The protein C pathway in necrotising enterocolitis

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Thesis submitted for the degree of Doctor of Philosophy
Declaration

I, Paula Hannah Lister, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

August 2012.
Abstract

Necrotising enterocolitis (NEC) is an acute inflammatory condition of the preterm intestine associated with significant morbidity and mortality. The pathogenesis remains unclear.

The Protein C (PC) pathway is the main anticoagulant system of the body with key anti-inflammatory, anti-apoptotic and barrier stabilising effects on the microcirculation, cells of the innate immune system and epithelial cells of the lungs, intestine and skin.

Neonates, and preterm neonates in particular, have a physiological deficiency in the PC pathway.

This thesis was aimed at discovering the effects of NEC on the PC pathway.

We demonstrated with immunohistochemistry that thrombomodulin (TM) and endothelial protein C receptor (EPCR) are strongly expressed in healthy preterm neonatal bowel; these receptors are the activation apparatus of PC. On examination of intestine affected by NEC, we discovered TM and EPCR expression was reciprocally reduced with increasing inflammation, but lost only in necrotic areas. Dual staining showed that intravascular deposits of fibrin occurred in vessels with weak or absent TM expression.

The effect of NEC on the concentrations PC pathway proteins was assessed in an observational study. We report for the first time that activated protein C (APC) levels in healthy preterm infants are low; in keeping with the physiologically low levels of PC. In NEC the levels of PC, free and total PS are reduced. The levels of APC varied; some infants were able to boost levels 10-fold while others remained unchanged.
We studied the effect of APC and PC supplementation in an animal model of bowel ischaemia-reperfusion injury designed to simulate prophylaxis and treatment schedules. APC given after reperfusion reduced intestinal injury; PC had less effect.

The results presented in this thesis show that PC pathway activity is reduced in NEC and may contribute to the pathogenesis of the condition; introducing the possibility of new therapeutic options.
This is for Ivan and Dad, the men of my life, thank you.

Acknowledgements:

I would like to thank Mark Paters and Nigel Klein for their guidance, encouragement and support, and infinite, good-natured patience.

Many, many thanks to Heli Uronen-Hansson and Nigel Hall for the collaborative work on the animal model. Thanks also to Saul Faust, Virpi Smith, Nigel and Mark who have assisted as histology observers.

I am grateful to my ‘day-job’ intensive care colleagues who have been particularly supportive during the writing of this thesis.

And lastly, I wish to thank my husband Ivan, without whom this, quite literally, would not have been possible.
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Abbreviations

A2M  \( \alpha-2 \) Macroglobulin
AP  Alkaline phosphatase
APC  Activated protein C
AT  Anti-thrombin III
C4BP  C4b binding protein
CLP  Caecal ligation puncture
EC  Endothelial cell
eNOS  Endothelial nitric oxide synthase
ELBW  Extremely low birth weight (<1000g)
FFPE  Formalin fixed, paraffin embedded
IBD  Inflammatory bowel disease
Ig  Immunoglobulin
IL-  Interleukin
iNOS  Inducible nitric oxide synthase
HIER  Heat induced epitope retrieval
HMGB-1  High mobility group box protein - 1
LBW  Low birth weight (1501-2500g)
LPS  Lipopolysaccharide or endotoxin
MAPK  Mitogen activated protein kinase
MMP  Matrix metalloproteinase
MODS  Multiorgan dysfunction syndrome
MPO  Myeloperoxidase activity
NF\( \kappa \beta \)  Nuclear factor \( \kappa \beta \)
NEC  Necrotising enterocolitis
PAF  Platelet activating factor
PAR  Protease activated receptor
PBS  Phosphate buffered saline
PC  Protein C
PCI  Protein C inhibitor
PF1+2  Prothrombin fragment 1+2
PF4  Platelet factor 4
PIER  Protease induced epitope retrieval
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<td>PS</td>
<td>Protein S</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<td>SIRS</td>
<td>Systemic inflammatory response</td>
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<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
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<tr>
<td>TAT</td>
<td>Thrombin anti-thrombin complex</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>VLBW</td>
<td>Very low birth weight (&lt;1500g)</td>
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The protein C pathway in necrotising enterocolitis

Chapter 1: Introduction
Introduction

The past decade has been an exciting time to be an intensivist. We have witnessed the rise, and subsequent fall, of activated protein C (APC): the first licenced non-antimicrobial therapeutic agent in the treatment of severe sepsis. This story has been central to the maturation of critical thinking of intensivists.

We have a better understanding that dysfunctional complex systems require multi-modal therapies; there are no single, magic bullets, a shotgun or multi-modal approach may be more effective. We are beginning to understand the key role of the dysregulated endothelium in the development of multi organ dysfunction syndrome (MODS), a common feature of critical illness regardless of the initiating trigger. Supporting the endothelium may enable the host to endure the inflammation and immune paresis associated with critical illness\(^1\). And lastly we have a clearer understanding of the potential harm we can cause with intensive care support and how this can drive critical illness.

Intensivists have revisited their approach to clinical management in the light of this new knowledge and outcomes have improved in the last decade. For example, the ‘Surviving Sepsis campaign’ has reduced adult mortality from severe sepsis by 6% (comparable to the effect of APC in the first PROWESS study); an effect that ended the brief APC reign\(^2\). The withdrawal of the market licence for APC is fortunately not, however, the end of its story.

In 2001, the PROWESS trial in adults with severe sepsis showed a 6.1% absolute reduction in mortality with APC treatment. The trial was stopped early because treatment benefit was high, although there was concern about an increased rate of severe haemorrhage\(^3\).

A burst of intense research interest has followed; the annual Pubmed citation rate for APC has risen from under a hundred in the mid-1990’s to a steady 380 - 400 since 2003. In ten
years, basic science research has widened the profile of the protein C (PC) pathway, from primarily an anticoagulant system, to one with multifunctional effects: anticoagulant, anti-inflammatory, anti-apoptotic, cytoprotective and barrier preserving.

Clinical research into the utility of APC in diverse populations also followed licensing of the drug, and it is in this context that this project was undertaken.

Necrotising enterocolitis (NEC) is an inflammatory condition of the intestine in premature neonates that frequently leads to MODS, and not uncommonly, death. It is usually accompanied by thrombocytopenia and coagulopathy. It is a condition I face in my daily practice. It is frustrating that neither the treatment, nor the outcomes, of the condition have changed in 20-30 years. The similarities between the MODS seen in severe sepsis and NEC are striking and led to my interest in investigating the role of the PC pathway the pathogenesis of NEC.

This project began nearly 10 years ago. I mention this, not to bemoan the difficulties in undertaking and completing research in a full-time clinical job, but to place this project in the context of the subsequent discoveries that have emerged about this pathway. One could be concerned that the passage of time may have made this project irrelevant; but the opposite has happened. Discoveries about the functions of the PC pathway in stabilising the endothelial and epithelial barriers, in epithelial wound healing, in preventing apoptosis, in reducing inflammation and leucocyte infiltration, in neuroprotection and the roles in adult inflammatory bowel disease; have all served to make the investigation of the role of the PC pathway in NEC more relevant.

In this chapter I will review the current understanding of the pathogenesis of NEC and the multiple functions of the PC pathway. I will indicate areas where these overlap, in support
of, or evidence against, the concept of the PC pathway contributing to the pathogenesis of
NEC.

1. Necrotising enterocolitis

1.1. The burden of NEC: epidemiology, morbidity and mortality.

These paragraphs serve to emphasise that NEC is a growing public health concern with
important socioeconomic, morbidity and mortality consequences.

NEC occurs predominantly in premature infants. Preterm birth rates account for nearly 10%
of live births worldwide, with the rates being higher in less developed countries\(^4\). In 2005
there were 12.9 million preterm births globally. In the UK, in 2005, 7.6% of live births were
preterm (48 799 infants) and 6.7% of these were less than 28 weeks gestation (3285
infants)\(^5\). In some countries, like the USA, the rate of pre-term deliveries is increasing (10-
13%).

The intensive care of preterm neonates has advanced significantly over the last 20 years
particularly with the introduction of new therapies, most notably exogenous surfactant.
The survival rates of preterm infants have steadily improved as a result\(^6\)\(^\text{-}^9\). There are now
more very low birth weight (VLBW) and extremely low birth weight (ELBW) preterm infants
surviving to protracted neonatal intensive care courses, increasing the population of infants
at risk for NEC.

NEC is the most common cause of gastrointestinal surgical emergencies in neonates,
accounting for 1 - 7.5% of all neonatal intensive care unit admissions\(^10\)\(^\text{-}^18\). Population based
studies reveal the rate of NEC hospitalisation is 1-3/1000 live births in developed
countries\(^19\). In 2005, there were 645 700 live births in the UK\(^5\).
The incidence and mortality from NEC is inversely related to gestational age and birth weight\textsuperscript{10,19,20}. NEC occurs in 5-15% of the VLBW population, mortality ranges from 10 to 50% and this population has higher rates of surgery\textsuperscript{8,17,21}. Higher mortality is associated with surgically treated NEC, smaller and more immature infants, and in the USA, mortality is higher in the non-Hispanic black population\textsuperscript{19,22}. In the USA, 1 in 7 NEC hospitalisations were fatal in 2000 and in the UK, NEC accounted for 9.5% of neonatal intensive care unit (NICU) mortality\textsuperscript{10,19}. Despite improvements in outcomes for the VLBW population over time, there has been very little improvement in the outcomes of infants affected by NEC\textsuperscript{6,18,23}.

NEC progresses to a surgical intervention in 20-50% of cases and is more frequent in the more premature infants\textsuperscript{8,10}. The surgical care of these infants has not changed significantly in 20 years. Treatment options are discussed later.

The requirement for emergency abdominal surgery has other ‘hidden’ costs for these infants. In the UK, centralization of neonatal surgical services may dictate that these infants have to be transferred between hospitals at the time of worsening clinical condition, although this has not been associated with increased mortality\textsuperscript{24}. Anaesthesia, a prerequisite for surgery, in the neonatal population carries a risk of causing long-term neurodevelopmental sequelae\textsuperscript{25-27}. As discussed more fully below, infants with NEC that require surgery have poorer neurodevelopmental outcomes\textsuperscript{28,29}.

NEC survivors suffer ongoing morbidity. Forty to 47% of NEC survivors may suffer complications, many of which are postoperative, including stoma prolapse, stoma retraction, wound dehiscence and stricture formation\textsuperscript{30-34}. Approximately 10% of infants will suffer from a recurrent episode of NEC\textsuperscript{33}. Infants may require long-term (>90 days) total or partial parenteral nutrition (TPN or PPN) for relative or absolute short bowel syndrome. Long-term PN was has been reported in 21% - 43% of NEC survivors and is a known cause of liver dysfunction\textsuperscript{35,36}. NEC has become the leading cause of short bowel syndrome, affecting
8% of infants who survive the acute illness. Persistence of intestinal failure requires small bowel transplantation.

Perhaps the most important consequence of NEC is the association with adverse neurodevelopmental outcome. Systematic reviews show that infants with NEC are significantly more likely to develop neurodevelopmental impairment compared to infants of a similar gestation without NEC and the risk was 2.3 times higher in children with severe NEC or requiring surgery (figure 1.1). Hintz et al showed that infants with surgical NEC suffered significant growth delay and had adverse developmental outcomes at 18 – 22 months compared to controls. Blakely showed that only 50% of ELBW infants with surgical NEC survived, and 43% of these had neurodevelopmental impairment at 18 months. The poor neurodevelopmental outcome in infants with surgically treated NEC is not associated with intraventricular haemorrhage. The development of new, or the progression of previous intraventricular haemorrhage, is rare in infants with NEC. However, when it does occur, it is more likely to occur in the surgical cohort and it can have a poor prognosis.
Figure 1.1: Meta-analysis of neurodevelopmental impairment and NEC.
Forest plot of (A) Neurodevelopmental impairment in controls (No NEC) and NEC patients and (B) Neurodevelopmental impairment in medically- or surgically treated patients. n=number affected, N=total number, OR = odds ratio, CI = confidence interval. Reproduced from Rees CM. Arch Dis Child 2007;92:F193-198.

NEC significantly increases the length of hospital stay of infants. Median increases of 22 days for medical NEC and 60 days for surgical NEC have been calculated⁴⁷. The increased length of stay in intensive care exposes the infant to iatrogenic risks and nosocomial infection and generates a higher financial burden²².

In summary, the burden of NEC is significant; nearly half the infants with severe disease will not survive, and the long-term sequelae can be devastating on neurodevelopment and growth. The short-term and long-term morbidity is greater in the surgical cohort. The ability to modify and halt disease progression prior to requiring surgery would improve outcomes from this disease.
1.2. Inflammatory response: clinical features of NEC and current management

The pathogenesis of NEC, and its clinical features, are related to the interplay of immature intestinal function (motility, digestion, barrier, circulation, defence), immature host defence system with a reactive intestinal mucosa, abnormal colonisation, feeding and possibly genetic pre-disposition. The interplay of so many predisposing factors can result in an inflammatory response in the intestine that can spread to distant organs to become systemic\textsuperscript{48,50}.

The majority of neonates with NEC are premature and the incidence is inversely proportional to gestational age\textsuperscript{19,20}. NEC occurs less frequently in more mature neonates; in population based studies in Canada the incidence of NEC fell from 6.6% in VLBW neonates, to 0.7% in LBW and term infants\textsuperscript{14}. Clinically, the onset of NEC occurs earlier in the more mature infants, within a few days of delivery, but later in the premature infants at 10-21 days. It takes 2-3 weeks for bacterial colonisation of the intestinal tract in the preterm infants and this is a pre-requisite for NEC to develop\textsuperscript{51}. Mature infants are more likely to have an underlying co-morbidity; infection, patent ductus arteriosus or a period of perinatal asphyxia; possibly indicating a different pathophysiology\textsuperscript{52}.

The neonate with NEC is more likely to have been enterally fed (90-95%)\textsuperscript{48,53}.

NEC remains a clinical diagnosis based on symptoms, signs and radiographic findings. The modified Bell’s staging system uses clinical and radiographic features to describe severity (table 1.1)\textsuperscript{54}. Biomarkers of NEC are discussed later in this section.

Infants with NEC initially display non-specific clinical signs of inflammation such as lethargy, temperature and glucose instability, episodes of apnoea and bradycardia. These signs would not differentiate between neonatal sepsis and NEC. Physical signs of progressive
intestinal disturbance develop; feed intolerance, increased volumes of gastric aspirate that become bilious and abdominal distension due to the development of ileus. Rectal bleeding can occur as a result of mucosal damage and the abdomen becomes tender as generalised peritonitis evolves. The abdominal wall becomes shiny and discolouration can occur with cellulitis or internal haemorrhage. Abdominal masses can sometimes be palpable, usually in the right iliac fossa. Severe abdominal distension can cause abdominal compartment syndrome, with further restriction on microvascular perfusion.

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<th>Intestinal signs</th>
<th>Radiological signs</th>
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</tr>
<tr>
<td>I A</td>
<td>Temperature instability, apnoea, bradycardia</td>
<td>↑ gastric aspirates, mild abdominal distension, occult blood in stool</td>
<td>Normal or mild ileus</td>
</tr>
<tr>
<td>I B</td>
<td>Same as IA</td>
<td>IA and gross blood in stool</td>
<td>Same as IA</td>
</tr>
<tr>
<td>II – Definite NEC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II A</td>
<td>Mildly ill</td>
<td>Stage I and absent bowel sounds, abdominal tenderness</td>
<td>Ileus, pneumatosis intestinalis</td>
</tr>
<tr>
<td>II B</td>
<td>Moderately ill with mild metabolic acidosis, mild thrombocytopenia</td>
<td>Stage II A with definite abdominal tenderness, abdominal cellulitis, right lower quadrant mass</td>
<td>Same as II A, plus portal vein gas, with or without ascites</td>
</tr>
<tr>
<td>III – Advanced NEC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: Bowel intact</td>
<td>Severely ill. Stage II B with hypotension, bradycardia, respiratory acidosis, metabolic acidosis, DIC, neutropenia</td>
<td>Stage II B with signs of generalised peritonitis, marked tenderness and distension of abdomen.</td>
<td>Stage II B with definite ascites</td>
</tr>
<tr>
<td>B: Perforated bowel</td>
<td>Same as Stage III A</td>
<td>Same as Stage III A</td>
<td>Stage II B with pneumoperitoneum</td>
</tr>
</tbody>
</table>

*Table 1.1: Modified Bell’s staging criteria for NEC*.54

Many infants will develop a requirement for ventilation because of recurrent apnoeas or altered respiratory mechanics caused by abdominal distension as well as reduced lung compliance due to capillary leak in the lung parenchyma.53
Abdominal radiographs may initially be normal or show only mild distension of gas-filled, bowel loops, but this worsens as the disease progresses. Thickened bowel walls can be seen, particularly on ultrasound\textsuperscript{55,56}. Pneumatosis intestinalis is pathognomonic of NEC, but is not always present (37% in a recent series\textsuperscript{53}) and is caused by translocation of gas-producing bacteria into the intestinal wall. Portal venous gas may also be found, but is more rarely seen (12%)\textsuperscript{53}. Pneumoperitoneum on abdominal x-ray indicates intestinal perforation. As the condition progresses, the gas in the intestinal loops becomes replaced with fluid and the radiograph becomes gasless and featureless\textsuperscript{55}.

Infants with progressive NEC develop a systemic inflammatory response, and the majority of infants with advanced disease on Bell’s staging have multiple organ dysfunction (84%)\textsuperscript{57}. Capillary leak with the development of anasarca is a prominent feature of NEC associated MODS\textsuperscript{57-59}. Mechanical ventilation is frequently required to support the failing respiratory system. The need for inotropic support is a sign of progressive disease\textsuperscript{53}. Infants with 4 or more dysfunctional organ systems have high mortality\textsuperscript{57}.

\textbf{Figure 1.2:} Critically ill preterm neonate with severe NEC pre-surgery at our institution. The distended, discoloured abdomen is causing an abdominal compartment syndrome. The child is ventilated and on inotropic support. Courtesy of Mr J. Curry, Great Ormond Street Hospital.
Infants with NEC develop metabolic derangements as inflammation progresses; acidosis, lactic acidosis, hyponatraemia (Na <130mm/L), hyperglycaemia and hypoalbuminaemia. NEC can be accompanied by either an increased, or decreased, white blood cell count (WBC), however overall, there is usually an increase in the proportion of immature forms. Coagulopathy and a fall in platelet count are common features of NEC, thrombocytopenia is found in 65-90% of cases. Thrombocytopenia, neutropenia, disseminated intravascular coagulopathy and hyperglycaemia have been associated with poor outcome.

The mainstays of clinical management remain gastric decompression, bowel rest, antibiotic treatment, support of failing organs and surgery to remove non-viable bowel. Perforation is the only absolute indication for surgery; and deterioration despite optimised medical therapy is a common relative indication. Surgical management depends on the site and extent of disease. Multifocal disease often requires a temporary diverting stoma. Different surgical approaches do not appear to affect outcome. There are no current therapies that are disease modifying in the intestine, preventing progression to surgery.

1.2.1. Biomarkers of NEC

NEC is an inflammatory condition of the intestine and is accompanied by marked changes in the serum levels of immunoreactive molecules. Circulating endotoxin, tumour necrosis factor (TNFα) and IL-6 levels increase early in the disease process and are followed by the release of additional cytokines, chemokines and acute phase reactants. Platelet activating factor (PAF), TNFα, IL-6, IL-1β, IL-8, IL-12, IL-4, IL-10 have all been shown to be increased in NEC, often correlating with disease severity and contributing to the inflammatory stimulus.
The changes to cytokine, chemokine and acute phase reactant levels can be used as indicators or biomarkers of systemic inflammation, but they are unable to distinguish between sepsis and NEC. Unfortunately, there are currently no specific biomarkers that can reliably diagnose early NEC with assays that are readily available to clinicians at the bedside. However there are a few promising potential candidates on the horizon that are discussed later.\textsuperscript{72,79-82}

C-reactive protein (CRP) is a non-specific, acute phase reactant that increases in NEC, but levels are unable to differentiate NEC from neonatal sepsis with ileus. Serial measurements of CRP are useful to monitor disease progress, as levels should fall with improvement in intestinal inflammation.\textsuperscript{83,84} Serum amyloid A (SAA) is another acute phase reactant that increases in NEC. The severity of NEC is associated with higher levels of SAA\textsuperscript{85,86}. The combination of raised CRP and SAA may be more effective in the diagnosis of NEC than the individual levels alone.\textsuperscript{87} The levels of procalcitonin (PCT) in NEC seem to be variably affected in case series reports.\textsuperscript{87,88} The complement activation product C5a (anaphylatoxin) is increased in NEC and levels correlate with severity.\textsuperscript{89} The assays for CRP, PCT, and SAA are commonly available to frontline clinicians.

Interleukin-6, neutrophil CD64 (as well as SAA) levels rise early with systemic inflammation while C-reactive protein (CRP) levels increase approximately 6 - 8 hours later. IL-6 levels also fall early as inflammation resolves and therefore the peak may be missed, while neutrophil CD64 levels remain elevated for more than 24 hours.\textsuperscript{90} IL-6 assays are not universally readily available and neutrophil CD64 determination by flow cytometry remains a research tool for most centres.

Research is ongoing to find a specific biomarker for the early diagnosis of NEC that will enable earlier intervention. Potential biomarkers specific to NEC tend to be related to the intestinal injury rather than the inflammatory cascade; distinguishing NEC from neonatal
sepsis. The most promising potential biomarkers for NEC currently include intestinal fatty acid binding protein (I-FABP), faecal calprotectin, claudin 3 and exhaled hydrogen levels. These markers have been identified in a number of small studies; validation in larger cohorts is required to confirm their predictive value.

Intestinal fatty acid binding protein (I-FABP) is a small, water-soluble protein released from enterocytes injured by NEC. It has a short half-life in plasma and is present in the urine. Plasma and urinary levels of I-FABP have been shown to increase in NEC and the urinary level, expressed as a ratio with creatinine, has been found to be predictive of severity of disease\textsuperscript{91-95,96}. The marker is of limited value in infants with renal failure. Currently the investigation is hampered with the requirement for collecting timed volumes of urine; a task that can be difficult for a number of reasons in critically ill neonates often nursed under radiant heaters.

Calprotectin is a protein that constitutes 30-40\% of the cytoplasm in neutrophils and can be measured in faeces. Faecal calprotectin is increased in NEC due to neutrophil infiltration of the inflamed intestine. The value of this marker is reduced in patients with ileus; which can occur in neonatal sepsis as well as NEC\textsuperscript{97-102}.

Platelet activating factor is raised in plasma and faeces in the course of NEC, however further work is needed to explore its potential as a biomarker\textsuperscript{72,74,103,104}.

Claudin-3 is a membrane protein integral to epithelial tight junctions. Urinary levels of claudin-3 are increased in NEC as tight junctions are destroyed and may serve as a biomarker for NEC, but may be of limited value in infant with renal failure\textsuperscript{102}.

Bacterial fermentation of carbohydrates produces hydrogen gas (H\textsubscript{2}), which is exhaled in the breath. In NEC, the concentration of H\textsubscript{2} increases early and significantly in infants with
NEC, enabling early diagnosis before clinical signs develop. Unfortunately it is technically very difficult to measure H₂ and therefore is not applicable outside of the research setting.\(^{105}\)

Emerging new molecular techniques will enable the discovery of novel biomarkers. Mass screening methods, including microarray and proteomic technologies have revealed the changes in the proteome associated with NEC, providing insight to NEC pathogenesis as well as identifying potential biomarkers.\(^{106}\) Genomic technology has enabled the discovery of how gene-expression patterns are altered by disease. In NEC, 26 proteins have been found to be up-regulated; their functions were most commonly related to the inflammatory cascade (pro and anti), cell growth, angiogenesis, wound healing, cell adhesion, anti-apoptosis and extracellular matrix organisation.\(^{107}\)

1.2.2. **Neurodevelopmental effects of NEC**

I have previously mentioned the association between NEC with poor neurodevelopmental outcomes; increased risk of cerebral palsy, cognitive impairment, psychomotor impairment and severe visual impairment. These risks increased with severity of illness and were greatest in infants that required surgery for NEC.\(^{28,29}\) Assessment of the ORACLE study participants at 7 years of age using the Health Utilities Index -3 revealed that children who had suffered NEC as a neonate were more likely to have ongoing functional impairment (OR 1.55 [95% CI 1.05-2.29]).\(^{108}\)

Neurodevelopmental impairment is also associated with other systemic inflammatory conditions in preterm neonates; most notably neonatal sepsis, either in the early or late postnatal phase.\(^{109-111}\) Chorioamnionitis, whether histological or clinically overt, is associated with periventricular leukomalacia (PVL) and cerebral palsy (clinical chorioamnionitis only).\(^{112}\) Some observational studies include NEC as a category of
postnatal sepsis, in the examination of the effect on systemic inflammation on longer-term neurological outcomes. In a large cohort of ELBW neonates, clinical infection, sepsis with or without NEC and meningitis were all associated with cerebral palsy, psychomotor or cognitive impairment and severe visual impairment at 18-22 month follow-up. Neonates in these groups were more likely to have a head circumference below the 10th centile for age, but only those with sepsis, with or without NEC, were also more likely to have poor growth (weights below the 10th centile). Children with surgical NEC, with or without sepsis, had increased rates of neurodevelopmental impairment as compared to the medical NEC or NoNEC groups in the ELGAN study.

Structurally, neonatal sepsis and NEC have been associated with white matter abnormality on magnetic resonance imaging (MRI), coupled with delayed psychomotor development. Neonates with a history of NEC, rather than neonatal sepsis, were also more likely to have additional grey matter abnormalities (delayed cortical folding and increased subarachnoid space). As previously mentioned it is rare for patients with NEC to develop, or extend, an intra-ventricular haemorrhage, but is a poor prognostic indicator if it does occur.

The pathogenesis of the brain injury associated with systemic inflammation, and NEC in particular, is not fully understood in the preterm infant. The fact that brain injury does not occur in every case of systemic or CNS inflammation, or to the same degree in every child with similar disease severity, suggests a complex mechanism influenced by numerous variables. The hypothesis of the pathogenesis of inflammation-associated, neonatal brain injury is evolving; but key elements include:

- The central nervous system is an immune privileged site maintained by an intact blood-brain barrier (BBB), except in the circumventricular organs where a more permeable BBB allows their function. These areas preferentially express toll-like
receptor 4 (TLR 4) enabling endotoxin to induce an inflammatory response. Microglia and perivascular macrophages are key components of the innate immunity in the central nervous system (CNS) and these cells propagate inflammatory signals into the CNS on activation\textsuperscript{121}.

- Inflammatory triggers generate a response on interaction with pattern recognition molecules, either systemically or within the CNS, resulting in the production of inflammatory mediators. Examples in NEC are endotoxin, or histones released from necrotic and apoptotic cells that will generate an increase in TNF\(\alpha\), IL-1, IL-6, IL-8 systemically.

- Systemic cytokines can act directly on the CNS to induce metabolic and neuroendocrine responses; for example IL-1 and TNF\(\alpha\) induce fever

- Endotoxin and pro-inflammatory mediators also disrupt the blood brain barrier making it more permeable and allows the extension of inflammation beyond the barrier. The disruption of the BBB allows cross-talk between the CNS and systemic compartments modifying the inflammatory response.

- Activated microglia release cytokines, trophic factors and other mediators. They target infectious agents and clear debris. It is postulated that stimulated microglia may have different activation states: pro-inflammatory classical activation, an alternative activation state that is anti-inflammatory directed towards repair and an acquired deactivation state that is associated with immunosuppression and phagocytosis of apoptosed cells. Dysregulation of these states could induce pathology; for example increased destruction could be linked to classical activation dominance while chronic inflammation could be linked to alternative activation. Activated microglia effect many functions and pathways in the CNS; neuronal and glial proliferation and apoptosis, oligodendrocyte precursor depletion, axonal
damage, dendritic structure abnormalities, leading to disordered white matter tracts and reduced ‘connectivity’

- CNS inflammation can lead to altered vulnerability to additional insults, either sensitisation or pre-conditioning
- Host genetic factors that may influence immune-inflammatory response\textsuperscript{122-124}.
- CNS inflammation could be potentiated by systemic insults that occur in in a critically ill neonates in intensive care; hypotension, hypoxia, hypoglycaemia.
- All these factors can alter brain development in the short and potentially, long term\textsuperscript{117,119}.

As previously mentioned, NEC is associated with altered plasma levels of pro and anti-inflammatory cytokines, indicating a systemic inflammatory response and these have been linked to neurodevelopmental sequelae. The ELGAN study group found that in the first 14 days, neonates with IVH, white matter injury or both had increased levels of CRP, IL-8, TNF\textgreek{a} and SAA\textsuperscript{125}. This group also found that sustained increases were also associated with impaired motor and cognitive development at 2 years of age\textsuperscript{126}. Lodha et al found an association between high levels of TNF\textgreek{a}, IL-6 and IL-8 in neonates with NEC and severe cognitive and motor delay. Half of the NEC cohort also had abnormal cranial ultrasound scans (CUSS) as compared to 8% in the group without NEC\textsuperscript{43}. Silveria et al however, did not find an association between cytokine levels and developmental outcomes once confounding factors were controlled\textsuperscript{127}.

Anaesthesia is an additional factor that may influence the development of brain injury in neonates with NEC. Infants with NEC that require surgery have an increased likelihood of adverse neurological outcomes compared to the conservatively managed group. Anaesthesia is a prerequisite for surgery, and there are concerns that it may be an independent risk factor for adverse neurological outcomes in preterm neonates\textsuperscript{25-27,128}.
Preclinical studies have shown a vulnerability of the primate brain to anaesthesia-induced apoptosis, and the possible potentiating effect of inflammation\textsuperscript{128}. Clinical studies in humans have been heterogeneous in design to date, but have not reliably shown an association between anaesthesia and adverse neurodevelopmental outcomes. Nevertheless, robust clinical studies are underway to answer this important question\textsuperscript{128}.

To summarise, important clinical features of NEC include a pro-inflammatory milieu, ileus with oedematous, thickened bowel walls, the translocation of bacteria into the intestinal tissue (pneumatosis), and the development of a systemic capillary leak syndrome with multiple organ dysfunction. The condition has a yet-to-be defined and dose-dependent effect on the central nervous system.

1.3. Pathological features of NEC: ischaemia, inflammation, microvascular thrombosis.

The predominant anatomic lesion of NEC is coagulative or ischaemic necrosis of the intestine, most commonly involving the distal ileum and ileocolic region, that is also the most distant point of the mesenteric vascular tree. The intestinal involvement can be continuous or discontinuous, resulting in focal, multifocal or pan-intestine disease. In about half of the cases necrosis involves both small and large intestine. The macroscopic appearance of the intestine depends on the severity of the disease; mildly affected areas can appear almost normal. In more severe disease the bowel wall is friable, may have dark areas of discolouration containing haemorrhage and pneumatosis may be visible\textsuperscript{129-131}.

The hallmarks of NEC on histological examination of resected bowel include coagulative necrosis, inflammatory cell infiltrate, mucosal oedema and ulceration. These features are similar to those found in the intestine of adult patients with MODS, characterised by widespread microvascular thrombosis and coagulative necrosis. The earliest histological
feature of NEC is intense congestion of the superficial mucosa, focal necrosis of villous tips and epithelial sloughing into the bowel lumen. This is similar to the injury seen with ischaemia-reperfusion injury (IR). Capillary microthrombi and thrombi in larger submucosal or serosal veins are common\textsuperscript{129-131}.

There is a marked inflammatory cell infiltrate, migration into the tissue that is aided by the endothelial expression of adherence molecules\textsuperscript{132}. However, micro-abscesses and crypt abscesses, characteristic of infectious colitis, are only present in 10% of patients with NEC.

### 1.4. The pathophysiology of NEC.

The pathophysiology of NEC is complex and still not completely understood. Nevertheless, current hypotheses agree that there is a crucial interplay between the immaturity of intestinal function, the immaturity of the host defence responses and abnormal bacterial colonisation. Most theories propose that varied insults (enteral feeds, infection, ischaemia) contribute to epithelial disruption in the immature intestine with subsequent bacterial translocation triggering an inappropriate over-stimulation of inflammation and over-suppression of inhibitory pathways by the immature epithelium, endothelium and innate immune system. Inflammation is amplified, causing intestinal damage and harmful systemic effects.

In support of these theories, the main risk factors for NEC remain prematurity and bacterial colonisation. NEC occurs almost exclusively in the premature neonate, and as previously mentioned, the epidemiological features of the disease in term infants suggests a separate pathogenesis\textsuperscript{52}. 
The following features contribute, and are additive, to the overall vulnerability of the premature infant to breaches in the epithelial barrier, development of an exaggerated inflammatory response and the development of NEC.

1.4.1. Bacterial colonisation

Classically, the role of infection in the pathogenesis of NEC, while considered important, was not thought to be central, as only 60-65% of patients had positive blood cultures. Nevertheless antibiotic therapy has remained a part of standard treatment.

Evidence now supports the tenet that bacterial colonisation of the intestine is necessary to develop NEC. In utero, the intestinal tract is sterile and intestinal ischaemic events result in atresia, not NEC. Outbreaks of NEC have been reported in NICUs, related to nosocomial, transmitted infections. In the premature neonate, NEC commonly occurs 10-14 days after birth, after bacterial colonisation of the intestinal tract has occurred. Fast et al showed that prophylactic oral gentamycin reduced the incidence of NEC. Lastly, NEC did not occur in germ-free animal models, even when fed formula milk in utero or after birth in a sterile manner.

After birth, in healthy term infants, the intestine becomes colonised in a predictable series of phases known as succession. During the first 2 weeks (phase I) coliforms and Streptococci predominate, but during the next phase of colonisation, commensal obligate anaerobes become established, such as Bifidobacteria. The third phase only begins once the child weans onto solid food and the final phase matches the flora of the adult. During the first phase, the relative lack of anaerobes in the flora leaves the newborn vulnerable to the overgrowth of pathogenic bacteria. Breast milk promotes the establishment of commensal bacteria, particularly Bifidobacteria and Lactobacillus species, usually detectable within the first week in term infants. The colonisation process is prolonged in preterm neonates and
disrupted by the nosocomial bacterial strains that predominate in the hospital environment; the invasive procedures preterm neonates endure and repeated antibiotic courses. This results in a reduced diversity of bacterial species in the intestinal ‘microecology’ (only 2-5 species), allowing an opportunity for overgrowth of pathogenic species; and these features are associated with NEC. NEC has not been linked to a single bacterial species, but *Clostridia, Enterobactericeae* including *Klebsiella, Escherichia coli* and *Staphylococci* are commonly found. NEC has also been associated with viral infections\textsuperscript{136,137}.

The central role played by bacteria in the pathogenesis of NEC may influence the signs and symptoms of the clinical condition; aligning it more closely with severe sepsis and presents opportunities to consider therapeutic modalities that have been successful in sepsis.

1.4.2. Exaggerated inflammatory response: TLR signalling, PAF, NO, EGF, IL-10.

Bacteria interact with host cells using pathogen-associated molecular patterns (PAMPs) that are recognised by pattern recognition receptors (PRR). Toll-like receptors are classical PRR; TLR-4 interacts with endotoxin or lipopolysaccharide (LPS) on gram-negative bacteria and TLR-2 interacts with lipoteichoic acid on gram-positive bacteria. Interaction results in intracellular signalling that activates nuclear factor-κβ (NFκβ) and mitogen-activated protein kinase (MAPK) p38 pathways with the subsequent transcription of a myriad of downstream inflammatory cytokines, chemokines, adhesion molecules and metalloproteinases (MMP), including tumour necrosis factor-α (TNFα), interleukins IL-1β, IL-6, IL-8, inducible nitric synthase (iNOS), MMP-9\textsuperscript{138}.

Adult enterocytes express very little TLR-4 although it is present in the cytoplasm and can be transported to the surface if required. Fetal enterocytes have abundant expression of TLR-4 and TLR-2; they are believed to have an in-utero developmental role in dorsal/ventral cellular orientation. After birth, during bacterial colonisation, the maturing enterocyte is
suddenly exposed to large quantities of PAMPs. This should result in an inflammatory response, but term infants develop tolerance to TLR signalling by down regulation of TLR-4 surface expression, down regulation of the intracellular signalling molecules and up regulation of the intracellular signal inhibitors. The regulation of the process by which enteocytes develop tolerance to bacterial colonisation in the postnatal period of is not yet understood\textsuperscript{19}.

As an aside, the PC pathway is known to mediate anti-inflammatory responses by the down regulation of NFκβ and MAPK p38 signalling, as detailed later in this chapter.

Studies in enterocyte cell lines and xenographs have demonstrated that the protective mechanisms of tolerance development are deficient in preterm infants, and particularly in infants with NEC. TLR 4 and TLR2 are overexpressed in premature enterocytes and inhibitory signalling pathways are down regulated. The levels of Iκβ, the inhibitor of NFκβ, are also developmentally regulated, with low levels seen in prematurity. The xenographs of immature intestine had exaggerated cytokine production in response to stimulation by LPS or IL-1β.\textsuperscript{140,141} In a rat model, enterocyte TLR-4 expression was also increased by hypoxia and formula feeds in rat pups, amplifying the inflammatory response even further. TLR-4 expression was reduced by breast milk feeds\textsuperscript{142}. This evidence suggests that the over-expression of TLR-4 and the immaturity of the mechanisms to develop tolerance may be key in the pathogenesis of NEC in preterm infants\textsuperscript{143}.

Platelet activating factor (PAF) is a phospholipid produced by endothelia, platelets, inflammatory cells and bacteria. PAF, on interaction with its receptor (PAFr), is able to induce epithelial necrosis, apoptosis, leucocyte and platelet aggregation and vascular permeability. PAF is cleared by PAF-acetylhydrolase (PAF-AH), which is also present in breast milk, signifying the importance of this pathway in the newborn. PAFr’s are
abundantly expressed in the ileum; less so in the jejunum (56% ileal expression) and are minimally expressed outside the intestinal tract (heart, lung and kidney have <1% ileal expression)\textsuperscript{144}. PAF is able to increase the expression of TLR-4 by epithelial cells, amplifying the inflammatory response\textsuperscript{145}. The increased expression in the intestine underscores the role of PAF in intestinal inflammatory conditions.

Neonates with NEC have high levels of plasma PAF and low levels of PAF-AH\textsuperscript{74}. The ileum is the area of bowel most frequently affected by NEC. Animal models have shown that blocking PAFr or supplementing enteral PAF-AH prevented NEC\textsuperscript{146}. The evidence suggests a central role for PAF in the pathogenesis of NEC.

EPCR, a receptor for PC and APC, binds with phospholipids, commonly phosphatidylcholine. Phosphatidylcholine can be exchanged for PAF which impairs the ability of EPCR to bind to PC\textsuperscript{147}. This may be of relevance in NEC.

Nitric oxide (NO) has a dual role in the pathogenesis of NEC. In health, it is constitutively produced in low levels by endothelial nitric oxide synthase (eNOS) in the intestine and plays a vital role in the vascular tone of the microcirculation, maintaining perfusion by vasodilation. APC is known to up-regulate the transcription of eNOS\textsuperscript{148}.

However, there is a sustained increase in NO production in inflammatory conditions, including NEC, by the up-regulated expression of inducible nitric oxide synthase (iNOS). High levels of NO, and its intermediary peroxynitrate, are cytotoxic causing direct intestinal epithelial injury and epithelial cell apoptosis. NO also prevents epithelial restitution; the process of proliferation and migration of epithelial whereby apoptotic or injured are replaced to maintain the integrity of the barrier\textsuperscript{149-151}. Enhanced NO production is believed to be important in the pathogenesis of NEC.
Epithelial Growth Factor (EGF) and heparin-bound EGF (HB-EGF) are trophic factors secreted in amniotic fluid, human milk and saliva; and low levels have been found in NEC. EGF is important in epithelial barrier repair, promoting epithelial cell proliferation, migration and preventing apoptosis. Administration of EGF in animal models of NEC reduces the severity of injury and epithelial apoptosis. Interestingly, despite EGF depletion, the EGF receptor (EGF-R) expression was preserved in the intestine of infants with NEC; while this would allow exogenous EGF to function, but may suggest other roles for the receptor in EGF-deficient states. APC has been found to promote tight junction stability in keratinocytes, via transactivation of EGF-R.

IL-10 is an anti-inflammatory cytokine, produced by Th2 lymphocytes to balance the pro-inflammatory host response. The role of IL-10 in NEC is not clear as high levels are found in NEC. However, IL-10 knockout mice are prone to developing inflammatory colitis. In a mouse model, iNOS levels were increased in NEC, but intraperitoneal IL-10 suppressed iNOS production and improved outcome. IL-10 is present in breast milk, suggesting an anti-inflammatory role in the neonate. IL-10 has also been shown to reduce the production of metalloproteinases; responsible for tissue destruction as well as the cleavage of cell surface receptors like TM. APC is able to increase IL-10 production in monocytes.

High mobility group protein box 1 (HMGP-1) binds to DNA released from dying or injured cells. It is a potent inflammatory mediator produced late in the host response to LPS. HMGP-1 levels are increased in the intestine of infants with NEC, and in animal models, inhibitors of HMGP-1 reduced the intestinal injury. APC has been shown to inhibit the production of, and signalling of, HMGB-1. HMGP-1 also prevents the migration of enterocytes during the restitution process in a TLR-4 dependent manner, delaying mucosal repair. This is specific to enterocytes as HMGP-1 has no effect on the migration of
inflammatory cells\textsuperscript{163}. APC, a protease, is able to cleave extracellular histones, as detailed later in this chapter.

These paragraphs highlight some important mechanisms that contribute to the exaggerated inflammatory host response in the pathogenesis of NEC; but these are also mechanisms influenced by the counter-regulatory PC pathway. This is not intended as an exhaustive list, other mediators are also involved, for example the cyclooxygenase-2 (COX-2), IL-8, IL-6, IL-12, IL-18.

1.4.3. The role of commensal bacteria in intestinal maturation and homeostasis

The symbiotic relationship between host and commensal bacteria is important for the development and homeostasis of the intestine. Commensal bacteria are able to regulate gene transcription in relation to tight junction proteins in the epithelium to promote barrier stability, angiogenesis in the villi and digestive enzyme secretion to improve digestion.

Commensal bacteria block NFκβ transcription to dampen inflammatory signals. Commensal bacteria also enhance intestinal motility. As mentioned previously, preterm infants have a poor diversity of intestinal flora and a higher proportion of pathogenic strains. The loss of the symbiotic interactions mentioned increases the risk of NEC\textsuperscript{49,143,164,165}.

Breast milk promotes the diversification of intestinal flora and the establishment of anaerobic commensal species, \textit{Bifidobacteria} and \textit{Lactobacilli}. The beneficial effects of commensal flora have promoted a flurry of research into the effects of probiotics (and pre- and postbiotics) in the prevention of NEC in preterm infants. Probiotics have been shown to reduce the risk of NEC and improve outcomes but there is little consensus on which probiotic formulation or dosing schedule should be used\textsuperscript{166-168}. 
1.4.4. Immaturity of intestinal functions

Almost every function of the intestine is immature in the preterm neonate and contributes to the vulnerability of the epithelium to injury and NEC\textsuperscript{49,169}.

A number of features extrinsic to the epithelium contribute to its protection, including intestinal motility, mucin production, luminal secretion of antimicrobial peptides, digestive enzyme secretion and gastric acidity.

Gastric acidity is protective; but it is developmentally reduced in the preterm neonate. \( \text{H}_2 \)-blocker antacid medication is associated with an increased risk of NEC\textsuperscript{20,170}. The production of mucin matures by 27 weeks, in quantity and consistency; commensal bacteria accelerate this process. The production of antimicrobial peptides by the immature intestine is reduced, including immunoglobulin A (IgA)\textsuperscript{49,165,171}.

Peristalsis in the immature intestine does not mature until into the 3rd trimester as the migratory motor complexes only appear after 34 weeks gestation\textsuperscript{172}. This results in stasis of luminal contents providing the opportunity for bacterial overgrowth and prolonged contact of noxious substances with the epithelium.

The immature intestine has decreased secretory, digestive and absorptive functions before 26-28 weeks and this may contribute to the build up of harmful substances, like short chain fatty acids, that can damage the epithelium\textsuperscript{169,173}.

The intrinsic, physical barrier of the epithelium is maintained by the tight junctions between adjacent epithelial cells, by the selective restriction of antigen movement through the polarised epithelial cells and lastly, by the restitution process that maintains the integrity of the epithelial layer by ensuring that apoptotic or injured cells are replaced.
All of these functions are reduced in the premature intestine; antigen transport is less
restricted and the barrier is more permeable than in adults. In animal models of NEC, the
barrier functions were further eroded as evidenced by a reduction in tight junction
proteins, increased permeability of the epithelium and reduced mucin production. The
epithelial barrier is thought to be regulated by prostaglandins, epithelial growth factor
(EGF) and nitric oxide (NO). As previously mentioned, NO prevents epithelial restitution,
while EGF and prostaglandins promote it. This may explain the association of intestinal
perforation in premature neonates treated with non-steroidal anti-inflammatory drugs to
close the patent ductus arteriosus.

APC has been shown to have membrane stabilising effects, increasing tight junction
proteins and reducing barrier permeability; both in the endothelium and intestinal
epithelium. This will be described more fully later in this chapter.

1.4.5. Intestinal epithelial susceptibility to apoptosis

Epithelial apoptosis is a common feature on histological examination of intestine affected
by NEC and it is visible early in the development of the condition. Apoptosis can be
induced by TLR-4 activation and this has been shown to progress to NEC in animal studies
As already mentioned, the proliferation and healing of the epithelium is reduced in NEC
by increased NO levels, TLR-4 and reduced EGF levels.

APC has been shown to reduce apoptosis by altering gene transcription to balance the
Bax/Bcl-2 pathways. APC has been shown to aid wound healing by preventing
intestinal epithelial apoptosis, promoting angiogenesis and epithelial proliferation and
migration.
1.4.6. Immature intestinal vascular control

Perturbations in the perfusion of the intestine were believed to be central to the pathogenesis of NEC. However, patchy ischaemia as a terminal inflammatory event caused by occlusion and thrombosis of the microcirculation, provides a better explanation for the patchy, multifocal lesions of NEC. Consumptive thrombocytopenia and coagulopathy are common clinical features of NEC that indicate thrombin generation in the microcirculation.

The control of intestinal perfusion in the preterm infant is immature; the blood flow at rest is near to maximum, leaving little ability to increase blood flow following feeds. The mucosal capillary network is most affected. This places the infants at risk of intestinal ischaemia, particularly the tips of the villi, during enteral feeds.\textsuperscript{182,183}

The PC pathway is a key mediator in the maintenance of the anticoagulant and anti-inflammatory phenotype of the endothelium in the microcirculation.

1.4.7. Breast milk feeds.

Many preterm infants do not tolerate enteral feeds in the first days of life. Current recommendations are to give trophic feeds as a minimum. Amniotic fluid, colostrum and breast milk all contain a wealth of trophic and defence factors, including EGF, PAF-AH, transforming growth factor-β (TGFβ), IL-10, and immunoglobulin A.\textsuperscript{159,184} Breast milk, even at low levels, promotes the maturation of the immature intestine; increasing villous height, reducing permeability and enzyme secretion. Breast milk protects against NEC in a dose dependent manner.\textsuperscript{185}
1.4.8. Genetic susceptibility

Polymorphisms or mutations in genes related to circulation regulation that predispose to the development of NEC, have been rarely reported; including vascular endothelial growth factor (VEGF) and the urea cycle enzymes involved in the production of arginine, the precursor of NO. Most cytokine-related genetic polymorphisms are not associated with NEC, except IL-18 (increased severity) and IL-4 (protective)\(^49\). There are no reports of NEC in infants with PC pathway mutations.

To summarise, NEC is an important cause of neonatal morbidity and mortality. It has features in common with severe sepsis, including progression to coagulopathy, shock and multiple organ dysfunction. NEC is caused by a cascade of multiple pro-inflammatory and pro-thrombotic mediators. Many of these mechanisms are counter-regulated by the PC pathway.

Having described the myriad inflammatory mediators in NEC, it is important to explain why I chose to concentrate on the role of the PC pathway in the pathogenesis of NEC. There were several reasons and I have discussed them in detail in the next section. In short, several NEC characteristics pointed towards dysregulation of the anti-coagulant PC pathway: consumptive coagulopathy is a key feature of the clinical disease and the histological injury to the bowel includes widespread thrombosis of the intestinal microvasculature. Acquired PC deficiency had also been repeatedly described in other populations with systemic inflammatory conditions and was consistently associated with adverse outcomes. Preterm neonates have lower levels of PC in health and I wondered if this relative PC deficiency made preterm neonates vulnerable to NEC and sustained thrombotic challenge. PC levels in NEC had not been previously reported. Lastly, PC concentrate is available as a therapeutic option if my project revealed a significant role for the pathway in the pathogenesis of NEC.
2. The Protein C Pathway.

The following pages will summarise our current understanding of the functions of the PC pathway and the mechanisms by which it achieves these actions. The subject is vast, and I have concentrated on those elements potentially relevant to the pathogenesis of NEC.

I have included:

- A summary of our current understanding of endothelial cell functions at rest and during inflammation and the role of the PC pathway in these changes. Endothelial dysfunction with tissue oedema is a constant and early feature of NEC.
- A description of the effector molecules and receptors involved in the PC pathway: thrombin, protein C (PC) and activated protein C (APC), protein S (PS), thrombomodulin (TM), endothelial protein C receptor (EPCR). A number of ‘basic science’ biological facts that may be relevant in the preterm neonate developmentally deficient in PC and also important in considering the effects of PC pathway supplementation.
- The functions of the PC pathway and the mechanisms by which they are achieved:
  - Activation of PC
  - Anticoagulant functions of the PC pathway
  - Anti-inflammatory, anti-apoptotic, barrier stabilising functions
- A description of conditions associated with PC deficiency relevant to this project and discussion of treatment recommendations
  - Congenital PC deficiency
  - Physiological PC deficiency of infancy and childhood
  - PC deficiency associated with inflammatory states, focusing on severe sepsis, critical illness and inflammatory bowel disease
The PC pathway is pivotal in microvascular homeostasis, maintaining the anti-thrombotic and anti-inflammatory endothelial phenotype at rest and limiting the host microvascular inflammatory response to infection, danger and damage. The importance of endothelial dysfunction in the pathogenesis of MODS is becoming increasingly recognised.

The significance of the role of the PC pathway is evident in patients with congenital abnormalities of the system; homozygous PC deficiency is a lethal condition of microvascular, venous and arterial thrombosis that responds to treatment with PC replacement.

Inflammation and coagulation go hand-in-hand. Most of the cellular and humoral modulators of each system participate in both processes, enabling cross-talk, amplification and regulation. This implies, in evolutionary terms, that coagulation became integral to the innate immune response to signals of danger, damage and infection.

The natural anti-coagulants have evolved functions that limit inflammation to assist in their anticoagulant role; and conversely, inflammatory stimuli down regulate the anticoagulants in order to boost the inflammatory response.

The protein C pathway is the main anticoagulant system of the body. Thrombin is the only known physiological activator of PC, highlighting the importance of the pathway in regulating the host response to inflammatory stimuli that result in thrombin generation.

The PC pathway has a number of highly significant anti-inflammatory effects, designed to keep inflammatory host responses in check. APC production and functions are regulated on the vast endothelial cell surface; emphasising the role of the pathway in the localised response within the microvasculature.

2.1. The key components of the PC pathway
2.1.1. The endothelium

The endothelium covers the entire vasculature, forming a vast lining to a blood filled container. Under resting conditions this lining promotes the fluidity of blood, preventing intravascular coagulation and the leakage of fluid and cells and it regulates perfusion of the surrounding tissue. The PC pathway is a key intermediary in the promotion of this phenotype.

The surface area of the endothelium is immense, estimated at 3000-7000 m² in adults; consists of a single cell layer and is estimated to weigh only 720g. The majority of endothelial cells (EC) are found in the microvasculature (~600g). Blood-endothelial interactions can only occur if small volumes of blood are exposed to a large surface area; the ratio of surface area to blood volume is 100- to 500-fold less in arteries and veins as compared to capillaries (figure 1.3) 186-188.

APC is ubiquitously produced by, and carries out its main functions on, the entire surface of microvascular endothelial cells (EC) throughout every organ of the body; the area is vast, emphasising the importance of the PC pathway in maintaining microvascular homeostasis and regulating host response to inflammation.
2.1.1.1. \textbf{Endothelial functions:}

All ECs share similar general functions, but specialised adaptations occur depending on the blood vessel type and size (capillary, vein, artery) and the type of tissue or organ involved (for example pulmonary, intestinal, brain cortex).

Traditionally, EC are described as two phenotypes, the resting and activated states (figure 1.4), but this is an oversimplification. Firstly there is a spectrum of ‘activation’ and secondly, the functions of ECs are heterogeneous and site-specific, so that functions associated with ‘activation’ in one site may be the equivalent of ‘resting’ at another. For example, intestinal ECs have increased permeability at rest that would be considered a feature of activation in lung or brain ECs. Nevertheless; the ability to change function in response to the microenvironment, in a dose-dependent manner, is an essential function of ECs and underlies the concept of “activation”\textsuperscript{188,189}. The PC pathway is involved in mediating these effects as well as being affected by them.

At ‘rest’ the endothelium maintains the fluidity of blood and tissue perfusion:

- The endothelium separates circulating blood from the sub-endothelial tissues that express tissue factor (TF). TF is a potent stimulus for thrombin generation via factor
FVII. ECs at rest express tissue factor pathway inhibitor (TFPI) which rapidly inhibits and clears TF-VIIa \(^{190}\).

- Endothelial cells prevent intravascular thrombus formation and platelet aggregation using the three anticoagulant systems; TFPI, anti-thrombin III (AT) with heparan co-factor II (HCII) and the PC pathway. ECs synthesize and express TM and EPCR. The endothelium produces tissue plasminogen activator (tPA) to lyse fibrin.

- The endothelium is a barrier with an active glycocalyx and regulated tight junctions between adjacent cells that control the paracellular movement of fluid, solutes, macromolecules and cells\(^{191,192}\).

- Endothelial cells at rest prevent the adherence and migration of leucocytes.

- Endothelial cells regulate local perfusion by producing vasoactive substances like NO and endothelin-1 (ET-1)

Endothelial cells are activated by direct injury to the vessel exposing TF to the circulation, or as part of the host innate inflammatory response. Activated ECs promote clot formation to limit haemorrhage from damaged vessels or the spread of microbes. Activated ECs vasodilate to increase the recruitment of immune cells and to raise the local working temperature to for optimal immune function. Activated ECs ‘loosen’ tight junctions to increase permeability of the barrier to facilitate tissue oedema and the recruitment of inflammatory cells to the damaged area and, at a later stage, participate in the healing process. These are the classic signs of inflammation; rubor, calor, dolor and tumor. This can only be achieved by simultaneous down-regulation of the physiological anticoagulant and anti-inflammatory systems, with an up-regulation of inflammatory mediators\(^{188}\).
Figure 1.4: The endothelium at rest (A) and after activation (B).

(A) At rest, the endothelial anticoagulant phenotype is delivered by the PC pathway (activation of APC and APC-EPCR-PAR-1 anti-inflammatory signalling), TFPI, AT and tPA.

(B) Endothelial activation by TF, monocytes and thrombin results in the promotion of coagulation by reduced surface expression of TM, EPCR, TFPI, GAGs, release of vWF leading to platelet aggregation. APC and AT are consumed. PAI-1 inhibits tPA reducing fibrinolysis. Barrier function is lost by apoptosis and detachment of ECs. Complement activation is enhanced by reduced TAFI activity. Thrombin promotes the release of inflammatory cytokines by PAR signalling.


Endothelial cells are activated by a several stimuli. TF and thrombin are potent stimulants. Thrombin acts via protease-activated receptors (PAR-1 in low doses and PAR 3 & 4 at higher doses). Other activators include endotoxin, toll-like receptors, TNF-α, IL-1β, vasoactive agents (histamine, bradykinin, vasopressin), activated monocytes, hypoxia, metabolic stress, and trauma.
Activated endothelial cells,

- Increase production of NO causing vasodilation
- Release the contents of Weibel-Palade bodies; von Willebrand Factor (vWF) and P-selectin; causing platelet aggregation and leucocyte infiltration.
- Have an altered gene transcription profile. NFκβ and MAPKs induce transcription of proinflammatory genes and suppress anti-inflammatory genes.
- Have increased production of leucocyte adhesion molecules, cyclooxygenase 2, tissue factor (TF), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), urokinase-type plasminogen inhibitor (uPA).
- Reduce production of TM and EPCR\(^{196}\)
- Increase TM and EPCR shedding from the EC surface by metalloproteinases.
- Alter the glycocalyx, reducing the binding sites for AT and promoting leucocyte adhesion.
- Synthesise and release tPA, uPA and PAI-1, in higher concentration than tPA so the overall effect is anti-fibrinolytic.
- Release microparticles; a phospholipid surface expressing TF that amplifies the coagulant cascade and the inflammatory response

Endothelial activation can become dysregulated and dysfunctional, extending beyond a localised response to become systemic and causing harm to the host. This is central to the systemic inflammatory response syndrome (SIRS) that can progress to shock, multiorgan failure and death\(^{193}\). Dysregulation of the PC system may contribute to endothelial dysfunction.
2.1.2. Molecules of the PC pathway: Protein C and APC.

APC is the main effector protease of the PC pathway. It is produced by activation from its zymogen, PC. Thrombin is the only known physiological activator of PC.

The liver produces the bulk of PC, although it is also formed by intestinal epithelial cells, alveolar epithelial cells, endothelium, keratinocytes and the kidney\textsuperscript{181,197-199}. Hepatic dysfunction and the acute phase response reduce plasma protein C levels\textsuperscript{200}.

Under resting physiological conditions, PC has a concentration of 3-7 µg/mL (65-70nM) and a half-life of 6-10 hours. However in situations of thrombin generation, the half-life of PC is shortened to 2-3 hours. APC circulates in the resting state at approximately 1/2000\textsuperscript{th} of the concentration of PC; 1-3ng/mL (<40pM), and the half-life is ~20-25 minutes\textsuperscript{201}. Under resting conditions, circulating levels of APC are dependent on the concentration of PC and thrombin, however, the production of APC can increase dramatically in response to thrombin challenge, 100-280 fold increases have been in the plasma. These levels are still 10-20x lower than PC levels at rest. The half-life of APC is also shortened in these circumstances\textsuperscript{202,203,204}.

The implications of these findings are that the molar excess of PC can support a sudden increase in demand for APC: and that PC would ‘drown’ APC in the interaction with receptors with equal affinity for both, like EPCR.
Figure 1.5: The structure of APC. The heavy chain (light grey) includes the protease domain (red). The RGD domain binds β1- & β3 integrins (orange). The light chain (dark grey) includes the Gla and EGF domains. The Gla domain is essential for interactions with phospholipid membranes and EPCR. Substituting amino acids creates APC mutants; substitutions at L38 or L8 abolish interactions with PS and EPCR, substitutions in the FVa site abolish the anticoagulant properties without affecting EPCR interactions. From Weiler H. Hamostaseologie 2011;31:185-195.

PC is a small 62kD protein with heavy and light chains in 5 domains that include the anionic Gla- domain containing glutamic acid residues, 2 EGF-like domains, an activation peptide that is removed on activation, allowing the serine protease domain to become active. PC undergoes several post-translational modifications, including vitamin k-dependent γ-carboxylation of glutamic acid residues within the Gla domain; vitamin k deficiency prevents the formation of structurally functional PC. The Gla domain is vital to the anticoagulant function of APC and binds calcium, negatively charged phospholipid membranes and EPCR.

The protein C gene (PROC) is located on chromosome 2q13-14, and several polymorphisms account for variations in PC levels. The genetics are discussed in ‘congenital PC deficiency’.
Genetic engineering has produced APC mutants with altered domain structure changing the relative anti-coagulant or anti-inflammatory activities. For example, mutants in the Gla-domain selectively effect interactions with EPCR and phospholipids, either abolishing EPCR interactions or increasing anticoagulant activity by increasing the affinity of the mutant for phospholipids. Mutant APC has been used in preclinical studies to clarify the relative importance of APCs various activities\textsuperscript{206,207,208}.

The anticoagulant activity of APC shows significant species specificity\textsuperscript{209-211}.

**The inhibitors of APC:**

APC has a half-life or 20-25 minutes in health. It is cleared by several inhibitors: the serine proteases include Protein C inhibitor (PCI), plasminogen activator inhibitor -1 (PAI-1), $\alpha$-1-antitrypsin; and the non-serpin, $\alpha$-2 macroglobulin (A2M).

Complexes of APC-PCI, measured by ELISA, are markers of thrombosis\textsuperscript{212}. At rest, 10-50\% of APC is complexed with PCI\textsuperscript{213}. PCI inhibition of APC is accelerated by heparin. This biological relationship could theoretically reduce the effect of exogenous recombinant APC (rhAPC) if simultaneously administered with heparin; and was explored in a randomised controlled trial (RCT) of heparin in adult patients treated with rhAPC for severe sepsis, the XPRESS study\textsuperscript{214}. They did not find excess mortality or VTE in the heparin groups suggesting the effect of heparin on the acceleration of APC inhibition is clinically insignificant.

The binding of APC to PAI-1 is accelerated 300-fold by vibronectin, released from activated platelets. The interaction of these molecules inhibits both their activity. The PAI-1 concentration in plasma is several orders of magnitude lower than the other inhibitors of APC, therefore the clinical effect of APC-PAI-1 binding may not be clinically important or may be only significant in the local area of a platelet plug\textsuperscript{215-217}.
All of the inhibitors are positive acute phase reactants, rapidly increasing in concentration during inflammation, thereby increasing the clearance of free APC from the circulation\textsuperscript{212,218}. PC and APC are also degraded by neutrophil elastases, a process that is enhanced in inflammation\textsuperscript{219}.

### 2.1.3. Molecules of the PC pathway: thrombin

Thrombin is a central regulator of haemostasis and inflammation with effects on platelets, the endothelium and leucocytes.

It is a vitamin K dependant, trypsin-like serine protease formed by the cleavage of prothrombin (FII), releasing active thrombin and prothrombin fragment 1+2 (PF1+2).

Tissue Factor (TF) is the major trigger for the formation of thrombin. TF is exposed by vascular injury or expressed by activated platelets or activated cells of the innate immune system in response to infection or damage. TF triggers the extrinsic coagulation cascade, activating FVIIa, and in turn FXa. The prothrombinase complex (FVa, FXa, calcium (Ca\textsuperscript{2+}) and phospholipid) activates prothrombin\textsuperscript{205}. TF exposure is vastly increased during the mucosal injury of NEC.

Thrombin has multiple functions\textsuperscript{220}:

- Thrombin is pro-coagulant: proteolysis of fibrinogen leads to the formation of the fibrin clot. It further activates factors V, VIII, XI and XIII, amplifying the coagulation cascade. It is a potent activator of platelets enhancing aggregation and the formation of the platelet plug. Activated platelets also secrete inflammatory mediators (IL-1, PAF, and PF4).
• Thrombin is proinflammatory: endothelial, monocyte and platelet activation with up-regulation of NFκB and MAPK resulting in the production of proinflammatory cytokines, chemokines and endothelial adhesion molecules.

• Thrombin is barrier disruptive, increasing vascular permeability, facilitating leucocyte traffic.

• Thrombin produces growth factors for fibroblasts and promotes angiogenesis.

• Thrombin activates thrombin inducible fibrinolysis inhibitor (TAFI) that has anti-fibrinolytic effects and is a powerful inhibitor of C5a and C3a.\textsuperscript{205}

• Thrombin initiates anti-coagulant and anti-inflammatory regulation. On binding to TM, the thrombin exosite 1 is shielded and thrombin “switches” its activity from stimulation of inflammation and coagulation, towards the production of APC with anti-inflammatory and anticoagulant down-stream effects.\textsuperscript{205}

\textit{The inhibitors of thrombin:}

The half-life of thrombin is very short, just 2s. Direct inhibitors include the proteases AT and PCI. The inactivation of thrombin is significantly accelerated on binding to TM.\textsuperscript{205} α2-macroglobulin (A2M) accounts for approximately 7% of thrombin inhibition in adults.\textsuperscript{221}

AT is the main inhibitor of thrombin, and this reaction is enhanced 1000-fold by heparin co-factor II (HCII) and endogenous glycosaminoglycans produced by ECs in the glycocalyx, such as heparan sulphates. AT is a negative acute phase reactant; levels decrease markedly during inflammation. Low levels are also caused by increased consumption and increased degradation by neutrophil elastase.\textsuperscript{222}

PCI is a potent inhibitor of thrombin. The reaction with PCI is accelerated 140-fold if thrombin is bound to TM.\textsuperscript{213}
2.1.4. Molecules of the PC pathway: Protein S (PS)

PS is a vitamin k-dependent glycoprotein with a plasma level of 320nM. The carboxylated Gla domains enable Ca$^{2+}$-dependent interaction with phospholipid membranes. Approximately 50-60% of the circulating PS is bound with high affinity to the complement regulatory protein, C4b-binding protein (C4BP); leaving a free PS concentration ~130nM. This is in excess of PC and great excess of APC concentrations. Only the free PS is biologically functionally active. C4BP is an acute phase reactant, increasing in concentration as part of the host response to inflammation, reducing the free PS levels.

2.1.5. Molecules of the PC pathway: Thrombomodulin (TM)

Thrombomodulin (CD141) is a 60kD type 1 transmembrane protein of several domains: a lectin domain, 6 EGF-like domains, serine rich domain, a transmembrane section and a short cytoplasmic tail. The extracellular domain is modified post-translation with chondroitin sulphate glycosaminoglycan, which varies according to tissue type.

The lectin domain is involved in the anti-inflammatory roles of TM; including inhibition of high mobility group box protein 1 (HMGB-1), inhibition of neutrophil and monocyte adhesion to endothelial cells, inhibition of complement activation. The lectin domain enables endocytosis of TM from the endothelial surface. EGF domains are key for binding thrombin and the subsequent activation of PC and TAFI, and inactivation of uPA (urokinase type plasminogen activator). The EGF domains are mitogenic. The serine threonine domain is key to the inactivation of thrombin by PCI, AT and HCII. This domain also it binds platelet factor 4 (PF 4) and thereby accelerates APC production.

The dominant role of TM is binding thrombin and the subsequent production of APC. TM binds thrombin with high affinity; Kd 0.5 -10nmol/L, depending on the presence of
chondroitin sulphates\textsuperscript{205}. These levels of thrombin are similar to the levels found during the very initial of phase of clot formation, as platelets become activated, before the propagation phase has begun; emphasising again the important, early role the PC pathway plays in regulating thrombin generation.

\textit{Figure 1.6 Thrombin generation during TF-initiated whole-blood experiments in healthy adults (Thrombin concentration in log scale vs. time; mean ±SEM).}
The time points indicate when products were detected; platelet activation, factor V activation, fibrinopeptides A & B. The average clot time (C.T.) was 4.7 ±0.2 min. From Mann KG CHEST 2003; 124:4S–10S\textsuperscript{224}

TM is constitutively and abundantly expressed by the endothelium of blood and lymphatic vessels, particularly in the microvasculature and at points of flow disturbance\textsuperscript{225}. The estimated concentration of TM in the microvasculature is 100-500nmol/L assuming 100,000 copies of TM per endothelial cell\textsuperscript{205,226}. TM is especially prominent in the pulmonary microvasculature, brain cortical capillaries are the exception as they express very little TM.\textsuperscript{218,205}

TM is also expressed by platelets\textsuperscript{227}, monocytes\textsuperscript{205}, neutrophils\textsuperscript{228}, alveolar epithelial cells\textsuperscript{199}, astrocytes, osteoblasts, chondrocytes and syncytiotrophoblasts lining maternal placental blood vessels\textsuperscript{223,229}. TM is not expressed by intestinal epithelial cells\textsuperscript{198}. 
Cell surface expression of TM is regulated by transcription, endocytosis and shedding.

During inflammation there is increased endothelial shedding of soluble TM (sTM) and EPCR (sEPCR) by neutrophil elastase, activated metalloproteinases and bacterial proteases.

Activated neutrophil products also oxidize and inactivate TM\(^{230}\). TM undergoes endocytosis from the surface of the cell\(^{231}\). All of these factors are active in NEC, but sTM levels have not been reported.

Soluble TM is present in plasma under normal conditions (3-50 ng/mL) and levels are increased in conditions associated with vascular disruption; sTM can be used as a biomarker for vascular damage\(^{232}\). The physiological role of sTM is not certain, but it is thought to be vasculoprotective\(^{223}\).

TM transcription and production is influenced by a large number of factors indicating a role for the activation pathway of APC in many cellular processes. To list a few for interest; TM is increased by heat shock, statins, prostaglandin E1, 1,25 dihydroxyvitamin D3, VEGF and thrombin \(^{223}\). TM transcription is reduced by inflammatory mediators; endotoxin (LPS), TNF \(\alpha\), IL-1\(\beta\), CRP, NF\(\kappa\)B \(^{231,233}\) as well as other related stresses; hypoxia, free fatty acids, oxidised LDL, and shear stress \(^{196}\). As previously discussed, NEC is associated with increased levels of LPS, TNF\(\alpha\), IL-1\(\beta\), CRP and activation of NF\(\kappa\)B.

TM expression is reduced in inflammation in endothelial cell lines\(^{196}\), animal models\(^{234,235}\) and humans \(^{236}\) TM expression was reduced in the purpuric skin lesions of children with meningococcemia\(^{237}\), atherosclerotic plaques, and in the intestine of patients with inflammatory bowel disease\(^{238,239,240,241}\).

The effect of age on TM expression was recently reported in an acute endotoxaemia mouse model. Aged mice had a high mortality rate with sub-lethal LPS doses and had profound suppression of pulmonary TM expression. Younger mice required higher doses of LPS to
achieve similar mortality rates and pulmonary TM expression remained greater than the aged mice\textsuperscript{242}. Aged mice were also unable to increase levels of APC.

Genetic mutations of TM are rare; several have been described, but the clinical significance is not known. They have been linked to atypical haemolytic uraemic syndrome\textsuperscript{243}.

\subsection*{2.1.6. Molecules of the PC Pathway: EPCR}

EPCR (CD201) is a membrane protein of the MHC/CD1 family. The other members of this family are involved in immunity and inflammation.

EPCR is localised on the cell surface in lipid rafts with TM and PAR-1. It has a short cytoplasmic tail, and palmitoylation of the C-terminal localises EPCR to caveolae, which is important in APC signalling.

EPCR expression on the endothelium shows a reciprocal pattern of expression to TM, being more abundant on the endothelium of larger vessels than in the microvasculature, except in the liver and spleen.\textsuperscript{244-246} The need for altered expression of TM and EPCR in different vascular beds is not clear if only the anticoagulant effects of APC are considered in maintaining the patency of the microvasculature. It is likely that the myriad of alternative signalling pathways both receptor participate in, have an effect on the relative tissue bed distributions. For example EPCR my play an enhance role in immune signalling in the liver and splenic vascular bed.

EPCR is expressed by intestinal epithelial cells, skin keratinocytes, monocytes, macrophages, neutrophils, eosinophils, CD 56+NK cells, haemopoetic stem cells; and CD8+ dendritic cells (present in the intestine).\textsuperscript{181,226, 198,247-249}
1.7: EPCR and TM expression on cultured arterial (AEC) or venous endothelial cells (VEC). The antigen expression was determined using flow cytometer analysis. The x-axis represents fluorescence intensity (log scale) and the y-axis represents relative cell numbers. From Fukudome K. J.Exp Med. 1998;187:1029-1035.

Figure 1.8: EPCR (left) and TM (right) expression in normal human heart (A) and lung (B). EPCR and TM appear brown.

(A) Heart: A small coronary artery (arrow) is EPCR positive, but capillaries are negative. TM is strongly positive in larger vessels (arrow) and capillaries.

(B) Lung: Positive EPCR staining is seen in an artery. The alveolar capillary endothelial cells are negative. Strong TM expression is present both in larger vessels (arrows) and alveolar endothelial cells (open arrow). Reproduced from: Laszik Z et al. Circulation 1997;96:3633-3640
EPCR expression, like TM expression, is regulated by transcription, endocytosis and cell surface shedding.

Mouse caecal ligation and puncture (CLP) and LPS models have shown that EPCR mRNA, in liver, lung, heart, and kidney increased in response to the inflammatory insult; in compensation for increased levels of sEPCR in the plasma and tissue expression of EPCR remained constant in the heart and lungs\textsuperscript{250,251}.

In humans, EPCR expression in skin lesions associated with severe sepsis, was depressed\textsuperscript{237}.

Soluble EPCR circulates in the plasma (100ng/mL, \textasciitilde2.5nM, in excess of APC) and binds to APC preventing its anticoagulant functions but does not interfere with APC inactivation by PCI or \(\alpha\)1AT. There are conflicting reports on the effect of sEPCR on PC activation; some report no effect while others report inhibition. PC/APC bound to sEPCR prevents binding to membrane bound EPCR and intracellular signalling via PAR\textsubscript{1}\textsuperscript{245,252,253}.

Genetic mutations are described of the gene for EPCR (PROCR). They are associated with an increased risk of VTE, myocardial infarction and late foetal loss in pregnancy (Online Mendelian Inheritance in Man (OMIM)).
2.2. Activation of PC by TM and EPCR

The functions of the PC pathway largely depend on the production of APC by thrombin on the endothelial cell surface, involving the receptors TM and EPCR.

TM is aptly named; it modulates the activity of thrombin from the promotion of thrombosis and inflammation, towards the generation APC with its anticoagulant and anti-inflammatory effects. PC, in the presence of calcium (Ca\(^{2+}\)) and phospholipid membranes, binds to the 4\(^{th}\) EGF domain of TM, while thrombin binds to the 5\(^{th}\) and 6\(^{th}\). TM allosterically alters the structures of thrombin and PC, enabling proteolytic cleavage and the production of APC. The T-TM complex increases the activation rate of PC approximately 1000 fold\(^{205}\). The activation rate is increased a further 10-20 times by EPCR; which binds PC, calcium and places them in close proximity to the T-TM complex and the phospholipid membrane, accelerating activation\(^{254}\).

Activation of PC by the T-TM complex is a low affinity reaction (Kd =0.7-1\(\mu\)M; ~ 15x higher than plasma PC concentration). PC activation is therefore unlikely at physiological PC concentrations without the action of EPCR. EPCR binds both PC and APC equally with higher affinity; the Kd is in the range of PC plasma concentration (Kd ~30 - 60nM)\(^{187}\). The affinity between EPCR and PC/APC is increased by zinc ions\(^{255}\).

PC and APC levels correlate strongly during resting conditions, but this relationship is lost during thrombin challenge; the excess of PC is required to respond to an increased APC demand\(^ {203}\). The availability of PC becomes rate-limiting during prolonged episodes of thrombin generation.
The enzyme kinetics and abundance of TM and EPCR expression have a number of important consequences:

- PC concentrations are ~2000 times greater than APC in health; therefore the majority of EPCR will be occupied by PC. APC levels in plasma can be boosted in response to thrombin, but only levels up to 100-280 fold have been recorded. This is relevant in considering the relative contribution of PC and APC plasma concentrations to EPCR occupancy in disease states, and it is important in the consideration of replacement therapy.

- Under physiological conditions, the majority of EPCR will be occupied by PC; EPCR is ‘primed’ for interaction with T-TM complex or PAR-1. Low levels of plasma PC will reduce EPCR-PC binding, jeopardising cytoprotective PAR-1 signalling and APC production.

- TM is abundantly expressed (100-500 nmol/L) and has a high affinity for thrombin (Kd~ 0.5-10nmol/L); this is a very sensitive mechanism for detecting minute amounts of thrombin throughout the body.

- Cells co-expressing EPCR-PC and TM are able to rapidly produce APC after minute amounts of thrombin are captured by TM. This is a localised response to local generation of thrombin.

- The extent of PC activation is determined by the bioavailability of thrombin, PC, and the relative abundance of TM and EPCR expression.

Platelet factor 4 (PF 4) is stored by platelets and is released on activation. PF 4, like EPCR, can accelerate APC production by the T-TM complex (~20 x), enhancing the generation of APC locally at sites of platelet activation.

Activation of APC by TM is inhibited by histones H3 and H4, although these are also inactivated by APC.
EPCR-bound PC has no anticoagulant activity, therefore once generated, APC can either
dissociate from EPCR to undertake its anticoagulant functions, or it can remain bound to
EPCR and undertake the multiple activities associated with its cytoprotective functions.

Studies in mice estimate that approximately 40% of APC remains bound to EPCR and does
not enter the systemic circulation; LPS challenge in EPCR negative mice resulted in normal
APC plasma levels but poor outcomes due to the deficiency of the EPCR-APC pool\textsuperscript{262,263}.
Plasma APC levels therefore, may not be representative of PC activation.

2.3. Anticoagulant actions of the PC pathway.

\textit{Protease actions of APC}

APC is the main effector protease of the anticoagulant functions of the PC pathway (figure
1.9).

This primarily involves proteolytic inactivation of FVa and FVIIIa; thereby reducing the
generation of thrombin. These reactions are enhanced by protein and lipid co-factors. APC
dissociates from EPCR and co-locates with free PS on negatively charged surfaces;
negatively charged phospholipids, HDL or sphingolipids. Cleavage of FV (not activated)
produces a fragment that acts a cofactor for FVIIIa inactivation by APC. Cleavage of FVa
occurs at two sites: Arg 506 and Arg 306. The reaction at Arg506 is quicker, but is inhibited
by FXa bound to FVa (prothrombinase) and results in only partial inactivation; cleavage at
Arg306 is required for full inactivation. Point mutation at Arg506 results in the FV Leiden
mutation with APC resistance; there is impaired degradation of FVa by APC as cleavage
cannot occur at Arg506, only at Arg306. The mutation is prevalent in \textasciitilde5% of populations of
European descendent and gives a higher risk of venous thrombosis.
Cleavage of FVa by APC is reduced on the surface activated platelets, due to Platelet factor 4 (PF-4). As mentioned previously, PF 4 increases APC production, but it also inhibits the cofactor interaction of free PS with APC, thereby suppressing platelet-associated FVa inactivation. Nevertheless, APC retains the ability to promote cytoprotective PAR-1 signalling and it retains potential anticoagulant function on circulation away from the platelet plug.

**Other anticoagulant effects of the PC pathway**

- EPCR binds FVIIa, and to a lesser extent, FVIIIa. On binding, the complexes are rapidly internalised, clearing the factors from the circulation.
- TM captures thrombin and facilitates inhibition by PCI and AT with HCII.
- APC indirectly reduces thrombin generation by preventing the thrombin amplification of the coagulation cascade.
- The T-TM complex activates thrombin activated fibrinolysis inhibitor (TAFI). TAFI inhibits fibrinolysis, however this action is thought to be physiologically less important than its anti-inflammatory action of inhibiting the anaphylaxotoxins C3a and C5a.
- Neutrophil oxidation of the TM methionine388 inactivates TM ability to activate APC but has no effect on the ability of TM to activate TAFI. PF 4 inhibits TM activation of TAFI.
- Free PS stimulates TFPI; this molecule immobilises an intermediate complex, TF-FVIIa-FXa, preventing release of FXa.
- APC is inhibited by, but in turn, inhibits the action of PAI-1, promoting fibrinolysis.

Thrombosis and coagulative necrosis are common histological features of the mucosal injury in NEC. Occlusion of the microcirculation by fibrin deposition and platelet aggregation is believed to cause terminal ischaemia of the mucosa, contributing to necrosis.
Figure 1.9. Diagram of the coagulation cascade (depicted in black) and the anticoagulant inhibitors (depicted in red).

Tissue factor (TF) is the main trigger of coagulation via the extrinsic pathway (FVIIa). The intrinsic pathway is initiated by FVIIa or contact with negatively charged surfaces. There are 3 anticoagulant inhibitors; APC, Antithrombin III (AT) with heparin cofactor II (HCII) and Tissue Factor Pathway Inhibitor (TFPI). Plasmin undertakes fibrinolysis, and is inhibited by Thrombin Activated Fibrinolysis Inhibitor (TAFI). From Segel GB Blood cells, Mol Dis 2000; 26(5):540-560
2.4. The cytoprotective, anti-inflammatory PC pathway functions

Nearly twenty years ago, Esmon et al used a baboon model of *E.Coli*, LPS-induced sepsis to demonstrate that APC, PC and PS infusions all protected against the lethal effects of *E.Coli*, and that blocking EPCR, PS, or PC exacerbated the host response; inducing cytokine production, capillary leak, neutrophil infiltration, DIC, organ failure and death \(^{256,269}\).

These initial findings of the anti-inflammatory effects of the PC pathway have been reproduced, and the underlying mechanisms explored, in many models of inflammatory disease, both acute and chronic. These include sepsis and septic shock \(^{218,270,271}\), critical illness \(^{272,273}\), pancreatitis, and conditions associated with ischaemia-reperfusion injury such as myocardial infarction, stroke, intestinal reperfusion injury, acute kidney injury, spinal cord injury and liver reperfusion injury \(^{274}\). The PC pathway has been shown to have an anti-inflammatory role in many chronic inflammatory diseases; inflammatory bowel disease (IBD) is of interest to this project (ulcerative colitis and Crohn’s disease) \(^{174,198,238,239}\), but also includes asthma, atherosclerosis, diabetic nephropathy, glomerulonephritis, Alzheimers disease, rheumatoid arthritis, chronic wound healing.

The anti-inflammatory effects of APC are independent of its anti-coagulant activity. In mouse models, mutant APC variants that retain intracellular signalling properties without anticoagulant properties were as effective as wildtype APC in preventing LPS-induced septic shock, while APC mutants without signalling properties had no benefit \(^{208,275,276}\).

In general terms, the PC pathway directly suppresses inflammation by interactions with endothelial cells, cells of the innate immune system (monocytes/macrophages, neutrophils, eosinophils \(^{244,249,277}\)) and some epithelial cells (lung \(^{278}\) and intestine \(^{198}\)). The PC pathway also has non-cellular direct anti-inflammatory effects (for example extracellular histone
removal) and lastly, the system indirectly reduces inflammation by reducing thrombin generation.

The cell-interaction anti-inflammatory mechanisms used by the PC system include modulating the gene expression profiles with subsequent altered quality and quantity of cytokine production, reducing leucocyte chemotaxis and trafficking, preventing apoptosis, stabilising the barrier function of the endothelium and other epithelia including the intestinal mucosa and alveoli, and lastly the pathway stimulates cell proliferation to promote wound healing.

All of these actions are highly relevant to the disease process in NEC.

The cytoprotective effects of APC and the PC system are mediated through the innate immune system and endothelium, and not the adaptive immune system. This is of particular relevance to the term and preterm neonatal population, as the adaptive immune system is particularly immature.

### 2.4.1. PC/APC mechanisms of cell signalling

The cytoprotective functions of APC are mainly mediated by interaction with membrane receptors triggering intracellular signals. The notable exception is the direct proteolytic action of APC on extracellular histones.

Cells subject to cytoprotective signalling by APC include endothelial and epithelial cells, various lymphocytes, dendritic cells and neurons. There is also a growing list of APC receptors, implying that a unifying mechanism for APC-cytoprotection is unlikely; host responses more likely will depend on the relative activities of APC receptors in specific cell types.
The best known APC receptors include the EPCR-APC-PAR-1 axis (endothelial cells, monocytes and macrophages, epithelial cells, neurons and astrocytes, myocytes and fibroblasts\textsuperscript{207,208,241} the Mac-1-APC-PAR-1 axis and EPCR-APC and \(\beta_1\)- and \(\beta_2\)-integrin interactions.

The detailed descriptions of the receptor interactions are not strictly necessary for relating to pathogenic processes in NEC for this project, however they do give insight into the complexity and the vast sphere of influence of the PC pathway. And the elegance of the EPCR-APC/PC-PAR-1 signalling mechanism is fascinating.

\textbf{Figure 1.10}: Cellular receptors for PC/APC. The transmembrane receptors are displayed at the top of the figure, and the major responses are below. Dashed lines indicate EPCR plays an accessory, but not necessary, role in the pathway. From Weiler H. Hamostaseologie 2011;31:185-195.
2.4.1.1. EPCR-APC-PAR-1-S1P signalling

The molecular events involved in the EPCR-APC activation of PAR-1 are not completely understood, but novel mechanisms for receptor-substrate interactions have been discovered\textsuperscript{279}.

Protease activated receptors (PAR 1-4) act as their own ligands on proteolysis. PAR-1 G-protein coupled receptor can be cleaved by thrombin or APC, although the affinity for thrombin is very much greater. Activation of PAR-1 occurs by proteolysis of the extracellular N terminal (by APC or thrombin), leaving an activated “tethered ligand” that triggers signals in coupled intracellular pathways.\textsuperscript{280} The downstream effects of activation depend on the particular signalling pathway or platform coupled to the receptor at the time of activation. Therefore activation can produce either pro- or anti-inflammatory effects; these are not dependent on the protease as initially thought (APC or thrombin), but are dependent on the occupancy of EPCR by its ligands, APC or PC. Unoccupied EPCR is associated with caveolin-1 lipid rafts and PAR-1 is coupled with disruptive G-proteins (G12/13 & Gq), however when PC or APC binds to EPCR, it disassociates from Caveolin-1 and switches PAR-1 signalling to couple with Gi protein. The net effect is that proteolysis of PAR-1 by either APC or thrombin in this position will demonstrate a cytoprotective response.

The duration of the active signal from the “tethered ligand” is also affected by the couple platform; the ligand is rapidly removed from the cell surface by endocytosis when the signal is proinflammatory (unoccupied EPCR) but it is active for a longer period when activated by an anti-inflammatory signal\textsuperscript{280-282}.

Given that under resting physiological conditions with normal PC levels, EPCR is largely bound to PC, therefore cells that co-express EPCR-PC and PAR-1 will be directed to a
protective pathway. The endothelium of the microcirculation will be “anti-inflammatory” and “anti-coagulant”, maintaining the fluidity of the microcirculation. Low plasma PC levels, as found in the preterm neonate, will reduce this function.

The downstream mediator essential to the barrier-stabilising activity of APC, Sphingosine-1-phosphate (S1P), is increased by up-regulation of sphingosine kinase 1 (sphk 1) caused by PAR-1 triggering. The difference in signalling between occupied and unoccupied EPCR proteolysis correlates with cross-talk between PAR-1 and S1P receptors 1 and 3. Protective effects on endothelial cell permeability are mediated through S1P-R1 while S1P-R3 increases endothelial permeability.

Barrier stabilising effects also occur with EPCR-APC-PAR-1, and via EGF transactivation, up-regulating the angiotensin1/Tie2 axis, increasing tight junction proteins and causing smooth muscle cell migration.

EPCR-APC/PC-PAR-1 signalling also decreases the release of vWF and P-selectin from Weibel Palade bodies in endothelial cells.

In neuronal cells, the protective effects of APC requires that EPCR-APC interacts with PAR-1 and PAR-3; indicating refinement of this signalling axis according to cell type.

2.4.1.2. Mac-1-APC–PAR-1 interactions.

This paradigm is similar to EPCR-APC-PAR-1 previously discussed. The anti-inflammatory effects of APC in murine macrophages were not dependent on the presence of EPCR, but rather on mac-1 (CD11b/CD18) present in cholesterol-rich lipid rafts. In murine models of sepsis, APC proteolysis of PAR-1 resulted in the production of S1P, the suppression of IL-6 production, and suppression of inflammatory response genes including NF-κβ.
Therapies designed to block CD11b/CD18 to reduce inflammation will also, paradoxically, block this anti-inflammatory function of APC. This may account for the failure of these medications in inflammatory bowel disease and asthma\textsuperscript{289}.

2.4.1.3. EPCR-APC and $\beta_{1}$- and $\beta_{3}$ – integrins.

APC interacts with the $\beta_{1}$- and $\beta_{3}$ – integrins on neutrophils, inhibiting their migration\textsuperscript{290}. This process is enhanced by EPCR, but it is not essential.

2.4.1.4. Other APC receptors:

Other APC signalling interactions include:

- **Epidermal growth factor receptor (EGFR)**\textsuperscript{207}. The migration of lymphocytes in response to chemokines is inhibited by APC and PC, in an EPCR- and EGFR-dependent manner\textsuperscript{291}. Similarly PC and APC stimulate proliferation, migration and survival of skin keratinocytes in an EPCR- and EGFR-dependent way\textsuperscript{197}.

- **APC-apolipoprotein E receptor 2 (apoER2) interactions** on monocytes, independent of EPCR and PAR-1, suppress LPS-activated TF expression on monocytes and monocyte derived microvesicles\textsuperscript{218,271}. Immobilised PC/APC interacts with apoER2 on platelets under sheer conditions causing activation and spreading.

- **Glycoprotein1b$\alpha$ (GP1b$\alpha$) adhesion molecule** on platelets. PC and APC *in vitro* support platelet aggregation in a GP1b$\alpha$ and apoER2 dependent manner. The significance of these findings is not known\textsuperscript{292}. 
2.4.2. Cytoprotective actions of the PC pathway.

2.4.2.1. Proteolysis of extracellular histones and HMGB-1

APC has inhibitory effects on histones and HMGB-1\textsuperscript{162,261,293}. These are intranuclear proteins released by dying cells or secreted by immune cells. Histones and HMGB-1 emerge late in the host response to infection, after several hours. These are potent inflammatory agents; capable of eliciting a cytokine storm. They are associated with organ failure and disease severity.

Histones are cationic proteins that form nucleosomes with DNA, they can be secreted by immune cells as “extracellular traps” for microbes, enhancing their removal by the immune system. They are directly cytotoxic. Histones reduce T-TM-PC activation, contributing to thrombin generation in the microcirculation. APC is able to cleave histones H3 and H4.

HMGB-1 binds to immune cell receptors to recruit proinflammatory, and cell proliferation pathways (TLR 2 and 4, and receptor for advanced glycation end products (RAGE)) and it regulates neutrophil recruitment. EPCR-APC-PAR-1 axis is able to down-regulate expression of HMGB-1 receptors and inhibits the release of HMGB-1\textsuperscript{261,293}. 
Figure 1.11: (A) APC anti-inflammatory activity. Inflammatory gene expression is inhibited by EPCR-APC-PAR-1 (green arrow). APC down-regulates the expression of adhesion molecules (red block). APC reduces inflammatory mediator release from leukocytes (red block). sEPCR and proteinase 3 (PR3) interfere with CD11b/CD18 (Mac-1) on activated neutrophils.

(B) APC anti-apoptotic activity mediated via EPCR-APC-PAR-1. Green arrows indicate gene modulation. APC down-regulates pro-apoptotic p53 and Bax protein (red blocks) and maintains protective anti-apoptotic Bcl-2 protein levels (green arrow), balancing the Bax/Bcl-2 ratio. APC inhibits activation (red block) of intrinsic and extrinsic cascades (caspase-8 and -3). From Mosnier LO. Blood. 2007;109: 3161-3172.

2.4.2.2. Altered gene expression:

Transcriptional profiling of endothelial cells and monocytes has shown that APC treatment modulates gene expression of the major pathways of inflammation and apoptosis, with suppression of proinflammatory cytokine, chemokine and adhesion molecule production. Examples of altered gene expression:

- Suppression of NF-κβ, cRel (NF-κβ family), activator protein -1 (AP-1) family in monocytes and ECs at rest and after stimulation with LPS or TNFα.
• Suppression of genes for the production of adhesion molecules: E-selectin, ICAM1, VCAM1, thrombospondin, P-selectin

• Suppression of cytokines and chemokine production in ECs and monocytes stimulated by LPS, IL 8, TNFα: tissue factor, monocyte chemoattractant protein-1 (MCP-1), monocyte TNFα, IL-1β, IL-6, IL-8.

• Upregulation of anti-inflammatory cytokines, IL-10.

• Suppression of some apoptosis related genes: p53, Bax

• Upregulation of anti-apoptosis: Bcl2A

• Upregulation of cytokine and chemokines: IL-1R, eNOS

• Promotion of endothelial barrier function: Rac 1. Intestinal epithelial barrier function is also promoted by APC; in mechanisms that are S1P – independent, but can be enhanced by S1P. Pulmonary epithelial barrier function is promoted by the PC pathway.

The precise mechanisms of how APC affects gene expression are not known but much of the effect is mediated by EPCR-APC-PAR-1 activation.

2.4.2.3. Reduced leucocyte infiltrate.

As already mentioned, APC reduces the production of cellular adhesion molecules and chemokines and stabilises the endothelial barrier, reducing leucocyte chemotaxis, rolling, adhesion and tissue infiltration. The combined anti-inflammatory effects result in the inhibition of neutrophil, eosinophil and lymphocyte migration. APC controls the maturation and activation of CD8+ DCs in an EPCR-dependent manner.

The lectin domain of TM directly interferes with leucocyte-endothelial interactions and reduces leucocyte tissue infiltration and EPCR interactions with neutrophil proteinase 3 (PR-3) inhibit the migration of neutrophils into the tissues.
2.4.2.4. Anti-apoptosis activity and promotion of cell proliferation

This activity involves the EPCR-APC-PAR-1 axis.

Apoptosis can be induced by the death receptor (extrinsic pathway involving Bcl-2 family) or mitochondrial pathway (intrinsic pathway involving caspase 8). APC modulates both pathways; up-regulates Bcl-2 and suppresses Bax and p53, reduces nuclear translocation of apoptosis-inducing factor (AIF) and APC inhibits caspase-3 and -8 activity. APC induces phosphorylation of extracellular signal related protein kinases 1/2 (ERK1/2) and MAPK; resulting in reduced cytochrome c release from the mitochondria to the cytoplasm\textsuperscript{179,180}. As mentioned earlier, APC also reduces TNFα production, so indirectly reduces death receptor signalling. APC has been shown to be anti-apoptotic in endothelial cells, epithelial cells, monocytes and neurons\textsuperscript{294,295}. APC has been shown to counteract the neurotoxicity of tPA which induces apoptosis via the extrinsic pathway\textsuperscript{306}.

Blocking apoptosis may indirectly reduce inflammation by reducing the quantity of histones and HMGB-1 released by cells.

APC promotes re-epithelisation and healing of breaks in the epithelial barrier by increasing DNA synthesis and inducing the proliferation of endothelial cells and skin keratinocytes\textsuperscript{181}.

These functions are particularly relevant in NEC, where epithelial apoptosis is an initiating factor and failure of restitution delays healing. HMGB-1 levels are increased in NEC and contribute to the pro-inflammatory cascade.

2.4.2.5. Barrier-stabilising effects

APC has potent barrier stabilising effects, mediated by EPCR-APC-PAR-1. This stimulates sphingosine kinase-1 (SphK-1) to form sphingosine-1-phosphate (S1P). S1P activates S1P receptor-1 (S1P1), activating Rac 1; stabilising the cytoskeleton, maintaining the tight
junctions and preventing permeability and oedema formation. There is variation in S1P-1 distribution in the tissues; it is abundant in the lung and brain but almost absent in the kidneys, an organ whose function is dependent on the permeability of the endothelium. This suggests modulation of the biological functions of APC occurs at the tissue level by varying receptor distribution.

It is noteworthy that barrier stabilisation, and prevention of vascular permeability, is much more effective if APC is endogenously derived.

2.4.2.6. Preservation of the microcirculation

The PC pathway is a key mediator in the preservation of a functioning microcirculation; loss of the microcirculation leads to organ dysfunction. Intravital microscopy in animal models of sepsis confirmed that APC preserves the density of functioning capillaries and maintained microcirculatory flow with reduced leukocyte-EC rolling and adhesion.

2.4.2.7. Other anti-inflammatory effects of the PC pathway

**TM binds HMGB-1**

The lectin-like domain of TM sequesters, and may lyse, HMGB-1 protein (HMGP-1), preventing interaction with downstream receptors. This action is highly effective because of the abundance of TM on endothelial cells.

**TM lectin domain interferes with leucocyte trafficking**

The lectin domain of TM interferes with neutrophil–endothelial interactions, reducing migration. The lectin domain also binds to the Lewis Y antigen on Gram-negative bacteria, inducing aggregation and opsonisation. This interaction also interferes with LPS signalling, dampening the host inflammatory response to gram negative bacteria.
**TM and TAFI inhibit complement and bradykinin**

TM inhibits complement in at least 2 ways, effectively uncoupling complement activation from thrombin generation. TM enhances C3b and C4b inactivation by complement inhibitors I and H. Secondly, TM activates TAFI, which inactivates C3a and C5a in turn. The clinical importance of TM’s role in complement regulation is demonstrated in individuals with mutations in TM; they are at higher risk of complement-mediated atypical haemolytic syndrome. TAFI also inhibits bradykinin, reducing vasodilation and vascular permeability.

**Free PS–C4BP promotes phagocytosis of damaged cells.**

Free PS binds to exposed, negatively charged phospholipid membranes on apoptotic cells. Subsequent binding to C4BP concentrates it on the surfaces of damaged cell surfaces, enhancing phagocytosis.

PS is one of two ligands for the TAM (Tyro/axl/Mer) family of receptors; activation results in downregulation of signalling cascades downstream of TLRs, in particular myD88 and NFκβ, down grading the inflammatory response. This may be useful in the development of tolerance to colonising bacteria in the newborn.

In the brain, PS has preserved endothelial barrier function and reduced the effects of hypoxia/ischaemia injury.

**sEPCR**

Soluble EPCR, sEPCR-PC and sEPCR-APC bind to activated neutrophils via proteinase 3 (PR3) released from α granules. PR3 and EPCR in turn interact with mac-1, preventing leucocyte adhesion and migration.
2.5. **PC deficiency states**

Protein C deficiency can be congenital or an acquired phenomenon in response to inflammation. The clinical effects of PC deficiency underscore the importance of the PC pathway; the homozygous congenital condition is lethal without treatment. Neonates, infants and young children tolerate a physiological PC deficiency without increased thrombotic risk; but they may be vulnerable to sustained increases in demand for PC.

Transgenic PC-deficient mice that express very low endogenous PC levels (1-18%) show an exaggerated inflammatory response and higher mortality after CLP or LPS challenge\(^\text{251,318}\).

2.5.1. **Congenital PC deficiency:**

In animal models, lack of a functioning PC pathway in-utero is associated with fetal and perinatal loss\(^\text{319-321}\).

In humans, congenital PC deficiency is caused by mutations in the PC gene on chromosome 2q13—14 (PROC, OMIM*612283 with phenotypes #1768690 and #612304)). The human gene mutation database has 260 PC mutations registered associated with PC deficiency (accessed June 2012). The inheritance patterns vary; autosomal recessive and dominant patterns have been described. Deficiency can be heterozygous, homozygous or compound heterozygous.\(^\text{322}\)

Type I deficiencies are more common (~80%) and are characterised by reduction in PC antigen levels and activity, while Type II has near normal PC antigen levels but reduced functional activity.

The reported incidence of asymptomatic PC deficiency is estimated at 1 in 200-1 in 500 healthy individuals; and has been found in 0.4% of healthy blood donors. The incidence of
clinically significant PC deficiency is estimated at 1 in 20 000. \(^{323}\) Homozygous PC deficiency is rare; the incidence is 1 in 500,000 – 750,000 live births; the prevalence is lower than carrier rate estimations would predict. There may be a number of explanations, including fetal loss, early postnatal death, poor recognition and under-reporting of the condition.

Congenital PC deficiency is associated with an increased risk of thromboembolic events; in homozygous or compound heterozygous PC deficiency this presents as life-threatening purpura fulminans and large vessel thrombosis that requires urgent PC replacement therapy. These infants are not been reported to have NEC.

Heterozygous PC deficiency is associated with a 10-fold increased risk of venous thromboembolism (VTE) and had been found in 5% of patients with VTE. A prospective cohort study on asymptomatic patients with PC deficiency found an increased risk of VTE at 2.5% per year.\(^ {324}\) Venous thrombosis is often spontaneous, and usually involves the veins in the lower limbs, brain, renal or mesenteric vessels. These patients may also present with coumarin-induced skin necrosis, which resembles purpura fulminans and is caused by microvascular venous thrombosis. It occurs in the first few days of treatment with vitamin K antagonist anticoagulants because the already low levels of PC are depleted faster than the vitamin K procoagulant factors, causing a paradoxical prothrombotic state during the initiation of therapy\(^ {323,325,326}\).

Severe PC deficiency is associated with homozygous or compound heterozygous inheritance; PC levels are very low, usually below 0.01 U/mL.

Intrauterine manifestations of severe PC deficiency have been uncommonly described including retinal artery thrombosis and vitreous venous thrombosis with haemorrhage, ischaemic stroke and intracranial haemorrhage\(^ {327}\). Infants usually develop symptoms after
birth; this has raised the possibility of small amounts of transplacental transfer of PC, but this has not been confirmed or considered a potential route of therapy.

In the newborn, the condition manifests shortly after birth as purpura fulminans; acute haemorrhagic necrosis of the skin caused by dermal microvascular thrombosis. DIC, and arterial and venous thrombosis often develop. The severity of the initial presentation is dependent on the residual PC levels; levels above 0.03-0.05U/mL prevent spontaneous development of purpura fulminans, although these patients present with other thromboembolic phenomena.

This is a lethal condition without treatment. It is not understood why there is preferential thrombosis of the skin microvasculature in this condition and relative sparing of other vascular beds; as previously mentioned there are no reports on NEC in this group. This suggests that PC deficiency alone does not induce intestinal vascular thrombosis or NEC. The relative distribution of activation receptors are not known on the endothelium of neonatal microvasculature that could effect local APC production.

Treatment during the acute thrombotic phase, involves replacement of PC to achieve levels that will inhibit and ultimately balance the prothrombotic and proinflammatory stimulus. PC can be replaced with large volumes of fresh frozen plasma (FFP) or PC concentrate. FFP contains 1U/mL of PC, while Ceprotin® (PC concentrate, Baxter) contains 100U/mL. Doses are titrated according to the plasma PC levels; aiming for a minimum trough level of 0.25 U/mL. PC concentrate is the preferred treatment as large volumes of FFP are associated with volume overload and exposure of the patients to ABO iso-agglutinins.
The aim of longer-term therapy is to maintain the patients on oral anticoagulation with PC concentrate supplementation for situations with increased thrombosis risk. Patients can have breakthrough episodes of purpura fulminans beyond the neonatal period associated with infection or trauma, and they are at risk of developing recurrent VTE.  

Liver transplantation is the only curative treatment for these patients; four successful transplants have been reported and PC activity was fully reconstituted in all the patients.

The anti-inflammatory and cytoprotective functions of the PC pathway should also be lacking in patients with congenital deficiency. Patients with genetic PC deficiency have not been described as presenting with an altered immune response; it is possible that the acute life-threatening thrombotic events dominate the clinical presentation. The innate immune response has many redundant pathways, therefore it is also possible that the immune functions of PC can be undertaken by alternative pathways if levels of PC are low. PC supplementation in this group will mask any effect that low levels could have on the immune response.

APC is unable to cleave FV at Arg506 in patients with a FV Leiden mutation; they have ‘APC resistance’ to the anticoagulant function of APC that is associated with an increased risk of VTE as mentioned previously. The mutation is found in 2-15% of the general population of European descent and is present in 60% of patients presenting with VTE. The high prevalence of the mutation, despite its prothrombotic effects, suggests an evolutionary benefit; the carrier status may be a balanced polymorphism that confers survival advantage.

There are opposing effects that the FV Leiden mutation may have in the setting of inflammation; firstly the resistance of FV Leiden to proteolysis will cause higher levels of circulating thrombin that, in turn, will augment APC generation and promote the anti-
inflammatory effects of APC. There is evidence that low dose infusion of thrombin was protective in dogs challenged with endotoxin. Secondly, one could argue that these patients may be at increased risk of an exaggerated host response and worse outcome, as they have reduced anticoagulant activity.

Endotoxin rodent models support a survival benefit of the FV Leiden heterozygous state in sub-lethal doses. The effect of FV Leiden mutation was examined in 3894 patients enrolled in the PROWESS and ENHANCE trials. There were no homozygous patients in the trial, which may have been chance or due to the low prevalence of the homozygous state (0.06-0.25%). The number of FV<sup>+</sup> carriers was slightly, but significantly, lower then a conservative estimate of expected patients; this could represent a decreased risk of developing severe sepsis. Baseline clinical characteristics were similar, not worse, in carriers and non-carriers; there was a trend to decreased cardiovascular compromise in the carriers. There was a trend to survival benefit, but numbers were small with wide confidence intervals (RR 0.82%, CI 0.57-1.17).
2.5.2. Physiological PC deficiency in early childhood: developmental haemostasis

The concept of developmental haemostasis describes the predictable maturation pattern of the human coagulation system from the fetus through to adulthood. Dr Andrews, who was pivotal in producing age-related neonatal, infant and childhood reference ranges for coagulation parameters, introduced the term in the 1980s.\textsuperscript{332-335}

Initial work concentrated on describing the quantitative differences of proteins at varying ages, but later work has been aimed at understanding the qualitative differences induced by aging and the possible rationale behind the need for such changes during development. Developmental changes are not isolated to the coagulation cascades; they have been seen throughout the proteome.\textsuperscript{336} It is likely that the non-coagulation functions of these proteins during fetal development determine the changes in developmental haemostasis.

Key findings from the literature can be summarised as follows:

- Protein C is quantitatively deficient at birth, and remains so until adolescence. PC levels are a third of adult levels in term neonates, and 12-20% in VLBW preterm infants. In relative terms, PC is affected the more than other factors; levels are consistently lower and take longer to recover.\textsuperscript{333,335,337}

- Spontaneous haemorrhage, thrombosis, bruising or petechiae are rare events in healthy newborns, even after the haemorrhagic risk and procoagulant stimulus of delivery. This implies that despite prolongation in global coagulation assays (PT and aPTT) and deficiencies in factor levels, healthy newborns have a functionally adequate haemostatic system that is appropriately balanced with the inhibitors of coagulation. However, sick neonates that are septic, inflamed or asphyxiated often present with coagulopathy or thrombotic complications suggesting that the
haemostatic system lacks capacity to cope with non-physiological or prolonged thrombotic stimuli\(^{338-341}\)

- The epidemiology of VTE in children is different to adults; children seem to be protected from thromboembolism. For equivalent risk factors, either acquired or inherited, the thrombosis risk in children is reduced compared to adults until the late teenage years; for example, heterozygous PC, PS and AT deficiency rarely present with VTE before adolescence, and the risk of VTE is 2% in children with nephrotic syndrome compared to 20% for adults with the same condition.\(^{342}\)

- The mechanisms for the control of developmental haemostasis include regulation of translation, post-translational modifications, and differences in protein clearance; in general children have increased protein clearance, particularly infants and neonates.\(^{338}\). In the phase I trial of recombinant APC (rhAPC) in children, the drug clearance in infants ≤ 3 months age was 22% greater than in children 3m-18 years of age, and 19% greater than adults.\(^{343}\). This is in contrast to the findings that APC has delayed clearance with an extended half-life of 50 minutes in cord blood of term infants.\(^{344}\).

- The liver, despite its role as major producer of plasma proteins, does not regulate plasma protein levels during childhood. The haemostatic profiles of children after liver transplantation with an adult graft, continue to be paediatric\(^{345}\). The vascular endothelium has been proposed as the site for developmental haemostasis regulation.\(^{342}\).

- In the fetus:
  - Coagulation factors do not cross the placenta; the fetus begins to produce coagulation factors independently by the 5\(^{th}\) week of gestation. These are measurable by the tenth week and, in general, rise in concentration as the
gestational age increases. The rise in concentration is slower from mid
gestation until term.\textsuperscript{335,346}

- Some factors are essential for human development where the homozygous
deficient state has never been described and animal models show
embryonic lethality. These factors include TF, TFPI, TM, EPCR. Prothrombin
(FII) and FV show excess foetal wastage in animal models, but some do
survive to term, only to succumb from haemorrhagic complications shortly
after birth\textsuperscript{320,346}.

- Platelets appear in the 11\textsuperscript{th} week and reach adult levels by the 20\textsuperscript{th} week of
gestation. Platelet receptors are detected by 12-16\textsuperscript{th} week and, except for
low levels of epinephrine receptors, they are equivalent to those on adult
platelets. Fetal and neonatal platelets are hyporesponsive, showing less
activation in response to thrombin and nitric oxide; they show less
aggregation in response to thrombin and epinephrine and neonatal
platelets have lower expression of adhesion receptors, P-selectin. Platelet
reactivity increases with gestational age; in the VLBW, platelet
hyporesponsiveness is maximal on day 3-4 with recovery over 10 days\textsuperscript{347}.
NEC in the VLBW group has an increased incidence after this timeframe.

- In the fetus, coagulation proteins are regulated at the level of translation,
rather than transcription. Only one unique fetal protein has been
described; it is a physiological anticoagulant similar to dermatan sulphate
and is produced by the placenta. Foetal anticoagulant catalyses thrombin
with heparin cofactor II (HCII). It is also found in the plasma in pregnant
women and disappears by day 5-post delivery. The length of time it
circulates in neonates is not known.\textsuperscript{346}. 
• Vitamin K is involved in post-translation carboxylation of the vitamin K dependent proteins (FII, VII, FIX, FX, PC, PS). In the absence of vitamin K, non-functional forms of the proteins accumulate and can be measured; PIVKA (Proteins Induced by Vitamin K Antagonism or Absence). The placenta ensures a steep gradient of vitamin K, with fetal levels kept below 10% of maternal levels\textsuperscript{346}. Vitamin k is a ligand for tyrosine kinases and promotes mutagenesis; therefore relative foetal deficiency may be a protective mechanism against mutagenesis in the rapidly dividing cells. The mRNA, and stores, of vitamin k dependent proteins are increased in fetal hepatocytes considering the low plasma levels and this may be related to reduced carboxylation. Traces of non-functioning, under-carboxylated thrombin (7%) and Protein C (27%) have been found in cord blood; however high levels of non-functioning PIVKA PC are found in only 2% of term neonates and 3% of preterm neonates\textsuperscript{348,349}.

• TF is widely expressed in the fetus by ectodermal, endodermal and neuroepithelial cells and in cell lineages that do not express TF later in life (skeletal muscle, pancreas). FVII availability is very low early in embryonic life and coupled with the knowledge that homozygous TF deficiency is lethal in mouse models, it is speculated that TF has a non-coagulant, critical role in cell proliferation, differentiation and angiogenesis\textsuperscript{350}. TFPI is also widely expressed from weeks 8-24. Levels of TFPI are low in neonates\textsuperscript{351}.

• Levels of sTM are high in the foetus, peaking at 24-26 weeks gestation (165ng/mL)\textsuperscript{352}. Levels gradually reduce to ~4ng/mL by 1-5years but only reach adult values by adolescence. Soluble TM is generally a marker of endothelial cell activation or disruption; the significance of raised levels
during childhood is not known. The effect of aging on endothelial expression of TM has not been established.\textsuperscript{353}

- After 10-11 weeks gestation, coagulation and fibrinolysis pathways produce equivalent, or shorter, whole blood clotting and euglobin clot lysis times as compared to adult values.\textsuperscript{338}

- Some proteins undergo post-translational modification to produce fetal forms. Fibrinogen, plasminogen, PC and vWF are found circulating in fetal forms:
  - Foetal fibrinogen contains increased sialic acid and phosphorus content. Thrombin clotting times are prolonged in neonates, suggesting differences in polymerisation in foetal fibrinogen.\textsuperscript{354}
  - A foetal form of PC has been discovered in the ovine foetus that has an increased proportion of single-chain molecules. The physiological significance of fetal PC is not known. If a fetal form is present in humans, it does not affect the functional activity of the protein.\textsuperscript{337}
  - Fetal plasminogen has a slower activation rate as well as reduced enzymatic activity. The activity of $\alpha2$ – antiplasmin in inhibiting plasminogen is also reduced, balancing the effects.\textsuperscript{338}
  - In the fetus, VWF circulates as ultra-large molecular weight multimers (ULVWF) until 35 weeks, then the transition begins over 2-3 months to the smaller circulating vWF molecules as found in adults.\textsuperscript{346} The foetal ULVWF shortens bleeding time and enhances platelet-endothelial interaction.
• Quantitative changes in neonates
  
  • Coagulation:

At term, in quantitative terms, the levels of most vitamin k-dependent factors (II, IX, and X) and the contact factors (XI, XII, prekallikrein (PK) and high molecular weight kininogen (HMWK)) are less than half the adult values at birth, rising to the adult range by 6 months of age. The global coagulation assays are prolonged (PT and aPTT). FVII levels are 70% adult values at birth and rise to low adult levels within a week of birth. Fibrinogen, FV and FXIII are within normal adult values from birth, while FVIII and vWF levels are higher. The absolute level of vWF is increased for the first 3 months of life, as well as the level of high molecular weight multimers of vWF.

• Clearance of plasma proteins

Thrombin generation is both decreased and delayed in neonates, and comparable to adults treated with heparin or warfarin. The amount of thrombin produced is proportional to the levels of available FII and the production rate reflects the concentration of the other procoagulant factors.

Amounts of clot-bound thrombin are reduced in neonates due to low levels of FII and FPA, reducing the local amplification effect of thrombin on platelets, slowing clot propagation.\(^{338}\)

• Inhibitors:

The levels of AT and HCII, are reduced at birth to levels similar to heterozygote deficient adults who develop spontaneous VTE. Levels rise to adult values over 6 months. AT has potent anti-angiogenic properties; therefore physiologically low levels in the foetus may promote healthy angiogenesis during a period of prolific growth.\(^{353}\) Replacement AT therapy in preterm infants may have unexpected deleterious effects; and excess mortality was seen in the only controlled trial of AT therapy in preterm infants with RDS.\(^{356}\)
In contrast the levels of α2-macroglobulin (A2M) are increased at term and continue to rise to twice-adult levels by 6 months. A2M is a major plasma protease, forming 2-4% of the total protein content in adults, where it accounts for 7% of thrombin inactivation. In neonates, A2M accounts for 33-64% of thrombin inactivation, compensating for low AT levels.221,357 Paradoxically Cvirn et al found A2M in the cord blood also interferes with APC-PS interaction, limiting APC anticoagulant activity358.

The levels of PC are low at birth and relatively more reduced than the pro- or other anticoagulants. PC levels rise the slowest of all the factors, only reaching adult levels at adolescence. PS levels are low at birth, but the functional, free PS level is preserved by low levels of C4BP.

The mechanism for APC activation appears intact in the newborn. PC levels measured in cord plasma were reduced compared to adult levels; however APC levels were 5.2 fold greater than resting adult levels344.

Levels of TFPI in cord plasma are low, 64% of adult values351.

- **Fibrinolysis.**

Plasminogen levels are reduced in the neonate and increase to adult levels by 6 months of age. The reduced levels and activity of the fetal form are balanced by reduced inhibition338. Levels of t-PA are also reduced in the neonate and remain reduced throughout childhood. Levels of PAI-1 are increased in childhood333-335,338.

- **Effect of birth**

Coagulation parameters based on fetal sampling are consistently lower than those based on healthy, preterm neonates. The last weeks of intrauterine life and the first few days after delivery are associated with an increase in levels of coagulation-related plasma proteins337.
Birth is a procoagulant stimulus, triggered by TF. Levels of TF are raised in cord blood and markers of thrombin generation (TAT complexes and PF1+2) and fibrinolysis (D-Dimers) are elevated cord blood and in the newborn for 24-48 hours after delivery. As mentioned previously, APC production is increased in response to thrombin generation.

- **Markers of thrombin generation and fibrinolysis:**

Despite lower levels of AT, neonates and even VLBW neonates, are able to generate raised TAT levels when critically ill with severe sepsis, or RDS. The levels of TAT complexes are increased in healthy children up to 5 years of age as compared to adults. Similarly, neonates are able to generate D-Dimers, and levels are higher than adult values throughout childhood. The higher levels suggest a constitutive low level production of thrombin in the early years.

It is important to note that the lack of thrombosis in healthy neonates despite the relative deficiency of the anticoagulants PC, AT and TFPI, may reflect an increased role for a substitute thrombin inhibitor, for example α2- macroglobulin; the levels which are doubled in the neonatal period. The effect of maturation on the potential capacity of the PC pathways is not known.

The premise that neonatal physiological levels of PC and APC are below saturation of the PC pathway was proven in an ex-vivo study using adult and neonatal plasma; thrombin generation was controlled by PC in a dose-dependent manner. The effect of maturation has not been investigated with regard to the expression or function of TM or EPCR.

All of these developmental effects decrease the activity of the PC pathway in preterm infants, and the reduction is relatively greater than the effects on the other factors. Preterm neonates are vulnerable to sustained thrombotic stimuli, having reduced capacity in the PC pathway to counter-balance.
Acquired PC deficiency in acute inflammatory states

Acute inflammation down-regulates every aspect of the PC system.

2.5.2.1. PC and APC plasma concentrations

There are multiple factors involved in the development of low PC levels during acute inflammation; some have been mentioned previously.

To summarise, the liver down-regulates production of PC as part of the acute phase reaction driven by IL-6, there is reduced expression, and increased shedding, of TM from the endothelial cell surfaces, and increased TM expression on neutrophil and monocyte cell surfaces. There is increased shedding of EPCR, endocytosis and variable expression of EPCR on cell surfaces. There is increased consumption of PC and APC and increased clearance from the circulation by inhibitors.

Acquired Protein C deficiency develops in more than 80% of adults with severe sepsis. The levels of PC fall early, even before the development of clinical signs. Severe PC deficiency (≤40%), is associated with adverse outcomes; shock, DIC, longer ventilation times, ICU stay and higher mortality.

The time taken to recover PC levels also has predictive value, with non-survivors failing to recover PC levels over 2-3 weeks (figure 1.12). This finding is common to observational and interventional studies using rhAPC.

It has been suggested that the absolute PC level and the time course of PC recovery may be useful biomarkers for severity and mortality; potentially stratifying patients for interventions, and serving as a surrogate clinical endpoint.
Figure 1.12: PC levels of adults with severe sepsis enrolled in the placebo and treatment arms of the PROWESS trial, classified according to the timing of their death or survival. Early mortality (days 1-5) was associated with severe PC deficiency and later mortality was associated with failure to recover PC levels. (Reproduced from: Shorr et al Critical Care 2008,12:R45 370)

A number of observational and interventional trials in sepsis have shown a similar pattern in children and infants; low PC levels are prevalent in severe sepsis 371-373. The term and preterm neonatal population also show a fall in PC, PS, and AT, in response to sepsis. Baseline PC levels below 10% are associated with mortality and severity of illness (shock and DIC) 340,374-376.

In neonates, low PC, PS, and AT levels were also found in critically ill neonates with hypoxic/ischaemic injury following perinatal asphyxia and lower levels were associated with severity and mortality 339.

Low PC levels are also common in critically ill adult patients without sepsis; and associated with increased severity in organ failure and mortality. Brunkorst et al showed that 50% of surgical critically ill adults had PC levels below the reference range on admission to
intensive care, although not as low as patients with septic shock. PC levels fell to a nadir on day 3-4 and rose over 2-3 weeks.\textsuperscript{273,370} Borgel et al compared the PC pathway in patients with organ failure, with and without sepsis, to healthy controls. PC and PS levels were significantly lower in both critically ill groups and not different to each other. The PC system is implicated in the development of coagulopathy in early, severe trauma; although the mechanisms need clarification. High levels of APC during acute stabilisation were associated with mortality, organ failure and longer ICU stay in patients with major trauma and low PC levels in the ER were associated with the development of ventilator associated pneumonia\textsuperscript{377,378}.

Low PC levels in non-septic, as well as septic, adult patients with organ failure are associated with ARDS, length of ventilation, organ failure and death.

To summarise, low plasma PC levels are an early indicator of critical illness in all age groups and levels have prognostic significance in terms of severity of illness and survival. Levels that do not recover are also associated with mortality. These findings suggest that either, or both, ongoing consumption of PC and/or inadequate supplies of PC are associated with severity of illness and survival. This places the PC pathway in a key, central position in managing the host response to inflammation, with most of the pathway actions occurring in the microvasculature at the at the endothelial surface. Deficiencies or derangements in the PC pathway may be causal to, and caused by the inflammatory response. The levels of PC in NEC are not known.

The levels of APC have not shown a similar, clear, directional change with inflammation in human observational studies. An enzyme capture assay to determine plasma APC levels was first developed in the 1990’s; it was able to detect to lower limits of 0.3ng/mL but this took up to 10 days to achieve\textsuperscript{379}. As a consequence many studies used a less sensitive technique with a higher detection limit of 5-10 ng/mL, which is above the resting circulating
concentration of APC (1-3ng/ml). Not surprisingly, APC was not detected in two adult phase I trials of rhAPC; and in a similar phase I trial in septic children, the pre-infusion APC levels were below 10ng/mL in 88% of patients[^343,^380,^381].

**Figure 1.13:** Comparison of PC levels in patients with organ failure to healthy controls.

(A) PC levels are low in patients with ALI/ARDS (medians ±IQR (box) & 10th & 90th centiles: *p<0.001).

(B) PC levels in ALI/ARDS group from (A) classified by additional organ failures. (mean± SD; *p<0.05).

(C) PC levels classified according to measures of severity of illness (means ±SD; *p<0.05).


The APC assay has been refined using a new monoclonal antibody, reducing the laboratory time. Liaw et al discovered that patients varied in their ability to produce APC. In general, septic patients had lower PC and higher APC levels than controls (PC: 0.48 U/ml (±0.23 SD).
vs. 0.86U/mL (±0.16 SD) and APC: 4.36 ng/mL (±2.06 SD) vs. 1.18 ng/mL (±0.64 SD)). Levels of APC were not related to PC levels. Survivors had higher levels of APC\textsuperscript{257}. Borgel et al found this pattern was reproduced in non-septic critically ill patients and higher APC levels were also associated with survival\textsuperscript{272}.

Liaw et al found sEPCR and sTM were raised in septic patients but there was no relationship to mortality\textsuperscript{201,257}. Borgel found raised sTM in all critically ill patients. This was associated with decrease in TM mRNA related to neutrophils and monocytes in septic patients, but an increase in TM mRNA and increased monocyte TM expression in non-septic critically ill patients. This implies potential fine-tuning of the PC pathway in the host response to different triggers\textsuperscript{272}.

De Kleijn et al measured the baseline APC levels in 40 children with meningococcaemia. The median levels of APC in the groups at study entry were raised above normal levels, and the maximum value was 55 times the upper limit. All of the groups had very low median PC levels\textsuperscript{382}.

As previously mentioned, some 40% of APC remains bound to EPCR and is not in circulation, therefore this may be a reason why plasma levels of APC may not reflect activation of the pathway\textsuperscript{262,263}.

The levels of APC in healthy neonates is not known, and PC or APC concentrations in neonates with NEC are not known.


2.5.3. Interventional studies using rhAPC or PC concentrate.

In humans, PC concentrate has been used to supplement PC levels in acquired PC deficiency states since the early 1990s. However, recognition that the activation apparatus of PC (endothelial TM and EPCR) is down regulated in severe sepsis, lead to the preferred use of exogenous rhAPC in interventional studies involving the PC pathway.

A number of landmark RCTs have taken place over the last decade examining the efficacy of APC supplementation in severe sepsis. All trials used the same dose of rhAPC (96-hour infusion of rhAPC at a dose of 24µg/kg/hour).

The PROWESS (rhAPC Worldwide Evaluation in Severe Sepsis) trial was reported in 2001. It was a randomised, placebo controlled trial (RCT) in 1690 adults with severe sepsis, defined as infection with signs of systemic inflammatory response (SIRS) and one sepsis-induced organ failure. The trial was halted at the second interim analysis because the treatment benefit exceeded the a priori guidelines for halting the study. The trial reported a 6.1% mortality benefit. The steady-state APC plasma level was 45ng/mL, a 15-40-fold increase in resting physiological levels and approximately 1/1000th normal PC levels. The baseline median PC levels were 50% of normal levels. The IL-6 levels in the rhAPC treated group were significantly lower. There was a non-significant increase in severe bleeding events in the treatment group (3.5% vs. 2%)\(^3\)

FDA approval for the use of rhAPC in severe sepsis was granted in November 2001 and drug-licensing boards across the globe followed suit\(^383\).

A safety, pharmacokinetics and pharmacodynamics trial (2004) of escalating doses of rhAPC was undertaken in 83 children with severe sepsis (term neonates - <18years age)\(^343\). The
study confirmed that the rhAPC dose/kilogram bodyweight was the same as for adult
patients. The incidence of serious bleeding during the study period was 4.8%.

Post-market surveillance continued in the open-label trial, ENHANCE. This trial recruited
adults and children (term neonates - <18 years) with infection, SIRS and single-organ failure
within 48 hours to rhAPC infusion. The results were reported separately for adults (2005)
and children (2006), as there were clear differences\textsuperscript{372,384}. The data from 2375 adults
supported the findings of PROWESS (28 day mortality 25.3%), but they observed an
increased serious bleeding rate (6.5%) particularly CNS haemorrhage (1.5%). This study
suggested that rhAPC was more efficacious if given early within 24 hours\textsuperscript{384}. The data from
187 paediatric patients showed a 4 day mortality of 7% and 28 day mortality of 13.4%,
there was no placebo group to draw efficacy conclusions. However, more importantly, they
observed a higher rate of serious bleeding events (27.7%) and a higher rate of CNS
haemorrhage (2.7%)\textsuperscript{372}.

Concerns arose that the interaction of prophylactic heparin with rhAPC could either;
increase the bleeding risk or paradoxically increase the thrombotic risk due to increased PCI
for treatment with heparin in prophylactic doses or placebo. Heparin was not associated
with increased mortality; it was associated with increased bleeding events but not serious
bleeding events. The placebo group had more thromboembolic events\textsuperscript{214}.

Sub-group analysis of PROWESS suggested that the benefits of rhAPC appeared greater if
the therapy was started earlier (within 24 hours) and was targeted to sicker patients with
multi-organ failure\textsuperscript{385}. The ADDRESS trial in 2613 adults with severe sepsis and low risk of
death showed lack of efficacy of rhAPC in this group, and was stopped at interim analysis
(2005). There were increased serious bleeding events in the treatment group, both during
rhAPC infusion and afterwards over the 28-day follow-up.\textsuperscript{386}
An RCT of rhAPC infusion in 477 children with septic shock was reported in 2007 (RESOLVE)\(^3\). Participants included term neonates to children less than 18 years of age with sepsis induced cardiovascular and respiratory failure. The primary outcome was a composite score of the time taken to organ failure resolution. The trial was stopped at the second interim analysis as the efficacy endpoints could not be reached. There was no difference in the composite score or 28 day mortality between rhAPC and placebo groups. There was a trend towards increased serious bleeding events (4.6% rhAPC vs. 2.1% placebo) and there were numerically more CNS haemorrhages in the infants of the treatment group. In the whole cohort, fewer children under 6 months of age had abnormal PC levels at baseline as compared to older children and adults\(^3\).

Lastly, an RCT was undertaken in 1697 adults with septic shock (PROWESS-SHOCK), rhAPC did not reduce mortality at 28 (26.4% rhAPC vs 24.2%) or 90 days. Bleeding events were more common in the treatment group, but serious bleeding events were equal between the groups\(^3\). This later study showed the baseline improvement in mortality rates over the last decade (24.2% in the placebo group) and this ‘swallowed’ any benefit that may have been offered by APC in earlier years.

Eli Lilly withdrew rhAPC (Xigris) from the market in October 2011. They declared that the PROWESS-shock trial had failed to demonstrate improved patient survival. They reported there were no new safety concerns but the data called into question the benefit-risk profile of treatment\(^3\). The drug is not recommended in the new surviving sepsis campaign guidelines\(^3\).

It is disappointing that the RCTs of the use of rhAPC patients with severe sepsis showed lack of clinical efficacy. There are a number of considerations that may contribute to understanding this failure. Much of the ‘pre-clinical’ exploratory work into the mechanisms of PC/APC functioning has occurred after the clinical trials. It has become clear that the
anti-coagulant and anti-inflammatory effects are distinct, if over-lapping. The crucial roles, and distribution, of the cell surface receptors are also more defined; although not fully understood. The importance of the relative concentrations of PC and APC at the cell surface in the microcirculation, or in and around a thrombus, is not fully understood; however it is known that plasma levels of APC do not reflect the bound, active pool, and endogenous APC has been shown to be more efficacious\textsuperscript{262,263,279}. It is also important to note that the doses of rhAPC used in many of the proof-of-concept, pre-clinical investigations were supra-pharmacological at 1000-20 000 x the endogenous plasma APC level. The rhAPC dose (24μg/kg/hr x 96 hours) in human RCTs was 15-45 x the endogenous level and was associated with bleeding events; therefore increased doses can not be considered\textsuperscript{219}.

The increased bleeding events associated with exogenous rhAPC treatment has fostered an renewed interest in the possibilities of PC concentrate replacement, harnessing the endogenous production of APC; as well as the promise of developing genetically mutated APC for therapeutic use, with retained anti-inflammatory effects but reduced anticoagulant potential.

The published evidence of PC concentrate (PCC) supplementation in humans includes individual case reports, case series, and a single randomised, controlled, blinded dose-finding study in children with meningococcal sepsis\textsuperscript{382}. These are summarised in the study protocol that is attached as an appendix to this thesis. PCC is reported in 358 patients; at least 229 are children and 61 neonates, most of whom are premature. The reports are mainly limited to populations of septic patients. There were no reported serious adverse events associated with PCC administration and, not surprisingly with positive publication bias, most report benefit; reduced coagulopathy, reduced morbidity and/or improved survival compared to predicted.
In the single phase II trial, administration of PCC resulted in a dose-dependent increase in APC levels. Markers of DIC resolved more rapidly in higher PCC dose groups. It is clear from this study that despite reduced expression of TM and EPCR in the skin lesions of children with meningococcaemia, patients are able to activate PC when levels are supplemented in a dose-dependent manner\textsuperscript{237,382}.

2.6. The PC pathway in Inflammatory Bowel Disease:

Ulcerative colitis (UC) and Crohn’s disease (CD) are major forms of inflammatory bowel disease (IBD) in adults in which the role of the innate immune system is becoming increasingly recognised. NEC is a inflammatory condition of the premature bowel that results in part from the over-activity of the innate immune system. There may be sufficient similarity or overlap in the pathogenesis of the conditions such that preclinical research results to understand mechanisms of disease and therapeutic possibilities in the adult disease should be taken into consideration in NEC research\textsuperscript{392}.

UC and CD are characterised by chronic relapsing inflammation of the intestinal mucosa; the pathogenesis is complex and involves dysfunctional interaction of the microflora and the mucosal immune system leading to leucocyte infiltration, progressive destruction and defective repair\textsuperscript{393,394}.

The pathogenesis of IBD was previously thought to be entirely due the actions of a dysfunctional immune system. More recently, importance of the interplay between the immune response and the endothelium and epithelium has become clear. This direction of investigation is similar to that being seen in NEC.

The PC pathway modulates the functions of the endothelium and intestinal epithelium and is important in the maintenance of intestinal homeostasis\textsuperscript{238,394}. TM and EPCR expression is
decreased in acutely inflamed areas of bowel on histological examination of human specimens. The expression remained strong in less inflamed or non-inflamed areas.\textsuperscript{174,239}

\textbf{Figure 1.14:} Immunohistochemical staining of EPCR and TM in the colon of normal and actively inflamed IBD adult patients. EPCR and TM appear brown in the microvasculature of colonic mucosa and submucosa. Normal control (A and B), active inflammatory bowel disease (C and D), and non-inflamed tissue from patients with inflammatory bowel disease (E and F).

The similarity to the immunohistochemistry undertaken in neonatal intestine in this thesis can be seen in colour plates in chapters 3 and 4. Red arrows indicate intestinal vessels. Reproduced from Scaldaferri J Clin Invest 2007; 117:1952.

Human intestinal microvasculature endothelial cells (HIMEC) express TM and EPCR; TNF$\alpha$ down-regulated the cell surface expression and transcription of TM and EPCR and increased shedding of EPCR, although not TM. IL-10 increased TM expression on HIMECs, but not EPCR expression. HIMECs were able to convert PC to APC \textit{in vitro}; this was impaired by TNF$\alpha$. HIMECs activated by TNF$\alpha$ \textit{in vitro} produced adhesion molecules and chemokines. Exogenous rhAPC had a potent anti-inflammatory effect reducing the adhesion molecule and chemokine production. In a murine model of chemically induced colitis, affected mice had a reduced ability to convert PC to APC. Intravenous recombinant APC ameliorated the
severity of the colitis on histology and reduced leucocyte-endothelial adhesion on intravital microscopy\textsuperscript{124}.

Figure 1.15: (Left) TNF$\alpha$-activated HIMECs have reduced capacity to activate PC. 
(Right) APC production in healthy mice compared to mice with chemically induced colitis. 
The relative capacity for PC activation is shown as change in absorbance over time. 
HIMEC=human intestine microvasculature endothelial cells. 
* $p<0.05$. From Scaldaferri F. J Clin Invest.2007; 117:1951-1960.
Chapter 1: Introduction

**Figure 1.6:** Effect of TNFα and IL-10 on the HIMEC expression of TM and EPCR. HIMEC monolayers were incubated for 24 hours with TNFα and IL-10 and TM and EPCR expression were measured by flow cytometry. TNFα reduced TM and EPCR expression, IL-10 increased EPCR expression. Black filled curves represent signal from isotope control. Numbers are the net percentage of positive cells. HIMEC = human intestine microvascular endothelial cells. From Scaldaferri F. J Clin Invest. 2007; 117:1951-1960

The role played by the PC pathway in the intestinal microvasculature is similar to the role it plays throughout the rest of the body. The surprise recent finding is the expression of PC in the intestinal epithelium and its role in maintaining the intestinal barrier function.

Intestinal epithelial cells (IECs) express PC, EPCR and PAR-1 but not TM. IECs from bowel with active ulcerative colitis or Crohn’s disease had reduced expression and mRNA of EPCR. IECs from quiescent bowel had normal levels of expression. Transgenic mice with PC deficiency were used to determine the effect of low PC expression. Mice expressing only 3% PC had spontaneous colitis; and an exacerbated response when exposed to chemicals to induce colitis. These mice had more severe injury on histology, higher levels of pro-inflammatory cytokines, increased intestinal epithelial barrier permeability and reduced expression of tight-junction proteins. In vitro, rAPC (with EPCR) prevented the permeability effects of TNFα and preserved the expression of tight junction proteins in an IEC monolayer. APC (with EPCR) was able to induce IEC migration and proliferation; and to a similar degree as epithelial growth factor (EGF). rAPC also improved the healing of an
intestinal surgical anastomosis in a CLP-surgical murine model\textsuperscript{395}. Topical intrarectal rAPC had a beneficial effect in mice with colits; promoting mucosal healing\textsuperscript{198}.

The function of the intestinal epithelium is to provide a selective barrier to solutes, antigens, macromolecules and cells, and to regulate the passive movement of solutes between adjacent cells. Loss of barrier function can lead to inflammation due to translocation of luminal antigens and molecules causing inappropriate activation of the mucosal immune system. The PC system is a key modulator in epithelial barrier function, enhancing the expression of tight junction proteins\textsuperscript{198}. 
3. **Hypotheses underlying this thesis.**

This review of our current understanding of the pathogenesis of NEC and the functions of the PC pathway reveal the highly complex, web-like nature of the PC pathway and its vast sphere of influence.

I have discussed the many compelling reasons to hypothesise that the PC pathway will play a role in the multi-faceted pathogenesis of NEC.

The overarching hypotheses underlying the work in this thesis are two-fold:

1. **NEC in is associated with deficiency in the PC Pathway in neonates.**
2. **Supplementation of the PC pathway will improve outcomes from NEC.**

We undertook retrospective and prospective observational clinical studies to address the first hypothesis, and explored the second in an animal model of NEC.
Chapter 2: General methods and materials
Materials and general methods

1 Introduction

The experimental work in this thesis required adaptation and optimisation of existing scientific methods of investigation, either for novel use in neonatal intestinal tissue or novel use in a rodent model.

This chapter describes the underlying principles of my approach and common protocols. Chemicals and materials are listed at the end of this chapter in Table 2.10 and 2.11

2 Histology and immunohistochemistry techniques

2.1 Sample Fixation:

All human tissue samples and some rat ileum samples were fixed in 10% neutral buffered formalin solution for 18-36 hours. Tissue samples were subsequently embedded in paraffin. The remaining rat specimens were snap frozen and stored at -80°C.

2.2 Histology sections

The intestinal tissues from neonates and the animal model were friable. We used APES (3-aminopropyltriethoxysilane) coated slides to improve adherence of the tissue to the slide during processing. APES coating gives the slide surface a positive charge and the tissue tends to have a net negative charge in aqueous medium (pH range 5-7), due to an excess of acidic amino acids in the structural proteins.

Formalin fixed, paraffin embedded tissue (FFPE): 4 μm sections were cut with a microtome and mounted on APES coated slides using a water bath and air dried.
Cryostat sections: 4 μm sections were cut from frozen tissue embedded in optimal cutting temperature (OCT) embedding medium. Multiple sections from each tissue bloc were mounted on APES coated slides and air-dried. Sections were stored at -80°C.

2.3 Dewaxing and rehydration protocol for FFPE sections

Slides were incubated for 10 minutes in a histoclear bath to remove the paraffin wax, repeated once. Sections were re-hydrated through a series of downgrading ethanol baths (100% ethanol x 3min twice, 70% ethanol 3 min twice) and taken to deionised water. Care was taken throughout the handling of the slides to ensure they did not dry out. All slides are prepared by encircling the tissue with a hydrophobic line using a PAP- marker pen. The PAP pen applies the hydrophobic circle around the mounted tissue, keeping applied liquids and solutions pooled over the tissue. This reduces waste of reagents and reduces the risk of specimens drying out during staining. It is soluble in histoclear.

2.4 Haematoxylin and eosin staining

Haematoxylin stains basic structures (nuclei) blue/black, and eosin stains acidic structures (cytoplasm), in shades of red and pink.

Sections were dewaxed according to the protocol above. The slides were incubated with Haematoxylin QS for 5 min and rinsed until they ceased running ‘blue’ in running tap water. Slides were then dipped in 1% acid alcohol for differentiation and rinsed in running water again. They were incubated in 0.5% eosin for 5 minutes and rinsed in running water for 2 minutes. They were taken to deionised water and dehydrated through a series of upgrading concentrations of ethanol (70% ethanol x 3 min twice, 100% ethanol x 3 min twice) and taken to a histoclear bath for 10 minutes, twice. The coverslips were mounted with a non-aqueous mounting medium.
2.5 Immunohistochemistry: general principles applied in this project

Immunohistochemistry exploits the specificity of antigen-antibody reactions to localise tissue and cellular antigens at the level of light microscopy. The antigen-antibody reaction is visualised by markers such as fluorescent dyes or enzymes acting on a chromogen substrate.

The direct immunohistochemistry technique involves only one step; the labelled antibody reacts directly with the antigen in tissue sections. This procedure is short and quick, but it is relatively insensitive as it does not allow signal amplification and therefore is not often used.

The in-direct or two-step method allows for signal amplification. The primary antibody (first layer) is unlabelled and binds to the tissue antigen to be studied. The primary antibody becomes the antigen for the secondary antibody that is conjugated with an enzyme or fluorescent dye (secondary layer). This method is more sensitive as there is signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody.

Common enzyme conjugates include the streptavidin-biotin-horseradish peroxidase (HRP) combination and alkaline phosphatase. These are both problematic in immunohistochemistry of the intestine as the high levels of endogenous biotin, peroxidase and alkaline phosphatase increases the non-specific background staining.

2.6 Polymer conjugate technology.

One way to reduce non-specific background staining is to use dextran polymer conjugates, as utilised by EnVision™. An inert backbone of dextran is bound to ~70 enzyme molecules (horseradish peroxidase (HRP) or alkaline phosphatase (AP)) and ~10 secondary
antibodies, forming the second layer. The secondary antibodies are species specific, as in the indirect method above and are directed against the species of the primary antibody.

There are a number of benefits of using dextran polymer conjugates as the second layer including, increased sensitivity because the secondary antibody is bound to many enzyme molecules, as well as a reduction in the total number of assay steps as compared to conventional techniques, thereby reducing the risk of the tissue drying out. The secondary antibody polymer conjugates do not use the streptavidin-biotin method and therefore nonspecific staining caused by endogenous biotin is eliminated. However, the potential disadvantage of the large dextran backbone is that it may obstruct penetration into the tissue, reducing specific antibody-antigen interactions.

The ImmPRESS™ second layer systems from Vector Laboratories are based on a newer method of polymerizing enzymes and attaching the polymers to antibodies. The novel approach forms enzyme "micropolymers" and avoids the potential shortcomings of using large dextrans or other macromolecules as backbones. The antibody - micropolymers are sensitive, and also do not use the streptavidin-biotin method and therefore eliminates nonspecific binding to endogenous biotin.

I used the EnVision secondary antibody dextran polymers in the earlier study described in chapter 3 as it was the only polymer system available at the time. The ImmPRESS secondary antibody micropolymers became available later and we elected to use these for the immunohistochemistry described in chapters 4 and 5 because of the theoretical advantages of improved tissue penetration. The staining was improved with the ImmPRESS system, particularly for EPCR, as can be seen in the colour plates in the relevant chapters. The change in method did not introduce bias because sections are graded against positive tissue controls and negative antibody and reagent controls that are processed in the same
experiment. Sections from the different studies in chapter 3 and 4 were not directly compared.

2.7 General immunohistochemistry protocol for FFPE sections with polymer HRP- (EnVision™ System (HRP) and ImmPRESS™ Peroxidase).

Slides were dewaxed, rehydrated and marked with a PAP pen. Dehydration of the slides during incubation periods was prevented by using a humidification chamber. Antigen retrieval steps were undertaken if required (please see specific methods for individual antibodies). This may have included the application of heat (heat induced epitope retrieval, HIER) or proteolytic enzyme digestion (proteolytic induced epitope retrieval, PIER). The slides were then rinsed in 0.1% PBS –tween and incubated with peroxidase blocking agent (3% hydrogen peroxide in phosphate buffered saline) for 20 minutes. The hydrogen peroxide was rinsed off and the slides washed twice in 0.1% PBS-tween for 3 minutes. Next, the slides were incubated with protein blocking serum for 30 minutes to reduce non-specific staining, rinsed and washed twice in a bath of 0.1% PBS-tween for 3 minutes. The slides were incubated with the primary antibody at the appropriate dilution for the period of time determined during optimisation of the protocol. The slides were then rinsed and washed twice in a bath of 0.1% PBS-tween for 3 minutes. Next, the polymer solution was applied for 30 minutes, containing the appropriate secondary antibody to react with primary antibody species. The slides were rinsed again and washed twice in a bath of 0.1% PBS-tWEEN for 3 minutes. The peroxidase substrate chromogen (3,3’ Diaminobenzidine (DAB) was applied to the slides for 3 minutes for optimal visualisation, then rinsed and washed in a bath of 0.1% PBS-tween for 3 minutes. The slides were counterstained using Haematoxylin QS for 2-3 minutes and ‘blue-d’ in running water for 5 minutes. Slides were dehydrated in upgrading concentrations of ethanol to 100% and then immersed twice in a
histoclear bath for 10 minutes. The coverslips were mounted using a non-aqueous mounting medium.

2.8 General immunohistochemistry protocol for FFPE sections with polymer conjugates with AP (Envision™ System (Alkaline Phospashatase))

In general the same steps were followed as for the as the HRP polymer conjugate method described in point 2.7 above. The endogenous peroxidase block was replaced with an endogenous alkaline phosphatase block; incubation with levamisole for 15 min. The chromogens for alkaline phosphase included Fast Red and permanent Red and the visualisation was optimal with an incubation time of 20 minutes. Slides were not dehydrated in an upgrading ethanol series as this removes the chromogen. Coverslips were mounted using aqueous mounting medium.

2.9 General protocol for simultaneous multiple antigen staining using polymer conjugate systems on FFPE sections of neonatal intestine (EnVision and ImmPRESS)

The simultaneous staining of two or more antigens with different chromogens was useful to determine the co-location of antigens within a tissue; TM and fibrin. The second layer polymer was conjugated with both HRP and alkaline phosphatase enzymes and secondary antibodies of two species. The method involved sequential staining of each primary antibody after complete colour development. After the visualisation step of the first primary antibody, an elution step ensured that only the deposits of chromogen from the previous steps remained, reducing the potential for cross-reactivity.

The steps of the HRP polymer conjugate method described in 2.7 were followed in the first instance. Antigen retrieval steps for both primary antibodies were undertaken immediately after rehydration of the slides. The protocol in 2.7 was followed up to and including the visualisation stage using DAB for 3 minutes. Slides were then rinsed and washed twice in a
0.1% PBS-tween bath for 3 minutes. The slides were blocked with levamisole for 20
minutes, rinsed and washed twice in a 0.1% PBS-tween bath for 3 minutes. The slides were
incubated for the optimal time with the next (or second) primary antibody at the
appropriate dilution and thereafter rinsed and washed twice in a 0.1% PBS-tween bath for 3
minutes. Slides were incubated with the AP-conjugated polymer for 30 minutes, rinsed and
washed twice in 0.1% PBS-tween. Visualisation occurred with incubation with Fast Red for
15 minutes. After rinsing and washing twice in bath of 0.1% PBS-tween, slides were
counterstained with haematoxylin QS and ‘blue-d’ in running water. Coverslips were
mounted using aqueous medium.

The process for optimising the protocols for multiple antigen immunostaining, or dual
staining, was dependent first on determining the optimal staining protocols for the primary
antibodies individually. Optimisation involved choosing the antibody with the best results,
manipulating antibody concentration, incubation time and temperature, antigen retrieval
and blocking steps in order to maximise specific immunostaining. Thereafter the running
order of the two primary antibodies was determined by comparing results of a reversed
running order. These steps also confirmed if any of the pre-treatments (blocking steps and
antigen retrieval steps) affected the subsequent or second antigen-antibody reactions in
the procedure.
2.10 Controls

Reagent and tissue controls are necessary for validation of immunohistochemical staining results. They are necessary to identify tissue specific, temporal (day-to-day), reagent - or user-dependent variations in staining results. I used the following controls in each experiment.

2.10.1 Positive tissue controls

Tissues that are known to express the antigen under investigation were used to optimise the immunohistochemistry protocols. Human tonsil sections were positive tissue controls for TM, fibrin and P-selectin. Normal human skin sections are positive tissue controls for TM and EPCR\(^1\). FFPE sections of blood clot were positive controls for fibrin, CD41 and P-selectin.

TM and EPCR were found to be constitutively expressed on intestinal endothelium, therefore the unblinded controls in chapter 3 also served as positive tissue controls for later experiments in chapter 4 and unblinded sham animals served as positive tissue controls in chapter 5.

2.10.2 Negative controls for the primary antibodies and reagents.

Antibody and reagent controls were used to detect binding unrelated to the specific antibody-antigen interaction under investigation.

In the negative control of the primary antibody, immunoglobulin of the same species and subtype, and in the same concentration, replaced the primary antibody during the staining procedure.

In the reagent negative controls, 0.1% PBS –tween is used to replace the primary antibody layer.
2.11 Optimisation techniques for immunohistochemistry protocols

The choice of antibody is important in optimising the detection of an antigen using immunohistochemistry. Where possible, monoclonal antibodies were used as these have reduced non-specific staining. Multiple antibodies to different epitopes of the molecule under examination were worked up in an effort to develop an optimised protocol for staining. In addition, the protocols for individual antibodies were optimised using the techniques aimed at increasing the signal from specific antigen-antibody reactions and reducing any background, non-specific signal; improving the signal-to-noise ratio.

Antibody-antigen reactions were enhanced by: unmasking the epitope in FFPE sections following fixation using heat or proteolytic enzyme digestion, by manipulating the antibody dilution and both the incubation time and temperature. A number of blocking treatments to reduce non-specific background staining or ‘noise’ were trialled for each antibody\textsuperscript{2,3}.

None of the primary antibodies under investigation had been previously used in the neonatal intestine, although some had been used in adult intestine. TM, EPCR and P-selectin had not been previously used in the rat intestine. Published methods and manufacturer recommendations for the primary antibodies had also not used the newer, more sensitive polymer-conjugate systems.

Therefore, the optimum protocol for each primary antibody had to be determined for use with the polymer-conjugate system in neonatal human and rat intestine. A systematic approach, recommended by the Cell Markers Committee of the College of American Pathologists, was used\textsuperscript{2}. A series of experiments was undertaken for each antibody, assessing the individual impact of the manipulation of antibody dilutions, antigen retrieval, incubation times and the use of blocking steps, on specific immunostaining. All experiments
had positive tissue controls and negative antibody and reagent controls in order to interpret the results.

Background staining was particularly problematic in the necrotic areas of NEC or IR intestine and required significant adaptation to maximise the ‘signal to noise’ ratio.

2.11.1 Antigen retrieval.

- **Heat Induced Epitope Retrieval (HIER)**

The exact mechanism of how heat unmarks epitopes is not known but is thought to involve the reversal some of the cross-links formed during fixation, possibly by chelating calcium\(^3,4\).

Sections were immersed in citrate buffer solution (pH 6.0-6.15), covered with cling film to prevent dehydration, and microwaved on full power in a 900watt microwave. The citrate buffer boiled at this power. Sections were cooled over 3-5 minutes by the gradual addition of PBS-tween at room temperature.

The optimum microwave time was 5 minutes; longer times caused increasing tissue destruction and 10 minutes caused complete disintegration of the sections. Shorter times were ineffectual. This demonstrates the fragility of the intestinal tissues, as HIER treatments are usually 10-20 minutes.

- **Protease Induced Epitope Retrieval (PIER)**

Proteolytic digestion is thought to expose epitopes that become masked during fixation\(^3\).

Proteinase K, diluted in 0.05 mol/L Tris-HCl and pH7.5, was used for 10minutes, incubated at 37°C. Longer times were associated with loss of tissue morphology and there was complete tissue destruction at 15 minutes.
2.11.2 Antibody dilution

The optimal antibody dilution for each primary antibody was determined in a series of experiments. The dilution recommended by the manufacturer served as an initial starting point for commercial antibodies, while methods described in the literature served to guide the initial experiments for the non-commercial antibodies\textsuperscript{15}.

Antibodies were diluted in 0.1% PBS-tween or serum from an unrelated species to the primary and secondary antibodies.

2.11.3 Incubation time and temperature.

A series of experiments for anti-TM, anti-EPCR and anti-fibrin primary antibodies, investigated the effect of varied incubation times (30 minutes to 4 hours) in a humidification chamber at room temperature. There was no increase in efficacy. The sections were at much greater risk of drying out at incubation times longer than 4 hours at room temperature.

A series of experiments for anti-EPCR and anti-P-selectin antibodies, investigated the effect of incubating the primary antibody at 37°C in a humidification chamber. Sections were prone to drying out if they were incubated at this higher temperature for periods longer than 30 minutes and there was no increase in signal-to-noise ratio.

A series of experiments determined P-selectin staining was optimal with incubation in a humidification chamber for 18 hours at 4°C.

2.11.4 Blocking treatments

Background staining can be caused by a number of factors for example; diffusion of the antigen is known to occur if there is delayed or inadequate fixation. As mentioned
previously, necrotic areas had higher levels of background staining, it is possible that the
diffusion of antigens occurred before resection in these tissues, and may mimic delayed
fixation.

• **Endogenous enzyme activity**

Non-specific staining was reduced in intestine with HRP immunostaining by including a
blocking step in the protocol of endogenous peroxidase activity before the first layer of
antibody. The blocking step involved quenching the sections with 3% hydrogen peroxide in
PBS for 20 minutes before the primary antibody was applied.

Similarly, reduced background staining was seen with alkaline phosphatase immunostaining
by inclusion of a blocking step of endogenous alkaline phosphatase activity in the protocol.
The block involved the application of levamisole before the first layer antibody for 15
minutes.

• **Non-immunological binding of antibodies: Hydrophobic & electrostatic reactions**

Serum blocks (or protein free serum blocks) reduced the background staining by competing
with the primary antibody against the weak hydrophobic forces in the tissues. The blocks
did not react with the secondary antibody and therefore they reduced non-specific staining.
The blocking protein was added to the primary antibody diluent for the animal model
immunostaining as well as incubated with the sections as a separate step as for the staining
procedures on human intestine. Protein free serum block has the advantage of being
universal; it can be used with any secondary antibody species.

• **Cross reactivity of antibodies.**
This occurs if the antibody epitopes are shared by other proteins within the tissue. The use of specific clones of monoclonal antibodies reduces this type of background staining. As mentioned previously, several antibodies for each antigen were worked up to determine the antibody with the best performance. These are listed in the table at the end of this chapter (table 2.10)

• **Antigen diffusion**

As mentioned previously, specific background staining can occur if the antigen diffuses from its usual location. This may be evident in damaged or disrupted tissue, as occurred in sections of neonatal intestine severely affected by NEC. Antigen diffusion causing background staining may occur if the antigen is present in plasma and has perfused through the tissue prior to fixation. Disrupted cellular adhesion and intestinal wall oedema occurs in NEC, allowing leakage of plasma into the interstitium. Several of the antigens studied are present in plasma (soluble TM, soluble EPCR, fibrinogen) and may have diffused through the intestinal tissues increasing the background staining. There are no specific blocking steps to reduce this.

• **Endogenous biotin activity**

The intestine has high levels of endogenous biotin that can cause non-specific staining. The polymer conjugate system does not use a streptavidin- biotin labelling system therefore avoided this problem.
Figure 2.1. Antigen diffusion in severe NEC. Both intestine sections are stained for TM (brown). Panel (a) shows background haemorrhage and diffusion of TM from the endothelium. Panel (b) shows loss of endothelial TM but clear expression of TM by cells of the immune system infiltrating the tissue. There may also be antigen diffusion in the tissue.
### 2.12 Summary of single-antigen immunohistochemistry protocols in neonatal intestine.

<table>
<thead>
<tr>
<th>Tissue section</th>
<th>TM</th>
<th>EPCR</th>
<th>Fibrin</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFPE neonatal intestine</td>
<td>FFPE neonatal intestine</td>
<td>FFPE neonatal intestine</td>
<td>FFPE neonatal intestine</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Monoclonal Mouse anti TM clone 1009 IgG1κ</th>
<th>Monoclonal Mouse anti EPCR clone1489 IgG1κ</th>
<th>Polyclonal Rabbit anti-fibrinogen Reacts with fibrinogen, fibrin &amp; degradation fragments D&amp; E</th>
<th>Monoclonal Mouse anti CD41 clone 5B12 IgG1κ</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Supplier</th>
<th>CT Esmon, Oklahoma medical research foundation</th>
<th>CT Esmon, Oklahoma medical research foundation</th>
<th>Dako UK Ltd</th>
<th>Dako UK Ltd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ely, UK</td>
<td>Ely, UK</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Working dilution</th>
<th>10 μg/ml</th>
<th>50μg/ml</th>
<th>1:30 000</th>
<th>1:50</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Polymer conjugate</th>
<th>Envision™ or ImmPRESS™</th>
<th>Envision™ or ImmPRESS™</th>
<th>Envision™ or ImmPRESS™</th>
<th>Envision™ or ImmPRESS™</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>HRP</th>
<th>AP</th>
<th>AP</th>
<th>HRP</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Antigen retrieval</th>
<th>None</th>
<th>None</th>
<th>HIER</th>
<th>PIER</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Endogenous enzyme block</th>
<th>3% H2O2/H2O x 20 min</th>
<th>Levamisole blocking agent x 15 min</th>
<th>Levamisole blocking agent x 15 min</th>
<th>3% H2O2/H2O x 20 minutes</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Serum Block</th>
<th>Protein free serum block x30min</th>
<th>Protein free serum block x 30 min</th>
<th>Protein free serum block x 30 min</th>
<th>Protein free serum block x 30 min</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Primary antibody incubation</th>
<th>30 min at RT</th>
<th>30 min at RT</th>
<th>30 min at RT</th>
<th>30 min at RT</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Positive Tissue control</th>
<th>Skin, tonsil, intestine</th>
<th>Skin, tonsil, intestine</th>
<th>Tonsil, blood clot</th>
<th>Tonsil, blood clot</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Negative antibody control</th>
<th>Mouse IgG1κ</th>
<th>Mouse IgG1κ</th>
<th>Rabbit Immunoglobulin fraction</th>
<th>Mouse IgG1κ</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Negative reagent control</th>
<th>0.1%PBS-tween</th>
<th>0.1%PBS-tween</th>
<th>0.1%PBS-tween</th>
<th>0.1%PBS-tween</th>
</tr>
</thead>
</table>

*Table 2.1: Protocol summary for single antigen immunohistochemistry in neonatal intestine.*

2.13 Summary of single antigen immunostaining protocols in rat intestine.

<table>
<thead>
<tr>
<th>Tissue section</th>
<th>TM</th>
<th>EPCR</th>
<th>Fibrin</th>
<th>P selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh frozen sections ileum &amp; lung Fixed in ethanol series</td>
<td>Fresh frozen sections ileum &amp; lung Fixed in ethanol series</td>
<td>FFPE sections ileum &amp; lung</td>
<td>Fresh frozen sections ileum &amp; lung Fixed in ethanol series</td>
<td></td>
</tr>
<tr>
<td>Polyclonal goat, anti- mouse TM IgG1</td>
<td>Polyclonal goat, anti-mouse EPCR IgG1</td>
<td>Polyclonal goat, anti-rat fibrinogen</td>
<td>Polyclonal rabbit, anti-human P-selectin</td>
<td></td>
</tr>
<tr>
<td>CT Esmon, Oklahoma medical research foundation</td>
<td>CT Esmon, Oklahoma medical research foundation</td>
<td>Accurate Chemical &amp; Scientific Corporation New York, USA</td>
<td>BD PharMingen San Diego, USA</td>
<td></td>
</tr>
<tr>
<td>5 μg/ml in 20% rabbit serum</td>
<td>10μg/ml in 20% rabbit serum</td>
<td>1:2500</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>ImmPress™</td>
<td>ImmPRESS™</td>
<td>ImmPRESS™</td>
<td>ImmPRESS™</td>
<td></td>
</tr>
<tr>
<td>HRP</td>
<td>HRP</td>
<td>HRP</td>
<td>HRP</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>HIER 5 minutes</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>3% H2O2/H2O x 20 minutes</td>
<td>3% H2O2/H2O x 20 minutes</td>
<td>3% H2O2/H2O x 20 minutes</td>
<td>3% H2O2/H2O x 20 minutes</td>
<td></td>
</tr>
<tr>
<td>20% rabbit serum x 120 min RT</td>
<td>20% rabbit serum x 120 min</td>
<td>10% rabbit serum x 30 min</td>
<td>10% goat serum x 30 min</td>
<td></td>
</tr>
<tr>
<td>120 minutes at 37°C</td>
<td>120 minutes at 37°C</td>
<td>50 minutes at RT</td>
<td>120 minutes</td>
<td></td>
</tr>
<tr>
<td>Lung or gut</td>
<td>Lung or gut</td>
<td>Placenta</td>
<td>Lung or gut</td>
<td></td>
</tr>
<tr>
<td>Goat IgG CT Esmon, Oklahoma medical research foundation</td>
<td>Goat IgG CT Esmon, Oklahoma medical research foundation</td>
<td>Goat IgG Abcam, UK</td>
<td>Rabbit IgG Dako, UK</td>
<td></td>
</tr>
<tr>
<td>0.1% PBS-tween</td>
<td>0.1% PBS-tween</td>
<td>0.1% PBS-tween</td>
<td>0.1% PBS-tween</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2: Protocol summary single antigen immunohistochemistry in rat intestine.

2.14 Dual immunostaining protocol for TM and Fibrin.

The single – antigen protocols should be followed for the two primary antibodies as described above, in terms of antigen retrieval requirements, antibody dilution, incubation time and temperature.

Slides were dewaxed, rehydrated, and HIER was used for antigen retrieval. The first primary antibody, anti-TM, was applied and the secondary antibody micropolymers were conjugated with HRP and DAB was the chromogen substrate. Anti-fibrin was the second primary antibody, using AP as the conjugated enzyme on the secondary antibody micropolymers and fast red was the chromogen substrate.

2.15 Histology scoring systems

Histological scores were used in this project to assess the severity of histological injury and to compare the expression of cell surface molecules in human and rat intestine.

2.15.1 Severity of intestinal injury in NEC in neonates.

The histological severity of NEC was graded on H&E sections using a simple score adapted from Ade-ajayi. This score describes the severity of injury according to the depth of the inflammation through the layers of the intestinal wall.

<table>
<thead>
<tr>
<th>Ade-Ajayi score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>no inflammation,</td>
</tr>
<tr>
<td>Mild NEC</td>
<td>inflammatory changes confined to the mucosa</td>
</tr>
<tr>
<td>Moderate NEC</td>
<td>changes affecting the mucosa and submucosa</td>
</tr>
<tr>
<td>Severe NEC</td>
<td>inflammation involving the mucosa, submucosa and serosa</td>
</tr>
<tr>
<td>Very severe NEC</td>
<td>transmural necrosis including perforation.</td>
</tr>
</tbody>
</table>

*Table 2.3: The Ade-Ajayi score to grade neonatal intestinal injury.*
2.15.2 Severity of intestinal ischaemia-reperfusion injury in rats

The Park-Chiu score is widely used to grade the intestinal injury caused by ischaemia-reperfusion injury in rats. This score was used to grade the severity of injury in our animal model.

<table>
<thead>
<tr>
<th>Park-Chiu score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal mucosa</td>
</tr>
<tr>
<td>1</td>
<td>Subepithelial space at villus tips</td>
</tr>
<tr>
<td>2</td>
<td>Extension of subepithelial space with moderate lifting</td>
</tr>
<tr>
<td>3</td>
<td>Massive lifting down sides of villi, some denuded tips</td>
</tr>
<tr>
<td>4</td>
<td>Denuded villi, dilated capillaries</td>
</tr>
<tr>
<td>5</td>
<td>Disintegration of lamina propria</td>
</tr>
<tr>
<td>6</td>
<td>Crypt layer injury</td>
</tr>
<tr>
<td>7</td>
<td>Transmucosal infarction</td>
</tr>
<tr>
<td>8</td>
<td>Transmural infarction</td>
</tr>
</tbody>
</table>

*Table 2.4: The Park-Chiu score to grade the IR intestinal injury in rats.*

2.15.3 Scoring system for grading the expression of TM, EPCR, P-selectin and fibrin deposition.

TM and EPCR surface expression was scored in a 5-point scoring system in comparison to the expression in control intestine from human subjects without NEC (investigations in chapter 3 and 4) or sham animals (investigations in chapter 5). In the animal model, TM and EPCR expression was graded in intestine and lungs.

A 5-point scale was used (instead of a simpler 4- or 3-point scale as described for other antigens) in order to reflect more subtle changes in levels of expression.
Table 2.5: Scoring system for the expression of TM and EPCR. The shaded area corresponds to the expression intensity in the experimental controls: sham animals (chapter 5) or control neonatal intestine without NEC (chapter 3 and 4)

Fibrin deposition was scored in comparison to the positive tissue control, placenta. The extent of intravascular deposition and extravascular deposition was taken into consideration.

Table 2.6: Scoring system for fibrin deposition. The fibrin deposition in placenta sections served as the positive tissue controls.
P-selectin expression was investigated in the animal model. Comparisons were made to sham animals.

<table>
<thead>
<tr>
<th>P-selectin staining intensity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Many vessels staining more strongly than sham, &gt;50%</td>
</tr>
<tr>
<td></td>
<td>(in the intestine: &gt; 1+ve vessel / 1-2 villi, involving most of the mucosa)</td>
</tr>
<tr>
<td>2</td>
<td>More vessels staining positively, &lt;50%</td>
</tr>
<tr>
<td></td>
<td>(in the intestine: 1 +ve vessel / 2-5 villi)</td>
</tr>
<tr>
<td>1</td>
<td>As sham: few vessels staining positively</td>
</tr>
<tr>
<td></td>
<td>(in the intestine: &lt; 1 +ve vessel / 5 villi)</td>
</tr>
</tbody>
</table>

*Table 2.7: Scoring system for P-selectin expression in lung and intestine. The shaded area indicates P-selectin expression in the sham animals.*

2.16 **Light Microscopy**

All microscopy was undertaken on a Leica DMLB microscope. Images were photographed with CoolSnap digital camera kit and ImagePro Plus software (Media Cybernetics Inc). The images have not been digitally processed.
3 Enzyme linked immunosorbant assays (ELISA)

ELISA’s were used to determine the plasma levels of the PC pathway proteins, thrombin-antithrombin complexes (TAT), prothrombin fragment 1+2 (PF1+2) and D-Dimers.

3.1 ELISA in the animal model

In the animal model, commercial human ELISA kits were optimised as discussed in more detail in chapter 5, in particular rat plasma standards replaced the human standards. However the general protocols are described here.

The TAT immunoassay, Enzyngost TAT micro (Dade Behring-Siemens), is a sandwich ELISA. The kit provided pre-prepared microtitre plates coated with human anti-thrombin antibodies, human standards and human control plasmas. The kit also provided washing and dilution solutions. The human standards were replaced by rat standards for optimisation of the assay.

Duplicates of study samples were analysed, providing sample volumes allowed, and every plate had substrate blanks. A 50µL volume of Tris buffer solution (100mmol/L) with tween (10mL/L) and EDTA (37g/L) was pipetted in to the wells followed by the addition of 50µL of plasma standard, control or sample. A multi channel pipette was used to rapidly transfer samples from a prepared template in order to reduce the time taken to prepare the microplate for the first reaction, as this assay is particularly sensitive to temporal drift across the plate. The microtitre plate was covered and incubated for 15min at 37°C. The wells were then aspirated and washed three times with a 0.1% phosphate buffer solution with tween; the excess moisture was removed by ‘knocking’ the plate on absorbent tissue. Peroxidase-conjugated antibodies against anti-thrombin III were diluted to 100µL with a citrate buffer solution and transferred to the wells, covered and allowed to incubate for 15min at 37°C. The plate was washed three times again in the phosphate buffered solution
and the excess moisture ‘knocked out’ from the plate. The chromogen, o-phenylenediamine in a phosphate buffered solution, was added to the wells, covered and incubated in the dark at room temperature for 30 minutes. The reaction was stopped by adding 100µL of 0.5M sulphuric acid. The optical densities of the well plates were read at 492nm using the Multiskan EX ELISA reader by Thermo scientific® and the data was processed using Ascent and MasterPlex software to produce standard curves. These are documented in chapter 5. The measurement range for the assay is 2-60µg/L, although the reference range is 1.0-4.1µg/L.

In the animal model, D-Dimers were assessed with the Asserachrom® D-Di assay (Diagnostica Stago). This is a sandwich ELISA. The kit provides micro titre well-plates coated with mouse monoclonal anti-human D-Dimer antibody. Study samples were tested in duplicate if sample volumes were sufficient. Standards and controls were duplicated. Each plate had substrate blanks. A 200µL volume of appropriately diluted sample, standard or control was incubated in the wells after covering the plate to prevent dehydration, for 1 hour at room temperature. The plate was washed 5 times and excess moisture removed by knocking the plate on absorbent tissue. The wells were then incubated with 200µL rabbit anti-human fragment D antibodies conjugated with peroxidase, for 1 hour at room temperature under a foil cover. The plate was then washed 5 times to remove unbound proteins. The chromogen TMB was used as the substrate for peroxidase, and the wells were incubated in the dark with 200µL TMB for 5 minutes. The reaction was stopped with 1M sulphuric acid. The optical densities of the plates were read at 450nm by the Multiskan EX ELISA plate reader (Thermo Scientific®) and the data was processed using Ascent and MasterPlex software to produce standard curves. The detection limit of the assay is 3ng/mL of D-Dimer.
3.2 Human neonatal plasma assays.

Commercial ELISA kits were used for the human plasma assays, except APC, and were used according to the manufacturers instructions. These tests are common investigations and the manufacturers have optimised the methods for human plasma. The standardised method allowed comparison of the test samples to population reference ranges.

Due to the very limited sample volume in our population, commercial laboratories carried out these tests, as indicated in the table below.

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Antigen</th>
<th>Assay</th>
<th>Source</th>
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<tr>
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<td>Asseachrom D-Di</td>
<td>Diagnostica Stago</td>
<td>Asnieres sur Seine, France</td>
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<tr>
<td>Rat model &amp; UCL</td>
<td>TAT</td>
<td>Enzygnost TAT micro</td>
<td>Dade Behring, Siemens</td>
<td>Erlangen, Germany</td>
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<tr>
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<td>Technoclone</td>
<td>Vienna, Austria</td>
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<tr>
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<td>Hyphen BioMed</td>
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<tr>
<td>Baxter</td>
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<td>Zymutest RK015A</td>
<td>Hyphen BioMed</td>
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<tr>
<td>UCL</td>
<td>PF1+2</td>
<td>Enzygnost F1+2</td>
<td>Dade Behring, Siemens</td>
<td>Erlangen, Germany</td>
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<tr>
<td>UCL</td>
<td>D-Dimer</td>
<td>D-Dimer plus</td>
<td>Dade Behring, Siemens</td>
<td>Erlangen, Germany</td>
</tr>
</tbody>
</table>

Table 2.8: Commercial ELISA assays used during this project. ‘Rat model’ denotes the optimisation and utilisation of the assay by PL in the animal model. ‘Baxter Laboratory in Vienna Austria, where the indicated assays were performed. ‘UCL’ denoted the Haemostasis Laboratory UCL, London UK, where the indicated tests were performed.

In general terms, many of the ELISAs performed by the Baxter and UCL Haemostasis laboratories were sandwich ELISA’s based on the same principles described in the TAT and D-Dimer assays I used in the rat model.

The method involves coating a microtitre well-plate with a capture antibody specific to the antigen under investigation. Appropriately diluted plasma is then added to the wells so the
surface-bound antibody can capture the antigen. A second antibody, specific to a different epitope of the antigen under investigation, and conjugated to an enzyme such as HRP, is introduced to the wells and binds to the captured antigen. The wells are washed after incubation to remove any unbound proteins and a chromogenic substrate for the conjugated enzyme of the second antibody is added to the wells. The reaction is timed and stopped by the addition of strong acid. The intensity of colour, or optical density, is read by an ELISA plate reader at an absorbance appropriate to the chromogen used. A standard curve is generated from the standard samples of known quantity of the antigen under investigation; the quantity of antigen in test samples can be read from the standard curve.

The Asserachrom Protein C ELISA is a sandwich ELISA using two clones of rabbit anti-PC antibodies. The conjugate enzyme is peroxidase and the chromogen used is tetramethylbenzidine (TMB). Absorbance is read at 450nm. The lower detection limit of the assay is 1% (0.01 U/mL).

The PC activity assay, Technochrom PC, involves incubating the plasma sample with Protac®, an extract of the venom from the snake *Akistrodon contortrix contortrix*, for 5-minutes at 37°C. Protac activates the PC in the sample. The activated Protein C then acts as the enzyme, cleaving a chromogenic substrate (Pad-Pro-Arg-pNA·AcOH). The substrate is incubated for 3 minutes at 37°C and the reaction is stopped with 20% acetic acid. Absorbance is read at 405nm.

The Zymutest assays for total PS and free PS are ‘sandwich’ ELISAs. The antibodies used in the free PS assay are specific only to free PS while the antibodies in the total PS assay react with free and C4b-binding protein (C4BP)-bound PS. HRP is the conjugate enzyme in both assays and tetramethylbenzidine is the chromogen peroxidase substrate; 0.45M sulphuric acid is used to stop the reactions and optical densities are read at 450nm.
The D-Dimer Plus automated assay was used to process the human plasma samples by the UCL Haemostasis lab. This is a latex enhanced turbidometric assay. Polystyrene particles are coated with monoclonal antibody to the cross-linkage region of D-Dimers (cross-linked fibrin degradation products). The epitope for the antibody occurs twice on the antigen. The particles agglutinate following reaction with the antigen and this is detected as an increase in turbidity. The reference range for the laboratory was 64-246µg/L.

The circulating APC level was determined in plasma collected in benzamidine, which prevents the inhibition of APC by its inhibitors. The assay is a sandwich capture ELISA based on the method of Gruber et al.8.
4 Animal model of ischaemia reperfusion.

The study described in chapter 5 was approved under the United Kingdom Home Office regulations for Animals (Scientific Procedures) Act 1986. All animals were handled in accordance with guidelines prescribed by UK and International regulations on protection, care and handling of laboratory animals. Mr Nigel Hall performed the anaesthetic and surgical procedures (figure 2.2).

In general terms, intestinal ischaemia reperfusion injury (IR) was achieved in anaesthetised Sprague-Dawley rats by 60 minute clip occlusion of the superior mesenteric artery (SMA) followed by 120 minutes of reperfusion prior to exsanguination by cardiac puncture.

Adult male Sprague-Dawley rats weighing 225 to 250 g were used. They were kept under standard conditions with free access to chow and water for 48 hours prior to the procedure. The animals were anaesthetised with a 50:50 oxygen/nitrous oxide mixture with 2% to 3% isoflurane inhalation via a nose cone. The heart rate and oxygen saturations were monitored with a pulse oximeter throughout the procedure. The rectal temperature of the animals was maintained between 36.0°C and 37.5°C throughout the experiment using a servo-controlled heating blanket. Following induction of anaesthesia, the right femoral vein was cannulated with a 24G plastic cannula (BD Neoflon™ Becton Dickinson Uk Ltd).

The sham group served as negative controls; a midline laparotomy was performed, the superior mesenteric artery (SMA) was identified and dissected, but no vascular occlusion was carried out. The abdominal incision was closed and the animal kept anaesthetised and monitored for 180 minutes until the end of the experiment.

In the other experimental groups, animals underwent a midline laparotomy, with exposure of the SMA as above. The SMA was occluded close to its origin by the application of a
microvascular clip (S&T AG, Neuhausen, Switzerland). Complete occlusion was confirmed by the intestine becoming white and pulseless. The incision was closed. After 60 minutes of intestinal ischaemia, the abdominal incision was reopened and the clip removed.

Reperfusion was confirmed by the restoration of the intestinal colour and pulsation of the mesenteric vascular arcades. The incision was closed again and the animal monitored for a further 120 minutes.

At the end of the experiment, animals in all groups were euthanised by exsanguination from cardiac puncture.

4.1 Tissue preparation for sampling

Tissue samples were collected for histology, immunohistochemistry and MPO analysis.

Immediately following the end of the experiment the entire small intestine and lungs were removed. The intestinal contents were gently flushed out with saline in order to remove faecal content without disrupting the intestinal mucosa. Four sections of terminal ileum were excised in a standardised way to minimise bias.

![Figure 2.3: Diagram of the standardised tissue collection method. Ileal samples were collected between 12-20cm proximal to the ileo-caecal (IC) valve and processed as described. FFPE – formalin fixed paraffin embedded, MPO – myeloperoxidase activity.](image)

To IC valve

- Gut 1: FFPE 12-14cm
- Gut 2: MPO 14-16cm
- Gut 3: Fresh frozen 16-18 cm
- Gut 4: FFPE 18-20cm

Two sections and one lung were fixed in 10% neutral buffered formalin and embedded in paraffin. The intestine specimens were cut longitudinally and laid out flat on filter paper to
facilitate processing. 4 µm sections of intestine and lung were cut with a microtome and mounted on APES coated slides.

A third section of ileum and the other lung were snap frozen and stored at -80°C. Fresh frozen sections (5µm) were cut with a cryostat and air-dried at room temperature. Sections were stored at -80°C.

The remaining intestinal section was snap frozen and stored at -80°C. It was used to determine intestinal myeloperoxidase (MPO) activity by Nigel Hall.

4.2 Plasma samples

Blood from the cardiac puncture was used for TAT and D-Dimer assay.

Blood (1ml) was collected into sodium-citrate buffer (final concentration 3.2%) and centrifuged at 2000 rpm at room temperature for 20-minutes. Plasma was removed and stored at -80°C until analysis.

4.3 Myeloperoxidase activity

Mr Nigel Hall measured the myeloperoxidase activity (MPO) in the intestine. I include his method and results to provide additional information on the cohort. The intestine sample (Gut 2) was homogenised using an Ultra-Turrax homogeniser in 2ml 50mmol/l potassium phosphate buffer, pH 6.0, containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide. 100ml of homogenate was removed for protein estimation and the remainder centrifuged at 18,500rpm for 30 minutes at 4°C. A total of 100ml of supernatant then was added to 2.9ml potassium phosphate buffer containing 0.53 mmol/l O-dianisidine hydrochloride and 0.0005% hydrogen peroxide. MPO activity was followed spectrophotometrically at 25°C at a wavelength of 460 nm and was expressed as milli-units (mU) per mg protein.
Figure 2.2: Adult rat intestinal ischaemia reperfusion (IR) animal model.
Panel (A) adult rats were anaesthetised, monitored (saturation probe right foot, rectal temperature probe) and underwent laparotomy.
Panel (B) Intervention drugs administered via femoral venous cannula (24G Neoflon™).
Panel (C) shows the vascular clip in relation to 5mL syringe (S&T AG, Neuhausen, Switzerland).
Panel (D) – (F) shows the intestine following reperfusion. The intestine is distended with poorly perfused, oedematous, dusky areas of macroscopic injury. The solid arrow indicates visible pneumatosis of the injured bowel wall.
4.4 Statistical analysis

Statistical analyses for this thesis were performed using Prism 5 for Mac OS X, by GraphPad Software Inc.

Methods are described in more detail within results chapters. In general, parametric methods were employed for Gaussian samples; t-tests and 1-way analysis of variance with Bonferroni’s correction for multiple comparisons, Pearson r for correlation. Non-parametric methods were used for non-Gaussian samples; Mann-Whitney test, Kruskal-Wallis with Dunn’s test of multiple comparison and Spearman r for correlation. Significance at p<0.05.
5 Chemicals and reagents.

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<th>Chemicals and materials</th>
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*Table 2.9: Table of chemical reagents and other material, and the sources, used in the experimental work reported in this thesis.*
Chapter 2. General methods and materials

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Table 2.10: Table of the antibodies used in the immunohistochemistry protocols, or in the development of the protocols, as described in this thesis. Some antibodies were worked up but not included in the final protocols.
Chapter 3:

Histological investigation of the PC pathway in neonatal intestine and the impact of necrotising enterocolitis
Histological investigation of the PC pathway in neonatal intestine and the impact of necrotising enterocolitis.

1 Introduction

The expression and distribution of the endothelial receptors of the PC activation pathway, TM and EPCR, have not been characterised in the immature human intestine. The effect of necrotising enterocolitis (NEC) on the distribution and expression of these receptors is also unknown.

The expression of TM and EPCR was characterised in neonatal intestine, with and without NEC, in a retrospective, blinded, case-control study.

2 Methods

Great Ormond Street R&D Project Number: 99AR65

2.1 Study Design

A retrospective case control study compared the histological characteristics of the PC pathway in intestine resected from infants with NEC to those with a congenital abnormality of the intestine, but without acute inflammation of the bowel.

A list was generated from the admissions database of all NEC and non-NEC patients who had undergone bowel resection over a 20-month period. A senior pathologist, not otherwise involved in this study, reviewed the routine histological sections from these subjects. Subjects (control and NEC) with representative histology of normal bowel and the typical features of NEC were chosen for inclusion in this study by the pathologist. The sections were blinded by the pathologist.

Five additional control patients remained unblinded in order to characterise TM and EPCR expression in non-inflamed intestine and to act as comparators for the blinded slides.
2.2 Study outcomes

2.2.1 Primary outcomes

• To characterise the distribution and expression of TM and EPCR in neonatal intestine

• To determine the effects of NEC on the distribution and expression of TM and EPCR in neonatal intestine

2.2.2 Secondary outcomes

• To determine the association between the expression of TM and fibrinogen distribution in neonatal intestine, with and without NEC.

2.3 Ethics Committee approval

Approval was given by the local ethic committee for this project. As some of the patient population had died, the chairman waived the need to seek retrospective parental consent for the research use of stored intestinal tissue blocks or clinical data from the medical records.

2.4 Study population

Infants admitted to the level 3 neonatal intensive care unit (NICU) at Great Ormond Street Hospital, and who underwent bowel resection, were identified retrospectively from the ward register over a 20-month period. Infants with a diagnosis of NEC were separated from those with other conditions requiring bowel resection. The latter subgroup formed the control group; these infants had conditions with minimal intestinal inflammation, but nevertheless required bowel resection. These included intestinal atresia, and the formation and closure of stomas for ano-rectal malformations. A senior pathologist reviewed the
tissue sections from these cases and selected the most representative intestinal tissue blocs from 13 of the NEC subjects and 11 controls.

2.5 Study outline

Figure 3.1: Flow diagram of study outline.
2.6 Clinical data collection

The following clinical data was retrieved from the medical records.

Demographic data: Birthweight, gestation at birth, sex, weight, age and corrected gestation at time of surgery.

Clinical information: mortality of the current admission, risk of mortality score, markers of organ impairment: ventilation status, inotropic requirement, transfusion requirement prior to surgery and urine output, and organ failures. Organ failure data was collected according to the definitions of Smith, as modified by Morecroft. Missing data were considered within the normal range. Mortality figures relate to death during the current episode of NEC.

Results of investigations taken pre-operatively on the day of surgery: coagulation screen; prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, markers of inflammation; CRP, white cell count and differential, blood culture results; markers of organ impairment: liver function, albumin, alanine transferase (ALT), renal function, creatinine

2.7 Histological investigations

Methods common to all investigations have been described in the previous chapter ‘general methods’

2.7.1 Histological section preparation

As described in general methods, 4 µm sections were prepared from FFPE issue blocks. Individual cases could give rise to several tissue blocks representing severely affected areas and resection margins, which would be mildly affected. Multiple sections were cut from
each block and labelled with the same code allowing for multiple investigations on each tissue sample.

2.7.2 Immunohistochemistry:

The optimisation techniques and final protocols for the immunohistochemistry investigations were described and summarised in chapter 2.

Positive tissue controls, unblinded control sections, negative antibody and reagent controls were run with each experiment.

2.7.3 Histological assessment and scoring

The investigators were blinded to the underlying diagnosis of the remaining tissue sections. Two investigators assessed all the histology sections. (PL and SF) Quality control and arbitration of inter-observer differences, was provided by an additional three blinded investigators (VS, MP, NK).

Unblinded, control samples of intestine, served as references for comparison to the blinded sections. These samples were mainly cases of intestinal atresia or ano-rectal malformation and were not acutely inflamed.

The scores for grading severity of injury and the expression of TM, EPCR and fibrin were detailed in chapter 2. Sections were also stained for CD41 to visualize platelet thrombi, these were not formally graded.

The scores for grading the surface expression of TM, EPCR and fibrin were described in Chapter 2.

Statistical analysis was undertaken as described in chapter 2.
3 Results

Eleven of 13 NEC patients had areas of severe or very severely affected bowel on histology. These included 5 cases of multi-organ failure, 3 of whom died. One of 2 patients in the moderate NEC group died. There were no deaths or episodes of organ failure in the control group. The clinical data is summarised in table 3.1.

One control case was 52 weeks at surgery, an outlier in the control group by 48 weeks. This case was omitted from the age at surgery analysis.

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<tbody>
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<tr>
<td>Birthweight (g)</td>
<td>1135 ±195</td>
<td>2495±318</td>
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<td>Corrected gestation at surgery (weeks)</td>
<td>32.5 ±4.4</td>
<td>41±1.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Age at surgery (weeks)</td>
<td>4.8 ±1</td>
<td>0.7±0.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Mortality</td>
<td>4 (30.8%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Platelet count x10^9/L (normal:150-450 x10^9/l)</td>
<td>115 ±29</td>
<td>300 ±37</td>
<td>0.0007</td>
</tr>
<tr>
<td>PT (s) (normal: 8.2-14.1s)</td>
<td>17.1 ±1</td>
<td>14.6±1.4</td>
<td>0.18</td>
</tr>
<tr>
<td>APTT (s) (normal: 28-50s)</td>
<td>66.9±6.9</td>
<td>56.8 ±7.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Fibrinogen (g/l) (normal: 1.7-4g/l)</td>
<td>2.2 ±0.4</td>
<td>1.9 ±0.3</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Table 3.1: Summary of clinical data (means ±SEM).*

All blinded control sections were correctly identified by all investigators.

Of the 23 NEC sections (from 13 patients), 4 sections demonstrated mild features of NEC, 6 were moderate, 10 were severe and 3 were very severely affected.

There was a high level of agreement between the observers in the interpretation of the slides. Differences in opinion occurred with 12% of the sections to a maximum of one
intensity level in any individual section. These sections were examined by a third investigator and in all cases the majority decision was followed.

3.1. Histology of the non-inflamed, control bowel:

3.1.1 Morphology on H &E staining of control sections.

The morphology of the bowel of the control sections was normal. No abnormalities were evident.

3.1.2 Immunohistochemistry of control sections:

Thrombomodulin was strongly expressed by the endothelium of vessels of all calibre throughout the bowel wall (figure 3.2). This was particularly evident in the vessels of the microcirculation, including the lacteal capillaries of the intestinal villi (figures 3.3 and 3.4).

EPCR was consistently expressed by the small, medium and large veins and arteries of the serosa and submucosa. EPCR was not however not detected in the microcirculation; namely the capillaries of the mucosa or submucosa, or the small venules and arterioles. (figures 3.5 and 3.6). All controls had similar expression of TM and EPCR in terms of staining intensity and distribution; corrected gestational age did not appear to have an effect. The majority of vessels that stained strongly for TM were patent and had no evidence of thrombosis (fibrinogen negative) on the dual staining.

No platelet thrombi were present on CD41 staining.

EPCR staining was present on the epithelium.
Figure 3.2. Thrombomodulin expression in control bowel (x100). TM appears brown (arrow). The endothelium of vessels of all calibre stain strongly for TM, including the lacteal vessels of the villi.

Figure 3.3: Mucosal TM (appears brown) in control intestine (x200). TM is strongly expressed by the microcirculation of the villi in control intestine.
Chapter 3: PC pathway on histology

Figure 3.4: TM (appears brown) is strongly expressed by the microcirculation (x400): arterioles, venules and capillaries.

Figure 3.5. EPCR expression in control bowel (x100). EPCR appears red (arrow). EPCR is expressed in the arteries and veins but not by the microcirculation.
Figure 3.6: EPCR expression in control bowel endothelium of the submucosal vessels (x400) EPCR appears red. EPCR is expressed on the endothelium of larger vessels but not by capillaries (dashed arrow). EPCR staining of an immune cell; CD+8 and macrophages express EPCR (block arrow)
3.2 Histology of NEC affected bowel (H&E)

The histological features of necrotising enterocolitis varied in severity within sections, reflecting the patchy, discontinuous nature of the inflammation.

**Mild NEC:** Areas of mild NEC affected the mucosa. Vessels within the villi appeared congested, or there was localized haemorrhage and epithelial cell separation at the villous tips. The submucosa appeared oedematous, with some congested, dilated blood vessels. *(figure 3.7 and 3.8).*

![Figure 3.7. H&E stain of mild NEC (x100). Haemorrhage and congestion is associated with sloughing of the villous epithelium. Dilated and congested vessels are seen in the submucosa.](image)

**Moderate NEC:** In moderate NEC, the mucosa was necrotic with an inflammatory cell infiltrate, thrombosis and loss of architecture. A luminal pseudomembrane was commonly present. The vessels of the submucosa and serosal were more frequently congested or thrombosed.

**Severe NEC:** In more severe disease, the necrotic process had ulcerated into the submucosa. The vessels showed greater degrees of congestion, haemorrhage, thrombosis...
with a dense inflammatory cell infiltrate in the surrounding tissue. Pneumatosis intestinalis, where present, caused disruption of the submucosa (figure 3.9). In the most severely affected sections, the structure of the bowel layers became lost in transmural necrosis.

**Figure 3.8: H&E stain of mucosa in mild to moderate NEC (x400)** Inflammatory infiltrate seen in the congested vessels and tissue of the villi.

**Figure 3.9. H&E section of severe NEC (x100).** The mucosa is necrotic and many of the submucosal vessels show congestion and thrombosis. There is an inflammatory cell infiltrate. Pneumatosis is seen in the submucosa
3.2.2 TM, EPCR and Fibrinogen immunohistochemistry of NEC affected bowel.

The key immunohistochemistry results are summarised in the following table 3.2.

<table>
<thead>
<tr>
<th></th>
<th>TM expression</th>
<th>EPCR expression</th>
<th>Fibrin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microcirculation</td>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arteries &amp; veins</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>NEC</td>
<td></td>
<td></td>
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<tr>
<td>Patent vessels</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosal capillaries</td>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Submucosa &amp; Serosa</td>
<td>+++</td>
<td>++/+++</td>
<td>0</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>Mucosal capillaries</td>
<td>0/+</td>
<td>0</td>
<td>++/++</td>
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<tr>
<td>Submucosa &amp; Serosa</td>
<td>+/+</td>
<td>0/+</td>
<td>++/++</td>
</tr>
<tr>
<td>Thrombosed vessels</td>
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<td>Submucosa &amp; Serosa</td>
<td>0/+</td>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>Necrotic Areas</td>
<td>0/+</td>
<td>0</td>
<td>++/+++</td>
</tr>
</tbody>
</table>

Table 3.2: Overview of TM & EPCR expression. An overview of the intensity of staining of endothelial TM/EPCR and fibrinogen in control and NEC sections. Abbreviations: SM = submucosa. NEC = necrotising enterocolitis. Symbols: 0 = absent stain, + = weak stain, ++ = moderate intensity, +++ = strong intensity.

The histological features of mild NEC were associated with reduced expression of TM in the congested lacteal capillaries when compared to controls. TM expression remained strong in other gut layers (figure 3.10, 3.11).
Figure 3.10: Mild NEC: mucosa (x400). Thrombomodulin expression by the mucosal capillaries is reduced in mild NEC (arrow). TM appears brown. The necrotic luminal pseudomembrane stains brown in a non-specific manner.

Figure 3.11: Mild NEC: mucosa (x400). Thrombomodulin expression by the submucosal vessels remains strong, TM expression in mucosal vessels remains strong in some areas. Inflammatory infiltrate is seen. TM appears brown.

In more advanced NEC, TM expression was retained in patent vessels, reduced in congested vessels and lost in frankly thrombosed vessels (Figure 3.12, 3.13). Loss from the endothelium was more pronounced in necrotic areas. Some of inflammatory cells stained positively for TM.
Figure 3.12. Moderate NEC with necrotic mucosa (x100). Thrombomodulin expression is preserved by vessels in non-necrotic areas in NEC (arrow).

Figure 3.13. Submucosa in moderate NEC (x400). Thrombomodulin (line arrow) is expressed by patent vessels in NEC, but is weak or absent in congested vessels (dashed arrow).

Dual staining for fibrinogen and TM showed that fibrin strand deposition was common in congested vessels, many of which displayed reduced expression of TM. The number of thrombosed vessels (strong fibrin stain) that had no TM expression increased with the severity of the NEC (figure 3.14). Small vessels were more frequently affected. Fibrin deposition in the tissue was common around focal haemorrhage or pneumatosis blebs.
Figure 3.14. Moderate NEC, dual stain for TM (brown) and fibrin (red) (x200). Patent vessels express TM (dashed arrow). Thrombosed vessels appear densely red and do not express TM (line arrow). There are some congested vessels with fibrin strands and weak TM expression.

Figure 3.15. Mucosa in moderate NEC stained for platelet CD41 (brown) (x200). Mucosal thrombi contain platelet plugs (arrow).

EPCR expression by submucosal and serosal vessels was lost in necrotic areas, but was present in the larger, patent vessels in less affected tissue (figure 3.16, 3.17)
**Figure 3.16. EPCR expression in severe NEC (x100).** EPCR expression (red) is reduced in necrotic areas.

**Figure 3.17. EPCR expression in the serosa of severe NEC (x200).** EPCR expression (red) remains in the large arteries and veins of non-necrotic bowel.
4 Discussion

This study has shown that the expression of critical endothelial receptors for protein C activation, EPCR and TM, are present in neonatal intestine although the distribution of EPCR is different to TM. The distribution was similar in all the cases across the corrected gestational age range examined; and this was similar to that described in normal adult intestine\(^2\). This finding is not unexpected as it is known from animal models that that a functioning PC activation pathway is required to survive fetal and perinatal life\(^3,4\).

This study showed that TM was expressed by the intestine mucosal microcirculation but EPCR was not. The reasons for differential expression are not immediately clear. The increased TM concentration relative to blood volume in the microcirculation may be adequate to activate sufficient APC without the catalyzing effect of EPCR; however it could leave the microcirculation vulnerable to thrombin generating stimuli. EPCR in the remainder of the circulation will generate APC that may circulate into the microcirculation. The lack of EPCR expression in the microcirculation may reduce anti-inflammatory dampening of the inflammatory response of the endothelium to danger signals. Further research is required to understand how the distribution of EPCR varies in vascular beds, varies in adults and children, and varies in relation to tissue-associated immune systems.

EPCR staining was also present on the epithelium. The function of intestinal epithelial EPCR remains under investigation but it seems to be involved in maintenance of the barrier integrity by enhancing tight junction expression and acting as a growth factor, promoting epithelial cell proliferation and migration\(^5\).

This study showed that TM and EPCR expression is reduced in NEC, however complete loss of TM and EPCR only occurred in necrotic areas. The expression of both receptors was
variably reduced in congested vessels and was associated with increased fibrin deposition in those vessels, indicative of thrombosis. Mild NEC was associated with decreased TM expression in the villi, associated with congested vessels, epithelial lifting and inflammatory cell infiltrate.

NEC is an inflammatory condition with a systemic inflammatory response of varying intensity. It is known that TM and EPCR is downregulated in other inflammatory states; for example in the intestine of patients with inflammatory bowel disease or more globally, as part of a SIRS response⁶. In NEC, the TM and EPCR expression are not uniformly reduced as suggested in severe sepsis⁷. Instead, there is a patchy reduction in expression common to areas of necrosis and this is associated with increased thrombosis of the local microcirculation. There is preservation of expression in non-necrotic areas.

This would suggest there might be residual capacity to activate PC, providing the levels of substrate and the functioning of the receptors were adequate. As discussed in the opening chapter of this thesis, preterm neonates have physiologically low levels of PC that may become rate-limiting during times of increased demand for APC, such as the inflammatory state of NEC.

There are several observations in relation to the absent, or much reduced, TM and EPCR expression in the worst affected areas of necrosis in NEC. The histological investigations cannot distinguish if the downregulation is causal to, or a consequence of (or indeed, even a mixture), the inflammation and thrombosis seen in the immediate surrounding area. It is known that inflammation down regulates TM and EPCR expression and promotes their endothelial shedding. The ensuing reduced local production of APC would promote thrombosis and obstruction of the microcirculation, reducing fibrinolysis and would
facilitate leukocyte recruitment and migration into the tissue; tipping the balance further towards a procoagulant, inflammatory milieu.

There are some theoretical biological benefits to the reduction and/or loss in TM and EPCR expression in the worst patches; reduced levels of APC may prevent bleeding from ulcerated and necrotic areas and promotes leukocyte migration and host defense. It has been shown that reduction of APC anticoagulant activity in the vicinity of platelet thrombi is mediated by PF4. Therefore the loss of receptors in necrotic, thrombosed and non-perfused areas may not represent pathology, but rather survival biology.

This study has a number of limitations. The uneven nature of the injury of NEC on histological examination precluded more detailed quantitative analysis. The expression of TM and EPCR could vary markedly within adjacent high power fields. In order to reduce bias, multiple blinded observers reviewed multiple sections and we found consistent interpretation of the clinical material between observers, as detailed above.

The resected intestine from subjects in the control group was not normal; however there were no signs of acute inflammation either systemically or on histological examination. Nevertheless, a large proportion of this group had bowel atresia, which may be associated with an in-utero ischaemic event. This process could theoretically affect the expression of TM and EPCR. The expression was similar in both atretic and non-atretic controls.

The control group was younger than the NEC group at surgery and could not have been successfully enterally fed due to their underlying intestinal atresia. Therefore the intestine of the control group would not have become colonized with bacteria at the time of surgery,
a process that is known to be key in the development of NEC\textsuperscript{9-11}. The effect of bacterial colonization on the endothelial expression of PC pathway receptors is not known, but the difference in colonization between the groups may have introduced bias.

Lastly, the location of an antigen with immunohistochemistry does not guarantee its function.

5 Conclusions and future work:

The premature infant has abundant expression of TM and EPCR in the intestine.

In NEC, TM and EPCR expression are reciprocally reduced as the inflammatory injury increases. Reduced expression is associated with fibrin deposition in the microcirculation. The inflammatory injury in NEC is not uniform, and consequently the reduction in TM and EPCR expression is also patchy, but expression is only completely lost in necrotic areas.

The main question to arise from this work remains; do the receptors that we now know to be present on the intestine, function adequately in NEC by producing APC?
Chapter 4:
Prospective observational study of PC pathway in NEC
1. Introduction

In the previous chapter, we showed that TM and EPCR are strongly expressed by the endothelium in the neonatal intestine of premature and term infants in a similar distribution to adult intestine. EPCR is not expressed in lacteal capillaries. This may indicate a role for the PC pathway in mediating the anti-coagulant and anti-inflammatory phenotype of the vasculature in health.

The circulating levels of PC and PS are known to be physiologically low in healthy preterm and term infants, but the levels of APC are not known. The overall rates of thrombosis are reduced in infancy and childhood for equitable risks; however the contribution of the PC system to this achievement is also not known. The increased concentrations of D-Dimers and TAT in healthy neonates and infants beyond birth suggest thrombin generation continues at a low level. This may suggest inadequacies in the anticoagulant systems or alternatively it could be argued that it may reflect a need for an increase in baseline stimulus of the PC system, perhaps to overcome low PC levels.

In NEC, the expression of TM and EPCR was reciprocally reduced with increasing inflammation; but was only absent or weak in severely affected areas. Dual staining immunohistochemistry techniques showed that reduction in TM expression was associated with increased intravascular fibrin deposition and thrombosis. The effect of NEC on the plasma concentrations of the PC pathway proteins is not known.

As discussed in the first chapter, low PC levels are associated with severe sepsis, acute respiratory distress, multiorgan dysfunction, and ischaemic-reperfusion injuries (for example birth asphyxia) in adults, children and neonates.

We undertook a prospective observational study to characterise the effects of NEC on the plasma protein concentrations of the PC pathway in a bid to improve our understanding of
the function of the pathway in healthy term and preterm neonates and the potential contribution to the clinical condition of NEC.

2. Method and materials

2.1. Overview

Our primary aim was to characterise the effects of acute necrotising enterocolitis on the concentration the PC pathway proteins.

We undertook an observational study in two, level III, neonatal intensive care units in the UK and compared PC, free and total PS and APC in a control group of healthy neonates to those in a group of neonates with NEC. We also assessed thrombin generation and fibrinolysis to try to determine the haemostatic consequences of any changes seen.

Secondary outcomes included characterising associations of PC pathway protein concentrations and the severity of NEC as demonstrated by coagulopathy, the development of multiorgan dysfunction and the need for surgery.

2.2. Study design

This was an observational study undertaken in two level III neonatal intensive care units that provide surgical care to preterm infants; Great Ormond Street Hospital, London, is mainly a surgical unit and Addenbrooke’s Hospital, Cambridge, is a combined medical and surgical neonatal unit.

R&D number: 02 AR 13

NRES reference number: 03/057.
The study received a favourable ethical review from the local research ethics committees for Addenbrooke’s hospital and Great Ormond Street Hospitals. I have included the parent information leaflets and consent forms in appendix 1.

2.3. Study outcomes

The primary outcome was to determine the effect of NEC on the concentration of PC antigen, PS (free and total), PC activity and APC and to assess the effect on thrombin generation and fibrinolysis.

Secondary outcomes included determining any association of the concentration of PC pathway proteins with indices of NEC severity; this could provide insights to inform future investigations. Multi-organ failure, DIC, shock requiring inotropic support, emergency surgery and infection are associated with severe NEC.

2.4. Study population

The study compared two groups: neonates with a diagnosis of acute NEC and a control group of healthy term and preterm neonates.

_Inclusion criteria for both study groups:_

Neonates, male and female, less than 44 weeks corrected gestational age were eligible.

Neonates must have received an intramuscular Vitamin K injection after delivery; this ensured that any perturbation of the coagulation-anticoagulation pathways involving vitamin K dependent factors was not due to deficiency of vitamin K.
NEC group inclusion criteria:

- A history of any of the following; bilious nasogastric aspirates, abdominal distension, rectal bleeding and/or radiological features of NEC, including, distended bowel loops, pneumatosis, portal free gas.

and

- A confirmed diagnosis of NEC by the attending neonatologist and paediatric surgeon (Bell’s stage II) such that the neonate was treated for NEC with antibiotics, gastric decompression and intestinal rest.

Control group exclusion criteria.

The control group were healthy neonates without evidence of inflammation, infection or organ failure. The following were exclusion criteria.

- Invasive ventilation
- Haemodynamic instability requiring inotrope support or volume resuscitation.
- Known bleeding diathesis or recent haemorrhage in preceding 7 days.
- Recent infection; positive culture within 5 days prior to sampling (blood, urine, CSF)
- Developing infection; positive culture within 2 days after sampling (blood, urine, CSF)
- Intolerance of enteral feed.
- Congenital anomalies

Routine Care

All patients received standard care for NEC including antibiotics for 7 days, gastric decompression by naso-gastric drainage, intestinal rest for 10 days and intravenous feeding. Intensive care and organ support was provided as required. All blood product transfusions were given at the discretion of the attending physician. Control patients received routine care.
Confounding factors:

Fresh frozen plasma (FFP) is a pooled plasma product that contains PC pathway proteins, coagulation factors, and inhibitors of the PC pathway. In neonates with, and without, congenital PC deficiency, FFP administration results in reduced thrombin generation by increasing PC levels\textsuperscript{1,2}. The effect of FFP on protein C concentration is dependent on consumption and the relative balance of the pro- and anticoagulant stimuli, and it cannot be easily predicted. We recognised that treatment with FFP could be a confounder, affecting the primary outcome variables, however we decided not to exclude neonates that had received transfusions in the 24 hours prior to study entry. We felt that it would be more clinically relevant to study the primary variables using standard intensive care treatments; to reflect the role of the pathway in our usual patient population. We did, however, monitor the use of blood product transfusions.

2.5. Study investigations

Patients were enrolled after fulfilling the entry criteria and parents had given informed consent.

2.5.1 Study plasma samples

Collection:

Neonates with NEC typically have central venous access to facilitate intravenous feeding; many will also have indwelling arterial catheters. Study blood samples were collected from the lines and transferred by syringe to collection vials in this cohort. Healthy neonates do not have indwelling venous access therefore sampling was done by venepuncture timed with routine blood draws using the ‘broken needle’ technique. Blood is allowed to drip from the needle into the collection vials under intermittent tourniquet pressure. The
needle frequently clots over time; therefore the first flow of blood was used for the study samples.

Sample volume was restricted to 1.4 mL in neonates <1000g in weight (7 participants) and PC antigen, PS, APC, TAT and D-Dimer investigations were prioritised in these individuals\(^3\). PC antigen concentration and activity are closely correlated except in the rare circumstance of congenital PC deficiency caused by the production non-functional PC. TAT and PF1+2 are both sensitive markers of thrombin generation, TAT assays have been reported in neonates previously and therefore this test was prioritised. Prioritisation resulted in a single measure of thrombin generation and PC concentration rather than two; and did not introduce bias.

Sample volumes of 2.4mL were collected from neonates >1000g.

Two mL (or 1mL in <1000g neonates) was collected into 3.2% citrated plasma and 0.4 mL was collected into 0.3M benzamidine - 3.2% citrated vials for APC.

Samples were centrifuged at 2000 rpm for 20 minutes and the plasma was collected and immediately refrigerated at -80°C. These were batched for investigation; a median 7.5 months after collection (range 1-18 months) and all samples had a single freeze-thaw cycle. Freezing for long periods can affect PF1+2 assays in particular.

**Investigations**

Assays for PC antigen and PC activity were planned; however PC antigen levels were prioritised. Some rare PC deficient states are associated with adequate levels of antigen but malfunctioning protein; while rare we hoped to be able exclude this where possible.

Thrombin generation can be assessed by the measurement of thrombin-antithrombin complexes (TAT) or Prothrombin fragment 1+2 (PF1+2). Thrombin is difficult to measure directly because of its short half-life and the plasma level may not reflect true generation
because of binding to membrane receptors, particularly in propagating thrombi. TAT complexes are a reliable measure of one of the main inhibition pathways of thrombin and reflect activation of coagulation. TAT complexes are known to rise in preterm neonates after delivery, with sepsis and respiratory distress. However AT levels are physiologically low in preterm neonates and may underestimate thrombin generation. PF1+2 is produced on activation of prothrombin, it can remain surface bound and levels may not always reflect thrombin generation. Thrombin can cleave PF1+2, also reducing the levels. We therefore decided to perform both assays to gather a full understanding of thrombin activation, prioritising TAT in smaller samples.

The human plasma samples were very small and precious. TAT, PF1+2 and APC tests in particular can be difficult to master with an adequate level of quality assurance. We therefore elected to have these investigations done in commercial laboratories familiar with the investigations.

ELISA for TAT, PF 1+2 and D-Dimers were undertaken as described in general methods by the Haemostasis Laboratory, University College London. QC samples were within range.

ELISA for PC antigen and activity, APC, PS free and total were undertaken as described in general methods by the Baxter Laboratory, Vienna. QC samples were in range.

2.5.2 Study histology samples

At the time of study enrolment, parents consented to additional immunohistochemical investigation of any intestine that required resection at surgery and that was sent for routine histopathology. The laboratory prepared 4µm sections from formalin fixed and paraffin embedded intestinal samples. Sections were stained with H&E and the expression of TM, EPCR and fibrin deposition was determined with immunohistochemistry as previously described in general methods.
2.5.3 Clinical data collection

2.5.3.1. Demographic data

Demographic and background data was collected on all participants to enable comparison of the groups according to criteria known to influence the development of NEC. As mentioned in the literature review, the incidence of NEC has a reciprocal relationship with gestational age and birthweight.

The following definitions were used for birthweight: extremely low birth weight (ELBW) <1000g, very low birthweight (VLBW) 1000-1500g and low birth weight (LBW) 1500-2500g.

We recorded other risk factors for NEC; intra-uterine growth retardation (IUGR), associated co-morbidities and haemodynamically significant patent ductus arteriosus (PDA).

2.5.3.2 Clinical data

Clinical parameters relating to organ failure and coagulopathy were collected for 48 hours around the sample, including the volume of platelet and FFP transfusions the child received. Data temporally closest to the study sample time were used for analysis.

Clinical parameters relating to sepsis were collected for 5 day prior to and 2 days after sampling; these included c-reactive protein (CRP), white blood cell (WBC) and neutrophil counts, any culture results and any commencement or change to antibiotics regimes. In the control group, clinical stability was also monitored, as judged by the attending team.

Population based risk of mortality scores have not been validated for this group of children. We collected the Paediatric Index of Mortality 2 score (PIM2) but recognise the limitations of this score in this population. Mortality associated with the acute NEC episode was recorded, as well as the final outcome for the cohorts at 6 months.

2.5.3.3 Organ failure definitions
There is no recognised organ failure scoring system in premature infants that is calibrated for outcome, beyond the first few days of life. We therefore defined organ failure criteria based on age- and gestation-appropriate reference ranges adapted from the following scoring systems: SOFA\(^9\), surgical neonatal multiorgan failure score\(^10\) and the DIC score from the ISTH\(^11\). I used the definitions below to calculate the number of dysfunctional organ systems at the time the study blood samples were taken.

**Respiratory failure:**
Defined by any of: a new requirement for invasive ventilation and, or a PaO\(_2\)/FiO\(_2\) < 300 mmHg, the definition of acute lung injury (ALI).

**Cardiovascular failure**
Defined as any requirement for inotropic support.

We recorded the volume (mL/kg) of colloid or crystalloid given as boluses for the 24 hours prior to study sampling, but did not include packed red cells, FFP, cryoprecipitate or platelets. The purpose of volume resuscitation is to restore circulating intravascular volume reduced by capillary leak syndrome; thereby supporting the circulation and restoring organ perfusion. In general terms it marks a failing circulation, but the thresholds for prescribing volume resuscitation vary and therefore we did not regard it as cardiovascular failure on its own. Packed red cell, FFP, cryoprecipitate and platelets may have the same intravascular effect as other colloid or crystalloid boluses; but they were excluded from the cumulative volume calculation as they are given primarily as therapy for coagulopathy or anaemia, even in circumstances when the intravascular volume is adequate.

The contemporaneous blood lactate estimations were recorded at the time of sampling in the NEC group. Blood lactate levels over the 24-hour period before, and after sampling,
were recorded if they were performed by the clinical team. In NEC, lactate can be a marker of generalised shock, but it can also be a marker of localised hypoperfusion of the intestine.

**Renal failure**

Defined by anuria for 6 hours or raised serum creatinine > 100 µmol/L if younger than 10 days of age, or > 88 µmol/L if older than 10 days.

**Thrombocytopenia and coagulopathy**

Severe thrombocytopenia has been identified as a poor prognostic factor in NEC\textsuperscript{12,13}. A platelet count below 150 X 10\(^9\)/L is defined as thrombocytopenia, and counts below 100 x 10\(^9\)/L as severe thrombocytopenia.

The reference ranges for PT and APTT are dependent on the gestation and age of the neonate. In order to compare results of neonates of varying age and gestation, I calculated the difference in time between the measured value and the upper limit of the age-and gestation matched reference range. Positive values represent prolonged assays. The reference ranges for fibrinogen are similar across the age and gestation range and therefore are directly comparable.

DIC has been reported in 28% of neonates with NEC and it is a poor prognostic factor\textsuperscript{14}. The International Society for Thrombosis and Haemostasis (ISTH) DIC score is based on adult parameters; we modified it using age- and gestation- matched upper limit of the normal reference range to calculate the PT prolongation time. Scores equal to or greater than 5 were consistent with DIC\textsuperscript{11,17}.

The volume (mL/kg) of platelet, fresh frozen plasma and cryoprecipitate transfusions were noted in the 48 hours around study blood collection.
Chapter 4: Prospective observational study of PC pathway in NEC

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<td>&lt;1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: ISTH DIC score. Age and gestation matched PT reference ranges were used to calculate prolonged PT. Neonates with NEC and a scores >5 were considered to have DIC.11

Hepatic dysfunction

Hepatic dysfunction was defined as any, or a combination of;

- Very low albumin levels below the laboratory minimum detection limit of 20g/L were considered abnormal. Albumin is produced by the liver but can be affected by a number of factors other than hepatic function; such as nutritional state and the acute phase reaction that down-regulates its production. Healthy preterm neonates have low albumin levels; studies vary on the reference 10th centile; Zoitkin suggests that 90% of preterm infants born at or after 27 weeks gestation will have a serum albumin level above 20g/L.15
- Alanine transferase levels above 32 U/L (ALT) were considered abnormal.
- Elevated bilirubin level.

Absolute bilirubin levels in neonates and preterm neonates can be raised without hepatic dysfunction; therefore a ratio was also calculated of the total bilirubin levels to the gestation- and age-adjusted upper thresholds for phototherapy.16 The thresholds are not an upper limit of a normal range, but represent the therapy threshold to prevent toxic neurological effects of high bilirubin levels. Therefore a ratio of >1 was considered a conservative estimate of abnormality.
2.6. Surgical data

The severity of NEC was staged with Bell’s criteria (chapter 3).

If the participants underwent surgery as part of the treatment for NEC, the findings at laparotomy and surgical details were noted. Histopathology results were recorded.

2.7. Analysis of the effect of confounders

The potential effect of FFP transfusion on the primary variables was explored in the NEC group.

2.8. Secondary outcome analysis

The potential association of PC pathway protein concentration and severity of illness was explored by subgroup analysis of the NEC group. Shock, as demonstrated by a need for inotropic support, the presence of DIC, the requirement for surgery and co-infection were considered indices of severity.

2.9. Histology of resected intestine

2.9.1 Severity of histological injury

The severity of the histological injury was assessed on H&E sections. The sections were anonymised so the study investigator was blinded to their origin. The investigator graded the severity of the NEC histological injury according to the scale described in general methods:

Normal: no inflammation

Mild NEC: inflammatory changes confined to mucosa

Moderate NEC: Inflammation of mucosa & submucosa

Severe NEC: Inflammation of mucosa, submucosa & serosa

Very severe NEC: transmural necrosis including perforation.
The formal pathology report and the study investigator findings were reviewed for the level of agreement.

2.9.2 Assessment of TM and ECPR expression and fibrin deposition

The endothelial expression TM and EPCR of was compared to positive control intestinal samples and graded on a five-point scale:

Stronger than control, strong (as control), moderate, weak or absent expression.

2.10 Statistics

The sample sizes (13 participants) were calculated with an estimated 80% power to detect a fall in PC antigen level from 0.3 U/mL to 0.2 U/mL to a 5% significance level.

Parametric methods were used to compare the means of samples with normal distributions, using unpaired t-tests or one-way analysis of variance (ANOVA) with Bonferroni’s correction for comparison of multiple means; the samples with Gaussian distributions included PC antigen, free PS, total PS, platelets, lactate, PT, WBC, neutrophil count, CRP.

Non-parametric methods were used for non-Gaussian distributions (APC, PC activity, TAT, D-dimers and PF1+2) and for sub-group analysis of the NEC group. Comparison of medians was undertaken with Mann-Whitney or Kruskal-Wallis tests with the Dunn’s correction for comparison of multiple medians. Significance is reported at p<0.05.
3. Results

3.1. Characteristics of the study population

*Control group:*

11 neonates were enrolled in the control group; 4 were later excluded as they developed clinical infection within 48 hours of sampling (2 patients) or had recent haemorrhage detected that would have been present prior to sampling (adrenal haemorrhage) or had a significant congenital cardiac anomaly diagnosed.

*NEC group:*

13 patients were enrolled in the NEC group; 9 required emergency surgery (surgical NEC) and the remaining 4 were conservatively treated with gastric decompression, intestinal rest and antibiotic therapy (medical NEC). All patients received standard intensive care therapy including intravenous feeding with total parenteral nutrition (TPN).

Study blood samples were taken within 48 hours of the diagnosis on NEC in 7/13 participants in the NEC group, 72 hours in 12/13, and at 96 hours in the remaining case. Study bloods were taken pre-surgery in surgical cases; and 6 were within 24 hours pre-surgery.
Table 4.2: Summary of the case histories of the 13 NEC participants.


<table>
<thead>
<tr>
<th></th>
<th>Gestation at birth</th>
<th>Age &amp; CGA at NEC</th>
<th>Clinical status pre-NEC</th>
<th>Risk factors</th>
<th>Organ/system dysfunction</th>
<th>Surgical findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40w</td>
<td>3 days term</td>
<td>SV, feeding</td>
<td>PROM with <em>Group B Streptococcus</em></td>
<td>Bell’s stage IIIB IPPV, hepatic</td>
<td>Perforation &amp; gangrenous left colon; hemicolectomy, end-end anastomosis</td>
</tr>
<tr>
<td>2</td>
<td>30.9w</td>
<td>4 days 31.6 w</td>
<td>CPAP, feeding CUSS normal</td>
<td>Coliforms on intra-operative peritoneal swab</td>
<td>Bell’s stage IIIA, IPPV, hepatic thrombocytopenia</td>
<td>Multifocal NEC, 2 resections, end-end anastomosis</td>
</tr>
<tr>
<td>3</td>
<td>24.4w</td>
<td>9 days 26 w</td>
<td>CPAP, TPN CUSS bilateral IVH &amp; ventricular dilation</td>
<td><em>Candida</em> sepsis, insignificant PDA, <em>Staphlococcus epidermidis</em> on blood culture</td>
<td>Bell’s stage IIIAIPPV, inotropes thrombocytopenia</td>
<td>Perforation, resection and ileostomy.</td>
</tr>
<tr>
<td>4</td>
<td>28w</td>
<td>10 days 29.7 w</td>
<td>CPAP, feeding CUSS normal</td>
<td>Previous feed intolerance, ?NEC <em>Enterobacter</em> on blood culture</td>
<td>Bell’s stage IIIAIPPV, inotropes,hepatic, thrombocytopenia.</td>
<td>Sealed perforation, inflammatory mass,, gangrenous bowel resected, ileostomy.</td>
</tr>
<tr>
<td>5</td>
<td>35w</td>
<td>11 days 36.6 w</td>
<td>SV, feeding CUSS normal</td>
<td>Twin, imperforate anus, stoma day 1, Haemophilus sp. ETT</td>
<td>Bell’s stage IIIAIPPV, inotropes</td>
<td>No surgery</td>
</tr>
<tr>
<td>6</td>
<td>33w</td>
<td>14 days 35w</td>
<td>CPAP, feeding, normal CUSS</td>
<td>Twin, VSD &amp; PDA, indomethacin (on day 11). <em>Escheridia coli</em> on intra-operative peritoneal swab</td>
<td>Bell’s stage IIIBIPPV, hepatic thrombocytopenia</td>
<td>Perforation. Extensive NEC affecting jejunum &amp; colon. Hemicolectomy and ileostomy.</td>
</tr>
<tr>
<td>7</td>
<td>32.7w</td>
<td>18 days 35.7w</td>
<td>SV, feeding CUSS normal</td>
<td>Bilateral choanal atresia – repaired day 6. Watery diarrhoea</td>
<td>Bell’s stage IIIAIPPV, DIC, hepatic, thrombocytopenia</td>
<td>Pan-intestinal gangrene. <em>Patient died.</em></td>
</tr>
<tr>
<td>8</td>
<td>26w</td>
<td>30 days 30.6w</td>
<td>CPAP, full feeds, PDA treated, CUSS normal</td>
<td></td>
<td>IPPV, HFOV, hepatic, DIC Bell’s stage IIIA</td>
<td>No surgery</td>
</tr>
<tr>
<td>#</td>
<td>w</td>
<td>days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>9</td>
<td>26.4w</td>
<td>52 days</td>
<td>SV, full feeds, CUSS normal</td>
<td>Twin, previous NEC conservative Rx. PDA on ECHO</td>
<td>Bell’s stage IIIA, IPPV, hepatic thrombocytopenia</td>
<td>Gangrenous inflammatory mass, resection end-end anastomosis</td>
</tr>
<tr>
<td>10</td>
<td>26.3w</td>
<td>52 days</td>
<td>IPPV for airway, feeding CUSS IVH &amp; ventricular dilatation</td>
<td>Cleft palate repair &amp; cricoid split day 50. Small PDA, <em>Staphylococcus epidermidis</em> on blood culture.</td>
<td>Bell’s stage IIB IPPV</td>
<td>No surgery</td>
</tr>
<tr>
<td>11</td>
<td>27.4w</td>
<td>53 days</td>
<td>CPAP, full feeds CUSS Bilateral IVH &amp; ventricular dilatation</td>
<td>Coliforms on intra-operative peritoneal swab</td>
<td>Bell’s stage IIIA IPPV, HFOV, inotropes, hepatic, thrombocytopenia</td>
<td>15 cm ileum resected, end-end anastomosis</td>
</tr>
<tr>
<td>12</td>
<td>26w</td>
<td>64 days</td>
<td>CPAP, full feeds. PDA ligation CUSS: L germinal matrix haemorrhage</td>
<td>Turner’s syndrome, horseshoe kidney, previous NEC. <em>Staphylococcus epidermidis</em> on blood culture</td>
<td>Bell’s stage IIIB IPPV, DIC thrombocytopenia</td>
<td>Ileal inflammatory mass, sealed perforation. Resection and ileostomy</td>
</tr>
<tr>
<td>13</td>
<td>26.9w</td>
<td>69 days</td>
<td>IPPV, TPN, Trophic feeds CUSS bilateral PV cysts</td>
<td>IUGR, co-arctation, VSD &amp; PDA. 6 x courses indomethacin, Prostaglandin E1 since D49.</td>
<td>Bell’s Stage IIIA IPPV, HFOV, inotropes, hepatic renal, DIC, thrombocytopenia</td>
<td>No surgery <em>Patient died acute renal failure</em></td>
</tr>
</tbody>
</table>
3.2. Demographic and background data:

The NEC and control group did not differ in gestational age at birth, birthweight, corrected gestational age or actual age in days at the time of study enrolment; however there was a trend towards a younger age in the control group (Table 4.3).

The control group had 1 VLBW and 6 ELBW infants and the NEC group had 6 ELBW, 2 VLBW, 3 LBW and 1 term infant.

One infant in each group had intrauterine growth retardation (IUGR) defined as birthweight below the 10th centile for the gestational age.

There was a trend towards more prevalent co-morbidities in the NEC group (6/13) than amongst the control cases (1/7) (Fishers p=0.17). One neonate in the control group had an intrauterine bowel perforation that was over-sewn at laparotomy 3 days after delivery and 10 days prior to study enrolment; and was tolerating full feeds. Six neonates had co-morbidities in the NEC group; Turner’s syndrome with horseshoe kidney, choanal atresia, co-arctation of the aorta, cleft palate, imperforate anus (stoma created 10 days prior to enrolment) and ventricular septal defect. Two neonates in the NEC group had previous episodes of NEC that were conservatively managed.

All of the control group were tolerating enteral feeds at the time of study blood sampling. Eleven infants in the NEC group had established some enteral feeding prior to developing NEC but 2 had not tolerated even trophic enteral feeds and remained on TPN.

Five neonates in the NEC group had PDAs on echocardiography; 3 were considered haemodynamically significant. A further 2 infants had undergone PDA ligation at least 3 weeks prior to study enrolment. None of the neonates in the control group had PDAs; 3 had successful treatment to close the PDA prior to enrolment.
Four neonates in the control group and 5 in the NEC group had abnormal cranial ultrasound scans prior to study enrolment; one neonate in the NEC group had progression during the NEC episode from bilateral intraventricular haemorrhage with ventricular dilation to include intracerebral extension of haemorrhage on one side. None of the neonates with previously normal CUSS developed haemorrhage during the NEC episode.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>All NEC</th>
<th>p</th>
<th>Medical NEC</th>
<th>Surgical NEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>13</td>
<td></td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Males</td>
<td>5</td>
<td>7</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Gestation at birth (weeks)</td>
<td>26.8 ± 1.06</td>
<td>29.5 ± 1.26</td>
<td>0.5</td>
<td>28.6 ± 2.26</td>
<td>29.9 ± 1.62</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>940 ± 72</td>
<td>1356 ± 212</td>
<td>0.44</td>
<td>1107 ± 267</td>
<td>1544 ± 283</td>
</tr>
<tr>
<td>IUGR (n)</td>
<td>1</td>
<td>1</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Corrected gestation at sampling (weeks)</td>
<td>30.3 ± 1.62</td>
<td>33.9 ± 1.02</td>
<td>0.21</td>
<td>34.3 ± 1.46</td>
<td>33.7 ± 1.37</td>
</tr>
<tr>
<td>Age at sampling (days)</td>
<td>25 ± 4.9</td>
<td>31 ± 6.9</td>
<td>0.68</td>
<td>41 ± 13</td>
<td>27 ± 8.2</td>
</tr>
<tr>
<td>Weight at sampling (g)</td>
<td>1121 ± 135</td>
<td>1652 ± 179</td>
<td>0.25</td>
<td>1522 ± 166</td>
<td>1734 ± 277</td>
</tr>
<tr>
<td>Multiple births (n)</td>
<td>4</td>
<td>3</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Co-morbidities (n)</td>
<td>1</td>
<td>6</td>
<td>0.17</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Previous episodes of NEC</td>
<td>0</td>
<td>2</td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.3: Comparison of baseline demographic data for the control and NEC groups; and the NEC medical and surgical subgroups. Mean ± SEM for birthweight, corrected gestation, age and gestation at sampling. P values refer to t-test of the means comparison between the NEC and control groups.

3.3. Clinical characteristics:

3.3.1. Timing of NEC

The timing of NEC development had a bimodal distribution; the first peak within 3 weeks of birth and the second peak some 8 weeks after birth. The corrected gestational age range of the neonates in the later peak was narrower (33.4-35.7 weeks), while the earlier peak had a wider range; 2 neonates <30 weeks, 4 neonates 30-36 weeks, 1 neonate> 37 weeks.
3.3.2. Organ failure

All of the participants in the NEC cohort had multiple organ failure involving at least the intestine and respiratory systems; none of the control group had any organ dysfunction.

The NEC cohort were severely critically ill; 12 of the 13 cases had 3 or more failing organ systems and 8 had 4 or more failing systems.

<table>
<thead>
<tr>
<th></th>
<th>Medical NEC (n=4)</th>
<th>Surgical NEC (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPPV</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Inotropes</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Renal failure</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Hepatic failure</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Thrombocytopenia (&lt;100 x10⁹/L)</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>DIC</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Platelet transfusion</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>FFP or cryoprecipitate</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

*Table 4.4: Summary table of organ failure, and markers severity at the time of study blood sampling*

3.3.2.1. Respiratory failure:

NEC was associated with deterioration in respiratory function in all cases.

11 NEC cases were either self-ventilating in supplemental oxygen or weaning from non-invasive CPAP prior to developing NEC, 2 had remained invasively ventilated since birth.
All the cases required invasive ventilation to support respiratory failure during their NEC course; 3 required High Frequency Oscillatory Ventilation (HFOV).

11/13 participants were invasively ventilated at the time of the study blood sampling.

10 participants had PaO₂/FiO₂ measurements; all were below 300 indicating acute lung injury (ALI).

3.3.2.2. Cardiovascular failure:

Five neonates in the NEC group required inotrope support at the time of study blood sampling and one additional case within the following 24 hours.

The NEC group required significant volumes of intravascular fluid resuscitation with crystalloid or colloid, excluding packed red cells, FFP, cryoprecipitate or platelets. The median volume for the group was 50mL/kg (25-65 IQR) in the 24-hour period of study blood sampling; ranging from 0 mL/kg to 130mL/kg. The intravascular volume of preterm neonates is estimated at 90-95mL/kg; therefore 50mL/kg represents approximately half the circulating volume.

Post hoc subgroup analysis according to markers of severity (inotrope requirement, presence of DIC or infection and the need for surgery) did not reveal differences between the groups in the median volumes required for intravascular resuscitation (p=0.73). (Figure 4.2)

Despite the large volume of fluid resuscitation, the mean haemoglobin values did not differ between the control and NEC cohorts at the time of sampling (control: 12.0g/dL ±0.9 SEM vs. NEC: 12.05g/dL ±0.6 SEM; p=0.97) indicating no haemodilution effect.
Figure 4.2: Volume of resuscitation fluid (median ±IQR) administered to the whole NEC group (left) and analysed according markers of NEC severity. The group median is indicated by the dashed line (50mL/kg). The subgroup medians were not different; Kruskal Wallis test p=0.73.

The mean plasma lactate concentration of the NEC group at the time of sampling was high, 3.83 mmol/L ± 0.59 SEM; which was greater than the mean reference range (0.7-2.1mmol/L)

There were no differences in the median lactate levels on analysis according to makers of NEC severity (inotrope requirement, presence of DIC or infection and the need for surgery; p=0.66). The high lactate levels in the medical subgroup at the time of sampling, fell rapidly to normal limits within 4-6 hours.

Figure 4.3: Lactate levels of the NEC group (left) and analysis according to NEC markers of severity (medians ± IQR). The reference range is depicted in grey and the dashed line represents the whole NEC group median (3.65 mmol/L). Subgroup medians were not different; p=0.66.
3.3.2.3. Renal Failure:

In the NEC group, one child developed acute renal failure and became anuric with a raised creatinine. She was too small for haemofiltration to be technically possible and NEC made peritoneal dialysis impossible. She did not survive the episode.

The remainder of the group maintained urine output >1ml/kg/hour, most with diuretic therapy. Three neonates had creatinine > 88μmol/L.

3.3.2.4. Thrombocytopenia, coagulopathy and transfusion requirements:

None of the NEC group had a normal platelet count and a normal coagulation profile for their gestation and age.

Four neonates had DIC according to the age- and gestation adjusted ISTH DIC score.

Only one patient was not transfused with platelets or fresh frozen plasma.

The platelet counts of the NEC group were lower than the control group (106 x 10^9/L ± 29 SEM vs. 426 x 10^9/L ± 30 SEM; p<0.0001).

Nine neonates in the NEC group were severely thrombocytopenic (platelet count <100 x 10^9/L); and 5 had platelet counts below 50 x 10^9/L including the 2 non-survivors. An additional case became thrombocytopenic within the following 24 hours. The entire control group had platelet counts above 150 x 10^9/L.

Four neonates received platelet transfusions prior to sampling and 4 within the following 24 hours. Transfusion volumes ranged from 10-15mL/kg. The mean platelet counts were similar in the transfused (Tx+) and non-transfused (Tx-) groups (Tx+ 39x10^9/L (30-60 IQR) vs. Tx - 65x10^9/L (29-253 IQR) p=0.31).
The median platelet count of the surgical NEC subgroup was lower than the medical NEC subgroup (48 x 10^9/L (28-65 IQR) vs. 253 x 10^9/L (79-316 IQR); p=0.02). The median platelet counts did not differ on analysis for other markers of NEC severity.

**Figure 4.4:** The mean platelet count was lower in the NEC group (p<0.0001). The bars show mean ± SEM. The grey area depicts the reference range. Open squares are participants that received a platelet transfusion during the 24 hours prior to sampling.

**Figure 4.5:** Median platelet count analysed by markers of severity (median ± IQR). The medical and surgical group platelet counts were different (p=0.02). The solid line is the control group median (440x10^9/L) and the dashed line is the median of the whole NEC group (55x10^9/L).
Two of 13 neonates had PT, APTT and fibrinogen levels within the normal range for their age and gestation; 10 neonates had prolonged PT, 3 had prolonged APTT and 5 had low fibrinogen levels. The control group did not have contemporaneous coagulation profiles available for comparison.

Figure 4.6: The difference between measured PT and APTT and the age- and gestation matched upper limit of the reference range was calculated for each individual. Positive values are abnormally prolonged and appear above the dashed line. Open squares and circles represent individuals that received FFP in the 24 hours prior to blood sampling.

Eight of 13 neonates in the NEC group received FFP transfusion in the 24 hours prior to study blood sampling, and a further 5 transfusions occurred in the following 24 hours, 2 of these were repeat transfusions. FFP transfusions ranged from 15-30mL/kg. One neonate received cryoprecipitate prior to sampling, and 2 after sampling.

The median difference in PT (measured PT - upper limit of age and gestation matched reference range) was not different between the transfused (FFP+) and non-transfused (FFP-) NEC groups (FFP+: 7.9s (3.5-9.6 IQR) vs. FFP-: 3.6s (-1.1 – 4.9 IQR) p=0.17).
3.3.2.5. Hepatic dysfunction:

All of the patients in both groups had low albumin levels as compared to infant and childhood levels. These were lower in the NEC group with levels below the detection limit of 20g/L in 6 patients.

Raised ALT levels were found in 7 of the NEC cohort (range: 33–91U/L) and 2 in the control group (range: 33–48U/L).

Twelve patients in the NEC group and 4 in the control group had total bilirubin levels greater than 20 μmol/L (upper limit of normal in infants, children and adults). If the levels were compared to the threshold used for phototherapy, matched for age and gestation, then this number fell to 5 cases in the NEC group and 1 control.

Figure 4.7: Cumulative number of transfusions received by participants in the 24 hours prior to- and after sampling.
3.4. Surgery

Twelve of 13 NEC participants had advanced disease on Bell’s staging (stage III). Nine patients had emergency surgery.

Surgery occurred within 48 hours in 5 neonates; 3 had intestinal perforation with active NEC and one patient had pan-intestinal gangrene in whom surgery was abandoned and intensive care support was later withdrawn.

Two cases underwent surgery at a later stage (days 5 and 6) because of failure of conservative management; in both cases inflammatory masses with sealed perforations were found, with ongoing active NEC.

Six patients had diverting ileostomies.

3.5. Associated infection

All of the cases had growth on at least one culture: blood, peritoneal or throat swab.

Four of the NEC group had positive blood cultures and were considered infected for the subgroup analysis. (3 *Staphylococcus epidermidis* and 1 *Enterobacter sp*.). In addition, 3 other cases had positive growth from peritoneal swabs taken at laparotomy (1 *Escheridia coli*, 2 coliforms). Throat culture showed 6 cases were colonised with coliforms and one with *Haemophilus influenza non-B species*.

The C reactive protein (CRP) was raised in 11 of 12 measured in the NEC group and the mean CRP was different to the controls (controls: 2.3 mg/L ± 0.52 SEM vs. NEC: 205 mg/L ±21 SEM; p<0.0001).
The WBC and neutrophil counts of the control and NEC group were similar (WBC control: $11.3 \times 10^9/L \pm 2.3$ SEM vs. NEC: $7.3 \times 10^9/L \pm 1.3$ SEM; $p=0.82$; and neutrophil count, control: $7.7 \times 10^9/L \pm 4.2$ SEM vs. NEC $5.3 \times 10^9/L \pm 1.3$SEM; $p=0.53$). Two cases were neutropenic.

### 3.6. Mortality

This group had a high risk of mortality as estimated by the PIM2 score.

Two neonates died during the NEC episode, one as a consequence of acute renal failure and the second from pan-intestinal gangrene.

Three neonates had died from unrelated causes at the 6-month follow up. There was one death in the control group and a total of 4 deaths in the NEC group.
3.7. Analysis of primary outcomes.

3.7.1. PC antigen:

The mean PC antigen levels were lower in the NEC group compared to the controls (0.18 U/mL ± 0.015 SEM vs. 0.3 U/mL ± 0.03 SEM; p=0.0007).
Comparison of individual values to the reference range containing 95% of the values of a healthy age- and gestation-matched population showed that the entire NEC cohort had PC antigen levels below the mean reference value; and 6 were below the 2.5\textsuperscript{th} centile.

Twelve of the NEC group had PC antigen concentration below the 40\textsuperscript{th} centile of the control group (0.245 U/mL). PC concentrations below the 40th centile threshold in adults with severe sepsis-induced PC deficiency are associated with increased organ dysfunction and mortality\textsuperscript{17}. In preterm neonates with severe sepsis, mortality is associated with levels below 10% (0.1U/mL)\textsuperscript{18,19}. None of the NEC cohort had PC antigen levels below 0.1U/mL.

PC concentration correlated to platelet count (Pearson’s r=0.53; p=0.017) and D-Dimer level (Spearman’s r= -0.51; p=0.026).

**Figure 4.11:** Ratio of measured PC Ag level to either: the mean (left) or the lower limit (right) of 95% age- & gestation-matched population reference range. Values <1 are abnormally low compared to the denominator. Open squares represent individuals transfused with FFP.

3.7.2. APC levels

The median APC levels in the control and NEC cohort were below the adult reference range (1-3ng/mL), but they were not different to each other (0.67 ng/mL (0.45-0.68 IQR) vs. 0.48 ng/mL (0.37-0.65 IQR); p=0.22).
There were 2 outliers of APC concentration in the group that were above the adult reference range (10.2 and 8.1 ng/mL). Clinically, these patients were ventilated with hepatic dysfunction but no inotropic requirement. They had thrombocytopenia and coagulopathy without DIC; they had FFP prior to sample collection. Both neonates required surgery and survived.

The quality assurance of the assay was within acceptable limits; therefore we continued to include the outliers in our analysis, as there was no biologically relevant or technical reason to exclude them. APC levels are known to correlate with PC antigen levels in health; however this relationship is lost with an inflammatory challenge. It is possible to boost APC levels as much as 100-280-fold above resting levels\textsuperscript{20,21}.

APC levels did not correlate with PC levels (Spearman's $r = 0.28; p=0.24$)
3.7.3. Free and total PS

The NEC group had lower free PS levels (control: 0.44 U/mL ±0.02 SEM vs. NEC: 0.32 U/mL ± 0.03 SEM; p=0.025) and total PS than controls (control: 0.45 U/mL ± 0.03 SEM vs. NEC: 0.37 U/mL ± 0.02 SEM, p=0.04).

The mean ratio of PS free/PS total was not different between the two groups and fell within the age and gestation matched reference range, (1.03 ± 0.1 SEM vs. 0.86 ± 0.06 SEM; p=0.15); however the ratio in 4 NEC cases fell ratio below the 2.5 centile, inferring higher levels of C4BP, an acute phase reactant.

**Figure 4.13:** Free and total PS levels were different in the control and NEC groups (means ± SEM). Grey areas indicate age-adjusted reference ranges; the preterm range cannot be depicted, as it is age and gestation specific. Open squares represent individuals transfused with FFP.
3.8. Fibrinolysis and thrombin generation:

The concentration of D-Dimers was increased in the NEC cohort (290 µg/L (196-522 IQR) vs. 43 µg/L (18-92 IQR); p=0.014). The levels in the control group were not raised compared to adult or suggested neonatal ranges\textsuperscript{22}. 

![Figure 4.14: D-Dimer levels were raised in the NEC cohort (median ± IQR). The grey area indicates the adult reference range. Open squares represent individuals transfused with FFP.]

The median levels of TAT complexes were high in both groups, but not different to each other; (control: 7.8 µg/L (96.6-24.6 IQR) vs. NEC: 5.6µg/L (93.2-7.9 IQR); p=0.06).

The median levels of PF1+2 were not different between the groups (control 1.13nmol/L (1.02-1.34 IQR) vs. 0.64nmol/L (0.41-1.48 IQR); p=0.21).
Figure 4.15: Plasma concentrations of TAT (A) and PF1+2 (B) in the control and NEC group were not different. Grey areas indicate the adult reference range (medians ± IQR). Open squares represent individuals transfused with FFP.

3.9. Analysis to examine confounder effect of FFP transfusion:

The results from the NEC cohort were analysed to examine the effect of FFP transfusion in the primary outcome variables; however the numbers in the subgroups are small increasing the risk of a type II error. We did not demonstrate an effect of FFP on the PC pathway proteins, however it was associated with a fall in D-Dimer concentration.

The median D-Dimer level of the transfused NEC subgroup was lower than the non-transfused group (FFP+: 206µg/L (140-348 IQR) vs. FFP-: 391µg/L (285-991 IQR); p=0.045).

Transfusion of FFP did not affect (medians (IQR)):

- PC antigen (FFP +: 0.17 U/mL (0.15-0.23) vs. FFP- 0.19U/mL (0.12-0.2) p=0.42)
- APC (FFP+: 0.63U/mL (0.41-6.2) vs. FFP- 0.4 U/mL (0.35-0.49); p=0.93)
- Free PS (FFP+0.34U/mL (0.21-0.42) vs. FFP-: 0.42U/mL (0.19-0.46); p=0.77)
- Total PS (FFP+: 0.37U/mL (0.31-0.41) vs. FFP-: 0.42U/mL (0.28-0.46) p=0.83)
Figure 4.16: PC antigen (A) and APC levels (B) did not differ between the NEC subgroups that had, or had not been transfused with FFP (medians ± IQR). The solid line is the median of the control group and the dashed line is the median of the whole NEC group.

3.10. Analysis according to markers associated with severe NEC

We analysed the primary outcome variables to determine any association with the severity of NEC. We found that DIC, shock requiring inotropic support, the need for surgery or sepsis demonstrated by positive blood cultures, were not associated with a change in median levels of the primary outcome variables.
Figure 4.17: Subgroup analysis of median PC antigen (A), APC (B), Free PS (C) and D-Dimer (D) according to markers for severity of disease. Solid lines indicate the median of the control group and the dashed lines depict the median of the entire NEC group.

It is interesting that the primary outcome variables were altered in the 2 control cases that were excluded from analysis as they developed sepsis requiring antibiotic treatment in the 24 hours after the study bloods were taken; one later had positive blood cultures for Staphlococcus epidermidis.
PC antigen, free and total PS were below the mean, and minimum values, for the control group:

- PC antigen: 0.16 U/mL and 0.13 U/mL vs. control: 0.3 U/mL ± 0.03 SEM;
- Free PS: 0.46 U/mL and 0.39 U/mL vs. control: 0.44 U/mL ± 0.02 SEM
- Total PS: 0.34 U/mL and 0.37 U/mL vs. control: 0.45 U/mL ± 0.03 SEM

The APC levels were similar to the rest of the control cohort: 1.34 ng/mL and 0.56 ng/mL vs. control 0.67 ng/mL (0.45-0.68 IQR)

The D-Dimers were of 262 µg/L and 134 µg/L; both above the median value and maximum value for the control group: 43 µg/mL (18-93 IQR).
3.11. Histology of resected intestine.

Nine neonates underwent emergency surgery and 8 had non-viable bowel resected. Surgery was abandoned in one infant without resection due to pan-intestinal gangrene.

Nineteen sections were available for histological examination from 7 of these cases; each case having 2-4 sections from differing areas of resected intestine available for examination. The sections from one case were missing.

3.11.1. Histological grading of the severity of NEC

All the cases had sections with full thickness, transmural necrosis indicating very severe NEC.

Most sections had areas of less severe disease interspersed with more severely affected areas; showing the typical pattern of multifocal disease. (Figure 4.16)

The mucosa had marked inflammatory cell infiltrate with lifting of the epithelium in milder areas progressing to shortening and destruction of the villi in more affected areas. Lacteal vessels become thrombosed as necrosis continued. The submucosa and serosa were involved in areas of more severe inflammation with marked inflammatory cell infiltrate, venous congestion, thrombosis and variable haemorrhage into the tissue as destruction continued.

Routine pathology review confirmed NEC in 8 cases. There was agreement between the severity grading of the pathologists and the blinded study investigator.
Figure 4.18. Representative histology sections of intestine from the NEC surgical group (H&E stain)
Panels (A) – (C) (x 100) show moderate NEC with progressive destruction of the villi of the mucosa, congestion and thrombosis of the lacteal and submucosal vessels, marked inflammatory cell infiltrate and haemorrhage. Panel (D) (x400) shows thrombosis of the microvasculature of the mucosa and
inflammatory cell infiltrate. Panel (E) demonstrates necrosis of the villi and panel (F) shows inflammatory changes extending to the submucosa with inflammatory cell infiltrate, thrombosis and haemorrhage. Panels (G) - (H) demonstrate very severe NEC with pneumatosis (G) and transmural necrosis (I) and peri-perforation of complete necrosis intestinal wall (I).

3.11.2. TM and EPCR expression and fibrinogen deposition

TM and EPCR expression followed the same pattern of expression as described in chapter 3.

TM stained strongly in all vessels, in all the layers of the intestine in areas of little inflammation. EPCR stained strongly in vessels of the submucosa and serosa in areas of little inflammation. The lacteal vessels of the mucosa did not express EPCR, as previously reported. Epithelial cells stained positively for EPCR.

Endothelial TM and EPCR expression was reduced in inflamed areas and absent in necrotic areas. Immune cells expressed TM and EPCR.

Fibrin deposition occurred extravascularly in areas of haemorrhage, coagulative necrosis and within luminal and serosal necrotic tissue. Intravascular deposition causing vessel thrombosis was commonly associated with reduced TM expression.
Figure 4.19: Thrombomodulin expression in the neonatal intestine affected by NEC. Thrombomodulin appears brown. Panels (A) – (D) represent progressively more severe stages of NEC. In (A) there is very little inflammation and there is strong TM expression by the vasculature including lacteal vessels in the villi. In (B) and (C) there is necrosis of the mucosa and an inflammatory cell infiltrate. Some vessel show reduced expression of TM (solid arrow). In (D) there is transmural necrosis with absent TM staining. (A-C x100, D x40).
Figure 4.20. Thrombomodulin expression of neonatal intestine with NEC.

Panels (A)-(H) are fields from sections of increasing histological NEC injury. In Panels (A) – (D) endothelial expression remains moderate or strong. Inflammatory cells are seen adhering to the endothelium and migrating through it (solid arrows). The dashed arrows indicate inflammatory cells expressing TM (monocyte/macrophages and neutrophils). As inflammation increases, TM expression becomes weak (E-G) or absent (H).(A-G x 400; H x 200).
**Figure 4.21.** Thrombomodulin expression in neonatal intestine affected by NEC (x1000).

TM is expressed on the endothelial surface of the microcirculation including arterioles (D), venules (A, C, E) and capillaries (B, F). Monocytes/macrophages express TM (B). An immune cell can be seen migrating through the endothelium in panel (C).
Figure 4.22. EPCR expression in neonatal intestine with NEC. EPCR appears red.
Panel (A) and (B) (x100) EPCR expression is preserved in many vessels in the submucosa in mild – moderate NEC. Epithelial cells also express EPCR. Panels (C) – (G) (x400). Endothelial expression of EPCR is variably reduced in vessels (solid arrow). Cells of the innate immune system express EPCR (dashed arrow).
Figure 4.23 Dual staining immunohistochemistry techniques were used to define the expression of TM in relation to intravascular fibrin deposits in the neonatal intestine affected by NEC. TM appears brown and fibrin appears red. Patent vessels without fibrin deposits express TM strongly (dashed line) while vessels with intravascular fibrin deposits have reduced TM staining. Panels (C) – (E) are not annotated. (A-E x100).
Figure 4.24. Dual staining immunohistochemistry techniques were used to visualise TM expression in relation to intravascular fibrin deposits. TM appears brown and fibrin appears red. TM is strongly expressed in patent vessels and expression is reduced in vessels with fibrin deposition. Panel (A) shows thrombosis of the lacteal vessels in the mucosa with epithelial cell lifting and villus tip destruction. Panels (B) – (D) show vessels in the submucosa. (A-D x 400).
4. Discussion

4.1. Overview

We report of the effects of NEC on the PC pathway in neonates for the first time. This is also the first report of APC concentrations in healthy pre-term infants; which are lower than the adult reference range.

In our cohort, NEC was associated with decreased PC, free PS and total PS plasma concentrations and raised D-dimer levels. Plasma levels of APC were not different to the control group, but were lower than the adult reference range. Significantly, two patients could increase their APC approximately 10 fold in response to the thrombotic challenge, while others did not. Histological examination of resected bowel showed down-regulation of endothelial TM and EPCR in acutely inflamed areas, but preservation of expression in less inflamed areas.

4.2. Patient cohorts

The NEC neonates were severely critically ill and the majority of the group had advanced NEC on Bell’s staging. All of the infants had 2 or more organ failures and 9 patients required surgery. The demographic and physiological characteristics of the group were representative of the population of neonates with advanced NEC that present to tertiary surgical NICU’s.

The group had a bimodal pattern of NEC incidence in keeping with epidemiology described in the literature. The majority of the group were premature; developing NEC either in the first 3 weeks, after bacterial colonisation, or much later in their ITU course. The only term infant in our cohort developed NEC within a few days of birth; and the pathogenesis in these infants is thought to be related to perinatal events (hypoxia, asphyxia or infection).
There was a trend towards lower birthweight, gestational age and corrected gestational age at the time of sampling in the control group. The physiological effect of increased prematurity would be to lower the PC pathway protein concentrations in the control group; making it more difficult to detect differences in protein concentrations with the NEC group as well as potentially underestimating the size of the effect. Nevertheless, the differences in concentrations were large enough to detect.

All of the patients were either colonised or actively infected during the acute episode; mainly with coliforms and *Staphylococcus epidermidis*. As mentioned in the introductory chapter, colonisation with pathogenic bacteria plays a significant role in the pathogenesis of NEC.

### 4.3. PC pathway in NEC

We have shown that the PC pathway is down-regulated during NEC with reduced plasma concentrations of PC, free and total PS and reduced endothelial expression of TM and EPCR in the intestinal vasculature. There is increased thrombin generation and fibrinolysis. We did not find altered plasma concentration of APC, and in two cases this was increased 10-fold.

These findings are not unexpected, as other acute inflammatory states have also shown down-regulation of the PC pathway, in both adults and children, as described in the first chapter. These include neonates with severe sepsis, respiratory distress and birth asphyxia. 18,19,23,24

#### 4.3.1. Protein C and protein S levels:

Our cohort had severe acquired PC deficiency. The majority of values were below the 40th centile of the control group. The 40th centile is associated with increased mortality in adults with severe sepsis. However, none of the NEC group fell below the lower, neonatal
threshold associated with increased mortality (0.1 U/mL). Analysis of data from the RESOLVE trial of rhAPC in children with severe sepsis (excluding preterm infants) showed that term infants younger than 6 months of age had the least decrement in PC level\textsuperscript{25}. Our data in preterm infants with NEC suggests that this may not be true for our population and may reflect maturational changes in the haemostasis system.

Low PC levels are caused by increased consumption as well as reduced production; however the fact that PC levels decrease at all, supports the notion that PC is actively consumed in the process of moderating the inflammatory response in NEC; and therefore deficiencies in the PC pathway may play a role in the pathogenesis of NEC.

We have also reported that the main activation mechanism for PC, TM and EPCR, is downregulated on the intestinal endothelium during NEC. PF4 may have an enhanced role in activating PC in neonates with NEC as it accelerates PC activation by TM by $\approx 20$ fold. It is possible the raised levels of PF4 may affect the activation of PC, particularly in the local environment of forming thrombi.

Nevertheless, the consumption of PC down to low levels suggests that there is sufficient expression of TM, and probably EPCR, in the body vasculature to consume PC, even if the low levels are also partially caused by inadequate replenishment.

The concept that the PC pathway retains some functional ability to activate PC in a dose dependent manner despite downregulation, was confirmed by de Kleijn et al in children with meningococcal sepsis; a condition known to have reduced expression of TM and EPCR in the skin\textsuperscript{26,27}.

Free and total levels of PS are reduced in neonates with NEC; this would reduce the anticoagulant functions of APC. The ratio of free:total PS reflects the concentration of C4BP in the circulation which is usually very low in neonates to preserve the bio-availability of
free PC. C4BP is an acute phase reactant, increasing in concentration with inflammation.

Some neonates in the NEC group had reduced ratios of free:total PS due to increased binding to C4BP.

The hepatic production of PC and PS is reduced as part of the acute phase reaction during SIRS. Our NEC cohort had raised CRP and very low albumin levels; providing evidence that the acute phase reaction had been activated in these patients, and this would contribute to the low PC concentrations we report.

### 4.3.2. APC levels.

The production of APC reflects the functional status of the PC pathway. However APC plasma concentrations may not accurately reflect production as a proportion of the pool (≈40%) remains bound to surface receptors.\(^{28,29}\) Nevertheless, APC levels have not been previously studied in neonates; either in health or disease.

We report that APC levels in the control group were all lower than the adult reference range.

A number of factors may contribute to this finding in healthy preterm infants. The Kd for the EPCR-T-TM complex is ≈30-60nM which falls approximately within the adult range of PC concentration. Plasma levels of all the anticoagulant and most of the coagulant proteins are lower in term and preterm neonates as compared to adults, and the PC levels are very low (~20%) therefore it might be expected that APC levels are also low.\(^{30}\) APC levels are known to correlate with PC concentration in health.

APC inhibitor concentrations are relatively greater in neonates; although PCI is 64% of adult values, \(\alpha_1\)-anti-trypsin concentration is equivalent and the level of \(\alpha_2\)-macrogloubulin is doubled in neonates.\(^{30,31}\) This finding might be expected to reduce the half-life of APC in neonates leading to lower levels but studies in neonates have not been undertaken to
confirm this. Petaja et al found the opposite effect in cord plasma; the APC half life was prolonged to 50 minutes\textsuperscript{32}.

APC levels may be affected by ongoing consumption caused by activation of coagulation. D-Dimer levels in healthy neonates are raised, particularly after birth and they remain raised throughout childhood, suggesting a constant, low level of thrombin generation.\textsuperscript{22 33}

In NEC we report that the plasma APC concentrations were not different to the control group. Significantly, two patients were able to boost the APC level approximately 10 fold; despite the reduced expression of TM and EPCR in inflamed areas of the bowel. The remaining patients however, did not have increased plasma levels of APC notwithstanding raised TAT and D-Dimer levels.

NEC is an inflammatory state commonly associated with consumptive coagulopathy, thrombocytopenia and raised D-Dimers levels that indicate the generation of thrombin\textsuperscript{14}. It is reasonable to assume that increased thrombin generation should result in increased APC generation, some of which may enter the circulation.

The ability to rapidly produce increased quantities of APC ‘on demand’ in response to a thrombin challenge has been reported in adults after liver transplantation\textsuperscript{20}; and is also reported after delivery in cord blood with a 5.2-fold increase in APC activity\textsuperscript{32}. The variable production of APC in response to a thrombin challenge has been noted previously in adult patients with severe sepsis\textsuperscript{21,34}. Liaw et al found that the APC concentration did not correlate with PC concentration, levels of APC inhibitors or PF1+2 levels; however baseline APC levels were higher in survivors (survivors: 5.27 ng/mL (±2.24 SD) vs. 3.66 ng/mL (±1.64 SD))\textsuperscript{34}. The causes of variation in APC production is not clear; many have felt it may represent an unpredictable variation in the downregulation of TM and EPCR, however as previously mentioned, it has been recognised that there is a large pool of surface bound
APC and therefore plasma levels of APC may not accurately reflect activation of the PC system\textsuperscript{28,29}.

### 4.3.3. TAT and PF1+2 levels.

TAT and PF1+2 levels were not different between the control and NEC groups; but levels in the control group were higher than expected.

Raised TAT levels have been described after delivery in healthy preterm infants and can remain raised for a week after delivery.\textsuperscript{5,6} The youngest infant in the control group was 9 days old at the time of sampling; the rest of the group were older than 18 days; therefore it is unlikely that delivery would have contributed to the elevated levels.

The raised TAT and PF1+2 levels in the control group were not associated with raised D-dimers; this suggests that thrombin was generated but fibrinolysis was not yet detected. These effects commonly occur with activation of thrombin during the collection and initial processing of the blood sample; the TAT and PF1+2 assays are highly sensitive to low-grade activation and collection techniques are known to influence results.\textsuperscript{4,35} The samples from the controls were collected using the ‘broken needle’ technique, blood drips from the broken end of the needle into the collection vial; the rate of blood flow gradually decreases over time as clots appear in the needle. Every effort was made to collect these samples from free flowing blood immediately after venepuncture; however activation of thrombin may have occurred. Samples from the NEC cohort were taken from indwelling central venous or arterial catheters. It is likely that the different blood sample collection methods affected the results of the TAT and PF1+2 assay and introduced bias.

Healthy preterm neonates have low levels of prothrombin and AT\textsuperscript{7}; however raised levels of TAT complexes have been reported in response to sepsis, respiratory distress syndrome
and delivery\(^6\). We did not measure AT levels in our population due to the restrictions on blood volume for sampling, but low levels may also have affected our results.

The median TAT level of the NEC group was above the reference range, however some values were not raised. Levels of AT are lower in preterm neonates with severe sepsis, asphyxia or respiratory distress compared to healthy neonates. As mentioned previously, we were unable to measure AT in our population due to sampling volume restriction; it is possible that low levels of available AT could have affected our results.

Low TAT and PF1+2 levels may have been related to the length of storage of some samples however we could not demonstrate this correlation (TAT p=0.73, PF1+2 p=0.74).

### 4.3.4. Signs of PC Pathway dysfunction

The group had a number of clinical features of the systemic inflammatory response (SIRS) that have been directly related to PC pathway dysfunction in other inflammatory conditions in the literature; and therefore PC pathway dysfunction could contribute to these features in advanced NEC.

- **Coagulopathy**

The majority of children in the NEC cohort were coagulopathic with thrombocytopenia and raised TAT and D-Dimers; suggesting enhanced thrombin generation and fibrinolysis, with consumption of the coagulation factors, platelet activation and inadequate counter-balance by APC, TPFI and AT pathways. Reduced free PS levels would contribute to reduced anticoagulant activity of the PC pathway promoting the coagulation cascade.

- **Multiorgan dysfunction syndrome**

The patients developed multi-organ dysfunction (MODS) including ALI. Microvascular dysfunction is believed to be the key determinant of MODS development and the PC
pathway is an important moderator of endothelial function to preserve the microcirculation.

- **Capillary leak**

The group required large volumes of intravascular resuscitation to maintain perfusion, indicating capillary ‘leak’ or increased vascular permeability and endothelial dysfunction. The EPCR-APC-PAR-1 axis with cross-activation of S1P-1 is key in maintaining the barrier function of the endothelium.

- **Leucocyte infiltration**

The histology of resected bowel revealed very severe disease with marked inflammatory cell infiltrate. EPCR-PC/APC-PAR-1 signalling axis reduces leucocyte migration into the tissues.

- **Epithelial mucosal disruption on histology**

Histology of the intestinal sections showed multifocal areas of inflammatory change that in the less affected areas could be confined to the mucosa. In these areas lifting of the epithelium and disruption of the epithelial barrier were common. The epithelial damage progressed with more severe injury. The EPCR-PC/APC-PAR-1 axis mediates epithelial barrier integrity, reducing apoptosis and maintaining tight junctions between adjacent epithelial cells.

4.4. **Confounding factors: blood product transfusion**

Patients in the NEC group received transfusions of FFP, cryoprecipitate, platelets and volume resuscitation with isotonic saline or colloid as clinically indicated. Some received these transfusions within 24 hours of blood sampling. In the clinical setting of NEC, cryoprecipitate is prescribed for low fibrinogen levels and FFP for prolonged PT and aPTT or clinical evidence of bleeding. However, FFP contains both pro- and anticoagulant factors
(PC levels ~1 U/mL) and transfusion in critically ill neonates has been shown to reduce thrombin generation (PF1+2 and D-Dimers) by improving PC levels and the PC pathway function\textsuperscript{1}. The increment and half-life of factors infused in FFP depends on the rate of their subsequent consumption. It is likely that the transfusions affected our results; however it is impossible to predict the direction of the effect on the relative balance of the anti- and pro-coagulant pathways either in individual patients or the cohort as a whole. The results are nevertheless clinically relevant as they provide information on the actual PC pathway activity in neonates with NEC receiving standard care, including the effect of FFP transfusion as customarily prescribed.

The NEC cohort received large volumes of resuscitation fluid. In general terms, fluid is administered to improve intravascular volume during the capillary leak phase of SIRS. Clinical evidence of poor perfusion occurs once compensatory mechanisms have failed; this usually occurs after 20-25% loss of the intravascular volume in children and volume replacement in 10ml/kg aliquots has become the standard of care. The intravascular volume is therefore rarely over-expanded; therefore simple dilution of the PC pathway constituents is an unlikely cause for the low values we report. It is possible that small proteins follow the osmotic gradient into the tissues if the endothelium is sufficiently dysfunctional; this process may have reduced the PC pathway proteins in the worst affected of our cohort; but the contribution to the overall low levels is likely to be small.

4.5. Limitations of this study

This study has several limitations; many are related to the restricted blood sample volume that can be taken from premature neonates:

• We restricted the study to the investigation of to the anticoagulant effects of PC pathway disturbance on thrombin generation and fibrinolysis. The cascade of inflammatory mediators associated with NEC development has been extensively
investigated and described. Therefore we elected not to measure indices of the acute inflammatory response, prior to confirming that NEC, in fact, affected the PC pathway.

- We examined the PC pathway in isolation, without investigating the effects of NEC on the other anticoagulant systems ATIII with HCII and TFPI. Physiologically, the functions of the three systems are interwoven.

- We examined the zymogen and effector proteins concentrations without consideration of the levels of their inhibitors, or sTM and sEPCR. These would have would have given a more complete picture.

- We did not investigate the response of the PC pathway protein concentration over time, particularly in relation to surgery as sample volume limitations precluded serial measurements of the PC pathway. Other studies have shown that the trends in recovery may be as important to the evolving clinical situation as the absolute level of acquired PC deficiency.

This study was also limited by the small sample size that while appropriate for the primary outcome, limits the reliability of the subgroup findings.

A further potential weakness of this study is that the cohort of NEC neonates was severely ill; the study did not include milder cases of NEC and therefore the evolution of PC disturbance in relation to the appearance of NEC symptoms, is not known.

Lastly, a fuller understanding of the role of the PC pathway in the pathogenesis of NEC could have been obtained if a comparison group of severe sepsis had also been included in the study design. Severe sepsis and NEC are both systemic inflammatory states, therefore unique alterations in the PC pathways associated with NEC could indicate a pathogenic role.
4.6. Conclusions and future work:

The results presented in this chapter provide new information on the effect of NEC on the concentrations of the PC pathway proteins; and it confirms the immunohistochemistry findings reported in chapter 3 in a second cohort of neonates.

In healthy neonates the levels of PC and PS are physiologically low and there is abundant expression of TM. APC levels are low compared to adult reference ranges; this is not due to lack of TM expression, but is mainly influenced by physiologically low PC levels. This could make preterm and term infants vulnerable to sustained thrombin challenge.

We have shown that PC and PS concentrations fell in NEC, in part due to active consumption to APC. The levels of APC were varied and did not correlate with PC levels. Similar findings have been reported in other acute inflammatory conditions.

The findings suggest that the PC pathway is active in attempting to modulate the inflammatory response in NEC.

It is not known however, to what extent deficiencies in the pathway contribute to the development of NEC; or the relative importance of maturational physiological deficits in the PC pathway in predisposing preterm infants to NEC.

It is also not known if supplementation of the PC or APC concentrations could influence the clinical course of NEC. In order to address this question we undertook an interventional study in an animal model, described in full in the next chapter.
Chapter 5

PC and APC supplementation in an animal model of intestinal ischaemia-reperfusion injury
Chapter 5: PC and APC supplementation in an animal model of intestinal IR injury

PC and APC supplementation in an animal model of intestinal ischaemia-reperfusion injury

1. Introduction

In the previous chapters, we have shown that the systemic levels of PC and APC were low in a healthy preterm population of infants at risk of developing NEC, and the levels of PC were further decreased in infants with NEC. TM and EPCR expression in the intestine were reduced in infants with NEC and this was associated with intravascular thrombosis at a local level. TM and EPCR expression was only absent in severely affected or necrotic areas. Levels of APC were variably affected with some neonates showing boosted levels, while others did not.

As previously discussed we can conclude that the PC pathway is diminished in NEC, but it is not known if this is a contributing factor to NEC development and severity, or if the reduction is simply ‘collateral damage’. It is also not known how much of the reduction in activity reduction is proportionally due to the rate-limiting effects of the low levels of PC or due to the reduced ability to activate PC.

Supplementation of APC levels will provide information of ‘proof of concept’ that the PC pathway reduction is integral to the pathogenesis of NEC.

Supplementation of PC will test the adequacy of the TM-EPCR activation mechanism in the face of active inflammation and down regulation of the surface receptor expression.

APC supplementation has beneficial effects in animal models of inflammatory conditions, including inflammatory bowel disease, and in animal models of IR injury, including IR of the intestine. In studies of intestinal ischaemia-reperfusion (IR) models, APC supplementation
prior to reperfusion has been shown to reduce mucosal injury on histological examination and attenuated the associated lung injury. APC had antithrombotic and anti-inflammatory activity; reducing thrombin generation and fibrinolysis (TAT, D-Dimer levels), fibrin deposition, inflammation and reducing oxidation enzyme activity (MPO), while increasing antioxidant enzyme activity. APC supplementation also reduced intestinal oedema and reduced the loss of intestinal function\textsuperscript{1,2}.

It is not known what the impact of supplemented APC would be in NEC.

APC is contra-indicated in the preterm population because of the high associated risk of serious bleeding events; these neonates have increased risk of intracranial haemorrhage at baseline due to central nervous system vascular immaturity and fragility.

We need to consider the ability of the microcirculation to activate PC in neonates with NEC as we know TM and EPCR expression is decreased in the intestine. We do not know if the expression of TM and EPCR is affected in other vascular beds in infants with NEC.

We therefore used a related model of rat intestinal IR to in order to:

- To assess the impact of APC supplementation, as a proof of concept, primarily on the severity of histological injury, but also on the endothelial expression of TM and EPCR in the intestine and lung, on the activation of coagulation and fibrinolysis, and lastly on markers of inflammation.
- To characterise TM and EPCR expression in intestinal IR injury, both locally in the intestine and remotely in the lung.
- To assess the impact of PC supplementation primarily on the severity of intestinal injury, but also on the activation of coagulation and fibrinolysis and markers of
inflammation. These assessments will reflect the function of the PC activation apparatus

- To investigate the impact of the timing of APC/PC supplementation in relation to reperfusion, primarily on the severity of histological injury but also on the thrombotic and inflammatory parameters.

2. Animal model: materials and methods

2.1. Development of the model and study design:

The animal model was described in Chapter 2. The following paragraphs outline the rationale for the study design.

2.1.1. Primary Outcome

The primary outcome was to detect changes in the intestinal IR histological injury score in the interventional drug groups as compared to the sham and saline groups in the model of intestinal reperfusion injury.

Secondary outcomes included assessing the impact of the intervention drugs on the endothelial expression of TM, EPCR, P-selectin and fibrin deposition in the lungs and intestine, assessment of TAT and D-dimer levels and the myeloperoxidase activity of the intestine. The latter assessment was undertaken by Mr Nigel Hall and is reported here for additional information.

2.1.2. Choice of animal model

We chose to investigate the effects of APC and PC in an adult rat model of intestinal ischaemia-reperfusion in the knowledge that this injury is not a complete model of NEC.
although some features are shared. We felt the advantages outweighed the disadvantages of this model in our hands.

Advantages:

- The animal model was already well established within the department\textsuperscript{3-5}.
- Adult rats are large enough for surgery to be technically feasible and to allow blood sampling in adequate volumes.
- Analogous rat intestinal IR models had been used to investigate other aspects of the PC pathway and reported in the literature\textsuperscript{1,2,6,7}. The use of a similar animal model allowed comparison of results and would contribute to the published body of knowledge.
- The protein C pathway had been investigated in rat IR models of different organ systems, including the intestine. We were able to use this data to inform our dosing schedule\textsuperscript{1,2,6-12}.

Disadvantages:

- IR injury does not embody all the features of NEC, as IR is only one aspect of NEC pathophysiology. This is a very limited model of NEC.
- At the time of this study there was more published experience of the PC pathway in mice, particularly with regard to adequate dosing schedules of APC and PC. However, the small size of mice precluded sufficient blood volumes to be collected for investigation and would have increased the technical difficulties of the procedures.
- Similarly, this model should have included neonatal or preterm rat pups to account for developmental influences; however suckling rat pups were too small to allow sufficient blood volume collection for samples and surgery was technically difficult; their weight is less than a tenth of an adult animal (15-20g v 225-250g).
2.1.3. Choice of intervention drugs:

Both PC and APC were investigated in this model. This allowed us to consider the state of PC activation following IR injury.

2.1.4. Timing of intervention drug administration.

Most study groups investigating the effects of APC in rat ischaemia-reperfusion models have administered APC before reperfusion, as proof of concept. We wished to mimic the clinical environment more closely: therefore interventions given both before reperfusion, to simulate prophylactic therapy as well as proof of concept, and after reperfusion to mimic treatment.

2.1.5. Choice of interventional drug doses

Human APC, PC and PS show species specificity with altered anti-thrombotic effects in non-humans. Species specificity in rats is known to reduce the antithrombotic effect of human APC, but it has not been quantified. Pharmacodynamic studies of APC in rats are limited and have not been published for PC. The effect of species specificity on the other cytoprotective activities of APC is uncertain; however anti-inflammatory and cytoprotective effects of APC have been documented in rat models.

Administration:

We chose to administer the intervention drugs as a bolus as it is difficult to infuse such small volumes.

APC Dose:

The therapeutic dose of recombinant human APC (rhAPC) in human clinical studies is 24µg/kg/hour. Dr S.B.Yan from the Eli Lilly Laboratories kindly shared their knowledge
of rhAPC dosing in rats (personal correspondence 2003); murine species, in their experience, were 40-80 times less sensitive to human APC in its antithrombotic activity, and human rhAPC needed to be administered in doses of 1-2 mg/kg in thrombotic rat models to match the effect in primates. They recommended the same concentration of drug should be used as for human administration.

Published studies have shown, however that a smaller bolus dose of 100µg/kg APC in rats would achieve the target therapeutic range for 2-4hours based on the pharmacokinetics of the drug and these studies reported efficacy at this dose\textsuperscript{1,5,7,9,10}. We chose this dose to enable comparison of results.

\textit{PC dose:}

In humans, the therapeutic dose range of Protein C is aimed at restoring plasma levels and is therefore dependent on consumption. Human studies in adults and children have used bolus doses of PC varying from 50 IU/kg/dose -200 IU/kg/dose and total daily doses 0f 100 IU/kg – 600 IU/kg\textsuperscript{1,2,6,7,19-21}. At the time of this study, there was no published data on the species specificity of human Protein C in rats to guide dosing.

The Baxter laboratories in Vienna kindly provided information (unpublished data in personal correspondence 2003) on their experience of the use of Protein C in rats. They recommended diluting PC with isotonic saline to ensure the same concentration as used in humans.

In summary, they found that PC was safe in acute toxicity studies in rats up to 2000 IU/kg. They had dose response curves in rats with doses up to 250 IU/kg; and the highest dose they had successfully used pharmacologically was 800 IU/kg in a volume of 8mL/kg bolus injection in a rat model of myocardial ischaemia. They determined the plasma half-life of PC was 90 minutes in healthy adult rats. They confirmed that APC was generated 5 minutes
after PC injection (24ng/mL) and the level remained constant for an hour, and then reduced to 15ng/mL at 6 hours.

We therefore initially designed the study to have low-dose and high-dose PC arms. The low-dose was known to boost plasma PC levels to the normal range in humans (80 IU/kg) and the high-dose was the highest dose Baxter had used in rats; 800 IU/kg. The first three animals in the high-dose group died within 10 minutes of the bolus injection, therefore we removed the high-dose arm from the study design.

In 2010, after we had completed the animal work in this study, Messaris et al published the use of PC concentrate, 100 IU/kg 6 hourly, in a caecal ligation and puncture (CLP) rat model with beneficial effects on their outcome measures\textsuperscript{1,5,8,12,22}.

**Volume of intervention drug:**

All groups received a total volume of 10ml/kg fluid during the procedure, ensuring that intravascular filling was equal across the groups as this may have influenced intestinal perfusion. 0.9% Saline was used in the positive control (saline) IR and sham groups, and the intervention drugs were diluted with 0.9% Saline to 10ml/kg in the APC and PC groups.

### 2.1.6. Choice of markers of thrombin generation, fibrinolysis and inflammation

Published studies have compared portal and systemic levels of haemostatic factors and cytokines\textsuperscript{1,2,6,13,14}. Systemic trends mimicked changes in the portal system but were much less pronounced. We chose to measure systemic levels of TAT and D-Dimers as these reflect routine clinical practice.
Groups have reported TAT and D-Dimers measured in murine models using commercially available human ELIZA kits with success, but without publication of optimisation techniques\(^1,^2,^7,^9,^10,^15-^17\). We elected after discussion with Prof Ian Mackie (UCL haemostasis laboratory) to optimise the human ELISA kit as rat antibodies to thrombin, anti-thrombin and D-Dimers were not easily available to establish an ELISA made with specificity for rat plasma, and there appeared to be sufficient reported species homology for this approach to be valid. It would also allow comparison to published work. Optimisation is described below.

MPO was measured by Mr Nigel Hall and the results included in this thesis for additional information.

### 2.1.7. Choice of histological investigation

The severity of intestinal injury was graded using the Park-Chiu score, a well-known scoring system for rat intestinal IR injury, allowing comparison across studies\(^23\).

Inflammation is known to reduce the endothelial expression of TM and EPCR, to increase the expression of P-selectin and the intra-and extravascular deposition of fibrin. We planned to assess the impact of IR, and the drug interventions, on the intestinal (local) and pulmonary (remote) vascular expression of these molecules in this model. P-selectin expression has a role in the pathogenesis of IR injury and APC is known to modulate its expression\(^24-^26\).

The effect of intestinal IR on remote organs, particularly the lungs, is clinically very relevant but had not been reported in the literature with animal models at the time of study design; Teke et al have subsequently described the pulmonary injury associated with intestinal IR injury\(^7\).
2.1.8. Intervention drugs in the animal model.

Protein C (Ceprotin) was obtained from Baxter Healthcare (Baxter AG, Vienna).

APC (Xigris) was obtained from Ely Lilly and company limited, Hampshire, UK.

0.9% Sodium chloride solution was obtained from Baxter Healthcare (Thetford, UK)

2.2. Study Approval

The study was approved under the United Kingdom Home Office regulations for Animals (Scientific Procedures) Act 1986. All animals were handled in accordance with guidelines prescribed by UK and International regulations on protection, care and handling of laboratory animals. Mr Nigel Hall performed the anaesthetic and surgical procedures.
2.3. Study design

2.3.1. Experimental groups

In total, 43 animals were randomly allocated to one of seven experimental groups as depicted in table 5.1.

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<tr>
<th></th>
<th>Number</th>
<th>Laparotomy</th>
<th>Dissection to expose SMA</th>
<th>IV Saline/APC/PC before clip application</th>
<th>Clip occlusion SMA 60min</th>
<th>Removal of clip, reperfusion 120min</th>
<th>IV Saline/APC/PC after 5min</th>
<th>Animal euthanized &amp; samples collected</th>
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Table 5.1: Summary of the interventions according to the experimental groups. Animals received either 0.9% saline, 100 µg/kg APC or 80 IU/kg PC made up to a final volume of 10mL/kg. Pre-R - pre-reperfusion, Post-R - post reperfusion, SMA-superior mesenteric artery, IV- intravenous.

One group of animals underwent sham operation without intestinal ischaemia-reperfusion injury and were maintained under general anaesthesia for 180 minutes (n=7). These served as negative controls.

Animals in the remaining groups underwent 60 minutes of intestinal ischaemia with SMA occlusion followed by 120 minutes reperfusion as described above.
Animals in the experimental groups received an isotonic intravenous infusion of either 0.9% saline, APC (100μg/kg) or PC (80 IU/kg) made up to 10mL/kg at one of two time points designed to simulate the effect of giving the intervention as either prophylaxis against IR injury, or as treatment following the reperfusion phase of the injury. The saline groups served as positive controls. APC and PC were administered with 0.9% saline to a total volume of 10ml/kg and all infusions were delivered over a period of 60 seconds via the femoral cannula. The groups designed to simulate prophylaxis had the drug 60 minutes before the SMA clip was removed (pre-reperfusion or Pre-R) and the groups simulating treatment received the drug 5 minutes after removal of the SMA clip (post-reperfusion or Post-R).

### 2.3.2. Tissue and plasma sample preparation

Tissue samples were prepared as described in chapter 2. FFPE and fresh frozen sections of lung and ileum were available for immunohistochemistry.

Blood was collected in citrate as described in chapter 2 and plasma was stored at -80°C.

### 2.4. Sample size

We calculated that sample size of 6 would have an 80% chance of detecting a 1.5-point difference in injury severity score (the primary outcome) at 5% level of significance.
2.5. Histological assessment of the intestinal injury severity.

Three blinded observers graded the severity of histological injury on H&E intestinal sections according to the Park-Chiu score listed in chapter 2\textsuperscript{23}.

The method of sample collection was described in chapter 2; each animal had 2 sections of ileum, 4 cm apart, processed as FFPE specimens (gut 1 and 4). These sections were stained with H&E, giving rise to a total of 86 sections. Each section was evaluated individually and scored by 3 observers (PL, NJH, HU) who were blinded to the group assignments.

\textit{Statistical analysis:}

The Park-Chiu score was analysed as a continuous variable because increased scores equated to increased severity of injury.

Each animal had 6 observations that gave rise to 3 scores. There were 3 observers, each observer scored two sections of intestine per animal and these were averaged, as they were not independent of each other.

The heterogeneity of the injury within individual animals was analysed using the difference between the scores of Gut 1 and Gut 4 for each observer.

Data was analysed with 1-way analysis of variance with Bonferroni’s correction for multiple comparison of means.

The inter-observer difference described the level of agreement between observers and the reliability of the results.
2.6. Immunohistochemistry of the Intestine

2.6.1. Optimisation of immunohistochemistry technique.

There were two FFPE and one frozen section available for immunohistochemistry per animal.

The techniques recommended by the manufacturer for each antibody were optimised in a systematic series of experiments, an approach recommended by the College of American Pathologists. The optimised protocols were summarised in chapter 2.

2.6.2. Assessment of immunohistochemical staining.

2.6.2.1. TM and EPCR of the intestine and lung.

Endothelial expression of EPCR and TM is reflected by the intensity and extent of staining on immunohistochemistry. Expression was graded on a 5-point scale described in chapter 2. The sections from the 7 sham animals were unblinded and served as the standards against which the intensity of staining in the remainder of the sections was compared and scored. The 5-point scale allowed more subtle changes to be described than a simple 3-point scale, and this was required because of the constitutive abundance of the molecules in unblinded controls. There was a single frozen section of lung and intestine per animal.

Intestine:

Two observers (PL and HU) remained blinded to the group assignments of the remaining sections. Both observers gave a crude score describing the overall TM and EPCR expression in the intestine.

However, in addition, in order to investigate if histological changes occurred differently in particular layers of the intestinal wall, one observer (PL) also scored the 3 intestinal layers
individually (mucosa, submucosa and the muscularis externa & serosa) for TM. This was not done for EPCR as expression in the mucosa was limited.

*Lung*: The lung sections for TM and EPCR were assessed by one observer (PL).

### 2.6.2.2. P-selectin in the intestine and lungs.

Sections from the 7 sham animals were unblinded and served as standards against which the intensity of staining in the remainder of the sections was compared and scored. P-selectin staining was graded using a simpler, 3-point scale as described in chapter 2. P-selectin is never absent therefore a 4-point scale was not required.

*Intestine*: Two observers (PL and HU) remained blinded to the group assignments of the remaining sections and gave an overall or crude score for P-selectin expression in the intestine.

*Lung*: Scored by a single observer (PL)

### 2.6.2.3. Fibrin deposition in the intestine and lungs

The extent of fibrin deposition in the lungs and intestine was assessed by scoring the staining intensity on a four-point scale as described in chapter 2. The scale had to include a grade of no fibrin or absent deposition; therefore a 4th grade was required.

*Intestine*: All sections remained blinded to 2 observers (PL and HU). Each animal had two FFPE sections assessed (Gut 1 and Gut 4) and these scores were averaged for each observer to give a single score for each animal per observer.

One observer (PL) also graded the extent of intravascular fibrin deposition.
Lungs: The lungs were assessed by a single observer (PL).

2.6.3. Statistical analyses

The scores were considered continuous variables because directional changes in scale represented directional changes in expression.

The medians of the experimental groups were compared with Kruskal-Wallis test with Dunn’s multiple comparisons post hoc test to compare all groups.

The correlation between injury severity and MPO activity and the endothelial expression of TM, EPCR and P-selectin was calculated giving the Pearson r statistic.

2.7. Markers of thrombin generation and fibrinolysis measured by Enzyme Linked Immunosorbent Assay (ELISA)

We measured the TAT and D-Dimer levels in the animal model after optimisation of commercially available kits with human anti-TAT or anti-D-Dimer antibodies. As previously mentioned, there is evidence of sufficient homology between the species for specific antigen-antibody interactions to occur and therefore ELISAs based on human antibodies should perform.\(^1,2,13\).

2.7.1. Generation of rat standards.

In order to interpret the assay results, it was necessary to generate rat standards, as it could not be assumed that rat TAT or D-Dimers would behave in the same way as their human forms in the assay. This approach supports the principle that ‘like’ should be compared to, or in this case derived from, ‘like’. We sought advice from Prof. Ian Mackie
(UCL Haemostasis laboratory) and Dade Behring. They advised against using commercial rat plasma as the strain of rat would not be known and the handling of the plasma may alter the TAT content.

We collected free flowing blood samples from two rats. One sample (plasma) was collected into 3.8% citrate and the other (serum) was collected into a gel tube and allowed to clot fully. The samples were centrifuged at 2000rpm for 20 minutes and the plasma or serum removed. Hirudin (10u/mL) was added to the serum sample to ensure complete inhibition of any residual thrombin or thrombin generating activity, preventing any potential ex vivo change in TAT and D-Dimer levels.

The serum-hirudin sample was assumed to have 100% TAT and the plasma 0% TAT. Serial dilutions of the serum sample with plasma created the rat standards for the TAT and D-Dimer ELISA’s.

**2.7.2. Thrombin-Anti-thrombin Complexes (TAT)**

TAT levels were determined using the Enzygnost®TAT micro immunoassay (Dade Behring). The test principles were described in chapter 2.

The highest concentration of rat standard was assigned the value of 60 as per the human standard.

Rat standards, quality controls and test samples were run in duplicate, provided test sample volumes allowed. Substrate blanks allowed subtraction of non-specific background. The Multiscan EX multiplate reader by Thermo Scientific® was used with Ascent and Masterplex software to process the data and produce the standard curves.
Figure 5.1: Standard curve for TAT complexes using Enzygnost® TAT micro ELISA, with rat standards (green triangles).

2.7.3. D-dimer

D-dimers were estimated using the human D-dimer immunoassay, Asserachrom® D-Di (Diagnostica Stago). The test principles were described in general methods.

Rat standards, quality controls and test samples were run in duplicate if test volumes allowed. Blanks were used to enable subtraction of non-specific background.

The manufacturer’s instructions were optimised for use with rat plasma by reducing the sample dilution (1:2 rather than 1:21).

Results are reported as arbitrary values of Fibrinogen Equivalent Units.
2.7.4. Statistical analysis:

The medians of the experimental groups were compared with Kruskal-Wallis test and Dunns multiple comparisons posthoc test to compare the groups.

Figure 5.2: Standard curve for D-Dimer ELISA using rat plasma standards (green triangles).
3. Results.

3.1. Histological assessment of intestinal injury

3.1.1. Sham group:

There was minimal injury on microscopic examination of the intestine from animals in the sham group, although two animals had evidence of mild damage to the mucosa. The mean Park-Chiu score was 1.1 (±0.23 SEM) with some subvillous space seen at the villous tips. The range of injury in the group was zero (normal) to 4 (denuded villi and dilated capillaries).

Figure 5.3: Intestinal IR injury in sham animals: representative fields of intestinal sections (H&E stain). Panels A and B show mild injury to the villous tips with a small amount of epithelial lifting (score 1) while the intestine in panel C shows no injury. Panel D is a higher magnification of the mucosa showing some epithelial lifting at the villous tips (score 1). (Panel A-C x 100 and Panel C x 200).
3.1.2. Ischaemia reperfusion groups

**Saline groups:**

The mean injury scores of the Pre-R and Post-R saline IR groups were significantly higher than the sham group (Pre-R: 5.4 ±0.22 SEM and Post-R: 5.5 ±0.20 SEM vs. Sham: 1.1 ±0.23 SEM; p <0.001) and they were not different to each other (figure 5.4). The mean score corresponded histologically with a moderate to severe injury of the mucosa. The range in Park –Chiu score was 4–7 and indicated, as a minimum, massive lifting of the mucosa exposing the lamina propria with denudation of the villi and, at worst, corresponded to transmucosal infarction (figures 5.5 and 5.6)

In general terms, some of the commoner histological features of necrotizing enterocolitis were not often reproduced in the IR injury. In particular, pneumatosis intestinalis was not seen, the submucosa appeared less oedematous and fewer blood vessels in the submucosa were thrombosed.

**Drug intervention groups:**

Treatment with APC after reperfusion (Post-R) resulted in a significantly lower mean Park-Chiu score when compared to animals treated with saline (Post-R APC 3.9 ±0.28 SEM vs. 5.5 ±0.2 SEM; p < 0.01), figure 5.4.

There was no difference in the mean score with the administration of APC before reperfusion (Pre-R APC 4.8 ± 0.35 SEM) or with the administration of PC either before (Pre-R PC 4.5 ± 0.24 SEM) or after reperfusion (Post-R 5.4 ± SEM) when compared to the respective saline treated groups
Figure 5.4: Scatter plot of the mean Park-Chiu Scores of the experimental groups. (means ± SEM are represented). The means of the group were different on 1-way ANOVA; p<0.0001. Individual group mean differences on testing with Bonferroni’s correction for multiple comparisons are displayed on the chart. Pre-R—intervention pre-reperfusion groups, Post-R—intervention after reperfusion groups.
In general terms, the intestinal injury severity in the experimental groups was heterogeneous and varied in intensity even within the length of individual sections. Severe affected areas were multifocal, interspersed with less affected areas.

Each animal had two sections of intestine, 4cm apart, assessed by each observer; the mean difference in injury scores between the two sections from the same animal was 1.0 (±0.08 SEM); but ranged from zero to as many as 4 points. This ‘within animal’ score difference was not significantly different between experimental groups, however a wider range was seen in the IR groups (0-4) than in the sham group (0-2). The “within animal” variation highlighted the multifocal distribution of injury severity; which mimics the injury seen in human intestine affected by NEC.

The mean inter-observer difference of the Park-Chiu score was less than a point (0.8; ±0.07 SEM) with a range of 0 – 2 points.
Figure 5.5: Intestinal IR injury in the Pre-R Saline group: representative fields (H&E stain, x100). The IR injury shows destruction of the villi, crypt layer injury and transmucosal infarction. Mean Park-Chiu score was 5.4 (±0.22 SEM) vs. sham 1.1 (±0.23 SEM; p<0.001).

Figure 5.6: Intestinal IR injury in the Post-R saline group (H&E stain, x100). The IR injury includes denuded villi, dilated vessels, and crypt layer injury and transmucosal infarction. Mean Park-Chiu score was 5.5 (±0.2 SEM) vs. sham 1.1 (±0.23 SEM; <0.001).
Figure 5.7: Intestinal IR injury in representative fields of the Pre-R APC group (H&E stain; A-C x100 and D x 200). Panels show destruction of the villous tips, dilated and thrombosed capillaries (2 examples indicated by arrows) and lifting of the epithelium down the sides of the villi but crypt injury and transmucosal infarction is reduced. The mean Park-Chiu score for the group was 4.8 ±0.35 vs. sham: 1.1 (±0.23; p<0.001); and vs. Pre-R saline 5.4 (±0.22 SEM; p>0.05).
Figure 5.8: Intestinal IR injury in the Post-APC group (H&E stain, A-D x 100). Panels A-C show mild to moderate injury; moderate lifting of the epithelium, dilated capillaries with loss of the villous tips. Panel D however shows more extensive injury as for the saline group with destruction of villi and injury extending to the crypt layer. The mean Park-Chiu score for the Post-APC group was 3.9 (±0.28 SEM) vs. Sham 1.1 (±0.23 SEM; p<0.001) and vs. Post-R saline 5.5 (±0.2 SEM; p<0.001).
Figure 5.9: Intestinal IR injury in the representative fields of the Pre-R PC group (H&E stain. X100). Panels A and B show moderate injury with denudation of the villi and destruction of the distal villi. Panels C and D show extension of the injury into the crypt layer. The mean Park-Chiu score in the Pre-PC group was 4.5 (±0.24SEM) vs. sham 1.1 (±0.23 SEM; p<0.001) and vs. Pre-R saline 5.4 (±0.22 SEM; p>0.05)
Figure 5.10: Intestinal IR injury in representative fields of the Post-R PC group (H&E stain x100). Panel A shows minimal injury, while panel B-D show more extensive injury with destruction of the villi and the injury extending down towards the crypt layer. The mean Park-Chiu score for the Post-R PC group was 5.4 (±0.29 SEM) vs, sham 1.1 (±0.23 SEM; p<0.001) and vs. Post-R saline: 5.5 (±0.2 SEM; p>0.05).
3.2. Immunohistochemistry

I was unable to refine a protocol for TM, EPCR or P-selectin immunohistochemistry on FFPE sections that allowed satisfactory staining; it was not possible to reduce the level of non-specific, background staining while retaining discernable specific staining. Three anti-TM (anti-human, anti-mouse, anti-rat) and three anti-EPCR (anti-mouse, anti-human) antibodies were trialled in a series of tests with multiple adjustments including: primary antibody concentration, length (30 minutes- 10 hours) and temperature of primary antibody incubation (-4°C - 37°C), antigen retrieval (HIER and PIER), serum block length of incubation and concentration and the addition of serum as primary antibody diluent.

We used confocal microscopy with immunofluorescence to confirm TM and EPCR expression on the endothelium prior to the extensive immunohistochemistry protocol adjustments described above. In separate experiments, the primary antibodies were incubated on the specimen for 2 hours at room temperature (goat anti-mouse TM [5mcg/mL] and goat anti-mouse EPCR [10mcg/mL]). The sections were then incubated with biotinylated rabbit anti-goat secondary antibody and finally incubated with fluorescein isothiocyanate (FITC) and streptavidin in a darkened chamber for 30min. Staining confirmed the presence of endothelial TM and EPCR. EPCR was also present on the epithelium (figure 5.11).

The difficulties with the FFPE sections may have been caused the sections being fixed for too long in buffered formalin; most sections were fixed for 24 hours, but some were longer.
Protocols were developed for fresh frozen sections as described in chapter 2. The disadvantages of fresh frozen sections include the relative lack of clear definition on microscopy and fragility of the tissue.

**Figure 5.11:** Confocal microscopy images of TM (A) and EPCR (B) endothelial expression in the ileum of sham animals. TM and EPCR appear green (FITC).

The negative controls (PBS and species appropriate Immunoglobulin replacing the primary antibody) were negative for all the immunohistochemistry results reported below.
3.2.1. Immunohistochemistry of the intestine: TM

**Sham group:**

TM was strongly expressed by the endothelium of capillaries, arteries, and veins of all calibre in the serosa, muscularis externa and submucosa of the ileum. In the mucosa, the capillaries of the villi stained strongly for TM but not in the distal third of their length (figure 5.12).

**Ischaemia reperfusion experimental groups:**

In general terms, endothelial expression of TM was reduced or lost in areas of severe inflammation, necrosis and tissue destruction, but the expression remained in areas of mild-moderate inflammation (figure 5.13).

The overall or crude TM staining intensity in all experimental groups was negatively correlated with the severity of injury (Pearson $r=-0.38$, 95% CI -0.52 - -0.21; $p<0.0001$); the relationship was more marked with the mucosal TM expression (Pearson $r=-0.45$, 95% CI -0.58 - -0.26; $p<0.0001$, figure 5.14).

In all experimental groups, the changes in TM expression were most marked in the mucosa as this tissue layer was the most affected by the IR injury.

In two specimens, one in the saline Pre-R and the other in the APC Post-R group, the expression of TM seemed stronger than that found in the sham group.
Chapter 5: PC and APC supplementation in an animal model of intestinal IR injury

Figure 5.12: TM expression in the intestine in the sham group (TM appears brown). Panels 1-3 (x100) show that TM is expressed in all layers of the intestine and extends into the mucosa for approximately half to two-thirds of the length of the villi. Panels 4-6 (x200) demonstrate these findings at higher magnification. Panels 7-9 (x200) demonstrate TM expression in the mucosa. Panel 8 is in cross section through the lower mucosa close to the crypt layer.
Chapter 5: PC and APC supplementation in an animal model of intestinal IR injury
Figure 5.13. Intestinal TM expression in the IR experimental groups (TM appears brown).
Panels 1-6 (Pre-R saline) and Panels 13-16 (Post-R saline) represent the positive controls. TM remains strongly expressed in the areas of milder inflammation (panels 6 and 13), however remains evident in more inflamed areas (3, 4, 5, 11, 12) until it is lost in necrotic areas (panel 2). TM expression in the PC or APC interventional groups was not different to the sham or saline groups, but there was a trend towards preservation. (Panels 1-3, 7-12 x100, 4-6, 13-19 x200)
**Figure 5.14:** Correlation between intestinal mucosal TM expression and the Park-Chiu severity of injury score.

*Saline groups:*

The median score for TM staining intensity was reduced in the mucosa of both saline groups as compared to the sham group; Pre-R saline 1.0 (1-3.25 IQR) and Post-R saline 2.0 (1-2 IQR) vs. 4.0 in the sham group; p=0.01. The TM staining in the submucosa, muscularis externa and serosa was not reduced compared to the sham group. The crude, overall mean TM expression in the intestine continued to reflect the changes in the mucosa: Pre-R saline 2.0 (1-3.5 IQR), Post-R saline 2 (2-2 IQR) vs. sham 4, p=0.012 (figure 5.15).

*Drug intervention groups*

All the drug therapy groups trended towards preservation of TM staining. In the APC groups, the reduction in TM expression was mainly seen only in the mucosa. The PC Post-R showed a trend towards preservation of TM staining but this was not evident in the PC Pre-R group (figure 5.15).
Figure 5.15:
Scatterplot of TM expression in the intestine according to experimental groups (medians ± IQR). Differences between group medians on Dunn’s multiple comparison tests are displayed on the chart. Panel A depicts TM expression in the mucosa. The medians were different on Kruskal-Wallis test, \( p<0.0001 \). Panel B depicts overall or crude TM expression in all the intestinal layers. Group medians were different, Kruskal-Wallis: \( p<0.0001 \).

There was concordance between the observers with a mean interobserver difference of 0.18 points (±0.064SEM).
3.2.2. Immunohistochemistry of the intestine: EPCR

Sham group

EPCR was distributed and expressed strongly the endothelium of arteries and veins in the serosa, muscularis externa and particularly in the vascular plexus in the submucosa. There was expression by the endothelium in the proximal part of the villous capillaries. The extension into the villous capillary network is different to that seen in human intestine. EPCR expression was also noted in the epithelial cells (figure 5.16).

Ischaemia reperfusion experimental groups

In general terms, EPCR expression was reduced in areas of tissue damage and necrosis, but preserved in areas of milder inflammation.

The EPCR staining intensity was negatively correlated with the Park-Chiu intestinal injury score (Pearson $r=-0.67; p<0.0001$).

![Figure 5.17: Correlation between intestinal endothelial EPCR expression and Park-Chiu score of severity of injury.](image)
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1. Sham
2. Pre-R Saline
3. Pre-R APC
4. Pre-R PC

5. 1
6. 2
7. 3
8. 4
9. 5
10. 6
11. 7
12. 8

Sham
Pre-R Saline
Pre-R APC
Pre-R PC
Figure 5.16: EPCR expression in the intestine of the sham and IR groups (EPCR appears brown). In the sham group (panels 1-3) EPCR expression was strong in the submucosa and was expressed by some mucosa capillaries. In the saline groups (panels 4-6 and 13-15) EPCR was weakly expressed in areas of severe inflammation (13) but was expressed in areas of milder inflammation (5, 6, 14, 15). A similar pattern was seen in the PC and APC interventional groups with reduction of expression in severe inflammation (panels 9, 12, 18 and 21). Some sections stained very strongly for EPCR suggesting an up-regulation or overexpression (panel 17).
**Saline Groups:**

The median scores for EPCR expression in the saline groups were lower than the sham group (Pre-R 2 (1-2.25 IQR) and Post R 2 (2-2 IQR) vs. sham 4; p<0.0001).

**Drug intervention groups.**

The medians of the groups were not different to the saline groups; and all except for Post-R APC were different to the sham group. (Pre-R PC: 3(2-3 IQR) and Post-R PC: 3 (2-3 IQR) and Pre-R APC: 2.5 (2-3 IQR) and Post-R APC 3(3-3 IQR) vs. Sham 4; p<0.0001).

The mean interobserver difference was low at less than a point difference; 034 points (±0.081 SEM).

![Figure 5.18: Scatter plot of EPCR expression in the intestine according to experimental group (medians ± IQR). The dashed line indicates the median for the sham group. Medians were different in the groups; Kruskal-Wallis p<0.0001. Significant differences on Dunn’s multiple comparison test are displayed on the chart.](image)
3.2.3. Immunohistochemistry of the intestine: fibrin

In general, fibrin deposition can be found intravascularly, or extravascularly in damaged tissues. Intravascular fibrin deposition varied from fibrin strands to occlusive thrombi.

The intravascular and overall fibrin deposition correlated with the severity of injury score (intravascular fibrin Pearson r =0.37 (95% CI 0.07-0.61); p=0.015 and overall fibrin deposition Pearson r =0.49 (95% CI 0.31-0.64) p<0.0001).

Sham animals

The sham animals had little fibrin detectable: either within the vasculature or the tissues (figure 5.19).

IR Experimental groups:

Saline groups:

The damaged, necrotic tissue of the mucosa stained strongly for fibrin, which was different to the sham group (Pre-R saline 3 (2-4 IQR) and Post-R saline 3 (3-4 IQR) vs. Sham 1; p<0.0001). Vessels in all the layers of the intestine had fibrin deposits varying from intravascular fibrin strands to more organised thrombi. Dilated vessels with thrombi were uncommon in the submucosa and muscularis externa but frequently seen in the serosa.

Drug intervention groups:

The damaged and necrotic tissue of the mucosa stained strongly for fibrin in all the drug intervention groups; the medians were not different to sham or saline sections. (Pre-R APC: 2 (2-4 IQR), Pre-R PC 2 (1.25-3 IQR) and Post-R APC: 3 (3-4), Post-R PC: 3(2.25-4) vs. sham)
The pattern and extent of fibrin deposition within the vessels was similar to the saline groups, but with a non-significant reduction particularly in the Pre-R group.

The mean interobserver difference was 0.69 points (±0.1 SEM) with a maximum difference of 2 points.

**Figure 5.20** Scatterplots of fibrin deposition in the intestine according to experimental groups (medians ± IQR). The dashed line denotes the median of the sham group.

Panel A depicts crude fibrin deposition in the intestine. Kruskal-Wallis test p<0.0001. The chart displays significant differences on Dunn’s multiple comparison test.

Panel B depicts the extent of intravascular fibrin deposition in the intestine according to experimental groups. Kruskal-Wallis test, p = 0.007; differences in medians on Dunn’s multiple comparison test are displayed on the chart.
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Pre-R APC

Pre-R Saline

Sham

Pre-R Saline

Pre-R APC

Pre-R PC

Pre-R APC

Pre-R Saline

Pre-R PC
Figure 5.19. Fibrin deposition in intestinal IR injury according to experimental groups. Representative stained for fibrin. Deposits of fibrin appear brown. Sham panels (1-3) show little deposition of fibrin, even on close examination of vessels in the mucosa (1) and submucosa (2-3). Drug and saline intervention groups show fibrin deposition as a luminal pseudomembrane near necrotic mucosa (4, 12, 14, 15) and as extravascular deposits in inflamed areas (10, 11, 21). Intravascular deposits of fibrin are seen in the serosa (5, 17) and submucosa and mucosa (6, 7, 8, 9, 10, 13, 16, 18, 19, 20) and the extent of fibrin deposition can vary from thrombus occluding the vessel, to a few strands of visible fibrin. Many of the vessels with intravascular deposits have an inflammatory cell infiltrate (5, 6, 8, 16, 18, 20).
3.2.4. Immunohistochemistry of the intestine: P-selectin

**Sham Group:**

In the sham group, P-selectin was expressed in a few vessels in the mucosa throughout the intestinal sections (figure 5.21).

**IR Experimental groups.**

P-selectin expression was significantly correlated to the histology injury score; Pearson r = 0.66 (95% CI 0.53-0.75), p<0.0001.

**Saline groups:**

The expression of P-selectin by the endothelium of intestinal vasculature was significantly increased in both saline IR groups compared to sham (Pre-R saline group: 3.0 (2.0-3.0 IQR) and Post-R saline: 3.0 (3.0-3.0 IQR); vs. sham 1.0; p<0.0001).

**Drug intervention groups:**

P-selectin expression was increased in all the drug intervention groups; Pre-R APC 2.5 (1.25-3.0 IQR) and Post-R APC 2.0 (1.25-3.0 IQR) vs. sham 1.0; p<0.05 and Post –R PC 2.0 (2.0-3.0 IQR) vs. sham 1.0; p<0.001. The Pre-R PC group was not different to the sham group.

The mean interobserver difference was low; 0.26 points (±0.075 SEM) with a maximum difference of 1 point.
Figure 5.21. P-selectin expression in the intestine of IR injury according to experimental group. Representative fields chosen (P-selectin appears brown). As the median scores for the groups were not different, each labelled panel of 3 photographs (excluding the sham group), include representations from the Pre-R and Post-R groups (x100). P-selectin was expressed by the endothelium in sham animals (top panel) but this was increased in the IR groups. The bottom panel depicts the affiliation between endothelial P-selectin expression and inflammatory cell rolling and adhesion to the endothelial wall.
3.2.5. Immunohistochemistry of the lung: TM

The expression of TM in the lungs correlated negatively with the severity of injury grading by the Park-Chiu score (Pearson r = -0.45, 95% CI -0.58 - -0.29; p<0.0001)

**Sham group:**

The endothelium of all vessels stained strongly for TM (figure 5.23).

**IR experimental groups:**

The median TM staining in all groups was reduced compared to the sham group on Kruskal-Wallis test (P= 0.01); however the individual medians were not different on Dunn’s multiple comparison test (figure 5.24).
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**Saline groups:**

The median TM staining in both saline groups was reduced as compared to sham. There were no sections with weak or absent staining. (Pre-R saline 3 (3-4 IQR) and Post-R 4(3-4 IQR) vs. sham 4; p=0.01).

**Drug intervention groups:**

There was reduced TM staining intensity; however the median intensity remained strong to moderately strong (Pre-R APC 4 (2.5-4 IQR), Pre-R PC 3 (2-3.5 IQR), Post-R APC 3 (2-3.3 IQR) and Post-R PC 3 (2.3-3 IQR) vs. sham 4).

**Figure 5.24:** Scatter plot of TM expression in the lung by experimental groups (medians ± IQR). The medians of the groups are different; Kruskal-Wallis test p=0.011.
**Figure 5.23**: TM expression in the lung in IIR. Representative fields of TM expression chosen (x400). TM appears brown. TM expression in the sham animals was strong in the lung parenchyma; the fields depict the terminal respiratory tree, alveoli and terminal bronchioles. TM is lost in some areas in the interventional groups as depicted by the photographs on the right, however remained strong in other areas, depicted on the left.
3.2.6. Immunohistochemistry of the lung: EPCR

Sham Group:

EPCR was not expressed by the pulmonary capillaries but was expressed on larger vessels (figure 5.25). This distribution is similar to that seen in adult humans\textsuperscript{28}. 

Experimental groups:

EPCR remained strongly expressed in the all the experimental groups in the areas of usual distribution, with no significant difference to the sham group (p=0.38). There was increased expression in the larger vessels in a few animals in all of the IR groups.

There was no correlation with severity of injury (p=0.11)

\textbf{Figure 5.26: Scatter plot of EPCR expression in the lungs by experimental group (medians ± IQR). The medians were not different. Kruskal-Wallis test p=0.38.}
Figure 5.25: EPCR expression in the lung, representative fields (EPCR appears brown). EPCR is not expressed by the microvasculature of the lung in sham animals, but is expressed by the larger vessels, similar to the pattern in adult humans\textsuperscript{28}. The expression remained strong in the drug intervention groups.
3.2.7. Immunohistochemistry of the lung: fibrin deposition

**Sham group:**

Some fibrin deposition was present in larger vessels and the capillaries of the alveoli in the sham animals (figure 5.27).

**Experimental groups:**

The deposition of fibrin was not different in the intervention groups to the sham group (p=0.55).

![Figure 5.28: Scatterplot of fibrin deposition in the lungs (medians ± IQR). The median of the sham group is depicted by the dashed line. The medians of the groups were not different; Kruskal-Wallis test p=0.55.](image)
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Figure 5.27: Fibrin deposition in the lung in IIR according to experimental group (fibrin appears brown). Fibrin deposition varied in all of the intervention groups from areas with little deposition to areas with significant deposition in the alveolar microvasculature.
3.2.8. Immunohistochemistry of the lung: P-selectin

**Sham group:**

P-selectin was expressed in a few vessels in the sham animals. The unblinded sections were scored 1.0 and served as standards for scoring.

**IR Experimental groups:**

**Saline Groups:**

The saline groups showed an increase in P-selectin expression in the lung as compared to the sham group; Pre-R saline: 2.0 (1.875 – 2.625 IQR); p<0.05 and Post-R saline: 2.5 (2.0–2.5 IQR) vs. sham 1.0; p <0.01.

**Drug intervention groups:**

The medians of the PC and APC groups were not different to the sham or saline groups. The PC groups trended towards a reduction in P-Selectin expression in the lung.

![Figure 5.29: Scatterplot of P-selectin expression in the lung according to experimental groups (medians ± IQR). The medians of the group are different, Kruskal-Wallis p=0.0017. Differences revealed by Dunn’s multiple comparison test are displayed on the chart.](image)
3.3. Markers of thrombin generation and fibrinolysis

Four samples were excluded from the TAT and D-Dimer analysis as they contained clots on defrosting (1x IR saline pre, 1x IR saline post and 2x IR APC post).

The levels of TAT and D-Dimers in these samples were very high and could be regarded as positive controls for the assays.

3.3.1. Thrombin-antithrombin complexes (TAT)

The TAT levels of the sham group appeared high; a second analysis excluding the two animals with mild mucosal damage and higher injury scores did not affect the results (data not shown).

The median TAT levels of the experimental groups did not differ significantly from the sham group or each other.

![Figure 5.30: Scatter plot of TAT levels by experimental group (medians ± IQR) shown. The medians were not different. Kruskal-Wallis test p=0.31.](image-url)
We contrasted the portal and systemic levels of TAT in two animals to determine if there was a reduction in levels of TAT caused by dilution into the systemic circulation. The TAT levels in the portal venous samples were higher than the systemic venous samples ratios 1.6:1 and 2.6:1).

Validity: The intra-assay co-efficient of variation (CV) was within acceptable limits; less than 8%. Quality control measurements fell within the confidence interval for the assay.

3.3.2. D-dimer ELISA

The D-Dimer levels were very low, requiring reduced sample dilution to optimise the assay.

The median D-Dimer level of the sham group was high compared to the experimental groups; the results were not altered by exclusion of the two animals with mild mucosal damage (data not shown).

The median D-Dimer levels of the experimental groups did not differ from the sham group or each other.

![Figure 5.31: Scatter plot of D-Dimer levels according to experimental group (median ± IQR). The Fibrinogen Equivalent Units (FEU) score represents the concentration of D-Dimers in arbitrary units. There was no difference in the medians; Kruskal-Wallis test p=0.46.](image)
The D-Dimer FEU score was greater in the portal blood samples than the systemic samples in two animals sampled (ratio 1.5:1 and 1.2:1).

**Validity:** The intra-assay co-efficient of variation (CV) was <13%. The quality control measurements fell within the interval for the assay.

### 3.3.3. Myeloperoxidase activity in the intestine

Intestinal MPO activity in the intestine was correlated to the severity of injury; Pearson r=0.58, p<0.0001. The levels were low in animals with little histological evidence of mucosal injury (Park-Chiu score ≤ 3) and increased as the injury severity increased.

Intestinal MPO activity was increased in all the experimental groups compared to the sham animals.

**Figure 5.32:** Scatter plot of intestinal MPO activity per experimental group (median ±IQR). The medians were different on Kruskal-Wallis test; p=0.0008. Group medians different on Dunn's multiple comparison test, are depicted on the chart.

Intestinal MPO activity correlated with the expression of P-selectin (Pearson r =0.86; p=0.01) and there was a negative correlation with overall or crude TM and EPCR expression (TM Pearson r = -0.33, p=0.004 and EPCR Pearson r = -0.31, p=0.009).
4. Discussion

4.1. Overview

This study reports new information on the PC activation pathway in the context of intestinal IR.

Firstly, we characterised TM and EPCR expression in intestinal IR injury; expression was reduced at 2 hours following reperfusion in the intestine, and TM expression was also reduced remotely (lungs) but EPCR expression was preserved in the lungs. These changes were accompanied by increased fibrin deposition in the intestinal microcirculation, increased expression of P-selectin both locally and remotely, which facilitated neutrophil recruitment to the tissue as evidenced by increased MPO activity; and lastly, reduction in TM and EPCR expression was associated with increased severity of the histological intestinal injury.

Secondly we examined the effect of APC and PC supplementation at two time points on the severity of the intestinal injury, and we examined the effects on markers of thrombin generation, fibrinolysis and leucocyte infiltration as these may be mechanisms by which APC and PC may exert influence. PC supplementation has not previously been reported in IR models, while APC has been used in a number of IR models with reported benefit\textsuperscript{12}. In our model, APC administered after reperfusion significantly reduced the histological injury, but APC-Pre-R and PC did not. Neither supplements showed an improvement in the other parameters measured as compared to the positive controls; however some trended towards improvement and were also not different to the sham groups. The trends were not consistent in any group. The evidence from this model to support APC or PC supplementation in clinical intestinal conditions characterised by ischaemia-reperfusion is limited, but should not be discounted.
4.2. The PC pathway in the context of Intestinal IR

Two other groups have investigated the PC pathway in the context of intestinal IR. And, where possible I have compared our results\textsuperscript{1,2,7}. Their models were slightly different; both administered the intervention drugs prior to reperfusion (15min) but after SMA occlusion, simulating very early treatment. Our model boosted the PC and APC level shortly before clip occlusion and 5 min after clip removal to simulate prophylaxis and treatment after the injury had begun. Both models delivered the drugs or placebo in small volumes (1-2mL/kg) while we gave 10mL/kg volume bolus. Both models had longer reperfusion times at 3 hours. Lastly, Schoots et al had shorter SMA ischaemic times (20 and 40 min) and measured TAT and D-Dimers in portal rather than systemic plasma.

In our model, the sham animals had minimal injury to the intestine on histological investigation. The positive controls had severe intestinal reperfusion injury that was different to the sham group; demonstrating the controls of the model had achieved the required standard for the primary outcome comparison.

In the sham group, TM was abundantly expressed throughout the microcirculation in the intestine and lungs. EPCR was expressed in the in larger vessels in the lungs and not the capillaries. In the intestine, EPCR was expressed mostly on the bigger vessels and less frequently on capillaries. This distribution is similar to adult humans\textsuperscript{8,28}. EPCR expression was strong in these sites.

In our model, reperfusion rapidly produced a severe intestinal injury at 2 hours in the saline groups that was different to the sham group. Histologically, this corresponded on average to disruption of the lamina propria and crypt layer, but was worse in some animals extending to transmucosal necrosis. The injury severity in our model was greater than the intestinal IR models in the literature. The upper range values of the saline groups in Schoots
study (Park-Chiu score of 5) are below our median injury severity (Park-Chiu 5.4 and 5.5); this could be explained by our longer ischaemic time. Our saline group median injury severity was also greater than the saline group range reported in the studies by Teke et al; however they cannot be directly compared as Teke used only the mucosal Chiu score with an upper score of 5, rather than the Park-Chiu score that incorporates crypt layer injury, transmucosal and transmural infarction, an upper score of 8.

The bolus of volume in our model, that may have boosted perfusion, did not prevent severe injury as compared to studies in which no bolus was given.

TM and EPCR expression has not been previously reported in the context of IR in any organ system of an animal model. We showed that the IR injury at 2 hours in the saline groups was associated with a reduction in the expression of endothelial TM and EPCR in the intestine, TM expression in the lungs but EPCR expression was preserved in the lungs. The reduction in expression was associated with increased severity of injury on histological examination. Nevertheless, TM and EPCR expression was weak or absent only in necrotic or severely inflamed areas.

The effect of reduced TM and EPCR expression would be to reduce the capacity to activate PC to APC locally in the intestine, causing a shift in the balance towards thrombin generation and inflammation.

Increased thrombin generation in the intestine was evident in the saline groups as they had significantly greater deposition of fibrin, both intra- and extravascularly, than the sham group. Intravascular fibrin deposition would decrease the perfusion of the intestinal microcirculation. These findings were similar to Schoots et al\textsuperscript{1,6}.\n
The large vascular bed in the lungs continued to express EPCR strongly in its usual distribution at 2 hours, but there was a reduction in TM expression. The reduction in median lung TM expression (Pre-R Saline 3 (3-4 IQR) and 4 (3-4 IQR) vs. Sham 4) was less than had been seen in the intestine (Pre-R Saline 1.8 (1-3.3 IQR) and Post-R saline 2 (202 IQR) vs. Sham 4).

It is possible that systemic APC levels might be only mildly reduced at this level of TM and EPCR expression; however poor intestinal perfusion of the microcirculation due to fibrin deposition could reduce delivery to affected areas. There was no significant increase of fibrin deposition in the lungs in the saline groups.

The saline groups demonstrated increased P-selectin expression in both the intestine and the lungs as compared to the sham group. P selectin expression facilitates leucocyte-endothelial adhesion and migration into the tissues. The intestinal MPO activity, which reflects neutrophil accumulation in the tissue, was significantly increased in both saline groups as compared to sham. These are similar results to Teke et al\(^2\).

The systemic levels of TAT and D-Dimers are difficult to interpret in this study for reasons that are discussed later; however there were no differences in the median TAT and D-Dimer levels in the systemic circulation between the sham, saline or interventional drug groups.

The portal samples from two IR animals showed that levels of both parameters were increased in the portal circulation as compared to the systemic levels. This is similar to the results reported by Schoots et al where portal and systemic levels of TAT and D-Dimers were significantly different to each other and to the sham group\(^1\). This indicates that both thrombin generation and fibrinolysis were active in the intestinal vascular bed in these animals, but that the markers became diluted when they spilled over into the systemic circulation in the early phase of IR injury.
The association of the increased severity of the injury on histology, increased thrombin generation, fibrin deposition and inflammation coupled with the reduced ability to activate PC locally in the intestine, suggests a role for the PC activation pathway in the pathogenesis of the IR intestinal injury.

4.3. PC and APC intervention groups in the context of IR injury.

We found that APC supplementation after reperfusion, but not before, reduced the severity of the intestinal injury. This is in contrast to Shoots and Teke et al who reported benefit with APC administration before reperfusion\textsuperscript{1,2}. PC supplementation did not significantly reduce the intestinal injury in our model.

Interestingly, although the APC Post-R group had reduced intestinal IR injury, the group did not show preservation of TM or EPCR expression, reduced fibrin deposition, TAT or D-Dimer levels or reduced P-selectin expression or MPO activity.

The drug intervention groups showed no difference to the positive controls in the expression of TM and EPCR, fibrin deposition, TAT and D-Dimer levels, P-selectin expression or MPO activity. Some groups showed a trend towards improvement such that they could also not be distinguished from the negative controls, however the trends could be inconsistent across the groups.

There was a trend towards reduced intravascular fibrin deposition in the intestine in all the PC and APC interventional groups, however the deposition of fibrin extravascularly in the necrotic tissue was preserved. It would be biologically and clinically important that fibrin continues to be deposited where it is needed, despite PC or APC supplementation.
4.4. Limitations of this model, study and causes for the lack of effect seen.

There are a number of limitations to the work presented here, some relate to the limitations of the model that could have contributed to the failure to demonstrate significant modulating effects of APC Pre-R or PC supplementation on injury severity, or consistent effects in the reduction of thrombotic and inflammatory parameters.

Two further limitations are discussed in more detail below, but include concern that small group numbers make interpretation of results difficult and lastly that the intestinal IR injury in adult rats is not representative of NEC.

4.4.1. Species Specificity:

The anti-thrombotic effects of APC have been shown to be species specific, although the specificities of the anti-inflammatory and anti-apoptotic effects have yet to be determined. The proteolytic inactivation of Va, the interaction with co-factor PS and the rate of APC clearance by inhibitors are particularly affected by species specificity.

Fernandez et al found that the anti-coagulant activity of human APC was 10-fold lower than rat APC in rat plasma due to a reduction in proteolytic activity against rat factor Va\textsuperscript{13,29}. APC requires PS as a co-factor to inhibit factor Va; in mouse plasma, the co-factor activity of rat PS for human APC has been shown to be very weak. The half-life of human and rat APC is also significantly shorter in rat plasma due to increased levels of inhibitors; this would increase the required dose of rAPC to achieve sufficient plasma concentration.

The activation of PC by TM to APC also demonstrates species specificity, with a reduction in activation of human PC in rat plasma\textsuperscript{30}.
The species specificity of APC and PC could have resulted in the reduced anti-thrombotic effect in the drug intervention arms in the doses we selected. The doses we chose had been used with reported success, but were lower than the doses recommended by the drug companies for use in rats (Baxter Healthcare and Eli Lilly and company)\textsuperscript{1,2,22}. Potentially this problem might be overcome with increased doses of human PC and rhAPC or, alternatively, obtaining purified rat PC and APC.

Species specificity must also be considered in the performance of the coagulation assays used in animal models; both the assays we selected had been successfully used in the literature. Ravanat et al showed good species cross-reactivity with Enzygnost TAT ELISA but poor cross reactivity with ASSERACHROM D-Di; this may account for the very low levels of D-Dimers we encountered\textsuperscript{31}. This problem could be potentially overcome with development of primary antibodies that do cross react with rat D-dimers.

4.4.2. Reduced activation of PC by thrombin-TM complex.

Apart from species specificity reducing the activation of human PC in rats, we showed a reduction in local TM and EPCR expression in the IR intestinal injury; this would reduce local activation of PC to APC, potentially reducing biological effect. However, we showed that in the lung vascular bed, EPCR expression remained intact while TM expression was reduced, although less so than in the intestine. This may contribute to the maintenance of the systemic activation of PC.

The relative importance of local, as compared to systemic, activation of PC is not clinically known. However it has been shown that 4x-higher levels of exogenous rhAPC were required to produce endothelial barrier stabilising effects compared to the locally produced APC from PC activation\textsuperscript{32}.
The reduced local capacity to generate APC in the PC supplemented groups could be a factor contributing to the lack of effect seen in these groups.

4.4.3. Dose of APC and PC:

As previously mentioned, we chose the lowest doses of APC that had been used in published studies with demonstrable effect. The doses were lower than those recommended by the drug companies for rats. The PC dose (80 IU/kg) is comparable to bolus doses in humans used to boost very low levels of PC; a ten-fold higher PC dose had been lethal in our model. A study published after we had completed the work reported benefits with a 25% increase in dose (100 IU/kg). The lack of effect we saw could be due to insufficient dosing of human PC and APC to overcome the effects of species specificity on their biological activity.

The reported toxic single-dose of PC concentrate in pre-clinical safety and toxicity trials is 2000 IU/kg. We were surprised and concerned that a single dose of 800 IU/kg was lethal in our model however we were not able to investigate the cause of death in these animals except to confirm they had not died of haemorrhage, including CNS haemorrhage.

4.4.4. Timing of intervention drug administration:

The impact of drug intervention may be influenced by the timing of the drug administration in relation to the development of the reperfusion injury. Generally, published studies had administered APC a short time before reperfusion (~15 minutes). In our study we found that APC administered before clamping the SMA, and an hour before reperfusion, had no significant effect but APC administered after reperfusion did attenuate the injury. This suggests there may be a therapeutic window during which drug treatment is effective. In our model, APC administered 60 minutes prior to reperfusion may have been too early in
consideration of the shortened half-life of human APC in rats; levels may have been non-therapeutic during the injury development. PC administered before or after perfusion may not have been activated in sufficient quantity by rat TM because of the species specificity, or rapidly enough in the Post-R group, to influence injury development.

4.4.5. **Influence of the severity of the intestinal IR injury:**

The injury observed in the positive controls had a rapid onset and was severe, with disruption of the mucosa extending to transmural infarction in some cases. It is possible that the model of 60 minutes ischaemia created an injury so severe that no intervention would be likely to meaningfully attenuate the damage. This is clinically similar to fulminating NEC with intestinal gangrene; the course is so rapid and severe that few interventions are able to preserve affected bowel. Schoots et al used shorter ischaemic times (20 and 40 minutes) and showed benefit with APC supplementation. It is possible that the longer ischaemic time in our model contributed to the lack of effect we showed in the drug intervention groups.

4.4.6. **Concerns with ELISA results:**

As mentioned above, the undetectable D-Dimers (at usual sample concentration) is likely to be due to the fact of poor cross-reactivity of the assay with rat plasma. The median levels of TAT and D-Dimer (undiluted) in the sham animals were as high, or higher, than the intervention groups.

The sham group did not show any other evidence of inflammation (MPO, histology, fibrin deposition, P-selectin) therefore we assumed this was due to activation of thrombin generation during the sample collection and handling. TAT assays are known to be
particularly sensitive to collection techniques; blood has to be free flowing and should be
collected into at least 3.8% sodium citrate.

It is possible that the collection of plasma samples by cardiac puncture during the demise of
the animal may have activated thrombosis in the samples; potentially this could be
overcome by sampling from the femoral cannula prior to the death of the animal. It may
also be of benefit to explore collection into more than 3.8% citrate, as the results from
three IR samples had to be discarded due to clot formation.

4.4.7. Statistical design of the study:

Our study design had small numbers in each group and was therefore only powered to
detect changes in the primary outcome, the severity of injury score.

The study was not powered to detect changes in the secondary outcomes, risking a type II
error. Therefore all the subgroup analyses must be interpreted with caution. The lack of
effect we have shown in the may be caused by the small numbers in each group.

The small numbers were unfortunately dictated by resource restrictions.

4.4.8. Intestinal IR model as a model for NEC

The factors influencing the choice of animal model in this project were discussed earlier in
this chapter.

Animal models of NEC should aim to reproduce the intestinal injury seen histologically. All
the key elements of the injury are difficult to reliably reproduce together in animal models;
the patchy distribution, the development of pneumatosis and the coagulative necrosis.
There are no spontaneous animal models of NEC, the injury has to be induced by
interventions⁴⁴-⁴⁶. Models have been developed in rats (adult and pups), piglets, rabbits, sheep and, increasingly in mice, allowing investigation in knockout mice. The models usually combine intermittent hypoxia with enteral feeding (with a chow or milk that may or may not be toxic) and often add an inflammatory insult such as bacterial infection, endotoxin, cytokines (for example PAF) or cold stress. Intermittent hypoxia can be administered to the whole animal or can be achieved by interrupting the perfusion to the intestine. Successful models use preterm or term animals, but technical difficulties increase in smaller species, both due to the size of the pups, as well as the rapid maturation rate of the animals. This is particularly pertinent if the model requires the young animal to become colonised prior to the intervention given to induce injury, because the animal matures during the delay.

These models are labour - and resource-intensive to develop, and to maintain. We elected to use a model already established with our department as our resource was limited.

The adult IR model we used is a very limited model for NEC. The model concentrates exclusively on the ischaemic aspects of NEC, ignoring the inflammatory and developmental features. At the time this study was undertaken, ischaemic events were given relatively more weight in the pathogenesis of NEC paradigm, than they currently are and therefore we felt the compromise would be acceptable. The model does not reflect or account for the additional factors now believed to be central to the development of NEC: immaturity of the intestine and host immunity, bacterial colonisation and enteral feeding. It is clear that this model is sufficiently different to NEC that findings in this model should not simply be extrapolated to NEC without further work.
5. Conclusions

We have shown using this animal model that the PC pathway is altered during IR injury to the intestine; loss of TM and EPCR expression is associated with fibrin deposition and inflammation of the intestinal wall. We have shown that APC given shortly after reperfusion can ameliorate the injury, but PC does not.

There are a number of factors that may have contributed to the lack of effect in this model; and some of these will be difficult to overcome in an animal model.

We also appreciate that while the animal model provides information relevant to a number of intestinal conditions that involve an element of IR injury such as volvulus, intussusception and NEC; we recognise that IR is only one element of NEC. Therefore the application of information gained from this model to NEC, is limited. This model does not account for any developmental aspects of NEC relating to the immaturity of the intestine or the host immune response.

Improving this model with some of the measures mentioned would be unlikely to improve our ability to translate the results to the bedside of the infant with NEC.
Chapter 6

Discussion, conclusions and future work
1. Discussion

This chapter has three main aims;

Firstly, to discuss the validity of the results presented in previous chapters, highlighting the limitations in this work.

Secondly, to discuss the significance of these results in the context of current knowledge; as an estimate of the value of this work.

Lastly, I will discuss the future work that will be necessary to answer some of the questions this project has generated.

2. The validity of the results presented in this project:

2.1. Histology and Immunohistochemistry

The underlying immunohistochemistry techniques used in this project are classic, but they were adapted for use with novel combinations of epitope, species, tissue, developmental age or condition. The approach used to develop the immunostaining protocols\(^1,2\), and the use of positive and negative tissue controls with reagent and antibody controls in each experiment, improved the validity and reproducibility of the technical staining results.

However, the interpretation of immunohistochemical staining remains qualitative and this potentially introduces bias; a shortcoming of the results presented in this thesis. Automated techniques of counting cells on processed digital microscopy images were considered, but were not suitably accurate in the assessment of the epitopes we were investigating. We reduced bias with multiple techniques; observers were blinded to the origin of sections, several observers were used, observer variance was reported, standardised scoring systems were used and sections were directly compared to unblinded
controls. Similar techniques to reduce bias are used in published work, but few studies have used all the techniques together. For these reasons we feel the qualitative results presented in this thesis are accurate and reproducible.

As the principle investigator in this work, and as a clinician, the ability to visualise the injury on histology was immensely valuable to improving my understanding of the nature of NEC.

2.2. ELISAs

There are a number of shortcomings in this project in relation to the ELISAs.

Firstly, the accuracy of the TAT and PF1+2 assays were very sensitive to the blood collection method and sample handling. In the human prospective study described in chapter 4, the blood collection method in the controls was different to the NEC group. This introduced bias, making the interpretation of the results difficult.

The TAT and D-Dimer assays in the animal model had a number of problems that may have affected the accuracy of the results. Firstly, the blood collection and processing method may have induced ex vivo thrombin and TAT formation, as three plasma samples developed clots. Samples were collected into 3.8% citrate, but higher doses may be required to fully anticoagulate rat blood. Secondly, we used rat standards to generate the standard curves to overcome the influence of species specificity; this increased the accuracy of these assays but this approach had not been reported previously in the literature, and therefore affected the comparison of our results with other studies. The CVs for these assays were within the accepted range.

Lastly, we elected to have the assays on the human preterm participants performed in professional laboratories. The laboratory QC processes and GCP certification ensured the accuracy of these results; however this could be considered a shortcoming in the laboratory
work I am required to perform for this thesis. This reflects on the difficult nature of research in the premature neonatal population\textsuperscript{3-5}. Sample volumes were very small and needed to be stretched as far as possible to undertake as many tests as feasible; these were prioritised as it was anticipated that all the tests would not be possible in the smaller infants. There was no 'left-over' sample volume and there was only one chance to get accurate results from each ELISA. In discussion with my supervisors, we felt that I would learn the necessary laboratory techniques optimising and performing the ELISAs in the animal model without damaging my prospects for a higher degree and that it was preferable, and ethical, for professional laboratories to perform the ELISAs on the human samples.

2.3. The animal model

The animal model had a number of limitations that were discussed in the previous chapter. The model of adult intestinal ischaemia-reperfusion is not a model of NEC, although many features are shared, and the injury created was very severe and of rapid onset. It is questionable if any intervention could meaningfully influence this severity of injury in the short time period. The unquantifiable impact of species specificity both on the biological effect of the interventions, and our ability to measure the outcomes, made it difficult to design the study and interpret the results of the interventional arms in this model. The use of positive and negative control arms enabled direct comparison of outcomes, ensuring that the influence of species specificity on outcome measurements were consistent across the groups. The question of determining the adequate dose of PC and APC to overcome species specificity can only be answered with additional pharmacokinetic work or work comparing the outcomes of increased doses of APC and PC. Nevertheless, we discovered important findings regarding the effect of IR on the expression of TM, EPCR, P-selectin and
fibrin in the intestine and remotely in the lungs; and these findings were accurate and reproducible.

2.4. Study design

The design of the prospective study presented in this thesis can be criticised for the small number of participants involved; making secondary outcome analysis more difficult to interpret. This reflects some of the difficulties of undertaking clinical research in the preterm neonatal population; the numbers of patients with NEC admitted to the institutions involved are low in terms of research recruitment; approximately 40 cases annually. On average half of the families initially approached to participate consented to enrolment; the severe nature of the condition, the need for intensive care transfer and the anxiety of the parents were factors involved in the refusal rate. Multiple sites are needed to recruit larger numbers of patients to improve the validity of clinical trials in this population.

The study design can also be criticised for the narrow focus of the investigations. The limitations on blood sample volume made it necessary to restrict the focus to the concentrations of proteins in the PC pathway, thrombin generation and fibrinolysis. We made a pragmatic decision during the study design to use clinical data and results of routine investigations to provide information on severity of illness, MODS, and markers of inflammation.

3. The value of this work and conclusions

The results presented in this thesis represent the first exploration of the PC pathway in healthy preterm neonates, and in those with NEC.

As presented in the opening chapter, there are compelling reasons to consider the role of PC pathway in the quest to understand the pathogenesis of NEC. Many of the pathways
that are known to be involved in in the pathogenesis of NEC, are counter-regulated or mediated by the PC pathway. These pathways include the APC and PC mediated endothelial and epithelial barrier stabilisation, reduction in permeability, reduction in apoptosis and promotion of repair. APC and PC are able to dampen NFκβ and MAPK p38 activation and reduce the downstream transcription of pro-inflammatory mediators, including NO, TNFα, IL-1β, IL-6 and IL-8, all of which are raised in NEC. APC and sEPCR reduce leucocyte chemotaxis, endothelial adhesion and migration into the tissues. APC inhibits HMGB-1 and degrades histones, which are raised in NEC. The anticoagulant activity of APC and PS reduces thrombin generation and preserves the microcirculation and lastly, APC is neuroprotective.

In addition, as detailed in the opening chapter, the preterm neonate has developmental reductions in PC and PS concentrations, and therefore may be vulnerable to sustained demand on the PC pathway. The receptor kinetics of EPCR and TM dictate that PC levels largely determine the levels of APC, although the plasma concentrations of APC are only a fraction of the total, as a proportion remains membrane bound. Low levels of PC, despite normal levels of APC, will result in reduced anti-inflammatory EPCR-PAR-1 signalling because of the receptor kinetics of EPCR. This may be relevant in the setting of the preterm neonate with physiologically low PC levels, at risk for an exaggerated inflammatory host-defence response in the immature intestine to colonising bacteria.

In this project, we have shown, in agreement with earlier published work, that healthy preterm neonates have low levels of PC and PS (free and total). The levels of APC have not been reported in neonates; we have shown that levels of APC are also low in healthy preterm neonates. We have shown that TM is abundantly expressed in the intestinal microvasculature of preterm infants, and EPCR is expressed in all vessels except the smaller arterioles, venules and capillaries.
PC, free and total PS levels were reduced in preterm neonates with NEC. Levels of APC were varied, with a few infants boosting their levels 8-10x. These are similar to findings in adults in critical care with severe sepsis. In the intestine, TM and EPCR expression was decreased in areas of inflammation and dual staining for fibrin showed that intravascular fibrin deposits occurred more commonly in vessels with reduced TM expression. These findings are similar to the findings in the intestine of adults with IBD.

We concluded from these findings that the PC pathway is downregulated in NEC. We could not be certain if this was merely a consequence of the pathological exaggeration in inflammatory response in NEC causing collateral damage to the PC pathway, or if the physiologically deficient pathway contributed to the inability to counterbalance the initial inflammatory response in these infants, amplifying it. Comparison to a group of neonate with systemic inflammation from sepsis may answer this question.

We attempted to address these questions in an animal IR model. Supplementing the levels of the pathway effector molecule, APC, reduced the intestinal injury; suggesting that deficiencies in the pathway may contribute to the overall, destructive inflammatory response. These findings are in keeping with findings in other animal models of IR injury.

We therefore concluded that the PC pathway was down-regulated in NEC and that this may contribute to the pathogenesis of the condition. This raised the prospect of therapeutic modification of the disease using PC pathway supplements.

The findings of the work in this thesis cannot be considered in isolation of the large randomised controlled trials showing the lack of efficacy of supplemented rhAPC in adults and children with severe sepsis. The findings of these trials do not negate our attempts to illuminate the pathways involved in the pathogenesis of NEC, however they do ensure that we act on this information with caution.
The syndromes of severe sepsis and NEC share many features in the advanced stages, however NEC in the premature host has some additional characteristics that may be influenced by PC pathway mediation, and that are different to the population with severe sepsis. It is because of these features that therapeutic supplementation of the PC pathway should not be discounted without further investigation, despite the findings in severe sepsis.

The epithelial and endothelial stabilising effects of the PC pathway are of particular interest. These infants develop gross oedema, anasarca, that contributes to multiorgan dysfunction, which in turn worsens the capillary leak, becoming a vicious cycle. It is usual intensive care practice to use extracorporeal renal replacement therapy to minimise volume overload and reduce capillary leak in critically ill patients; however this therapy is technically not possible in preterm infants due to their size. The reduction in organ oedema has been shown in animal models\(^{10,11}\). Extracorporeal renal replacement therapy was a standard intensive care therapy employed in the large RCTs of rhAPC in severe sepsis.

Secondly the epithelial barrier stabilising and reparative effects of the PC pathway, including the prevention of apoptosis, will be relatively more important in NEC than in severe sepsis. Intestinal injury is a key element of NEC. Disruption of the healing process contributes to the injury and amplification of inflammation. The PC pathway has been shown to promote healing of the intestinal mucosa and surgical wounds\(^{1,12-14}\).

Lastly, infants with NEC suffer long-term neurological deficits that are not seen in infants with severe sepsis. APC is known to have a neuroprotective role stabilising the blood-brain-barrier, reducing inflammation in the endothelium and microglia and preventing neuronal apoptosis\(^ {2,15}\). It has been suggested that APC may have a particular role to play in the treatment of preterm brain injury\(^ {11,16-18}\).
Appendix 1. Parent leaflet study chapter 4.

It is for these reasons that modulation of the PC pathway may yet prove to be of benefit to infants with NEC, despite the lack of efficacy shown with rhAPC supplementation in adults and children with severe sepsis.

4. Future work

The further investigation of the role and potential therapeutic manipulation of the PC pathway in NEC presents a huge task. This project has shown that the PC pathway is altered in NEC, but it has not confirmed whether this is a cause of NEC or an effect of the systemic inflammation. Further work comparing NEC to other systemically inflamed groups will need to be undertaken to understand this point.

As a first step, we would like to investigate if any of the PC pathway molecules are present in the breast milk of mothers of premature infants. Substances that are present in breast milk have a physiological benefit to the newborn infant on an evolutionary basis. If PC molecules (PC, APC, sTM, sEPCR) were present in breast milk it would lend weight to the hypothesis that the pathway has a protective role in the intestine.

Secondly, PC levels are developmentally regulated for unknown reasons, however it is possible that low levels are protective in the foetus and young child. Therefore considerable ‘proof of concept’ work remains to be done in the pre-clinical setting; including investigation of the impact of APC and PC intervention on the inflammatory nature of the intestinal injury, on the barrier functions of the intestine and, particularly, on the remote injury in the lung and brain.

We hope that the contribution of the work presented here will help to fire interest in collaborative research networks to carry the work forward. The therapeutic benefits of APC
mutants will need to be investigated and established beyond their current role limited to improving our understanding of the separate functions of APC.

Prior to the completion of this thesis, I had designed a pilot study to determine the dose of PC required in preterm infants with NEC, to raise and maintain PC concentrations to near adult levels for 7 days. However this project has shown that additional pre-clinical work is required before attempting clinical studies. I have included, for interest, the Investigator brochure for PC concentrate from this study in appendix 2.
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Chapter 2


Chapter 3


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Chapter 5


References: Chapter 6

Chapter 6


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PARENT INFORMATION LEAFLET

NECROTISING ENTEROCOLITIS (NEC) AND THE ROLE PLAYED BY THE PROTEIN C PATHWAY

Your baby has been diagnosed with necrotising enterocolitis, which is an inflammatory condition of the bowel. We would like to ask your permission to include your child in our project. We emphasize that if you should decide not to participate, it will not affect your baby’s treatment in any way.

What is the purpose of this study?
Protein C is a substance made by the body. It has two important functions; namely it prevents blood clotting and it dampens inflammation. It exists in an active and non-active form.

Protein C levels have been found to be very low in critically ill adults with severe infections. A recent study showed that treatment of these adults with activated protein C improved their recovery.

Necrotising enterocolitis is severe inflammation of the bowel that occurs in babies. Many of these babies can require intensive care and even bowel surgery. NEC is often associated with blood clotting abnormalities.

There has not yet been a study to determine the levels of protein C in healthy babies or those critically ill with NEC. We want to discover what happens to the levels of activated protein C in infants with necrotising enterocolitis (NEC).

Why has my child been chosen?
The doctors caring for your child have diagnosed NEC and have begun medical treatment for it.

What will happen to my baby if I consent to take part?
If you agree to take part in this study, your baby will have an extra blood sample taken soon after diagnosis. If your baby weighs less than 1000g the sample will be 1.4mls (1/4 teaspoon) and if your baby weighs more than 1000g the sample will be slightly bigger, 2.4mls (less than 1/2 teaspoon). In addition, if your baby needs to have surgery, we would repeat the blood test, if more than a day had elapsed since the first test.

The blood sample will be taken at the same time as the routine tests. The blood will be taken from existing central or arterial lines if they are in place. Your child will have no extra needle pricks.

We shall record clinical data relevant to your child’s medical condition. This information will be stored in a secured database in a non-identifiable format. The relationship between the clinical data and the blood results will be determined.
What tests will be done?
The levels of molecules of the protein C pathway and inflammatory markers shall be measured in the blood sample. We will also determine the gene type for three particular proteins important in the development of inflammation (ACE, MBL and IL-6). This genetic information will be anonymised in accordance with international guidelines and therefore cannot be traced back to your child.

What are the possible risks/side effects of taking part?
We anticipate no risks to your child. We shall not undertake any needle pricks purely for research blood samples.

What are the potential benefits of taking part?
This study will not bring any immediate benefit to your child. We hope to further our understanding of the causes of NEC. This will have implications for future therapies, such as treatment with activated protein C.

What happens at the end of the study?
The information generated by the study will be published in a medical journal. No details will be divulged from which your child could be identified. All stored data information will be destroyed.

Who will have access to the case/research records?
Only the researchers will have access to the data collected during this study. The use of personal information is safeguarded by the Data Protection Act 1998 (DPA). The DPA places an obligation on those who record or use personal information, but also gives rights to people about whom information is held. If you have any questions about data protection, contact the Data Protection officer via the switchboard on 01223 245151 ext 3768.

What are the arrangements for compensation?
This research has been approved by an independent Research Ethics Committee. They believe that it is of minimal risk to your child. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm occurs as a result of taking part in this study. No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This will require you to prove a fault on the part of the Hospital and/or any manufacturer involved.

Do I have to take part in this study?
No. If you decide, now or at a later stage, that you do not wish to participate in this research project, that is entirely your right. This will not in anyway prejudice any present or future treatment.
Who is organising the study?
The investigators and methods of contacting them:

Paula Lister
Clinical Research Fellow
The Portex Unit
6th Floor Cardiac Wing
The Institute of Child Health
30 Guildford Street
London
WC1N 1EH

Pager: 07644 069550

Rob McClure
Consultant Neonatologist
NICU
Rosie maternity Hospital
Addenbrooke’s site
Hills Road
Cambridge
CB2 2QQ

Via switchboard 01223 245 151.

Who do I speak to if problems arise?
If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher.

If the problems are not resolved, or you wish to comment in any other way, please contact:

The Chairman of the Research Ethics Committee,
The Research and Development Office,
Box 148
Addenbrookes NHS Trust
Hills Road
Cambridge
CB2 2QQ

Tel: R&D administrator: 01223 317 983.
PARENT INFORMATION LEAFLET

NECROTISING ENTEROCOLITIS (NEC) AND THE ROLE PLAYED BY THE PROTEIN C PATHWAY

(Co ntrol subjects)

We would like to ask your permission to include your child in our project. We know that your baby does not have necrotising enterocolitis (NEC) which is an inflammatory condition of the bowel. Your baby could provide us with important information that we could compare to the babies that do have NEC. We emphasize that if you should decide not to participate, it will not affect your baby’s treatment in any way.

**What is the purpose of this study?**
Protein C is a substance made by the body. It has two important functions namely it prevents blood clotting and dampens inflammation. It exists in an active and non-active form.

Protein C levels have been found to be very low in critically ill adults with severe infections. A recent study showed that treatment of these adults with activated protein C improved their recovery.

Necrotising enterocolitis is severe inflammation of the bowel that occurs in babies. Many of these babies can require intensive care and even bowel surgery. NEC is often associated with blood clotting abnormalities.

There has not yet been a study to determine levels of protein C in healthy babies or those critically ill with NEC. We want to discover what happens to the levels of activated protein C in infants with necrotising enterocolitis (NEC).

**Why has my child been chosen?**
Your baby does not have NEC or any other inflammatory condition that could affect his/her protein C activation pathway.

**What will happen to my baby if I consent to take part?**
If you agree to take part in this study, your baby will have an extra blood sample taken soon after diagnosis. If your baby weighs less than 1000g the sample will be 1.4mls (1/4 teaspoon) and if your baby weighs more than 1000g the sample will be slightly bigger, 2.4mls(<1/2teaspoon).

The blood sample will be taken at the same time as the routine tests. The blood will be taken from existing central or arterial lines if they are in place. Your child will have no extra needle pricks.
What tests will be done?
The levels of molecules of the protein C pathway and inflammatory markers shall be measured in the blood sample. We will also determine the gene type for three particular proteins important in the development of inflammation (ACE, MBL and IL-6). This genetic information will be anonymised in accordance with international guidelines and therefore cannot be traced back to your child.

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This study will not bring any immediate benefit to your child. We hope to further our understanding of the causes of NEC. This will have implications for future therapies, such as treatment with activated protein C.

What happens at the end of the study?
The information generated by the study will be published in a medical journal. No details will be divulged from which your child could be identified. All stored data information will be destroyed.

Who will have access to the case/research records?
Only the researchers will have access to the data collected during this study. The use of personal information is safeguarded by the Data Protection Act 1998 (DPA). The DPA places an obligation on those who record or use personal information, but also gives rights to people about whom information is held. If you have any questions about data protection, contact the Data Protection officer via the switchboard on 01223 245151 ext 3768

What are the arrangements for compensation?
This research has been approved by an independent Research Ethics Committee. They believe that it is of minimal risk to your child. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm occurs as a result of taking part in this study. No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This will require you to prove a fault on the part of the Hospital and/or any manufacturer involved.

Do I have to take part in this study?
No. If you decide, now or at a later stage, that you do not wish to participate in this research project, that is entirely your right. This will not in anyway prejudice any present or future treatment.
Who is organising the study?
The investigators and methods of contacting them:

Paula Lister
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Addenbrooke's site
Hills Road
Cambridge
CB2 2QQ

Who do I speak to if problems arise?
If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher.

If the problems are not resolved, or you wish to comment in any other way, please contact:

The Chairman of the Research Ethics Committee,
The Research and Development Office,
Box 148
Addenbrookes NHS Trust
Hills Road
Cambridge
CB2 2QQ

Tel: R&D administrator: 01223 317 983.
CONSENT FORM

LREC reference number: LREC 03/057

Title of Project:
Investigation of the role of the Protein C Activation Pathway in necrotising enterocolitis: A prospective study.

Name of lead investigator:
Paula Lister

Please initial the boxes:

1. I confirm that I have read the information sheet dated 10/03/2003, version 2, for the above study and I have had the opportunity to ask questions.

2. I understand that the participation of my child is voluntary and that I am free to withdraw my child at any time, without giving any reason, without my child's medical care or legal rights being affected.

3. I understand that sections of my child's medical notes may be looked at by the investigators, where it is relevant to my child taking part in research. I give permission for these individuals to have access to my child's records.

4. I understand that tests will be undertaken to determine the code for particular genes as described in the information sheet.

5. I agree for my child to take part in the above study

-----------------------------------------------------------------------------------------------
Name of Parent or Guardian             Date                      Signature

-----------------------------------------------------------------------------------------------
Name of Witness to signature           Date                      Signature

-----------------------------------------------------------------------------------------------
Name of Research team member           Date                      Signature
Investigator Brochure
Protein C Concentrate (Ceprotin)

MARKETING AUTHORISATION HOLDER
BAXTER AG Industriestrasse 67 A-1220 Vienna Austria

MARKETING AUTHORISATION NUMBER:
EU/1/01/190/002

EMEA Product Number:
EMEA/H/C/000334

Protein C in necrotising enterocolitis: a dose-finding pilot study.
EudraCT: 2010-021254-19
Sponsor trial number: 09AR21
Sponsor: Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London WC1N 3JH
Chief Investigator: Dr Paula Lister, Consultant Paediatric Intensivist, Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London, WC1N 3JH

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List of abbreviations and Definitions of terms:

APC  activated protein C
APTT activated prothrombin time
DIC disseminated intravascular coagulation
EMEA European Medicines Agency
FDA United states food and drug administration
GOSH Great Ormond Street Hospital
HAS Human Albumin Solution
IU/ml international units per milliliter
IVH intraventricular haemorrhage
PC  protein C
PCC protein C concentrate
PT  prothrombin time
TAT thrombin-antithrombin complex

1 U PC  = amidolytically measured activity of protein C in 1 ml of normal pooled human plasma.

Normal adult PC reference range = 0.8-1.2 U/ml.
1. Summary of Product Characteristics:

CEPROTIN is licensed for use in congenital PC deficiency. The EMEA and FDA summary of product details therefore refers to the licensed indication and not to the experience of “off-label” use of CEPROTIN in acquired deficiency states (section 2, page 19)

The following summary is taken from the EMEA website.


1.1. NAME OF THE MEDICINAL PRODUCT

CEPROTIN 500 IU or 1000 IU powder and solvent for solution for injection

1.2. QUALITATIVE AND QUANTITATIVE COMPOSITION

Protein C (PC) is from human plasma purified by mouse monoclonal antibodies.

CEPROTIN 500 IU is prepared as a powder containing nominally 500 IU of human protein C per container. The product reconstituted with 5 ml of Sterilised Water for Injections, and contains approximately 100 IU/ml human protein C.

The potency (IU) is determined using a chromogenic substrate method against the World Health Organisation (WHO) International standard.

Excipients:

Sodium chloride: 44 mg/vial

Sodium citrate. 2H2O: 22 mg/vial

For a full list of excipients see 1.6.1

* One International Unit (IU) of protein C corresponds to the amidolytically measured activity of protein C in 1 millilitre of normal plasma.

1.3. PHARMACEUTICAL FORM
Powder and solvent for solution for injection.

White or cream coloured powder or friable solid. After reconstitution the solution has a pH of between 6.7 and 7.3 and an osmolality of not lower than 240 mosmol/kg.

1.4. CLINICAL PARTICULARS

1.4.1. Therapeutic indications

CEPROTIN is indicated in purpura fulminans and coumarin-induced skin necrosis in patients with severe congenital protein C deficiency. Furthermore CEPROTIN is indicated for short-term prophylaxis in patients with severe congenital protein C deficiency if one or more of the following conditions are met:

- surgery or invasive therapy is imminent
- while initiating coumarin therapy
- when coumarin therapy alone is not sufficient
- when coumarin therapy is not feasible.

1.4.2. Posology and method of administration for congenital PC deficiency

A protein C activity of 100 % should be achieved initially and the activity should be maintained above 25 % for the duration of the treatment. An initial dose of 60 to 80 IU/kg for determination of recovery and half-life is advised.

The measurement of protein C activity using chromogenic substrates is recommended for the determination of the patient’s plasma level for protein C before and during treatment with CEPROTIN.

The dosage should be determined on the basis of laboratory measurements of the protein C activity. In the case of an acute thrombotic event these should be performed
every 6 hours until the patient is stabilised, thereafter twice a day and always immediately before the next injection. It should be kept in mind that the half-life of protein C may be severely shortened in certain clinical conditions such as acute thrombosis with purpura fulminans and skin necrosis.

Patients treated during the acute phase of their disease may display much lower increases in protein C activity. The wide variation in individual responses implies that the effects of CEPROTIN on coagulation parameters should be checked regularly.

Patients with renal and/or hepatic impairment should be monitored more closely (see section 4.4). Based on the limited clinical experience in children from reports and studies covering 83 patients, dosing guidelines for adult subjects are considered valid for neonatal and paediatric patient population (see section 1.5.1).

In rare and exceptional cases, subcutaneous infusion of 250-350 IU/kg was able to produce therapeutic protein C plasma levels in patients with no intravenous access.

If the patient is switched to permanent prophylaxis with oral anticoagulants, protein C replacement is to be discontinued only when stable anticoagulation is obtained (See 4.5). Furthermore, during the initiation of oral anticoagulant therapy it is advisable to start with a low dose and adjust this incrementally, rather than use a standard loading dose.

In patients receiving prophylactic administration of protein C, higher trough levels may be warranted in situations of an increased risk of thrombosis (such as infection, trauma, or surgical intervention).

In patients with combined severe congenital protein C deficiency and with APC resistance, there are limited clinical data to support the safety and efficacy of CEPROTIN.

CEPROTIN is administered by intravenous injection after reconstitution of the powder for solution for injection with Sterilised Water for Injections.
CEPROTIN should be administered at a maximum injection rate of 2 ml per minute except for children with a body weight of < 10 kg, where the injection rate should not exceed a rate of 0.2 ml/kg/min.

As with any intravenous protein product, allergic type hypersensitivity reactions are possible. For the events that allergic symptoms arise which are of an acute and life-threatening nature, administration should be made within reach of life-supporting facilities.

1.4.3. Contraindications

Hypersensitivity to the active substance or to any of the excipients or to mouse protein or heparin, except for control of life-threatening thrombotic complications.

1.4.4. Special warnings and precautions for use

As the risk of an allergic type hypersensitivity reaction cannot be excluded, patients should be informed of the early signs of hypersensitivity reactions including hives, generalized urticaria, tightness of the chest, wheezing, hypotension, and anaphylaxis. If these symptoms occur, they should inform the physician. Immediate discontinuation of product use is advised. In case of shock, the current medical standards for shock treatment are to be observed.

No experience in the treatment of patients with renal and/or hepatic impairment is available and therefore it is recommended that such patients be monitored more closely.

Standard measures to prevent infections resulting from the use of medicinal products prepared from human blood or plasma include selection of donors, screening of individual donations and plasma pools for specific markers of infection, and the inclusion of effective manufacturing steps for the inactivation/removal of viruses. Despite this, when medicinal products prepared from human blood or plasma are administered, the possibility of transmitting infective agents cannot be totally excluded. This also applies to unknown or emerging viruses and other pathogens.
The measures taken are considered effective for enveloped viruses such as HIV, HBV and HCV and for the non-enveloped virus HAV. The measures taken may be of limited value against non-enveloped viruses such as parvovirus B19. Parvovirus B19 infection may be serious for pregnant women (fetal infection) and for individuals with immunodeficiency or increased erythropoiesis (e.g. haemolytic anaemia).

Appropriate vaccination (hepatitis A and B) should be considered for patients in regular / repeated receipt of human plasma-derived Protein C products.

It is strongly recommended that every time that CEPROTIN is administered to a patient, the name and batch number of the product are recorded in order to maintain a link between the patient and the batch of the product.

CEPROTIN may contain trace amounts of heparin. Heparin induced allergic reactions, which can be associated with a rapid decrease of the number of thrombocytes, may be observed (heparin induced thrombocytopenia [HIT]). In patients with HIT, symptoms such as arterial and venous thrombosis, disseminated intravascular coagulation (DIC), purpura, petechiae and gastrointestinal bleeding (melena), may occur. If HIT is suspected, the number of thrombocytes should be determined immediately and if necessary therapy with CEPROTIN should be stopped. Identifying HIT is complicated by the fact that these symptoms may already be present in acute phase patients with severe congenital protein C deficiency. Patients with HIT should avoid the use of heparin containing drugs in the future.

In the context of clinical experience several bleeding episodes have been observed. Concurrent anticoagulant medication (such as heparin) may have been responsible for these bleeding episodes. However, it cannot be completely ruled out that the administration of CEPROTIN further contributed to these bleeding events. The quantity of sodium in the maximum daily dose may exceed 200 mg. This should be taken into consideration by patients on a controlled sodium diet.

1.4.5. Interaction with other medicinal products and other forms of interaction
No interactions with other medicinal products are currently known. In patients starting treatment with oral anticoagulants belonging to the class of vitamin K antagonists (e.g. warfarin), a transient hypercoagulable state may arise before the desired anticoagulant effect becomes apparent. This transient effect may be explained by the fact that protein C, itself a vitamin K dependent plasma protein, has a shorter half-life than most of the vitamin K dependent proteins (i.e. II, IX and X). Subsequently, in the initial phase of treatment, the activity of protein C is more rapidly suppressed than that of the procoagulant factors. For this reason, if the patient is switched to oral anticoagulants, protein C replacement must be continued until stable anticoagulation is obtained.

Although Warfarin-induced skin necrosis can occur in any patient during the initiation of oral anticoagulant therapy, individuals with congenital protein C deficiency are particularly at risk. (See 1.4.2.).

1.4.6. Pregnancy and lactation

Although CEPROTIN has been used safely in the treatment of pregnant protein C-deficient women, its safety for use in human pregnancy has not been established in controlled clinical trials. Furthermore no information on excretion of protein C in the milk is available. Therefore, the benefit of using CEPROTIN during pregnancy or lactation must be judged against the risk for the mother and baby, and should be used only if clearly needed.

1.4.7. Effects on ability to drive and use machines

CEPROTIN has no influence on the ability to drive and to operate machines.

1.4.8. Undesirable effects

As with any intravenous product allergic type hypersensitivity reactions are possible.

Patients should be informed of the early signs of hypersensitivity reactions, which may
include angioedema, burning and stinging at the injection site, chills, flushing, rash, generalised urticaria, headache, hives, hypotension, lethargy, nausea, restlessness, tachycardia, tightness of the chest, tingling, vomiting and wheezing. Patients should be advised to immediately contact their physician if these symptoms occur (see section 1.4.4).

During clinical studies with CEPROTIN, a total of 6 non-serious adverse drug reactions (ADRs) were reported in 3 of 225 patients enrolled. In total 21,988 administrations of CEPROTIN have been given. The distribution of the related ADRs is as follows: one report of pyrexia and one report of increased C-reactive protein in one subject, one case of urticaria in one subject, one report of pruritus, one report of rash and one report of dizziness in one subject. With a calculated adverse experience rate (per number of administrations) of 0.005% the frequency of these ADRs can be classified as very rare.

In the spontaneous, postmarketing database, there have been reports of restlessness, haemothorax, hyperhydrosis, increased body temperature and increased need of catecholamines to support blood pressure (verbatim term: increased need for catecholamines) in the course of the treatment.

If the preparation is used in patients with severe congenital protein C deficiency, antibodies inhibiting protein C may develop.

1.4.9. Overdose

No symptoms of overdose with CEPROTIN have been reported.

1.5. PHARMACOLOGICAL PROPERTIES

1.5.1. Pharmacodynamic properties

Pharmacotherapeutic group: group antithrombotic; ATC Code: B01AD12

Protein C is a vitamin K-dependent anticoagulant glycoprotein which is synthesised in the liver. It is converted by thrombin/thrombomodulin-complex on the endothelial surface to activated protein C (APC). APC is a serine protease with potent anticoagulant
effects, especially in the presence of its cofactor protein S. APC exerts its effect by the inactivation of the activated forms of factors V and VIII which leads to a decrease in thrombin formation. APC has also been shown to have profibrinolytic effects.

The intravenous administration of CEPROTIN provides for an immediate but temporary increase in plasma levels of protein C. Replacement of protein C in protein C deficient patients is expected to control or - if given prophylactically - prevent thrombotic complications. Twelve courses of short-term prophylaxis prior to surgery or invasive therapy and 7 courses of longterm prophylaxis were included in the efficacy analyses.

No formal clinical study in either paediatric or neonatal population with severe congenital protein C deficiency was ever conducted. However, several small retrospective and prospective studies investigating other clinical application areas have been published in this population. Indication was prevention and treatment of purpura fulminans and thrombotic disease, enrolling overall 14 subjects of 2 days old throughout adolescence.

Other experience with CEPROTIN covers case reports and a clinical study in overall 69 paediatric patients with acquired protein C deficiency. The study is a randomized, double-blind, placebo controlled dose-finding study, in the indication of acquired protein C deficiency due to meningococcal sepsis (IMAG 112). The reports suggest that CEPROTIN is well tolerated in children and small infants. Dosages of the above studies, covering 83 patients, indicate that dosing guidelines for adult subjects are also valid for neonatal and paediatric patient population.

In rare and exceptional cases, subcutaneous infusion of 250-350 IU/kg was able to produce therapeutic protein C plasma levels in patients with no intravenous access.

1.5.2. Pharmacokinetic properties

21 asymptomatic subjects with homozygous or double heterozygous protein C deficiency were evaluated for pharmacokinetic data. The protein C plasma activity was measured by chromogenic assay. The individual half-lives varied from 4.4 to 15.8 hours.
using a compartmental model and from 4.9 to 14.7 using the non-compartmental method. The individual incremental recovery ranged from 0.50 to 1.76 [(IU/dL)/(IU/kg)]. The patients differed significantly in age, body weight and plasma volume.

In patients with acute thrombotic disease, both the incremental increase in protein C plasma levels as well as half-life may be considerably reduced.

1.5.3. Preclinical safety data

Protein C contained in CEPROTIN is a normal constituent of human plasma and acts like endogenous protein C. Therefore experimental studies on tumorigenic or mutagenic effects - particularly in heterologous species - are not considered necessary.

Single dose toxicity testing showed that even doses of several times the recommended human dosage per kilogram body weight (10-fold) did not result in toxic effects on rodents.

CEPROTIN proved to have no mutagenic potential in the Ames test performed.

Repeated toxicity studies were not conducted because prior experience with coagulation preparations had shown them to be of limited value. Difference between the recipient species and human Protein C will inevitably result in an immune response with antibody formation.

1.6. PHARMACEUTICAL PARTICULARS

1.6.1. List of excipients

Powder

Human albumin
Sodium chloride

Sodium citrate . 2H2O

Solvent

Sterilised Water for Injection

1.6.2. **Incompatibilities**

In the absence of compatibility studies, this medicinal product must not be mixed with other medicinal products.

1.6.3. **Shelf life**

3 years if stored between 2-8°C

The reconstituted solution is stable for 3 hours at room temperature.

1.6.4. **Special precautions for storage**

Store in a refrigerator (2°C – 8°C). Do not freeze. Keep the vial in the outer carton in order to protect from light.

1.6.5. **Nature and contents of container**

500 IU and 1000 IU: CEPROTIN powder comes in vials of neutral glass of either hydrolytic type I (500 IU) or hydrolytic type II (1000 IU). The solvent comes in vials of neutral glass of hydrolytic type I. The product and the solvent vials are closed with butyl rubber stoppers.

Each pack also contains:
- one transfer needle
- one filter needle

Not all pack sizes may be marketed.

1.6.6. **Special precautions for disposal and other handling**

Reconstitute lyophilised CEPROTIN powder for solution for injection, with the supplied solvent (Sterilised Water for Injections) using the sterile transfer needle. Gently rotate the vial until all powder is dissolved. After reconstitution the solution is colourless to
slightly yellowish and clear to slightly opalescent and essentially free from visible particles.

The solution is drawn through the sterile filter needle into a sterile disposable syringe. A separate unused filter needle must be used to withdraw each vial of reconstituted CEPROTIN. The solution should be discarded if particulate matter is visible.

The reconstituted solution should be administered within 3 hours by intravenous injection.

Any unused product or waste material should be disposed of in accordance with local requirements.

1.7. MARKETING AUTHORISATION HOLDER

BAXTER AG Industriestrasse 67 A-1220 Vienna Austria

1.8. MARKETING AUTHORISATION NUMBER

EU/1/01/190/002

1.9. DATE OF FIRST AUTHORISATION/RENEWAL OF THE AUTHORISATION

Date of first authorisation: 16 July 2001

Date of last renewal: 16 July 2006
2. **Summary of post-marketing, “off-label” published use of CEPROTIN**

2.1. **Summary of Findings**

Protein C deficiency can be acquired in disease states associated with increased PC consumption, or increased degradation, and/or decreased PC synthesis; for example severe sepsis, septic shock and disseminated intravascular coagulation (DIC). PC concentrate (PCC) has been used to supplement PC levels in acquired PC deficiency states since the early 1990s.

The published evidence of the use of PCC in acquired PC deficiency includes individual case reports, case series, and a single randomised, controlled, blinded dose-finding study. All published evidence is listed in appendix 1 & 2. This “off-label” use of PCC is reported in a total of 358 patients; 229 are children and 61 are neonates, most of whom are premature.

Reported PCC supplementation has largely been limited to populations of septic patients; except for a case report in a patient with metastatic carcinoma and a study that reports the experience in paediatric stem cell transplant patients with veno-occlusive disease.

Severe sepsis causes a procoagulant milieu. Severe sepsis with or without laboratory evidence of DIC is associated with early, rapid depletion of protein C and mortality is associated with non-recovery of normal PC levels.\(^1\)\(^-\)\(^4\).

Summary of common findings across published reports:

1. The supplementation of PCC resulted in raised plasma PC levels.

2. Studies that measured haemostatic and DIC parameters, showed PCC supplementation resulted in normalisation of haemostasis parameters and resolution of DIC.

3. Studies that reported predicted risk of mortality showed that PCC supplementation was associated with lower actual mortality.
4. Studies that monitored thrombotic skin lesions or imminent peripheral ischaemia showed that PCC supplementation was associated with clinical improvement in perfusion.

2.2. Summary of Risks

The table in appendix 2 summarises the published evidence of adverse effects and adverse reactions of PCC concentrate administered "off-label" to 358 patients with acquired protein C deficiency.

To summarise the "off-label" data:

2.2.1. Ceprotin has an excellent safety profile

2.2.2. No cases of transmission of microorganisms have been observed

2.2.3. Serious adverse reactions are extremely rare and have been associated with the underlying DIC and condition or simultaneous use of thrombolytic drugs;
   pulmonary haemorrhage in patient concurrently treated with rTPA (thrombolysis);
   intracranial haemorrhage in 2 patients with severe, intractable DIC.

2.2.4. Mild AE or AR: rash (1), mild gastrointestinal haemorrhage (1), 21 others not further described by De Klein et al

3. References


### Appendix 1: Summary table of the findings from published literature using protein C supplementation in acquired PC deficiency in Humans.

<table>
<thead>
<tr>
<th>Publication Year</th>
<th>Authors</th>
<th>Study design</th>
<th>Study population</th>
<th>Study participants</th>
<th>Intervention (details in table 2)</th>
<th>Outcome Measures</th>
<th>Main Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990</td>
<td>Okajima k</td>
<td>Case Report</td>
<td>Metastatic Carcinoma</td>
<td>n=1 (54y)</td>
<td>PCC supp then APC Standard ICU care</td>
<td>Coagulation parameters</td>
<td>↑ fibrinogen, ↓ TAT</td>
</tr>
<tr>
<td>1993</td>
<td>Gerson WT</td>
<td>Case Report</td>
<td>Severe Sepsis &amp; PF &amp; DIC</td>
<td>n=1 (13y)</td>
<td>FFP then PPC x 11d Standard ICU care</td>
<td>PC level, survival</td>
<td>PC level better achieved with PCC vs FFP.</td>
</tr>
<tr>
<td>1995</td>
<td>Rivard GE</td>
<td>Prospective case series - PILOT</td>
<td>Meningococcal sepsis &amp; shock &amp; PF &amp; DIC</td>
<td>n=4 (3m-15y)</td>
<td>PCC supplementation Standard ICU care</td>
<td>Normalise PC act., Haemostasis &amp; DIC parameters Severity of ischemic limb lesions</td>
<td>Reversal of MODS &amp; DIC 0 deaths, 2 amputation</td>
</tr>
<tr>
<td>1997</td>
<td>Smith OP</td>
<td>Prospective case series</td>
<td>Meningococcal sepsis &amp; shock &amp; PF PC levels &lt;0.2IU/ml</td>
<td>n=12 (3m-27y~ 2 adults)</td>
<td>PC level maintained 0.8-1.2IU/ml with PCC Heparin 10-15u/k/h 11/12, Pts &gt; 50 x 10^7/L Fib &gt;2g/L CVVH 9/12 ATIII in 1 patient Wean PC &amp; heparin when PC, Plt, Fib &amp; DDimers normal Standard ICU care</td>
<td>PC levels 80-120IU/ml Length of treatment Haemostasis &amp; DIC parameters Predicted vs actual outcomes</td>
<td>PCC started within 18h in 10/12 PCC administered for mean 5.7d (4-8d). Inotropic support 4d (3-7d), ventilation 8.5d (4-17d) Predicted mortality 57%children &amp; 80% adults 0 deaths 2 patients had amputations &amp; one also had thrombotic CVA – both commenced PC &gt;48h after admission No bleeding</td>
</tr>
<tr>
<td>1998</td>
<td>Kreuz W</td>
<td>Case Series</td>
<td>Meningococcal sepsis &amp; shock &amp; PF</td>
<td>n=7 (2m-17y)</td>
<td>PCC &amp; ATIII suppl Standard ICU care</td>
<td>PC levels Haemostasis &amp; DIC parameters PF skin lesions</td>
<td>Haemostasis returned to normal signs of PF declined 5/7 survived 1 required limb amputation</td>
</tr>
<tr>
<td>1999</td>
<td>Ettinghausen CE</td>
<td>Prospective case Series</td>
<td>Meningococcal sepsis &amp; shock &amp; DIC &amp; PF</td>
<td>n=8 (3m – 18y)</td>
<td>PCC supplementation Standard ICU care</td>
<td>PC levels normalise Haemostasis &amp; DIC parameters PAI-1 level Skin lesion size &amp;</td>
<td>Partial or full correction of haemostasis in all. Improved microcirculation in all Improvement in skin lesions 2/8 died with MODS, 1/8 limb amputation</td>
</tr>
<tr>
<td>Year</td>
<td>Author</td>
<td>Study Type</td>
<td>Primary Diagnosis</td>
<td>Study Details</td>
<td>Case Numbers</td>
<td>Treatment</td>
<td>Haemostasis/DIC Parameters</td>
</tr>
<tr>
<td>------</td>
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<td>-----------------------------</td>
</tr>
<tr>
<td>2000</td>
<td>Clarke RCN</td>
<td>Case report</td>
<td>Meningococcal sepsis &amp; shock &amp; DIC &amp; PF</td>
<td>n=1 (17y)</td>
<td>PCC &amp; ATIII suppl, FFP</td>
<td>PC levels Haemostasis &amp; DIC parameters</td>
<td>Rapid improvement of shock, improved PC levels &amp; haemostasis parameters in 48h</td>
</tr>
<tr>
<td>2000</td>
<td>Rintala E</td>
<td>Case Series</td>
<td>Severe Sepsis &amp; PF &amp; DIC &amp; imminent peripheral necrosis</td>
<td>n=12 (adult)</td>
<td>PCC &amp; AT III until improvement in peripheral ischaemia &amp; normalised PC activity. 12/12 LMWH 10/12 CVVH Standard ICU care</td>
<td>Imminent necrosis Duration of supplementation Haemostasis &amp; DIC parameters Hospital &amp; 28 day mortality</td>
<td>Progressive reversal of peripheral ischemia, 2 patients required amputations, rapid improvement in DIC parameters, non-survivors did not have recovery in platelet count, mean length of mechanical ventilation 19d (6-73)</td>
</tr>
<tr>
<td>2000</td>
<td>White B</td>
<td>Case Series</td>
<td>Study gp = Meningococcal sepsis &amp; shock &amp; PF Control = meningococcal sepsis, no shock or PF</td>
<td>Study n=36 (12 ± 16; 3m-72y) Control n=23 (8±14y; 3m-72y)</td>
<td>PCC &amp; heparin Keep Plt &gt; 5x10^9/L Keep Fib &gt;2g/ATIII suppl in 2 patients 19/36 CVVH Heparin given to 26/36 Standard ICU care</td>
<td>PC &amp; ATIII levels Haemostasis &amp; DIC parameters PAI-1 level Predicted v actual outcomes</td>
<td>PC &amp; AT III levels significantly lower in MODS. PAI-1 ↑ in MODS. PC levels are inversely related to D-Dimers. Predicted mortality 50%; 3/36 died. Predicted risk of amputations 33%; 4/36 actual 2 were already non-viable before PCC given – one also had ischemic cerebral infarction.</td>
</tr>
<tr>
<td>2001</td>
<td>Faust SN</td>
<td>Case Series</td>
<td>Meningococcal sepsis</td>
<td>n=2 (children)</td>
<td>PCC supplementation Standard ICU care</td>
<td>PC and APC level</td>
<td>PC level normalised, APC not detected.</td>
</tr>
<tr>
<td>2003</td>
<td>Fourrier F</td>
<td>Retrospective Case series</td>
<td>Meningococcal sepsis &amp; shock &amp; PF</td>
<td>n=15 (10 children)</td>
<td>PCC &amp; ATIII suppl Standard ICU care</td>
<td>Pharmacokinetics of ATIII &amp; PC Haemostasis &amp; DIC parameters</td>
<td>PC &amp; ATIII in all patients on admission. Pharmacokinetic data into and intra individual variability as the DIC resolved. 9/15 died – 4 early refractory shock, 3 amputations in survivors.</td>
</tr>
<tr>
<td>2003**</td>
<td>Vaccarella G</td>
<td>Case report</td>
<td>Septic shock &amp; PF</td>
<td>n=1 (18yr male)</td>
<td>PCC &amp; AT suppl Standard ICU care</td>
<td>PC level Haemostasis &amp; DIC parameters</td>
<td>Increased PC level, Resolution of DIC improvement in cardiovascular stability improvement in skin lesions survival</td>
</tr>
<tr>
<td>2003</td>
<td>De Kleijn ED</td>
<td>Randomised, blinded, controlled dose finding study – Phase 2</td>
<td>Meningococcal sepsis &amp; shock &amp; PF</td>
<td>n=40 10 control 30 dose adjusted</td>
<td>3 PCC dose escalating groups. Standard ICU care</td>
<td>PC &amp; APC levels Haemostasis &amp; DIC parameters</td>
<td>9/40 died, 5 amputations activation of PC occurred in 27/28 patients activation of coagulation and D-Dimers normalised faster with increasing doses of PCC</td>
</tr>
<tr>
<td>2003**</td>
<td>Ramos Guerrero R</td>
<td>Case series</td>
<td>Severe sepsis</td>
<td>n=3 (children)</td>
<td>PCC suppl Standard ICU care</td>
<td>PC levels Haemostasis &amp; DIC parameters</td>
<td>Normalisation of haemostasis link D-Dimers 2/3 survivors</td>
</tr>
<tr>
<td>2004**</td>
<td>Linares –Tello F</td>
<td>Case Report</td>
<td>Meningococcal sepsis &amp; PF &amp; shock &amp; DIC</td>
<td>n=1 (12y)</td>
<td>FFP &amp; PCC Standard ICU care</td>
<td>PC level Haemostasis &amp; DIC parameters</td>
<td>FFP was unable to increase PC levels above 30% Resolution of DIC with PCC suppl Patient died</td>
</tr>
<tr>
<td>2004**</td>
<td>Pettenazzo</td>
<td>Case series</td>
<td>Sepsis &amp; shock &amp; DIC</td>
<td>n=8</td>
<td>PCC Suppl</td>
<td></td>
<td>6/8 rapid improvement in clinical status</td>
</tr>
</tbody>
</table>
### Table of Studies on PC Concentrate in NEC

<table>
<thead>
<tr>
<th>Year</th>
<th>Study Design</th>
<th>Setting</th>
<th>Case Description</th>
<th>Study Population</th>
<th>Treatment</th>
<th>Control</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>Retrospective case control</td>
<td>Severe sepsis &amp; Septic shock</td>
<td>n=29 (children) 11 treated 18 untreated</td>
<td>Study group given PCC suppl. Standard ICU care</td>
<td>Mortality, LOS, Predicted vs actual outcomes</td>
<td>Haemostasis &amp; DIC parameters</td>
<td>There was no difference in length of stay or mortality between the two groups</td>
</tr>
<tr>
<td>2005</td>
<td>Randomised, blinded, placebo cross-over</td>
<td>Human model of endotoxaemia</td>
<td>n=11 (healthy adult volunteers)</td>
<td>Placebo or PCC suppl to supranormal levels for 4 hours after endotoxin injection</td>
<td>PC &amp; APC levels Thrombin generation (TAT, F1+2), inflammation (TNFα IL-6)</td>
<td>Increased PC levels &amp; proportional increase in APC no major anti-coagulant, anti-inflammatory or profibrinolytic effects.</td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Prospective case series</td>
<td>Neonatal sepsis &amp; low PC</td>
<td>n=11 (25/40-term)</td>
<td>PCC suppl for 72 hours Standard NICU care</td>
<td>PC activity</td>
<td>Haemostasis &amp; DIC parameters</td>
<td>1PC activity levels ↑ Pits ↓D-Dimers 1 death</td>
</tr>
<tr>
<td>2005</td>
<td>Prospective case series PILOT</td>
<td>Neonatal sepsis + DIC &amp; low PC activity</td>
<td>n=12 Term &amp; preterm</td>
<td>PCC suppl daily Standard NICU care</td>
<td>PC level</td>
<td>Haemostasis &amp; DIC parameters</td>
<td>10 rapid improvement 2 deaths – 1 prenatal brain haemorrhage &amp; 1 fulminent septic shock died in few hours.</td>
</tr>
<tr>
<td>2005</td>
<td>Case series</td>
<td>Sepsis &amp; PF</td>
<td>n=8 (adults)</td>
<td>PCC suppl Standard ICU care Heparin, FFP, ATIII, fib, rTPA , plts as required</td>
<td>PC activity</td>
<td>Haemostasis &amp; DIC parameters</td>
<td>6 DIC, 5 shock, 6 renal &amp; 4 respiratory failure. DIC resolved in 7/8 by D3 2 deaths – 1 fulminent shock&amp; 1 late fungal sepsis.</td>
</tr>
<tr>
<td>2005</td>
<td>Caes series</td>
<td>Pediatric stem cell transplant &amp; veno-occlusive disease</td>
<td>n=6 (6m-11y)</td>
<td>PCC suppl (8-45days) Defibritione</td>
<td>PC levels, PAI-1 Haemostasis &amp; DIC parameters</td>
<td>Hepatic blood flow, clinical status</td>
<td>Normalised PC level Normalised PAI-1 level Improved haemostasis 5/6 prompt resolution of hepatic blood flow obstruction, 1/6 slower but detectable response</td>
</tr>
<tr>
<td>2005</td>
<td>Prospective case series PILOT Clinical contraindications for APC</td>
<td>Severe sepsis, shock, PF &amp; PC &lt;50% AND APC contraindiacted</td>
<td>n=20 (adults) 4 neurological patients 9 had plt&lt;30 3 bleeding risk 2 major surgery 2 anticoagulation</td>
<td>PCC suppl x 72h No ATIII suppl Standard ICU care</td>
<td>Haemostasis &amp; DIC parameters SOFA Mortality</td>
<td>PC levels normalised over 48 hours progressive and significant increase in Pts, fibrinogen, PT, ATIII progressive and significant decrease D-Dimers APTT DIC score lactate 7/20 died Mortality 35%, predicted 59% 1 with myocardial infarction &amp; 6 with MODS</td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Case series</td>
<td>Sepsis &amp; low PC level</td>
<td>n=3 (adult)</td>
<td>PCC suppl</td>
<td>PC level</td>
<td>Haemostasis &amp; DIC parameters</td>
<td>Supranormal levels of PC achieved improvement in DIC parameters {TAT, }PAI-1 All survived</td>
</tr>
<tr>
<td>2005</td>
<td>Case report (Turkish)</td>
<td>Severe sepsis</td>
<td>n=1 (preterm infant)</td>
<td>PCC suppl x 96h</td>
<td></td>
<td>Survival</td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td>Case Series</td>
<td>Neonatal septic</td>
<td>n=4</td>
<td>PCC suppl</td>
<td>NEOMOD organ</td>
<td>All improved in 24h</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>Authors</td>
<td>Design</td>
<td>Patients</td>
<td>Clinical Status</td>
<td>Coagulation Parameters</td>
<td>Outcome</td>
<td></td>
</tr>
<tr>
<td>------</td>
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<td>----------</td>
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<td>------------------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>Crivellari</td>
<td>Case series</td>
<td>Sepsis, shock &amp; ≥2 organ failure &amp; recent cardiac surgery so APC contraindicated</td>
<td>n=9 (adult)</td>
<td>PCC suppl x 72 h Standard ICU care 6/9 on heparin 300IU/h 4/9 on aspirin 100mg/d</td>
<td>PC &amp; APC levels Haemostasis &amp; DIC parameters</td>
<td>8 respiratory &amp; 7 renal failure, 6 DIC 1PC levels were achieved without APC levels Improved DIC and haemostatic parameters Predicted mortality 68%, actual mortality 11% (1/9)</td>
</tr>
<tr>
<td>Abstract only</td>
<td>Veldman a</td>
<td>Case series</td>
<td>Neonatal sepsis Acquired PC def – PC act 0.02IU/ml (0.01-0.1IU/ml)</td>
<td>n=7 premature (26-35/40)</td>
<td>PCC and AT supps Standard ICU care</td>
<td>Improved PF &amp; Coagulation</td>
<td>All survived</td>
</tr>
<tr>
<td>20101</td>
<td>Veldman A</td>
<td>Case series</td>
<td>Purpura fulminans associated with sepsis</td>
<td>n=94 (children, 8 newborns)</td>
<td>Varied</td>
<td>Clinical status Haemostasis &amp; DIC parameters Need for amputation or skin grafts</td>
<td>21 deaths 4 mild or moderate adverse events not attributed to PCC Improved PF and reduced need for amputation &amp; skin grafts.</td>
</tr>
<tr>
<td>2010</td>
<td>Decembrino L</td>
<td>Case series</td>
<td>Neonatal sepsis &amp; coagulopathy</td>
<td>N=18 (12 preterm, 6 full term)</td>
<td>PCC suppl x 72 h Standard NICU care</td>
<td>PC activity Haemostasis &amp; DIC markers Clinical parameters of organ failure</td>
<td>Improved coagulation and inflammatory markers Clinical and haemodynamic status improvement All survived No adverse reactions, bleeding or thrombosis</td>
</tr>
</tbody>
</table>

Key: PF= purpura fulminans; PC = protein C; PCC = protein C concentrate; AT III = antithrombin III; DIC = disseminated intravascular coagulation; FFP = fresh frozen plasma; CVVH = continuous veno-venous haemofiltration; APC = activated protein C; TAT= thrombin antithrombin complex; Plt = platelet count; PT= prothrombin time; fib = fibrinogen; suppl= supplement; MODS = multiorgan dysfunction syndrome;CUSS = cranial ultrasound scan.
Appendix 2: Summary of published data on Protein Concentrate supplementation in humans with acquired PC deficiency; relating to dose, dosing intervals, efficacy and adverse reactions.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Study design</th>
<th>Study population</th>
<th>Intervention</th>
<th>PC Response</th>
<th>Relevant Secondary Endpoints</th>
<th>Relevant Criteria relating to inclusion &amp; exclusion</th>
<th>Adverse reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>De Kleijn</td>
<td>2003</td>
<td>Randomised blinded, controlled dose- finding study Phase 2</td>
<td>n=30</td>
<td>Ceprotin: Placebo HAS 1% £ groups: 50IU/kg; 100IU/kg &amp; 150IU/kg 6h x 72h then 12h until resolution of symptoms for maximum total treatment period of 7 days.</td>
<td>Med Peak PC: 0.63 for 50IU/kg (0.38-0.92) 1.33 for 100 IU/kg (0.98-1.71) 1.74 for 150IU/kg (1.32-2.54) T1/2 PC: 6.8 for 50IU/kg (3.6-10.3) 8 for 100IU/kg (3.8-16.8) 8 for 150IU/KG (4.5-23.6)</td>
<td>APC index to baseline only increased in 100 &amp; 150 groups. D-dimers fell more in 150&gt;100&gt;50 groups.</td>
<td>None believed to be related to study drug - 13 serious AE – 9 deaths &amp; 4 amputations – all attributable to the underlying disease. 21 not serious AE – one mild GI haemorrhage in patient with DIC.</td>
<td></td>
</tr>
<tr>
<td>Rivard</td>
<td>1995</td>
<td>Prospective case series - PILOT</td>
<td>n=4</td>
<td>Ceprotin predecessor 100IU/kg 6h iv Daily Dose: 400 IU/kg Treatment period 2-9d</td>
<td>PC levels: Baseline: &lt;0.1 – 0.47 U/mL PC &gt; 1 U/mL thereafter Platelet transfusions: 0-4 per subject D-dimers normalized in 48h in ¾ subjects</td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Smith</td>
<td>1997</td>
<td>Prospective case series</td>
<td>n=12</td>
<td>Ceprotin predecessor 100IU/kg test, 100IU/kg bolus, then infusion 15IU/kg/h adjusted to PC 0.8-1.2IU/ml. Daily Dose: 360IU/kg Treatment period: 1-8d</td>
<td>Mean baseline PC 0.2IU/ml→1.1IU/ml in 24hours.</td>
<td></td>
<td>Heparin 10-15IU/kg/h to keep APTT ratio 1.5-2. Platelets &gt;50x10⁹/L Fibrinogen &gt;2g/L 9/12 CVVH; 1/12PD</td>
<td>None One patient had thrombotic CVA but had started PC &amp; heparin late (18h)</td>
</tr>
<tr>
<td>Kreuz</td>
<td>1998</td>
<td>Prospective Case Series</td>
<td>n=7</td>
<td>Ceprotin predecessor PCC Bolus 100IU/kg then 50IU/kg 4h adjusted to keep PC levels normal Daily dose:~300 IU/kg Treatment period 2-16d</td>
<td>Baseline PC 0.11IU/ml (0.02-0.48IU/ml) Normalisation of PC</td>
<td></td>
<td>ATIII as required 1/7 PD</td>
<td>None</td>
</tr>
<tr>
<td>White</td>
<td>2000</td>
<td>Open label, prospective case series</td>
<td>Study group n=36 (3m-72y)</td>
<td>Ceprotin predecessor test dose 100IU/kg bolus 100IU/kg infusion of 10IU/kg/h adjusted to PC level 80-120 IU/ml</td>
<td>Baseline PC lower in patients with MODS – 18vs41IU/ml PC and d-dimer levels were inversely proportional</td>
<td></td>
<td>Heparin 10iu/kg in 26/36 patients Plt &gt; 50x10⁹/L, Fib &gt;2g/L AT in 2 patients 19 CVVH</td>
<td>One intracranial haemorrhage in patient could not correct DIC.</td>
</tr>
</tbody>
</table>
### Decembri no L23 2005

**Prospective case series**

- **n=11**
- (25/40-term)
- Neonatal sepsis & low PC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ceprotin</th>
<th>PC activity %:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bolus 100 IU/kg then infusion 3 IU/kg/h x 72h</td>
<td></td>
<td>baseline 40% → 73% at 6h</td>
</tr>
<tr>
<td>Daily dose: 72 IU/kg Rx Period 72h</td>
<td></td>
<td>Remained &gt;65% for 84h</td>
</tr>
</tbody>
</table>

- **Platelets:** baseline 85 x 10^9/L, Recovered to > 150 x 10^9/L at 48h
- D-Dimers baseline 3750 mcg/L; fell but did not normalize.

### Crivellari M20 2009

**Prospective Case series**

- **n=9,** adult
- Sepsis, shock & ≥ 2 organ failure & recent cardiac surgery; APC contraindicated

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ceprotin</th>
<th>PC activity %:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bolus 50 IU/kg then infusion 3 IU/kg/h x 48h Daily dose: 72 IU/kg Rx Period 72h</td>
<td></td>
<td>Baseline 40% → 73% at 6h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Remained &gt;65% for 84h</td>
</tr>
</tbody>
</table>

- **Plt (x10^9/L):** baseline 78 → 120 (30h) did not normalize (130)
- **D-dimers:** did not normalize
- **TAT halved** 52h, did not normalise
- **PF1+2** did not normalise
- **IL6** – significant fall at 12h; IL8 at 48h, IL10 at 18h. They did not normalise
- **No FFP, platelet transfusion or ATIII APC activity remained unchanged** Fibrinogen remained unchanged.

### Gerson WT1993

**Case report**

- **N=1,** 13y Severe Sepsis & PF & DIC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ceprotin predecessor</th>
<th>PC activity %:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC 30 IU/kg 18h x 2 then 70IU 6 hourly x 2d then 10 IU/kg/h continuous infusion x 11 days</td>
<td></td>
<td>Initial PC activity 0% →50% (30IU) →&gt;100% with 70IU; kept at 150% with infusion</td>
</tr>
</tbody>
</table>

- **Fall in D dimer – did not normalize byday21** Baseline: 59200ng/ml; 48h 20210ng/ml; 96h 16330ng/ml; d213280ng/ml
- **Baseline FDPs 40mg/L (9-238; n<0.5) recovered day 7**
- **CARDFV patients had heparin 300IU/h** 4 patients had aspirin100mg/d

### Rintala E13 2000

**Case Series**

- **n=12** (adult)
- Severe Sepsis & PF & DIC & imminent peripheral necrosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ceprotin predecessor</th>
<th>PC activity %:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCC 100IU/kg 6 hourly adjusted after D1 to normal PC activity. Treatment period: med 5.5 (2-17d)</td>
<td></td>
<td>Baseline 26% (15-45%) PC activity &gt;100% within 24h</td>
</tr>
</tbody>
</table>

- **Baseline Plts 27x10^9/L (4-56)** In survivors recovery > 100 x 10^9/L by day8, >150 X 10^9/L day 10. . No recovery in non-survivors Baseline FDPs 40mg/L (8-238; n<0.5) recovered day 7
- **ATIII 50IU/kg 6 hourly then dose adjusted to normal activity. Daltaparin DVT prophylaxis**

- **4 episodes bleeding – 3 SAH not attributed to PCC**

### Faust SN15 2001

**Case Series**

- **n=2** (children)
- Meningococcal sepsis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PCC 50iu/kg 8h</th>
<th>PC activity %:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily dose: 150 IU Treatment period:3 days</td>
<td></td>
<td>PC Ag levels n</td>
</tr>
</tbody>
</table>

- **APC not detected. Pre infusion & at 1 hour**

### Fourrier Retrospective 2001

**n=15**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PCC 100IU/kg bolus then</th>
<th>PC activity: Baseline 13%</th>
</tr>
</thead>
</table>

- **Admission Plts 27 x 109/L in non-**
- **ATIII 100IU/kg bolus**

---

**Appendix 2. Investigator brochure of PC Concentrate mentioned in Chapter 6**

**Investigator Brochure for CEPROTIN**

**Version 1/4/ 2011**

**Protein C in NEC**

Sponsor number: 09AR21 EudraCT: 2010-021254-19
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Study Design</th>
<th>Patients</th>
<th>Condition</th>
<th>Treatment</th>
<th>PC Activity</th>
<th>Blood Parameters</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>F et al</td>
<td>2003</td>
<td>Case series (10 children)</td>
<td>Meningococcal sepsis &amp; shock &amp; PF</td>
<td>100IU/kg 6 hourly in infants &amp; 100IU/kg once a day in adults. Daily dose (infants): 400IU/kg</td>
<td>PC activity &gt; 21% NS</td>
<td>Only by D4 &gt; 1/2 of subjects PC &gt; 80%</td>
<td>D-Dimers low D5 PAI-1 low D3</td>
<td>then 100-150IU/kg/d x 4 days</td>
</tr>
<tr>
<td>Vaccarrell a G</td>
<td>2003**</td>
<td>Case report</td>
<td>n=1, 18yr Meningococcal sepsis &amp; shock &amp; PF</td>
<td>Bolus PCC 80 IU/kg then 2000IU 8 hourly Daily dose: 85 IU/kg</td>
<td>PC% Baseline 21% -- 78% first dose.</td>
<td>D-Dimer baseline 2400. Halved after first dose. All parameters normalized by day 7</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Ramos Guerrero R</td>
<td>2003**</td>
<td>Case series (Spanish) n=3 (children) Severe sepsis</td>
<td>Ceprotin Bolus 100 IU/kg then infusion 10 IU/kg/h to maintain plasma levels of 80-120 U/m Daily dose: 144 IU/kg</td>
<td>PC% Baseline 0-50% After bolus: 58-93%</td>
<td>Reduced D-Dimers Improved PT</td>
<td>1 patient insufficient dose given due to shortage.</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Linares – Tello F</td>
<td>2004</td>
<td>Case Report (Spanish) n=1 Meningococcal sepsis &amp; PF &amp; shock &amp; DIC</td>
<td>Ceprotin Bolus 70IU/kg then 6 IU/kg/h infusion to keep activity 95-128% Daily dose: 144 IU/kg</td>
<td>PC % baseline 27% 95-128% after 24 h</td>
<td>Pts 30 x 10^9/L baseline, 80 at 24h but did not recover to &gt; 150x 10^9/L Appt recovered to normal 24h D-Dimers returned to normal 7 days.</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silvani P</td>
<td>2004</td>
<td>Retrospective case control n=11 children 11 treated PCC 18 untreated – not given PCC Severe sepsis &amp; Septic shock.</td>
<td>PCC used in varied doses in treated patients Ave dose 324IU/kg/d (66-400IU) Continued 59h (24-120h)</td>
<td>Baseline PC activity &lt;50% at 24h &gt;50% by day 5.</td>
<td>PT normalized day 3 APPT normalized day 3 Platelets increased but did not &gt;150 x 10^9/L D-Dimers fell, normalized day 3-5</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiel AO</td>
<td>2005</td>
<td>Randomised, blinded, placebo cross-over n=11 (healthy adult volunteers) Human model of endotoxaemia</td>
<td>Ceprotin Bolus 150IU/kg/10 mins after LPS infusion then 30IU/kg/h x 4 hours Total dose: 270IU/kg</td>
<td>PC Ag and activity increased 4-5 fold.</td>
<td>APC activity paralleled PC activity TAT and PF1+2 levels lower in PC group TF mRNA lower in PC group. IL-6 no difference in PC group.</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schellong ow-ski P</td>
<td>2005</td>
<td>Case series n=8 (adults)</td>
<td>Ceprotin Dosing varied 5 subjects 10IU/kg/h</td>
<td>Baseline PC activity 29% (median) → 184% median</td>
<td>Coagulopathy resolved in 3 days in 7/8 patients</td>
<td>Heparin, FFP, ATIII&lt; rtPA, platelets as required</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>Study Type</td>
<td>n (Type)</td>
<td>Population</td>
<td>Treatment</td>
<td>PC% Baseline</td>
<td>Reduction in D-Dimers &amp; PAI-1 Levels</td>
<td>Outcome</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2006</td>
<td>Prospective Case series</td>
<td>6 (6m-11y)</td>
<td>Pediatric stem cell transplant &amp; veno-occlusive disease</td>
<td>Ceprotin Bolus PCC 40-100 IU/kg then either infusion 60-240 IU/kg/d or 60 IU/kg 12 hourly (120IU/kg/d). Treatment period: 3-18 days.</td>
<td>Baseline PC% 16-38% All PC% rose to &gt;80% over 48h</td>
<td>PC &gt;80% with dosing</td>
<td>All patients treated with defibrinogen. One also treated with rTPA. Patient treated with rTPA had respiratory tract haemorrhage, ceased after stopping TPA.</td>
<td></td>
</tr>
<tr>
<td>2007</td>
<td>Prospective Case series</td>
<td>n=20 (adults)</td>
<td>Severe sepsis, shock, PF &amp; PC &lt;50% AND APC contraindicated</td>
<td>Ceprotin Bolus dose calculated to increase activity to 100% PCC IU = (100-PC level/body weight kg) then infusion 3IU/kg/h adjusted to PC activity 70-120% Treatment period 3 days</td>
<td>Baseline PC 34.5% (22-49%) 75 at 24h, &gt;80 at 48h</td>
<td>Platelets baseline 69.3 x 10^9/L; &gt;150 by d10. DIC score by Day1 SOFA score decreased</td>
<td>4 neurological patients had pH&lt;30 3 bleeding risk 2 major surgery 2 anticoag</td>
<td></td>
</tr>
<tr>
<td>2008</td>
<td>Case series</td>
<td>n=3, adults</td>
<td>Sepsis &amp; low PC level</td>
<td>Ceprotin Bolus 100 IU/kg 6 hourly x 24 h (400IU/kg/d) then 50 IU/kg/day x 3 days</td>
<td>Supranormal PC levels described – no values given</td>
<td>Supranormal PC levels described – no values given</td>
<td>None reported</td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>Case Series</td>
<td>n=4 (28 – 34/40)</td>
<td>Neonatal septic shock &amp; microcirculation impairment</td>
<td>Ceprotin Bolus 200 IU/kg; then 50IU/kg 6 hourly x 72h. Daily dose: 200IU/kg Treatment period: 72h</td>
<td>Initial PC activity 12% (6-13) 24h: 30-60% 48h: 30-60% Did not reach 80%</td>
<td>Platelets 15-40mls/kg transfused FFP0-30ml/kg transfused Normalisation of heamostasis parameters?time Normalised MODS score 48h</td>
<td>1 patient previous IVH; 1 patient 3d post-laparotomy No Heparin. Platelets &gt; 50 x 10^9/L with transfusion ATIII&gt;30% with ATIII 50 IU/kg/d (3 patients None reported</td>
<td></td>
</tr>
</tbody>
</table>