.5-3)</td>
<td>1.36 (0.6-4.7)</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Figure 9.2: PT (A), APTT (B), fibrinogen (C), and ETP (D) in infants who clinically had signs of bleeding, bruising or extension of IVH compared to no evidence of bleeding.
Table 9.14: Characteristics of infants with APTT >100 s vs. those with APTT <100 s

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>APTT&gt;100 (n=30)</th>
<th>APTT&lt;100 (n=97)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gestation (weeks)</strong></td>
<td>27.1 ± 1.5</td>
<td>28.1 [26.5 – 28.7]</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Birthweight (g)</strong></td>
<td>929 ± 246</td>
<td>1063 ± 261</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Maternal Age</strong></td>
<td>34.2 ± 4.8</td>
<td>32.5 ± 6.2</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>17 (57%)</td>
<td>56 (58%)</td>
<td></td>
</tr>
<tr>
<td><strong>Vaginal Delivery</strong></td>
<td>7 (23%)</td>
<td>26 (27%)</td>
<td></td>
</tr>
<tr>
<td><strong>Five minute Apgar Score</strong></td>
<td>8 [6 – 9]</td>
<td>9 [8 – 9]</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Cord Ph</strong></td>
<td>7.33 ± 0.08</td>
<td>7.34 ± 0.08</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Antenatal Steroids</strong></td>
<td>26 (87%)</td>
<td>89 (92%)</td>
<td></td>
</tr>
<tr>
<td><strong>SNAPPE-II Score</strong></td>
<td>23.7 ± 16.5</td>
<td>9 [0-17.5]</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Coagulation Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Timing Blood Test (hrs)</strong></td>
<td>1.5 [1 – 2.8]</td>
<td>1.5 [1 – 2.8]</td>
<td>0.68</td>
</tr>
<tr>
<td><strong>Prothrombin Time (s)</strong></td>
<td>22.8 ± 4.8</td>
<td>17.4 ± 3.2</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>APTT (s)</strong></td>
<td>111 [102 – 130.5]</td>
<td>73 ±13.5</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Fibrinogen (g/L)</strong></td>
<td>0.98 [0.8-1.4]</td>
<td>1.4 [1.1-2.5]</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>ETP</strong></td>
<td>955 ± 290</td>
<td>1169 ± 299</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Peak</strong></td>
<td>83 ± 34</td>
<td>109 ± 31</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data Presented as mean ± SD, median [inter-quartile range 25th -75th], or count (%).
9.5 Discussion

Physiological variations must be considered in developing reference intervals as these may impact on the values obtained (Sikaris, 2014). Where there are considerable variations due to physiology that need to be accounted for, partitioning can be performed, where reference intervals are provided for particular subgroups. The Stockholm hierarchy provides consensus to determination of analytical quality, with the ideal state being a definition of analytical quality based on clinical outcomes and interpretation of results. The impact of partitioning on the quality of reference intervals relies on clinical opinion (Sikaris, 2014). Common examples of partitioning include effect of gender on reference intervals. Interestingly in this data, there appears to be no difference between male and females in relation to coagulation values. Other examples of subgroup analysis in our cohort are described below.

Maternal Smoking
It is well established that tobacco smoke can disturb haemostasis resulting in a procoagulant state (Mitsiakos et al., 2009a). As nicotine can cross the placenta (Sekhon et al., 2004), the infants haemostatic system may also be affected by maternal smoking (Mitsiakos et al., 2009a). Smoking during pregnancy can result in increased perinatal morbidity and mortality (Burguet et al., 2004) including increasing the risk of intracranial haemorrhage in preterm infants (Spinillo et al., 1995).

Preeclampsia
Preeclampsia is a clinical syndrome characterised by hypertension and proteinuria. The precise aetiology of preeclampsia is unclear, but
involves failure to establish an adequate uteroplacental blood flow and is associated with fibrin deposition in the kidneys (Lorquet et al., 2010). The condition usually resolves spontaneously following delivery. Disturbance of the maternal haemostatic system is a characteristic feature of preeclampsia. As components of the haemostatic system do not cross the placenta the effects on haemostasis in the fetal and newborn circulation remains unclear. Some infants do have an associated thrombocytopenia, although this may reflect the secondary growth restriction that occurs (Christensen et al., 2015a). It has been reported that neonates are protected to some extent from the adverse effects of preeclampsia on the haemostatic system, as only modest differences in haemostatic parameters have been identified in neonates born to mothers with preeclampsia compared with delivered after an uncomplicated pregnancy (Tanjung et al., 2005, Poralla et al., 2012b, Higgins et al., 2000).

**Sepsis**
Amnionitis or chorioamnionitis may induce premature birth (Pugni et al., 2015). Infection of the mother can transfer to the foetus before birth and cause sepsis in the preterm baby after birth (Pugni et al., 2015). Factors II, VIII and X are reduced in infants with maternal chorioamnionitis and fibrinogen levels are elevated. Fibrinogen belongs to the acute phase proteins, and might thus be subject to different regulatory control to other factors in this situation (Poralla et al., 2012b).

**Diabetes Mellitus**
Gestational diabetes can predispose to neonatal thrombosis formation and is often co-existent with maternal preeclampsia (Edstrom and Christensen, 2000, Sibai et al., 2000). The effect of gestational diabetes on the preterm coagulation system has not been described.
**Antenatal Corticosteroids**

Antenatal corticosteroids have been demonstrated to be effective in reducing complications associated with prematurity including RDS, IVH, and death (Falah and Haas, 2014). Preterm lambs with RDS who had received antenatal betamethasone, were observed to have a reduction in inflammatory response and coagulation activation (Jaarsma et al., 2004).

**Delivery**

Fetal values for coagulation pro- and anticoagulants are consistently lower than those observed for corresponding gestations in preterm and term infants (Reverdiau-Moalic et al., 1996). The birth process itself is thought to activate the coagulation system leading to up regulation of these proteins. The stress of delivery is observed by increasing cortisol levels in both maternal and fetal samples, with higher levels associated with vaginal compared to caesarean section deliveries. A large prospective cohort study of 154 term infants described the effects of labour on the fetal coagulation system (Kulkarni et al., 2013). In this study, significantly elevated levels of FVIII: C, VWF antigen, FIX, FXI, FXII and plasminogen levels were reported in cord blood from infants whose mothers laboured compared to those who underwent elective caesarean section (Kulkarni et al., 2013). Similar observations have been shown for FVIII in previous studies (Johnson et al., 1981). A corresponding increase was observed in protein C and anti-thrombin levels. Similarly elevated protein C, protein S, AT, fibrinogen and plasminogen activity have been reported in cord blood after vaginal delivery compared to caesarean section (Franzoi et al., 2002). In our cohort I was able to examine both infants in labour and not in labour to examine this effect in preterm neonates.
**Asphyxia**

Perinatal asphyxia is a well-described condition in neonatal medicine and clear definitions exist (Christensen et al., 2015b) using clinical condition, requirement for prolonged resuscitation, encephalopathy and cord pH values (pH < 6.99 ± base deficit >16). Megakaryocytes i.e. precursors for platelets are susceptible to asphyxia leading to thrombocytopenia in asphyxiated neonates (Christensen et al., 2015b). El Beshlawy et al. studied asphyxiated infants and found a marked decrease in the level of AT, Protein C, Protein S (El Beshlawy et al., 2004).

Standard unit practice is to obtain cord gases in situations where asphyxia has been anticipated, for example where infants are in poor condition at delivery or require unexpected levels of resuscitation. None of the infants in this study fulfilled definitions of perinatal asphyxia. Therefore cord gases were not performed by attending clinician on all patients as they were not clinically indicated in the majority of infants due to their clinical condition at birth.

**Multiplicity**

The greater the number of fetuses present, the higher the risk for the mother to have a premature labour (Fumagalli et al., 2015). Within multiple births, coagulation function may differ between infants. In a study by Salonvaara et al. the second twin had lower FII, FV, FVII, FX compared with the first twin, although platelet counts did not differ (Salonvaara et al., 2003).

**Birthweight**

SGA refers to infants whose BW is below a given centile (e.g. 10th) for a given GA. This may be due to placental insufficiency problems or maternal co-morbidities. A high proportion of very premature infants are
SGA making up 30-50% of the overall population. Premature infants who are SGA have increased rates of complications compared to premature infants who are not SGA, which may be accounted for by adverse in utero circumstances (Regev et al., 2003, Mamopoulos et al., 2015). The risk of haemorrhagic abnormalities increases in infants who are SGA (Rosenberg, 2008). Mistakos et al. compared coagulation parameters in AGA and SGA full term infants and found a significantly elevated PT in SGA infants although this was not associated with clinically significant events (Mitsiakos et al., 2009b). In contrast, Salonvaara et al. showed lower levels of FV and FVII without coagulation test prolongation in 39 SGA premature neonates (Salonvaara et al., 2003). The differences in the inclusion criteria could interpret the different results of the studies. Mistakos et al. further examined preterm neonates with mean GA of 34 weeks demonstrating APC resistance in preterm SGA infants compared to AGA preterm infants (Mitsiakos et al., 2010).

**Vitamin K**

Vitamin K is given routinely to all infants to prevent haemorrhagic disease of the newborn (HDN) (Shearer, 2009, Puckett and Offringa, 2000). Whether preterm neonates are at increased risk of HDN is not understood. The half-life of vitamin K1 in plasma is approximately 72 hours in neonates (summary of product characteristics; Konakion MM Paediatric Ampoules 2mg/0.2ml Solution for Injection). In a RCT of preterm infants less than 32 weeks GA, intramuscular and intravenous routes of administration were comparable (Clarke et al., 2006). The onset of action of vitamin K following intravenous injection is more rapid, but of shorter duration than intramuscular injection. In adults administered parenteral vitamin K, haemorrhage usually controlled within 3-6 hours, and normal prothrombin levels achieved in 12-14 hours.
In clinical practice, a further dose of vitamin K is frequently administered to neonates in response to high PT. However vitamin K levels were found to be normal in 22 preterm infants with a raised PT in the first month of life following vitamin K administration in a study by Clarke et al (Clarke et al., 2005). This suggests that a persistence of prolonged PT in the newborn may not be as a result of vitamin K deficiency and repeating vitamin K prophylaxis may not be justified (Clarke et al., 2005). Rather the coagulation profile might not be an accurate assessment of vitamin K status in infants.

**SNAPPE-II Score**

Multiorgan dysfunction impacts on aspects of the infant’s wellbeing. The SNAPPE-II score provides a validated illness severity score as a global endpoint of a variety of conditions and is known to predict morbidity and mortality. Severity of illness scores predicting mortality can be used as a means of benchmarking care in different units (Pollack et al., 2000, Gagliardi et al., 2004, Dorling et al., 2005). SNAPPE-II score is a composite of many variables with collection limited to the first 12 hours, to minimise early treatment bias (Richardson et al., 2001). It can be used in patients with all GA and BW. The score ranges from 0-162, with a higher score indicating a higher risk of mortality (Richardson et al., 2001). Severe illness may adversely affect haemostatic function. Very premature infants are often very unwell and it can be difficult to differentiate the effects of illness compared with the nature of prematurity. The use of this score in this regard can subdivide those infants whose coagulation parameters are altered by the effects of illness as opposed to simply the fact these infants are premature. The infants in this study did not have a normally distributed SNAPPE-II score, but was skewed in favour of the cohort with scores of 0-10 which indicates that the majority of these infants did not have severe illness and therefore are
representative of healthy premature neonates for determination of coagulation reference intervals.

Our results show that no factor apart from GA and BW had a significant impact on coagulation factors. SGA had significantly different coagulation parameters to that of AGA but this was due to confounding variable of GA. In addition these subgroups have small numbers and SGA could represent those that are intrauterine growth retardation as well as constitutionally small. This is consistent with findings of Salonvarra, who saw that beside GA, birth asphyxia was the only prenatal variable that affected coagulation status at birth in the linear regression analysis (Salonvaara et al., 2003).

Coagulation parameters in infants did not differ in infants thought to have active bleeding or documented bruising on day 1 of life. Bruising was documented as per clinical team usual method of recording bleeding on assessment of infant. A consideration of using the Neobat score by Venkatash et al was undertaken (Venkatesh et al., 2013). Briefly, the assessment tool is a checklist of dichotomous (yes/no) responses to 15 questions about pulmonary, gastrointestinal, cutaneous, and intraventricular haemorrhage (IVH), validated over a 2-4 week period. We felt that this tool was not suitable to be incorporated into this particular design given the lengthy questionnaire required. This is important as bruising or bleeding are often cited reasons for administering FP to very preterm neonates. Objective bleeding assessment tools in combination with standard laboratory coagulation tests have been advocated for use as criteria for decisions about clinical transfusion (Venkatesh et al., 2013). However I have shown that ETP does not vary in the clinical subgroups stratified as per bleeding status.

Limitations of this study include the fact that developing normative values for a cohort with varied underlying risk factors, pathologies and
morbidities can be challenging. In this cohort the idea of partitioning is further limited by means of diagnosing suspected underlying risk factors e.g. chorioamnionitis or suspected sepsis. In addition reporting biases (e.g. smoking) in this cohort limits its usefulness for partitioning. Partitioning may be more useful based on objective measures only e.g. mode of delivery or administration of antenatal steroids. In some cases, the small numbers of infants affected by the partition (e.g. PET/HELLP) may mask an effect. The data supports treating infants with APTT > 100 s with great caution given that the ETP in this population was also reduced compared with term infants. In the absence of clear evidence based upon randomized trials, we suggest continued individualized risk assessment by a senior multidisciplinary team when making decisions on plasma transfusion in preterm infants with prolonged coagulation times.
9.6 Conclusion

Not only gestational and postnatal ages but also the health status of the infant influences haemostatic balance (Pal et al., 2015, Saxonhouse and Manco-Johnson, 2009). There is hesitancy to describe reference ranges for premature infants because of the perceived heterogeneous nature of the population. However this study has demonstrated many of the co-morbidities commonly identified in VLBW infants do not significantly alter the reference ranges, even those who have perceived higher risk and pragmatically one reference range can be used for the entire cohort.
CHAPTER 10: SYSTEMATIC REVIEW AND META-ANALYSIS OF USE OF PLASMA TRANSFUSION TO PREVENT IVH IN VERY PREMATURE NEONATES

10.1 Background

This review, registered with the Cochrane neonatal review group, evaluated the role of plasma transfusion in the prevention of IVH in very preterm neonates, who are vulnerable to develop severe IVH and are observed to have apparent prolonged standard laboratory measurements of coagulation. FP is generally used in neonatal, paediatric and adult patients with coagulopathy who are bleeding or perceived to be at risk of bleeding (Stanworth, 2007). Despite the perceived benefit of FP in prevention of IVH, the benefit of this practice has not been clearly shown. Current guidelines for FP administration in neonates are mainly based on poor-quality evidence and this contributes to the high level of FP use which may be inappropriate. In addition, the age-related changes of coagulation proteins during infancy make it difficult to correctly diagnose coagulopathy in neonates and subsequently determine when FP should be used (Motta et al., 2015). Administration of FP carries potential risks due to volume overload, infection and transfusion reactions. Risk-to-benefit ratio in respect to adverse effects, volume, method and length of administration all need to be considered. It is unclear whether prophylactic administration of FP prevents IVH in all high risk infants or whether even infants identified as having prolonged PT and APTT times benefit from administration of FP to prevent IVH.
10.2 Aims

The aim of this review was to assess whether plasma transfusion administered within the first 24 hours of life can prevent IVH in preterm infants born at ≤34 week’s GA. The secondary objectives of this review were to determine if the use of plasma transfusion is associated with morbidities and complications of preterm birth. Subgroup analyses were planned according to BW and GA of the infants, postnatal age at treatment, type and volume of plasma transfusion used and the indication for plasma use (prophylactic or in response to abnormal coagulation profile).
10.3 Methods

10.3.1 Criteria for considering studies for this review

**Types of studies**
RCTs, quasi-RCTs or cluster RCTs that compare plasma transfusion with control (placebo or no treatment) to prevent IVH in preterm infants were reviewed. Abstracts were included as eligible if sufficient data regarding the study outcomes was available to reviewers.

**Types of participants**
Preterm infants less than 34 weeks GA who have demonstrated to have no IVH on cranial ultrasound or those who are unscreened were included in this review. Infants were excluded if they received first dose of plasma after 24 hours of age or if an infant had a known inherited coagulation defect. The review differentiated between infants receiving plasma based on abnormal coagulation results but with no evidence of bleeding versus empirical treatment early in the postnatal period.

**Types of interventions**
FP or solvent detergent plasma or methylene blue treated plasma transfusion versus placebo or no treatment. Studies of any dose, frequency, and duration of plasma infusion were considered. Timing of administration of the first dose of intervention was limited to within 24 hours after birth. After obtaining all possible eligible trials, it became evident that many of these trials continued to use FP after 24 hours of
I included any trial that where the initial dose of FP was given before 24 hours of age.

**Types of outcome measures**

**Primary outcome measures included any of the following:**

1. Any grade IVH (Papile’s classification) (Papile et al., 1978)
   - Grade I haemorrhage is confined to the subependymal germinal matrix with no blood clot in the lumen;
   - Grade II haemorrhage is blood within the ventricular lumen without ventricular dilatation;
   - Grade III haemorrhage is IVH with ventricular dilatation;
   - Grade IV haemorrhage is IVH plus parenchymal haemorrhagic infarction.

2. Neurodevelopmental disability at two years of postnatal age, defined as neurological abnormality, including cerebral palsy, on clinical examination, developmental delay more than two SDs below population mean on any standard test of development, or blindness (visual acuity < 6/60), or deafness (any hearing impairment requiring amplification) at any time after two years corrected age.

**Secondary outcome measures included any of the following:**

1. Death: Mortality will be defined as death before discharge from the primary hospital.

2. PVL (increased echogenicity or cysts) diagnosed on CRUSS or MRI before discharge home.
3. Post haemorrhagic ventricular dilatation: (Post haemorrhagic ventricular dilatation precedes hydrocephalus but may be self-limiting and is widely defined as IVH followed by progressive enlargement until the ventricular width at the intraventricular foramen exceeds 4 mm over the 97th centile for GA (Levene and Starte, 1981). This will be assessed by cranial imaging at 28 days.

4. Seizures: (assessed by clinical or amplified EEG/EEG within 6 hours of plasma transfusion).

5. Bleeding: (significant in terms of needing transfusion, volume replacement, or causing haemodynamic compromise) from skin, gastrointestinal tract, lungs, skin puncture, or any other site at any point after administration of FP transfusion within 6 hours following plasma transfusion.

6. Persistent correction of coagulopathy (This will be assessed by the proportion of coagulation values outside laboratory reference range, where available).

7. Documented acute transfusion reaction (during or within 6 hours of transfusion) (Pandey and Vyas, 2012)
   - transfusion related lung injury; characterized by acute hypoxemia and non-cardiogenic pulmonary oedema
   - transfusion associated circulatory overload
   - allergic reaction/anaphylactic transfusion reaction
   - haemolytic transfusion reaction
   - febrile reaction,
   - metabolic complications or
   - septic contamination
8. Necrotising enterocolitis: Clinical (systemic and intestinal signs) and radiological signs of NEC as defined by the ‘Modified Bell’s Staging Criteria’ (Walsh and Kliegman, 1986); as documented by 28 days.

Planned subgroup analyses included the following identified subcategories

1) Including only trials where FP was given a) in the first 6 hours after birth and b) between 6 and 24 hours
2) Comparing different types of FP was administered to determine which type is most effective
3) Comparing different volumes of FP administered to determine which type is most effective
4) According to GA and BW of the infant
5) According to whether trials enrolled:
   a. Infants with known coagulopathy versus
   b. Infants with unknown coagulation status
   c. Infants with normal coagulation status

All primary and secondary outcomes were included in subgroup analysis where available.

Search methods for identification of studies
A search was performed of Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE (1966 - 2015), EMBASE (1980 – 2015), previous reviews including cross references (all articles referenced), abstracts and conferences (Paediatric Academic Societies 2004 – 2015 and European Academic Societies 2004-2015). Electronic searches included searching websites of American Academy of Paediatrics, Australian Clinical Trials Registry, Medical Research Council Trials Register. The search strategy included MeSH terms Plasma AND
Cerebral Haemorrhage AND Blood Coagulation Disorders AND Infant Premature, and non-MeSH Keywords: Intraventricular Haemorrhage AND Plasma AND Neonates AND Blood Coagulation. These were limited to Human, Infant Newborn, and Controlled Clinical Trials. I ensured that both American and English spellings were searched. The standard search strategy of the Neonatal Review Group was used. No language restriction was used. The following articles were excluded: letters, editorial, commentaries, reviews and lectures.

Criteria and methods used to assess the methodological quality of the included trials:
Standard methods of the Cochrane Collaboration and its Neonatal Review Group were used. The methodological quality of each trial was reviewed independently by the three review authors. Risk of bias was assessed according to the standard methodology of the Cochrane neonatal review group. Particular emphasis was placed on allocation concealment, blinding, completeness of follow up and blinding of outcome assessment. In individual RCTs the unit of analysis is the infant and may be the NICU or the institution in cluster RCTs.

Methods used to synthesise the data:
Standard methods of Neonatal Review Group with use of relative risk were used. The fixed effects model using RevMan 5.3 was used for meta-analysis. Heterogeneity was explored using the Chi² statistic for heterogeneity, and quantified using the I² statistic. Sensitivity analysis was performed on the basis of methodological quality. The methodology used to score the literature and transform it into level of evidence and strength of recommendation was that proposed by the Grading of recommendation, assessment, development, and evaluation (GRADE) Working Group.
10.4 Results

10.4.1 Description of studies

(See Appendix: Characteristics of included studies; Characteristics of excluded studies)

The MEDLINE search retrieved 18 studies, and the EMBASE and CCTR search no studies. Review of abstracts from PAS and ESPR retrieved 5 abstracts. Review of the American Academy of Pediatric website, Australian Clinical Trials Registry, Medical Research Council Trials Register revealed no studies. Review of personal files and reading of documents obtained by the initial searches and by screening all references listed in manuscripts yielded 5 additional studies. After excluding ineligible studies and after merging multiple publications of the same studies, a total of three RCTs remained. Among these three trials, one was conducted in the 1980s and the other two studies in the 1990s. The number of excluded reports was twenty (see 'Characteristics of Excluded Studies'). Studies were placed in this group for the following reasons (some studies for more than one reason): trials in which infants were diagnosed with IVH at autopsy as it is possible that some cases of IVH may not have been diagnosed, trials were not randomised and none of the outcomes selected for this systemic review were analysed.
Frozen plasma versus placebo (Comparison 1)

Only one study was in this group. **NNNI 1996** study compared infants less than two hours of age randomised to either fresh FP 20 ml/kg, or a gelatin-based plasma substitute (Gelofusine) 20 ml/kg (given over 15 minutes and repeated after 24 hours) in live born infants with GA less or equal to 32 weeks (n=519). Entry criteria included that the infants enrolled had no specific indication for or known contra-indication to any of the policies and that the responsible clinician was uncertain whether or not to use volume expansion (with or without coagulation factors) for a particular baby. Infants were randomly allocated to the treatment group by a telephone call to the central randomisation service at the Clinical Trial Service Unit. During this telephone call, information was given to check eligibility and describe the sort of babies studied; to allow minimization to ensure balance of the most important prognostic variables (hospital of recruitment, gestation in weeks, gender, singleton or multiple pregnancy, condition of the baby at trial entry) and to assist follow up.

Frozen plasma versus no treatment (Comparison 2)

This group included three RCTs. FP was initiated within the first 24 hours in all treated patients. The duration of FP was up to three days. The total dose was 10-20mls/kg/day of treatment protocol. Studies enrolled very preterm infants on the basis of prematurity or low BW, not on the basis of coagulation parameters (Beverley 1985; Ekblad 1992; NNNI 1996).
**Beverley 1985** is a RCT assessing the effect of fresh FP 10ml/kg on admission and repeated at 24 hours of age on mortality and IVH in preterm infants. Entry criteria were: preterm infants with BW less than 1500g, or GA < 32 weeks GA. The concentration of FP was not documented. Controls received no treatment, although was noted that in some cases small doses of purified protein fraction was administered during the first 24 hours of life for blood pressure maintenance. Each infant was allocated to a treatment or control group by the opening of a pre-sealed envelope that randomised allocation to study groups. Further description of this process was not described. The total number of patients entered into the study was 85, including 38 in the treatment groups, 42 in the control group and 5 who were not randomised for administrative reasons. Mean gestation: Treatment group: 29.4 (SD 2.4) weeks; Control: 28.8 (SD 2.1) weeks. Mean BW: Treatment group: 1246g (SD 400); Control: 1216g (SD 320). IVH was diagnosed by CRUSS performed within the first 48 hours. Outcome variables included IVH, mortality, respiratory distress syndrome, ventilation, pCO2 > 7kPa, pH < 7.15, mean maximal peak inspiratory pressure, mean maximal inspired oxygen, pneumothorax, PDA, coagulation studies and platelet counts.

**Ekblad 1992** is a RCT comparing fresh FP 10ml/kg (given over two hours at less than five hours of age, then daily for three days) to no treatment. Entry criteria were that infants were less than 34 weeks GA. The method of randomisation is not stated. The total number of patients entered into the study was 40, including 21 in the treatment groups and 19 in the control group. Mean gestation: 27.8 (SD 1.7) weeks; Control 27.6 (SD 1.6) weeks. Mean BW: Treatment 1375g. Control 1448g. The stated primary outcomes for the study were water balance and renal function.
The **NNNI 1996** study compared infants less than 2 hours of age randomised to either fresh FP 20ml/kg, or to no treatment. The stated primary outcomes for the study included death before discharge or severe disability at two years. Data from the NNNI 1996 study for IVH and PVL were available from a subgroup of units with routine scan facilities. The data for severe neurodevelopmental disability included children who were blind, deaf, unable to walk, had a developmental quotient > 3 SD below mean or another severe disability.

### 10.4.2 Risk of bias in included studies

See the table 'Characteristics of included studies. Among the three studies included in this systematic review, none were double blinded or stated that the interventions were blinded in any way.

**Frozen plasma versus placebo (Comparison 1)**

**NNNI 1996** study reported adequate randomisation procedures and had adequate allocation concealment. Blinding measurement of outcomes was reported by the NNNI 1996 study (neurodevelopmental assessment). Studies reporting no losses to follow-up included NNNI 1996 study (neurodevelopmental assessment). Not all units in the NNNI 1996 study performed routine head ultrasound scans. Data for IVH were available for approximately 84% of 611 infants from centres with routine scan facilities. The NNNI 1996 study reported head ultrasound abnormalities in units with routine scanning facilities as IVH and periventricular abnormality in babies surviving at six weeks.
Frozen plasma versus no treatment (Comparison 2)

Beverley 1985 reported adequate randomisation procedures and had adequate allocation concealment. Blinding measurement of outcomes was reported for IVH. Beverley 1985 reported seven (12.5%) losses. Data for IVH for the excluded infants is available and used in the intention to treat analysis of mortality and IVH in this review.

Ekblad 1992 did not report the method of randomisation and reported outcomes for 35 of 40 infants recruited. Data for IVH and mortality for the excluded infants is available and used in the intention to treat analysis of mortality and IVH in this review.

NNNI 1996 as per outcome 1.
10.4.3 Effects of Intervention

Frozen plasma vs. placebo in very preterm infants (Comparison 1):

Primary outcomes:
One study involving 518 infants compared fresh FP 20mls/kg within 2 hours of birth and subsequently 10mls/kg after 24 hours with a placebo of comparable volumes and dosing which in this study was a gelatin-based plasma substitute (NNNI 1996). Of infants in units with routine scan facilities who had CRUSS imaging, IVH was diagnosed in 42/135 infants who received FP vs. 30/131 infants who received Gelatin (RR 1.36, 95% CI 0.91, 2.03). In the trial infants as a whole, of infants scanned, IVH was diagnosed in 44/147 infants who received FP vs. 33/142 infants who received Gelatin (RR 1.29 95% CI 0.87, 1.90) With respect to the second primary outcome of neurodevelopmental disability at two years of postnatal age, rates of severe disability in all survivors (RR 1.01, 95% CI 0.58, 1.75) were not significantly different.
Secondary outcomes:

No significant difference was found in mortality (RR 0.95, 95% CI 0.65, 1.39). In a subgroup of infants born in centres with routine scanning facilities, the rates of PVL in survivors (RR 1.24, 95% CI 0.58, 2.62) were similar. The rate of NEC was significantly lower (RR 0.20, 95% CI 0.06, 0.69) in infants who received FP. No data on rates of post haemorrhagic ventricular dilatation, seizures, bleeding complications, persistent correction of coagulopathy or acute transfusion reactions were available.

Frozen plasma vs. no treatment in very preterm infants
(Comparison 2):

Primary outcomes:

Three studies randomised infants to fresh FP or no treatment (Beverley 1985; Ekblad 1992, NNNI1996). No difference was found in rates of IVH between groups (RR 0.95 [0.69,1.33]. Two studies (Beverley1985, Ekblad 1992) reported data on all infants randomised. Beverley 1985 found a significant reduction in IVH (RR 0.34, 95% CI 0.14, 0.84) whereas Ekblad 1992 found no significant difference (RR 0.94, 95% CI 0.15, 5.97). From units with routine scan facilities, the NNNI1996 study reported no difference in IVH in survivors examined (RR 1.20, 95% CI 0.83, 1.74). Rates of severe disability (RR 0.83, 95% CI 0.5, 1.39) were not significantly different between infants receiving FP and those who did not receive treatment.
Secondary outcomes:

Meta-analysis of studies reporting mortality data (Beverley1985; NNNI1996) involving a total of 588 infants found no significant difference in mortality (Outcome 3 RR 0.95 [0.66,1.36]). In the NNNI 1996 study, NEC was significantly reduced (RR 0.22, 95% CI 0.06, 0.75) in infants receiving FP.

Other subgroup analysis

1) According to volumes of FP used
   a. Two trials used a dose of 10mls/kg (Beverly 1985, Ekblad 1992) and one trial used a dose of 20mls/kg (NNNI 1996). Meta-analysis of the two studies using a dose of 10mls/kg found a significant trend to reduced IVH with FP use (RR 0.42, 95% CI 0.19, 0.92) compared to the study using 20mls/kg which favoured no treatment (RR 1.2 95% CI 0.83, 1.74). Of these two studies examined mortality (Beverly 1985, NNNI 1996), with a reduction in mortality observed in Beverly 1985 (RR 0.64 95% CI 0.23, 1.78) with FP but no difference was seen in NNNI 1996 (RR 1.00 95% CI (0.68, 1.48).

No relevant data for the following subgroup analyses was found:

2) According to timing of treatment: Early treatment (<6 hours age) versus treatment (6-24hours age)
   a. All three trials randomised commenced FP infusion less than 6 hours of age

3) According to types of FP used
   a. All three trials randomised used fresh FP
4) According to GA and BW of the infant
   a. Two trials enrolled infants less than 32 weeks (Beverly 1985, NNNI 1996) and one trial enrolled infants less than 34 weeks (Ekblad 1992). Ekblad 1992 stratified results according to infants < 30 weeks and between 30-34 weeks.
   b. One study used BW as an entry criteria (Beverly 1985)

5) According to type of infants enrolled:
   a. Infants with known coagulopathy versus
   b. Infants with unknown coagulation status
   c. Infants with normal coagulation status

All studies enrolled infants on the basis of gestation or BW. Coagulation studies were performed on admission in one study but did not affect randomisation (Beverly 1985).

All outcomes are as for the comparison FP vs. no treatment.

**Heterogeneity**

No statistically significant heterogeneity was found for any analysis included in this review. Comparing plasma transfusion to no treatment: One small study (Beverley 1985) found a reduced rate of IVH. The other studies (Ekblad 1992; NNNI 1996) and the overall meta-analysis did not support a difference in IVH.

**Sensitivity analysis according to methodological quality**

The results of this review are not sensitive to excluding the study that did not state whether there was adequate allocation concealment
(Ekblad 1992) nor to excluding the study that authors viewed to be at high risk of bias (Beverly 1985). Comparing FP with no treatment, one study with results for IVH had incomplete head ultrasound data at six weeks (NNNI1996). Two studies had complete follow up of infants for IVH (Beverley1985; Ekblad 1992) and found a significant trend to reduced IVH with FP use (RR 0.42, 95% CI 0.19, 0.92). The NNNI 1996 study had complete follow-up for neurodevelopmental outcomes reporting no significant difference in severe disability (RR 0.83, 95% CI 0.5, 1.39).
10.5 Discussion

This review addressed the question of whether the use of prophylactic plasma transfusion in preterm infants is beneficial in reducing morbidity and mortality. Subgroup analysis was proposed to examine from RCTs the role of different regimens of plasma transfusion in different types of infants. Three RCTs were included in this review. One trial described adequate randomisation procedures (NNNI 1996) and a second study was unclear in its description of randomisation (Beverly 1985). Entry criteria was defined by GA and BW, which increases the risk of IVH and mortality, however as all the trials used similar GA they were comparable in this regard. Whether or not these infants had coagulation parameters outside the normal range was not measured in all infants. In all studies, blinding of intervention was not discussed and given the nature of the interventions, it is probable that caregivers were not blinded in any of the studies. Two studies reported attempts at blinding of outcomes but as the CRUSS imaging was performed by clinical staff in some scenarios, they would have knowledge of intervention received. Not all the outcomes proposed to be examined were listed in the trials studied.

With respect to primary outcomes, use of FP did not significantly affect development of IVH in preterm infants compared to placebo (gelofusion) or no treatment; the strength of this inference is strong, based on 521 randomized patients. There was incomplete ascertainment of head ultrasound findings in a subgroup of infants born in centres with routine scan facilities in the largest trial (NNNI 1996). The studies with complete ascertainment of head ultrasound abnormalities are much smaller (Beverley et al., 1985, Ekblad et al., 1991). Summary statistics using intention-to-treat analyses for Beverly 1985 and Ekblad 1992 did not alter
the findings of the overall meta-analysis in relation to development of IVH for (RR 0.98, 95% CI 0.7, 1.36). Only one trial provided data on long-term neurodevelopment (NNNI 1996). This trial had no losses to follow-up and blinded assessment of neurodevelopment at two years, including the Griffiths’ Scales of Mental Development and clinical examination.

With regard to secondary outcomes the observations from one study that infants who received FP had a lower incidence of NEC should be regarded carefully (NNNI 1996), as this was not a pre-stated outcome of the randomised study. The overall rate of mortality and rate of PVL were not different between infants who received fresh FP compared to placebo or no treatment in this study. The effect of sub-analyses on data can lead to statistically significant results that have occurred by chance alone. Subgroup analyses showed that dose of 10mls/kg had a better outcome than 20mls/kg; however this could be due to the bias observed in the studies with dosing of 10mls/kg.

Grading of the recommendations for FP compared to placebo is outlined in the summary of findings table (Table 10.5). There is no evidence to support the routine use of plasma transfusion given to very preterm infants on the basis of GA or BW in the first days after birth. There is no evidence that routine plasma transfusion decreases the incidence of IVH or mortality and no difference was seen in the rates of subsequent disability. This evidence was seen as high quality from a RCT with low risk of bias and as there was only one study, no imprecision was observed.

Grading of the recommendations for FP compared to no treatment is outlined in the summary of findings table (Table 10.6). Downgrading of the quality of evidence was attributed to serious risk of bias (selection bias, performance bias, detection bias and attrition bias are observed across the studies), and inconsistency in study findings. Evidence from
one study of increased rate of IVH is not supported by the overall meta-
analisis or any other study. As the control group in this study received no
treatment the effect due to volume cannot be ascertained. In this study, a
high level of bias was observed. This must lead to caution in inferring the
likely effects of treatment when estimated in this study. The study with
the high level of bias was conducted in the 1980s. Thus,
recommendations based on this study must be viewed with caution when
applied to the group of preterm infants inhabiting the modern neonatal
unit. Imprecision, directness and publication bias did not affect quality of
evidence.
10.6 Conclusions

In terms of implications for practice available data show that there is no evidence from RCTs to support the routine use of prophylactic plasma transfusion in preterm infants without evidence of bleeding. Regarding implications for research the question of whether plasma transfusion should be given prophylactically to prevent IVH in preterm infants has been addressed but evidence is based on one good quality study. However there is insufficient evidence regarding whether FP administration in response to prolonged coagulation times is beneficial in reducing IVH and limited data on adverse effects of FP. With FP carrying significant risk in the paediatric and adult populations for the development of transfusion related acute lung injury, a condition likely under recognised in neonates, the risk/benefit ration of plasma transfusions should be carefully considered. Collaboration across countries and research networks is needed to further understand the benefits and risk of transfusion in this vulnerable group (Keir et al., 2015).
APPENDIX

Table 10.1: Characteristics of included studies *(Ordered by study ID)*

- **Beverley 1985**

| Methods | Design: Randomised controlled trial. Method of randomisation: Each infant allocated to a treatment or control group by the opening of a pre-sealed envelope that randomised allocation to study groups. Blinding of intervention: no. Complete follow up: no 7 (9%) infants excluded from study due to death (1), IVH on admission (1), and treatment of hypotension or coagulopathy with fresh FP (5 controls); Data available for mortality and IVH in excluded infants. Blinding of outcome: The scans performed at 7-10 days and subsequent scans were performed by a radiologist who had no knowledge of the clinical state of the infant. Scans at day 1 and at 48 hours were performed by clinical team and therefore have known the clinical state of infant. |
| Participants | Total number of patients entered into study: 80; treatment group 38, control group 42. Data reported in each patient in each group. Entry criteria: BW<1500g or GA < 32 weeks, without IVH on cranial ultrasound performed on admission to NICU. Mean gestation: Treatment group: 29.4 weeks (sd 2.4); Control: 28.8 (sd 2.1). Mean BW: Treatment group: 1246g (sd 400); Control: 1216g (sd 320) |
| Interventions | Treatment: Fresh FP 10mls/kg given on admission and 24 hours. Control: Normal care + small doses of purified protein fraction during day 1 of life to help maintain blood pressure |
| Outcomes | Stated primary outcome: IVH. Other outcomes: mortality, respiratory distress syndrome, ventilation, pCO2 > 7kPa, pH < 7.15, mean maximal peak inspiratory pressure, mean maximal inspired oxygen, pneumothorax, patent ductus arteriosus, coagulation studies and platelet counts |

**Notes**

**Risk of Bias**

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<th>Item</th>
<th>Author’s Judgement</th>
<th>Description</th>
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<td>Random nature of pre-sealed envelopes not described.</td>
<td>Unclear</td>
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<tr>
<td>(performance bias)</td>
<td>Infants who received FFP known</td>
<td>High risk of bias</td>
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### Methods

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<tr>
<th>Item</th>
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<th>Description</th>
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<tbody>
<tr>
<td>Allocation concealment?</td>
<td>Unclear</td>
<td>B – Unclear</td>
</tr>
</tbody>
</table>

Adequate randomisation: method not stated  
Allocation concealment: unclear  
Blinding of intervention: no  
Blinding of measurement: no  
Losses to follow-up: yes. 5 infants were excluded. Data for IVH for the excluded infants is available and used in the analysis of these outcomes.

### Participants

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<td>&lt;30 weeks (n=19), 30-34 weeks (n=19). &lt; 5 hours of age</td>
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<tr>
<td>Mean gestation:</td>
<td>27.8 (sd 1.7); Control 27.6 (sd 1.6)</td>
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<tr>
<td>Mean BW:</td>
<td>Treatment 1375g, Control 1448g</td>
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### Interventions

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<td>Intervention (n=21): Fresh FP 10mls/kg over 2 hours, daily for 3 days, no additional sodium.</td>
<td>Control (n=19): no treatment, sodium added to fluids</td>
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</table>

### Outcomes

<table>
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<th>Item</th>
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<td>Stated primary outcome:</td>
<td>water balance and extracellular volume (bromide space), renal function.</td>
</tr>
<tr>
<td>Other outcomes:</td>
<td>ventilator assistance, respiratory distress syndrome, PDA, IVH</td>
</tr>
</tbody>
</table>

### Notes

Risk of Bias

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allocation concealment?</td>
<td>B – Unclear</td>
</tr>
</tbody>
</table>
NNNI Group (Elbourne) 1996

Methods
Design: Randomised controlled trial, multicentre. Method of randomisation: telephone call to the central randomisation service at the Clinical Trial Service Unit in Oxford. Allocation concealment: yes. Blinding of intervention: no. Complete follow up: none for death or disability, incomplete head ultrasound data (in units with routine scanning – 84% for 6 weeks survivors). Blinding of outcome: Scans performed by consultant neonatologist/radiologist who were usually unaware of (not formally blind) to the baby’s original trial allocation.

Participants
Total number of patients entered into study: 776; treatment group (FFP) 257, treatment group (gelatin) 261, control group 258. Data reported in each patient in each group. Entry criteria: GA< 32 completed weeks, <2 h, no specific indication for or known contra-indication to any of the three policies and clinician uncertain whether or not to use volume expansion (with or without coagulation factors) for a particular baby. Median gestation: 29 weeks (range 27-31) Median BW (interquartile range): Group 1: 1265g (981-1543); Group 2: 1254g (965-1535); Control: 1240g (980-1510)

Intervention
Treatment: Group 1: Prophylactic plasma volume expansion with IV infusion of 20mls/kg Group AB or Group O Rhesus negative CMV-antibody-negative FFP over 15 min given into line being used to infuse glucose as soon as possible and another 10mls/kg 24 h later. Group 2: Gelatin-based plasma substitute (Gelofusine) 20mls/kg over 15 min, 10ml/kg 24hrs later. Control: Maintenance fluids 60-120 ml/kg/day. Other management as per physician.

Outcomes
Death or disability amongst long term survivors at 2 years of age is the primary outcome. secondary outcomes: death before discharge or the presence of CRUSS abnormality 1/6 weeks after birth

Notes
77% eligible infants enrolled. IVH data from units with routine scans.

Risk of Bias
<table>
<thead>
<tr>
<th>Item</th>
<th>Author's Judgement</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(selection bias)</td>
<td>Information given to check eligibility and describe the sort of babies studied to allow minimization to ensure balance of the most important prognostic variables</td>
<td>Unclear</td>
</tr>
<tr>
<td>(performance bias)</td>
<td>Infants who received FFP known</td>
<td>High risk of bias</td>
</tr>
<tr>
<td>(attrition bias)</td>
<td>Not all infants had a cranial ultrasound</td>
<td>High risk of bias</td>
</tr>
</tbody>
</table>
Table 10.2: Characteristics of excluded studies *(ordered by study ID)*

<table>
<thead>
<tr>
<th>Study</th>
<th>Reason for exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catford 2009</td>
<td>This was not a RCT. The outcome was FP effect on coagulation</td>
</tr>
<tr>
<td>Christensen 2014</td>
<td>Observational Study, No Intervention</td>
</tr>
<tr>
<td>Dani 2009</td>
<td>This was not a randomised controlled trial</td>
</tr>
<tr>
<td>Ekblad 1991</td>
<td>None of the outcomes selected for this systemic review for analysed.</td>
</tr>
<tr>
<td>Forman 2012</td>
<td>This was not a randomised controlled trial. The outcome was FP effect on coagulation. Infants were not premature.</td>
</tr>
<tr>
<td>Gupta 1976</td>
<td>Observational Study, No Intervention</td>
</tr>
<tr>
<td>Hambleton 1973</td>
<td>Mean (range) GA is 35 weeks (27-42). Doesn’t report meaningful outcomes as only know IVH in post-mortem</td>
</tr>
<tr>
<td>Irish Blood Transfusion Service 2006</td>
<td></td>
</tr>
<tr>
<td>Johnson 1982</td>
<td>Examined the effect of FFP on coagulation abnormalities. This was not a randomised control trial, infants range 28-38 weeks, FFP given in first 48 hours</td>
</tr>
<tr>
<td>McDonald 1984</td>
<td>Observational Study, No Intervention</td>
</tr>
<tr>
<td>Mendicini 1971</td>
<td>Confounding variables in treatment group Doesn’t report meaningful outcomes as only know IVH in post-mortem</td>
</tr>
<tr>
<td>Muthukumar 2011</td>
<td>This was not a randomised controlled trial. The outcome was FP effect on coagulation</td>
</tr>
<tr>
<td>Neary 2015</td>
<td>Abstract. This was not a randomised controlled trial</td>
</tr>
<tr>
<td>Neary 2015</td>
<td>This was a prospective cross sectional study and not a randomised controlled trial</td>
</tr>
<tr>
<td>Neary 2014</td>
<td>Abstract. This was not a randomised controlled trial</td>
</tr>
<tr>
<td>Neary 2013</td>
<td>Observational Study, No Intervention</td>
</tr>
<tr>
<td>Northern Neonatal Nursing Initiative Trial Group (1996)</td>
<td>Prevent duplication of results</td>
</tr>
<tr>
<td>Piotrowski 2010</td>
<td>Observational Study, No Intervention</td>
</tr>
<tr>
<td>Tran 2012</td>
<td>This was not a randomised controlled trial</td>
</tr>
<tr>
<td>Van de Bor 1986</td>
<td>This was not a randomised controlled trial</td>
</tr>
<tr>
<td>Wright 1995</td>
<td>Published Abstract not available</td>
</tr>
</tbody>
</table>
### Table 10.3: Comparison 1; Plasma transfusion vs. placebo in very preterm infants

<table>
<thead>
<tr>
<th>Outcome or subgroup title</th>
<th>No. of studies</th>
<th>No. of participants</th>
<th>Statistical Method</th>
<th>Effect size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any IVH</td>
<td>1</td>
<td>266</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>1.36 [0.91, 2.03]</td>
</tr>
<tr>
<td>Neurodevelopmental disability</td>
<td>1</td>
<td>399</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>1.01 [0.58, 1.75]</td>
</tr>
<tr>
<td>Death</td>
<td>1</td>
<td>518</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>0.95 [0.65, 1.39]</td>
</tr>
<tr>
<td>Periventricular leucomalacia</td>
<td>1</td>
<td>266</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>1.24 [0.58, 2.62]</td>
</tr>
<tr>
<td>Necrotising enterocolitis</td>
<td>1</td>
<td>518</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>0.20 [0.06, 0.69]</td>
</tr>
</tbody>
</table>
### Table 10.4: Comparison 2; Plasma transfusion vs. no treatment in very preterm infants

<table>
<thead>
<tr>
<th>Outcome or subgroup title</th>
<th>No. of studies</th>
<th>No. of participants</th>
<th>Statistical Method</th>
<th>Effect size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any IVH</td>
<td>3</td>
<td>390</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>0.95 [0.69, 1.33]</td>
</tr>
<tr>
<td>Neurodevelopmental disability</td>
<td>1</td>
<td>408</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>0.83 [0.50, 1.39]</td>
</tr>
<tr>
<td>Death</td>
<td>2</td>
<td>588</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>0.95 [0.66, 1.36]</td>
</tr>
<tr>
<td>Periventricular leucomalacia</td>
<td>1</td>
<td>282</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>0.76 [0.40, 1.45]</td>
</tr>
<tr>
<td>Necrotising enterocolitis</td>
<td>1</td>
<td>518</td>
<td>Risk Ratio (M-H, Fixed, 95% CI)</td>
<td>0.22 [0.06, 0.75]</td>
</tr>
</tbody>
</table>
Figure 10.1: Comparison 1; FP vs. Placebo in infants less than 34 weeks, Outcome 1; Any IVH in survivors examined

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma</th>
<th>Placebo</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNM 1986</td>
<td>42</td>
<td>135</td>
<td>1.36 [0.91, 2.03]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>135</td>
<td>131</td>
<td>1.36 [0.91, 2.03]</td>
</tr>
</tbody>
</table>

Total events: 42 FP, 30 Placebo
Heterogeneity: Not applicable
Test for overall effect: Z = 1.49 (P = 0.14)

Figure 10.2: Comparison 1; FP vs. Placebo in infants less than 34 weeks, Outcome 2; Severe neurodevelopmental disability in survivors

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma</th>
<th>Placebo</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNM 1986</td>
<td>23</td>
<td>203</td>
<td>1.01 [0.58, 1.75]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>203</td>
<td>196</td>
<td>1.01 [0.58, 1.75]</td>
</tr>
</tbody>
</table>

Total events: 23 FP, 22 Placebo
Heterogeneity: Not applicable
Test for overall effect: Z = 0.03 (P = 0.97)
Figure 10.3: Comparison 1; FP vs. Placebo in infants less than 34 weeks, Outcome 3; Death

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma</th>
<th>Placebo</th>
<th>Risk Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Events Total</td>
<td>Events Total</td>
<td>M-H, Fixed, 95% CI</td>
</tr>
<tr>
<td>NINNI 1996</td>
<td>43 257</td>
<td>46 261</td>
<td>0.95 [0.85, 1.19]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>257 261</td>
<td>100.0%</td>
<td>0.95 [0.85, 1.19]</td>
</tr>
<tr>
<td>Total events</td>
<td>46</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Not applicable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect Z = 0.27 (P = 0.79)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Favours [Frozen Plasma] Favours [Placebo]

Figure 10.4: Comparison 1; FP vs. Placebo in infants less than 34 weeks, Outcome 4; Periventricular leucomalacia in survivors examined

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma</th>
<th>Placebo</th>
<th>Risk Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Events Total</td>
<td>Events Total</td>
<td>M-H, Fixed, 95% CI</td>
</tr>
<tr>
<td>NINNI 1998</td>
<td>14 135</td>
<td>11 131</td>
<td>1.24 [0.58, 2.62]</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>135 131</td>
<td>100.0%</td>
<td>1.24 [0.58, 2.62]</td>
</tr>
<tr>
<td>Total events</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Not applicable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect Z = 0.55 (P = 0.58)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Favours [Frozen Plasma] Favours [Placebo]
Figure 10.5: Comparison 1; FP vs. Placebo in infants less than 34 weeks, Outcome 5; Necrotising enterocolitis

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma</th>
<th>Placebo</th>
<th>Risk Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Events</td>
<td>Total</td>
<td>Events</td>
</tr>
<tr>
<td>NINNI 1996</td>
<td>3</td>
<td>257</td>
<td>15</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>3</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Not applicable
Test for overall effect: Z = 2.55 (P = 0.01)

Figure 10.6: Comparison 2; FP vs. No treatment in infants less than 34 weeks, Outcome 1; Any IVH in survivors examined.

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma</th>
<th>No Treatment</th>
<th>Risk Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Events</td>
<td>Total</td>
<td>Events</td>
</tr>
<tr>
<td>Benedict 1985</td>
<td>5</td>
<td>36</td>
<td>15</td>
</tr>
<tr>
<td>Elliott 1992</td>
<td>2</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>NINNI 1996</td>
<td>42</td>
<td>135</td>
<td>38</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>49</td>
<td>189</td>
<td>201</td>
</tr>
<tr>
<td>Total events</td>
<td></td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

Heterogeneity: Chi² = 6.45, df = 2 (P = 0.04); P = 60%
Test for overall effect: Z = 0.20 (P = 0.79)
Figure 10.7: Comparison 2; FP vs. No treatment in infants less than 34 weeks, Outcome 2; Severe neurodevelopmental disability in survivors

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma Events</th>
<th>Total Events</th>
<th>No Treatment Events</th>
<th>Total Events</th>
<th>Weight</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNHI 1966</td>
<td>23</td>
<td>203</td>
<td>28</td>
<td>205</td>
<td>100.0%</td>
<td>0.83 [0.69, 1.39]</td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td></td>
<td>203</td>
<td>28</td>
<td>205</td>
<td>100.0%</td>
<td>0.83 [0.58, 1.39]</td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>23</td>
<td></td>
<td>28</td>
<td>205</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 0.71 (p = 0.48)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Favours [Frozen Plasma] Favours [No Treatment]

Figure 10.8: Comparison 2; FP vs. No treatment in infants less than 34 weeks, Outcome 3; Death

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma Events</th>
<th>Total Events</th>
<th>No Treatment Events</th>
<th>Total Events</th>
<th>Weight</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beverley 1985</td>
<td>5</td>
<td>36</td>
<td>8</td>
<td>37</td>
<td>15.5%</td>
<td>0.64 [0.23, 1.79]</td>
<td></td>
</tr>
<tr>
<td>Elkind 1982</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>Not estimable</td>
<td></td>
</tr>
<tr>
<td>NNHI 1966</td>
<td>43</td>
<td>257</td>
<td>43</td>
<td>258</td>
<td>84.5%</td>
<td>1.00 [0.66, 1.48]</td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>283</td>
<td>295</td>
<td>100.0%</td>
<td></td>
<td>0.95 [0.66, 1.36]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>48</td>
<td></td>
<td>51</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Chisquare = 0.65, df = 1 (p² = 0.43); I² = 0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 0.28 (p = 0.77)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Favours [Frozen Plasma] Favours [No Treatment]
Figure 10.9: Comparison 2; FP vs. No treatment in infants less than 34 weeks, Outcome 4; Periventricular leucomalacia in survivors examined

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma Events</th>
<th>Total Events</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNHL 1986</td>
<td>14</td>
<td>135</td>
<td>0.76 [0.49, 1.15]</td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>135</td>
<td>147</td>
<td>0.76 [0.46, 1.25]</td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>14</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 0.93 (P = 0.36)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10.10: Comparison 2; FP vs. No treatment in infants less than 34 weeks, Outcome 5; Necrotising enterocolitis

<table>
<thead>
<tr>
<th>Study or Subgroup</th>
<th>Frozen Plasma Events</th>
<th>Total Events</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
<th>Risk Ratio M-H, Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNHL 1986</td>
<td>3</td>
<td>257</td>
<td>0.22 [0.06, 0.75]</td>
<td></td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>257</td>
<td>261</td>
<td>0.22 [0.06, 0.75]</td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>3</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterogeneity: Not applicable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for overall effect: Z = 2.42 (P = 0.02)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 10.5: Summary of findings table; FP vs. Placebo in infants less than 34 weeks

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>Anticipated absolute effects* (95% CI)</th>
<th>Relative effect (95% CI)</th>
<th>No of infants (studies)</th>
<th>Quality of the evidence (GRADE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Any IVH</strong></td>
<td>Risk with placebo</td>
<td>Risk with FP</td>
<td>RR 1.36 (0.91 to 2.03)</td>
<td>266 (1 RCT)</td>
</tr>
<tr>
<td></td>
<td>229 per 1000 (208 to 465)</td>
<td>311 per 1000 (208 to 465)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>229 per 1000 (208 to 465)</td>
<td>311 per 1000 (208 to 465)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neuro-developmental Disability</strong></td>
<td>Study population</td>
<td>Risk with FP</td>
<td>RR 1.01 (0.58 to 1.75)</td>
<td>399 (1 RCT)</td>
</tr>
<tr>
<td></td>
<td>112 per 1000 (65 to 196)</td>
<td>113 per 1000 (65 to 196)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>112 per 1000 (65 to 196)</td>
<td>113 per 1000 (65 to 196)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Death</strong></td>
<td>Study population</td>
<td>Risk with FP</td>
<td>RR 0.95 (0.65 to 1.39)</td>
<td>518 (1 RCT)</td>
</tr>
<tr>
<td></td>
<td>176 per 1000 (115 to 245)</td>
<td>167 per 1000 (115 to 245)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>176 per 1000 (115 to 245)</td>
<td>167 per 1000 (115 to 245)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PVL</strong></td>
<td>Study population</td>
<td>Risk with FP</td>
<td>RR 1.24 (0.58 to 2.62)</td>
<td>266 (1 RCT)</td>
</tr>
<tr>
<td></td>
<td>84 per 1000 (49 to 220)</td>
<td>104 per 1000 (49 to 220)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>84 per 1000 (49 to 220)</td>
<td>104 per 1000 (49 to 220)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI). CI: Confidence interval; RR: Risk ratio; OR: Odds ratio;
Table 10.6: Summary of findings table; FP vs. No treatment in infants less than 34 weeks

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>Anticipated absolute effects’ (95% CI)</th>
<th>Relative effect (95% CI)</th>
<th>No of Infants (studies)</th>
<th>Quality of the evidence (GRADE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Risk with no FP</td>
<td>Risk with FP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any IVH</td>
<td>Study population</td>
<td>RR 0.95 (0.69 to 1.33)</td>
<td>390 (3 RCTs)</td>
<td>★★★☆☆ LOW 1,2</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>259 per 1000 (178 to 344)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuro-developmental</td>
<td>Study population</td>
<td>RR 0.83 (0.50 to 1.39)</td>
<td>408 (1 RCT)</td>
<td>★★★★★ HIGH</td>
</tr>
<tr>
<td>Disability</td>
<td>Moderate</td>
<td>137 per 1000 (68 to 190)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>Study population</td>
<td>RR 0.95 (0.66 to 1.36)</td>
<td>588 (2 RCTs)</td>
<td>★★★★★ HIGH</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>173 per 1000 (114 to 235)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVL</td>
<td>Study population</td>
<td>RR 0.76 (0.40 to 1.45)</td>
<td>282 (1 RCT)</td>
<td>★★★★★ HIGH</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>136 per 1000 (54 to 197)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI). CI: Confidence interval; RR: Risk ratio; OR: Odds ratio;

1. Selection bias, performance bias, detection bias, attrition bias noted in studies
2. Statistical heterogeneity
CHAPTER 11: DISCUSSION

Differences in neonatal care are evident worldwide with respect to neonatal haematology and transfusion practices (Jain, 2015). The heterogeneous nature of clinical practice in this area underscores the need for further research in order to recognize best practice. Contributing to this lack of standardisation is poor understanding of the uniqueness of the neonatal haemostatic system. In this thesis we have contributed to evidence based medicine in this area by defining reference ranges for coagulation indices in very premature infants, reflecting on the impact of developmental and postnatal maturation on these parameters and examining the use of global haemostatic assays. The role of coagulation studies in the aetiology of IVH is further reviewed and the concept of prophylactic plasma transfusions for prevention of IVH is examined. The key findings of this thesis, which are summarised below, address the aims as stated in Chapter 2 and are that:

- Reference ranges for standard coagulation parameters in very preterm infants have been established
- Coagulation system is profoundly influenced by developmental changes
- Despite long clotting times, thrombin generated in preterm infants is comparable with term controls
- Although quantitative and qualitative differences are seen in preterm infants compared to term infants; haemostatic function is equivalent
- Coagulation values in isolation do not appear to be associated with IVH
- The future – evidence for plasma transfusion in VLBW infants has been addressed
11.1 Reference ranges for standard coagulation parameters in very preterm infants have been established

In this thesis, we aimed to provide information on normative values for coagulation indices in very preterm infants. The Stockholm Hierarchy provides grading system of quality of reference ranges (Sikaris, 2012). In this thesis we provide reference intervals for infants on admission to NICU categorised according to intra and inter individual physiological differences, which is level 2 of the Stockholm Hierarchy. A combination of direct and indirect methods was used. In the prospective study, infants were recruited, re-assessed for health (CRUSS was undertaken), blood samples processed and the corresponding coagulation parameters organised into reference ranges. The samples drawn on day 1 of life were clinical samples and results were available to attending clinician. Additionally indirect methods were employed to determine gestation specific reference ranges utilising the laboratory database in the larger cohort of infants less than 30 weeks to provide gestation specific reference ranges. In order to develop reference ranges in this manner, the population in question should match those for whom the reference ranges are to be applied to. Therefore the reference populations in this study are appropriate as the patient demographics and the process of phlebotomy, and analysis was comparable in all patients as given that samples were routinely processed on admission in this NICU. The cohorts were then subdivided based on the presence of IVH. Using published data although lower end of Stockholm hierarchy is acceptable in certain populations for example neonates (Sikaris, 2012, Chalmers, 2004). Reproducible methods and applicable reference populations are the two essential considerations when laboratories subjectively assess published reference intervals. By defining the methodology and
populations in this study, these reference ranges are generalizable to other hospitals using similar analysers and reagents.
11.2 Coagulation system is influenced by developmental changes

The dynamic and evolving process of the hemostatic system that occurs during infancy and childhood was first described by Andrew et al and was termed “developmental hemostasis” (Andrew et al., 1987). The primary concept of developmental hemostasis is that the levels of most coagulation proteins vary significantly with age (Monagle et al., 2010). These age-related changes result in corresponding changes in the standard coagulation screening tests, such as PT and APTT. Given the age-dependent specificities, the evaluation and interpretation of coagulation tests in neonates should be based on reference ranges that are appropriate for gestational and postnatal age (Henry and Christensen, 2015, Hathaway and Corrigan, 1991). This thesis provides data on neonates less than 30 weeks GA on admission to NICU and serially on day 3 and week 2 of life. We have provided evidence that in very preterm infants, coagulation indices decrease with increasing GA. In addition I have shown that these values reduce over the first few days of life and that this reduction occurs irrespective of FP administration.
11.3 Despite long clotting times in preterm infants, thrombin generated is comparable with term controls

Because of the complexity of the in vivo coagulation process, standard laboratory tests cannot accurately measure all the individual elements involved in hemostatic function. Consequently, standard coagulation tests in neonates must be interpreted with caution. A key limitation of standard coagulation tests is that they are poor predictors of bleeding. Global haemostatic assays such as CAT assay may be able to overcome some of these limitations. This assay differs significantly from conventional coagulation tests, as it evaluates the kinetics of the entire process of thrombin generation even after clot formation providing a composite picture reflecting the interaction of procoagulant and anticoagulant factors, and more closely reflects the in vivo condition than conventional tests. Despite striking differences in the levels of individual components of the haemostatic system, global coagulation assays have demonstrated that very premature neonatal coagulation is equal to that observed in term infants. There appears to be a balance in the haemostatic system in very premature neonates. However, a number of perinatal or neonatal conditions can disrupt this delicate balance and increase the risk for either haemorrhage or thrombus formation.
11.4 Although quantitative and qualitative differences are seen in preterm infants compared to term infants; haemostatic function is equivalent

Although the key components of the haemostasis system are present at birth, important quantitative and qualitative differences exist among preterm neonates and full-term neonates. Despite these physiological differences, healthy term and preterm babies do not tend to have problems with coagulation, indicating that these differences represent normal development of the human coagulation system (Pal et al., 2015).
11.5 Coagulation values in isolation do not appear to be associated with IVH

Level 1 of Stockholm Hierarchy involves examining the relationship with laboratory parameters and the incidence of disease (Sikaris, 2012). Severe IVH occurs through a variety of mechanisms, as discussed earlier in the thesis. The greatest risk factors are extreme prematurity and very low birth weight at delivery. In very premature neonates, the presence of abnormal coagulation tests is common. These tests are limited in ability to distinguish which infants will have haemorrhagic complications and do not necessarily correlate with a clinically relevant disease. It is widely accepted that administration of FP does not correct coagulation tests in all settings (Pal et al., 2015).

No convincing data have been published to date to indicate that immaturity of the coagulation system plays a role in the pathophysiology of IVH in very preterm infants. Of particular note, no correlation was observed in the prospective study between PT or APTT measured on day 1 of life and subsequent development of IVH during the first day or the first week of life in infants who did not receive plasma transfusion and in the group as a whole. While we did observe a reduction in endogenous thrombin generation in a cohort of infants with extreme APTT prolongations (> 100 s), there was no difference in IVH rates between these two groups. Moreover, the clinical relevance of this attenuation is uncertain.

However it is noticeable in the ambispective study of the entire cohort, we did observe that PT was significantly associated with rates of IVH. Limitations of the review of the overall cohort were that as some of the data was retrospective which may be associated with introduction of error. Alternatively as the rates of IVH were low in the prospective study this may have masked an association and larger prospective studies may
be needed to further answer this question. In the absence of clear evidence based upon randomized trials, we suggest continued individualized risk assessment by a senior multidisciplinary team when making decisions on plasma transfusion in preterm infants with prolonged coagulation times.
11.6 Evidence base for plasma transfusion in VLBW infants has been addressed

In order to facilitate a move to evidence based transfusion practices, an overview of current clinical care is warranted. Preterm infants frequently receive transfusions, irrespective of guidelines and recommendations. Within neonatal units, the indications for transfusion with FP are laboratory evidence of coagulopathy, correction of disseminated intravascular coagulopathy (DIC) and prevention of IVH (Pal et al., 2014). Previous common uses of FP that are now considered inappropriate include volume replacement, correction of hypoalbuminaemia, nutritional support and immunoglobulin replacement (Pal et al., 2014). Clinical audits continue to report a common use of FP in neonates outside evidence-based recommendations, ranging from 8-12% across all GA and BW (Motta et al., 2014, Puetz et al., 2009), raising questions about cost and risk. The main reasons for poor adherence to guidelines are the lack of clinical trials evaluating FP efficacy, and the limitation of standard coagulation tests as predictors of bleeding. A recent Canadian study reports an incidence of 15% of neonates less than 30 weeks GA receiving FP (Keir et al., 2015). This thesis reported an incidence of 34% use of FP which probably reflects the practice that coagulation tests were routinely performed in this unit which increases the potential of FP use as described by Catford et al (Catford et al., 2014).

Understanding physiologic hemostasis and interpreting coagulation tests are both needed to optimize neonatal FP management. The finding that thrombin generation is similar in term and preterm infants raises several questions. The following logical question is whether plasma transfusion is beneficial in neonatal management. There is a lack of data demonstrating that FP administration to non-bleeding neonates with abnormal coagulation tests reduces their risk of a subsequent
hemorrhage and the Cochrane review that I undertook is consistent with this hypothesis. Therefore, the most appropriate use of FP should be the treatment of active bleeding in neonates who have laboratory confirmation of coagulopathy. Prophylactic use of FP in non-bleeding neonates, on the basis that their coagulation test is abnormal, cannot be considered an evidence-based practice.

Prospective epidemiologic studies focused on clinical outcomes in high-risk neonates are needed to inform studies aimed at evaluating the efficacy of FP in specific conditions (Motta et al., 2015). FP is often administered in management of many neonatal conditions including necrotising enterocolitis, disseminated intravascular coagulation, sepsis, hypoxic ischaemic encephalopathy and may need to be re-evaluated. The paucity of RCTs focused on treatment strategies in neonatal hematological issues, leads to variations in clinical practice. The balance of risks versus benefit is particularly difficult with respect to hematological concerns as therapies for both haemorrhage and thrombosis have significant risks. Further research in this area is warranted (Saxonhouse and Manco-Johnson, 2009).
11.7 Limitations

Inherent to any study design are limitations which can contribute to bias. Some of the recognised limitations of this study are discussed below.

11.7.1 Cord Blood Sampling

A limitation of this study is that ethical considerations limited the volume of peripheral blood draw that was permitted. The circulating blood volume of an infant weighing 500 g is only 40 mL, and it was deemed inappropriate to take large volumes of blood for research purposes. Consequently, thrombin generation and coagulation factor assay measurement required successful cord blood sampling, which was not possible in many infants.

Cord blood sampling was attempted on all patients (almost always by the lead investigator); however, as many very premature infants are delivered because of placental pathology, sufficient samples of blood were not always obtained. In VLBW infants, cord blood coagulated rapidly after sampling despite efficient sampling procedures, leading to loss in the samples. This is a reflection of the reality of prospective data collection in this preterm cohort. The ideal approach is to have a cord blood banking system with a dedicated team to collect cord blood. Consequently, the number of selections made in the analysis of data is also a possible source of bias. The results obtained are in line with previously published data in retrospective case series and we believe them to be valid. As blood was obtained from venepuncture and cord blood, we recognised that this may have been a confounder of the laboratory results between first samples and the remaining ones. Therefore we compared the results from both sources and found that
they correlated. Where cord blood samples are used caution should be applied in interpreting them as neonatal values.

An additional limitation of our study is the lack of postnatal plasma samples from control infants, as was deemed unethical to draw blood from term infants who were not having clinical samples. Infants who were having clinical samples for other reasons such as bilirubin monitoring and sepsis management were deemed unsuitable given the concern that these morbidities could act as confounding variables.

11.7.2 Laboratory Analysis

The thrombin generation assay is a research tool at present. For accurate reliable results the assay requires standardisation. This assay was performed under same environmental conditions and repeat control samples were run with each preterm clinical sample to validate samples used on separate days.

Additionally alpha-2 Macroglobulin can alter results; Alpha-2-macroglobulin (a2M)-bound thrombin cleaves the fluorogenic thrombin substrate utilized in this assay and continues to do so following physiological thrombin inhibition. Initial a2M-mediated thrombin inhibition plays an important physiological role, particularly in neonates. The alpha-2-macroglobulin-bound thrombin is still amidolytically active towards the substrate, which is corrected for by the software.

11.7.3 Data Interpretation

Very premature infants are often very unwell and it can be difficult to differentiate the effects of illness compared with the nature of prematurity. The use of the SNAPPE-II score in this regard can
subdivide those infants whose coagulation parameters are altered by the
effects of illness as opposed to simply the fact these infants are
premature. The infants in this study did not have a normally distributed
SNAPPE-II score, but was skewed in favour of the cohort with scores of
0-10 which indicates that the majority of these infants did not have
severe illness and therefore are representative of healthy premature
neonates for determination of coagulation reference intervals.

In this observational study, individual attending neonatologists
made clinical decisions on plasma transfusion. This is a limitation of the
observational design of this study when addressing the question of IVH.
However, as plasma was administered after both cord and day 1
samples were taken, these infants do contribute to the overall dataset on
coagulation.

Reference ranges are specific to the analyser and reagent used in
sample analysis, but trends across laboratories should be comparable
(Monagle and Massicotte, 2011). Validation of reference ranges may be
performed by other laboratories using similar reagents but including
fewer cases.
11.8 Future Directions

11.8.1 Evidence for plasma transfusion in VLBWI needs to be re-visited

To definitively answer the question whether plasma should be administered or not prospective randomized trials are warranted. But in the context of the data we have presented raises questions whether this should be performed. The continuation of this study following completion of this thesis will further investigate the impact of plasma transfusion in VLBW infants. This question remains to be answered further. These studies will provide valuable insight into the therapeutic potential of plasma transfusion in NICU including necrotising enterocolitis, disseminated intravascular coagulation, sepsis, hypoxic ischaemic encephalopathy.

11.8.2 To investigate the molecular mechanisms underlying observed prolongations in clotting times in extremely premature neonates

What is the explanation for the discrepancy between ‘prolonged’ coagulation times and the observed normal thrombin generation in preterm infants compared with term infants? This remains poorly characterized. Neonates have lower levels of vitamin K-dependent clotting factors compared with term infants, which was confirmed in cord blood samples in this study. The APTT assay is sensitive to reduced plasma activity of these factors. However, coexisting defects in
anticoagulant pathways may result in a type of haemostatic ‘equilibrium’ in preterm infants. Moreover, the APTT is also prolonged by reductions in contact pathway factors, neither of which are associated with a bleeding tendency or reduced thrombin generation. Reduction in these contact pathway factors has been well characterized in older preterm infants (Andrew et al., 1988). Future work will focus upon elucidating the precise mechanism underlying coagulation time prolongation including identifying the contribution of contact factor pathways to this prolongation (i.e. factors not associated with a bleeding tendency).

11.8.3 To further examine thrombin generation in preterm infants

Interestingly, reductions in lag time to onset of thrombin generation and time to peak thrombin generation were observed in preterm infants. Previous studies have reported reductions in plasma TF activity in preterm compared with term infants (Schweintzger et al., 2010). An alternative potential explanation is a difference in either concentration or function of TFPI antigen (although we did not observe this). Further characterisation of thrombin generation in very preterm infants would be useful. Previous data (Schweintzger et al., 2010) have suggested that elevated concentrations of TF-expressing microparticles (MP) are present in preterm compared with term plasma. MPs are submicron fragments of the cell membrane, ranging in size between 100–200 nm, that are shed from the cell membrane into the circulation or other body fluids following cell membrane activation by a wide range of stimuli (inflammation, hypoxia or oxidative stress, irradiation, products of complement activation or shear stress) or apoptosis, i.e. a programmed cell death. Exosomes are another type of cell components, although they differ in several regards (they do not derive from the cell membrane but
from internal cell membranes; are smaller than MPs, 30–90 nm, their proteomes differ and their procoagulant potential is low)(Uszynski et al., 2011). In order to characterize potential mechanisms underlying observed differences in thrombin generation in plasma prepared from cord blood of very preterm infants compared with healthy term controls, the assay will be modified by omission of exogenously added TF. Thrombin generation observed in this modification of the assay is likely due to endogenous TF but may be observed due to contact pathway activation. To address each hypothesis, the assay will be repeated (again in the absence of exogenously added TF) but in the presence of an anti-TF monoclonal antibody and corn trypsin inhibitor (CTI; haematologic technologies inc., Vermont, MA).

11.8.4 To examine the role of primary haemostasis in very preterm neonates

In the case of primary haemostasis further studies examining the role of VWF and ADAMTS 13 levels and sialic levels on VWF and VWF multimers would be useful.
11.8.5 To further understand the role of coagulation testing in preterm infants

The results raise potential concerns regarding PT and IVH. As the rates of IVH were low in the prospective study this may have masked an association and larger prospective studies may be needed to further answer this question. Ideally future studies could examine coagulation studies from term infants to act as a control without the need to use cord blood.

In the future modern thrombin generation tests may become introduced in clinical practice and improve diagnostic accuracy of DIC in newborns (Veldman et al., 2010). Consequently it is possible to envisage individualised plasma transfusion guidelines on the basis of coagulopathy as opposed to empiric treatment. Similar approaches will be used to examining children with nephrotic syndrome. These findings have important clinical implications.
APPENDIX:
PUBLICATIONS/AWARDS/PRESENTATIONS
BASED ON DATA FROM THIS WORK

Peer Reviewed Publications


Published Abstracts


Awards

1. Irish Neonatal Symposium, November 2015 - Best Overall Presentation
2. Len Arlene Travel Award on basis of abstract submitted to Irish American Paediatric Society Annual Meeting September 2015
3. Paediatric Academy Society Travel Award on basis of abstracts submitted, May 2015
4. Nominated for Presidents Prize, Haematology Association of Ireland, October 2014
5. Irish Perinatal Society, 2014 – 2nd prize for Oral Presentation

International Presentations

Neonatal Society, London, November 2015, Oral Presentation:
- Coagulation system in very premature infants.
  E. Neary, F. Ni Ainle, M. Cotter, N, McCallion

Irish American Paediatric Society, Nashville, USA, September 2015, Oral Presentation
- Coagulation system in very premature infants
  E. Neary, F. Ni Ainle, M. Cotter, N, McCallion
Joint European Neonatal Society Meeting, Budapest, Hungary, September 2015,

**Oral poster presentation:**
1. The anticoagulant action of activated protein C in very preterm infants

**Poster:**
2. Cord blood investigation in premature neonates.
   E. Neary, F. Ni Ainle, M. Cotter, N. McCallion
3. Development of coagulation indices in infants less than 30 weeks with respect to gestational and postnatal age.
   E. Neary, F. Ni Ainle, M. Cotter, N. McCallion

Paediatric Academic Society, San Diego, April 2015, Poster:
   E. Neary, F. Ni Ainle, B. Kevane, M. Cotter, K. Egan, N. McCallion
   E. Neary, A. James, F. Ni Ainle, B. Kevane, D. Corcoran, N. McCallion, A. El-khuffash

European Academy Paediatrics Society, Barcelona, Spain, October 2014, Poster
- Baseline Coagulation Times Do Not Influence Likelihood of Intraventricular Haemorrhage (IVH) In Extremely Premature Neonates.
  Neary E, Ní Ainle F, Cotter M, McCallion N.
Paediatric Intensive Care Society, Newcastle, UK, October 2014
Oral poster presentation
  Neary E, Ní Ainle F, Forman E, Lis H, Cotter M, McCallion N.

Paediatric Academic Society, Vancouver, May 2014, Poster:
  Neary E, Ní Ainle F, Forman E, Lis H, Cotter M, McCallion N.

National Presentations

Irish Neonatal Symposium, Dublin, November 2015, Oral Presentations:
  1. Coagulation system in very premature infants.
     E. Neary, F. Ni Ainle, M. Cotter, N, McCallion
  2. The anticoagulant action of activated protein C in very preterm infants.

National Childrens Research Centre Seminar, November 2015, Oral Presentation:
- Coagulation system in very premature infants.
  E. Neary, F. Ni Ainle, M. Cotter, N, McCallion
Faculty of Paediatrics Autumn Study Day, October 2015, Oral Presentation
- Coagulation system in very premature infants.
  E. Neary, F. Ni Ainle, M. Cotter, N, McCallion

Interhospital Paediatric and Child health research Club, March 2015, Oral Presentation
- E. Neary, F. Ni Ainle, M. Cotter, N, McCallion

Haematology Association of Ireland, October 2014, Oral Presentation
Irish Perinatal Society, November 2013, Oral Presentation
Irish Neonatal Symposium, November 2013, Oral Presentation
Frequent local presentations for funding authorities and departmental meetings
- To characterise standard laboratory coagulation parameters and plasma thrombin generation in very preterm infants and to investigate their relationship to clinical outcomes. E. Neary, N McCallion, B Kevane, M Cotter, K Egan, I Regan, F NiAinle
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