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Necrotising Enterocolitis in Preterm Infants

Frequency, Risk Factors, Laboratory Diagnosis
and Microbiological Etiology



ACADEMIC DISSERTATION

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ABBREVIATIONS

BE	base excess
BPD	bronchopulmonary dysplasia
CPAP	continuous positive airway pressure
CRP	c-reactive protein
ELISA	enzyme-linked immunosorbent assay
GLC	gas-liquid chromatography
GLUC	glucose
HB	hemoglobin
IL	interleukin
I:T ratio	immature to total leucocyte count
IUGR	intrauterine growth retardation
IVH	intraventricular hemorrhage
LAP	laparotomy
LBW	low birth weight
LEUC	leucocyte
NEC	necrotising enterocolitis
NICU	neonatal intensive care unit
PAF	platelet-activating factor
PCR	polymerase chain reaction
PDA	patent ductus arteriosus
PLT	platelet
PPD	primary peritoneal drainage
PROM	premature rupture of membranes
PVG	portal venous gas
RDS	respiratory distress syndrome
RT-PCR	reverse transcription polymerase chain reaction
SBS	short-bowel syndrome
SD	standard deviation
TNF- α	tumor necrosis factor α
TPN	total parenteral nutrition
UAC	umbilical arterial catheter
VLBW	very low birth weight

ABSTRACT

Necrotising enterocolitis (NEC) is a neonatal disease characterised by gastrointestinal symptoms and various systemic manifestations, and by typical radiological findings and histological lesions from necrotic inflammation of the intestinal wall. It is most common among preterm infants in neonatal intensive care units (NICU).

The present aims were to investigate the frequency of and risk factors associated with NEC in preterm infants, to identify laboratory parameters predictive of NEC, to study the effects of mode of delivery and of NEC on the intestinal microflora and to evaluate the rate of nosocomial rotavirus and adenovirus infections in hospitalised neonates and to detect rotaviruses and adenoviruses in infants with NEC symptoms.

Frequency and risk factors in NEC were assessed prospectively among 140 infants born before 33 weeks gestation in Tampere University Hospital during the years 1998-1999. Twenty-six (18.6%) infants developed NEC stage I-III and 12 (8.6%) severe NEC (stage II-III). Maternal chorionamnionitis was associated with NEC. Intrauterine growth retardation (IUGR) and duration of assisted ventilation seemed to be associated with severe NEC. Early initiation of feeding with breast milk seemed to be associated with reduced risk and exposure to breast milk fortifier to be associated with increased risk of NEC. Increased duration of morphine administration seemed to be the strongest predictor of NEC and severe NEC. Further studies are needed to establish whether morphine is a causative factor in NEC.

The laboratory findings predictive of NEC were recorded in a prospective follow-up of 140 infants born before 33 weeks gestation. Twenty-six infants developed NEC (grades I-III) and for each infant two birth weight, gestational age and postnatal age-matched controls were selected. Blood counts, glucose and electrolyte levels, C-reactive protein and acid-base balance were recorded three, two and one day prior to and at the onset of NEC, and at corresponding ages from the controls. Metabolic acidosis occurred, platelet levels decreased and blood glucose levels increased on successive days in the infants with grade NEC II-III. At the onset of NEC, the infants had lower platelet and higher blood glucose levels compared to controls. More than half of those with intestinal perforation had leucocyte levels above $30 \times 10^9/l$ and pH less than 7.25, and their mean blood glucose levels increased >1.5 mmol/L in 24 hours. Such findings should alert the physician to look for signs of NEC in a preterm infant.

Symptoms suggestive of NEC in 140 infants born before 33 weeks gestation were followed up prospectively and stool samples for gas-liquid chromatography (GLC) and microbial culture were collected twice weekly. For each infant with NEC (n=21), two control infants matched for birth weight and gestational age were selected. In GLC analysis, the fecal bacterial microflora of infants born via cesarean section differed from the gut microflora of those born via the vaginal route, and the intestinal microflora showed alterations at the onset of NEC. Correspondingly, fecal colonisation with *Enterococcus* species and *Candida albicans* was more frequent at the onset of symptoms in NEC infants than in the asymptomatic matched controls. In infants with positive blood and/or intestinal biopsy cultures, concomitant stool samples revealed the same microbial pathogens. Pathogens detected in the stools at NEC onset might have a causative role in the development of the disease.

The rate of nosocomial rotavirus and adenovirus infections and the association between rotaviruses and adenoviruses detected in stools evincing symptoms of NEC were studied in 308 infants treated longer than one week in the neonatal unit during a period of 15 months from the beginning of February 1998, covering two rotavirus epidemics in the surrounding community. Altogether 1020 stool samples from the infants were collected weekly. Fecal samples were also obtained from infants with NEC at the onset of symptoms. All stool samples were tested for rotavirus and adenovirus by means of enzyme-linked immunosorbent assay (ELISA). The positive samples were further analysed by polymerase chain reaction (PCR). ELISA revealed five adeno-positive infants, who tested negative by PCR. Out of 16 NEC infants, one was adenovirus- and another rotavirus-positive when tested by PCR. Routine rotavirus and adenovirus screening in hospitalised neonates seems to be unnecessary. Viral diagnostic examinations should be considered in infants with NEC.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals.

- I. Hällström M, Koivisto A-M, Janas M, Tammela O. Frequency of and risk factors for necrotising enterocolitis in infants born before 33 weeks of gestation. *Acta Paediatr* 2003; 92: 111-113. Reprinted with permission from Taylor & Francis.
- II. Hällström M, Koivisto A-M, Janas M, Tammela O. Laboratory parameters predictive of developing necrotising enterocolitis in infants born before 33 weeks of gestation. *J Pediatr Surg*, in press. Reprinted with permission from Elsevier.
- III. Hällström M, Eerola E, Vuento R, Janas M, Tammela O. Effects of mode of delivery and necrotising enterocolitis on the intestinal microflora in preterm infants. *EJMID* 2004; 23: 463-470. Reprinted with permission from Springer-Verlag.
- IV. Hällström M, Vesikari T, Janas M, Ikonen S, Tammela O. Screening of rotavirus and adenovirus infections during prolonged hospitalisation in a neonatal unit. *Acta Paediatr* 2001; 90: 1196-1198. Reprinted with permission from Taylor & Francis.

INTRODUCTION

Necrotising enterocolitis (NEC) is well recognised among neonatologists as the most common acquired intra-abdominal emergency in the newborn infant. The characteristic feature of NEC is bowel-wall necrosis of variable length and depth, with perforation in up to one third of infants affected. This disorder primarily affects premature infants, and among them those of very low birth weight (VLBW; <1500g) constitute the majority of cases in many institutions (Kosloske 1994). As a result of the many advances in neonatal intensive care, NEC has emerged as a disease of neonatal intensive care unit (NICU) survivors.

A variety of studies have been undertaken to identify objective risk factors in infants with NEC, several of them from the era before surfactant (Stoll et al. 1980, Uauy et al. 1991, Beeby and Jeffery 1992), and there appears to be a lack of agreement in the literature. Various investigators have sought to establish laboratory measurements predictive of NEC (O'Neill et al. 1975, Hutter et al. 1976, O'Neill and Holcomb 1979, Buras et al. 1986, Gupta et al. 1994a, Ververidis et al. 2001, Ragazzi et al. 2003). Most previous studies are, however, from the pre-surfactant era and retrospective, and investigate only NEC infants, lacking control infants without NEC. To our knowledge no studies have been undertaken of the effects of mode of delivery in preterm infants nor the effects of NEC on the fecal microflora examined by means of gas-liquid chromatography (GLC) analysis. Although many organisms have been associated with NEC, most are frequently found to colonise the intestine. Investigation by standard microbiological methods has not identified any single causative microbe consistently associated with NEC (Gupta et al. 1994b). Adenovirus has been associated with symptoms of systemic infection and rotavirus with apneic-bradycardic spells, diarrhea and NEC in newborn infants (Rotbart et al. 1983, Riedel et al. 1996, Rosenlew et al. 1999).

Our study aimed to assess the frequency of NEC and risk factors associated with NEC among infants born before 33 weeks gestation. We also sought to identify laboratory findings predictive of NEC and to investigate the effects of mode of delivery and of NEC on fecal microflora. A further goal was to evaluate the rate of nosocomial rotavirus and adenovirus infections in hospitalised neonates and to detect rotaviruses and adenoviruses in cases with NEC symptoms.

REVIEW OF THE LITERATURE

Definition and clinical staging of NEC

Necrotising enterocolitis (NEC) is a serious medical and surgical problem in newborn infants, especially those born preterm. Despite over a century of clinical research and interest in this disease, its pathogenesis remains unproven, preventive strategies are controversial, and morbidity and mortality remain important problems.

Paltauf was the first to identify cases of this disorder in 1888, but the term “Necrotising enterocolitis” was first used by Schmid and Quaiser in 1953. However, it was not until the 1960s, when Santulli and associates reported a series of preterm infants with NEC at Babies Hospital, that it became recognised as a distinct clinical entity (Santulli et al. 1975). Over the past few decades there has been a marked increase in the incidence of NEC cases, mainly attributed to the increase in the number of premature births as a result of the more frequent use of cesarean section before 34 weeks of gestation for therapeutic reasons (Kramer et al. 1998), and to the fact that in the modern era of neonatal intensive care units (NICUs) and surfactant therapy, most premature infants are able to overcome a number of previously fatal health problems and survive, thus rendering themselves susceptible to NEC (Kosloske 1994).

NEC is characterised by gastrointestinal and systemic signs and symptoms including feeding intolerance, delayed gastric emptying, abdominal distention or tenderness, occult or gross blood in the stool, lethargy, apnea, respiratory distress and poor perfusion. In advanced cases, associated acidosis, shock, bacteremia and disseminated intravascular coagulopathy develop. The diagnosis is based on clinical presentation. Clinical findings must be confirmed by results of diagnostic radiographs, surgery or autopsy.

In 1978 Bell and coworkers proposed a clinical staging for infants with NEC (Bell 1978b). Infants are thereby classified as having stage I (suspect), stage II (definite) or stage III (advanced) disease. In stage I there is often a history of perinatal stress. Systemic manifestations include temperature instability, lethargy, apnea and bradycardia and gastrointestinal manifestations poor feeding, increasing gastric residuals, emesis, mild abdominal distension and occult blood

in stools. Abdominal radiographs show distension with mild ileus. In stage II NEC, persistent occult or gross gastrointestinal bleeding and marked abdominal distension are seen. Abdominal radiographs show significant intestinal distension with ileus, small-bowel separation, unchanging or persistent “rigid” bowel loops, pneumatosis intestinalis and portal vein gas. In Stage III disease, deterioration of vital signs, evidence of septic shock or marked gastrointestinal hemorrhage develop. Abdominal radiographs may show pneumoperitoneum.

Walsh and Kliegman 1986 modified Bell’s criteria to include systemic, intestinal and radiographic signs and to suggest treatment based on stage and severity of illness. Infants with suggestive clinical signs and symptoms but non-diagnostic results of radiographs are classified as stage I (suspect NEC). Infants with stage II disease (definite NEC) yield diagnostic abdominal radiographs (pneumatosis intestinalis) and are mildly ill (stage IIA) or moderately ill (stage IIB with acidosis, thrombocytopenia and ascites). Infants with stage III disease (advanced NEC) are critically ill with impending (stage IIIA) or proven (stage IIIB) intestinal perforation.

Table 1. Modified staging criteria for necrotising enterocolitis according to Walsh and Kliegman (1986).

Stage	Classification	Systemic Signs	Intestinal signs	Radiologic signs
IA	Suspected NEC	Temperature instability, apnea, bradycardia lethargy	Increased pre-gavage residuals, mild abdominal distention, emesis, quaiac-positive stools	Normal or intestinal dilation, mild ileus
IB	Suspected NEC	Same as above	Bright-red blood from rectum	Same as above
IIA	Proven NEC- mildly ill	Same as above	Same as above, plus absent bowel sounds, with or without abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
IIB	Proven NEC- moderately ill	Same as above, plus mild metabolic acidosis and mild thrombocytopenia	Same as above, plus absent bowel sounds, definite abdominal tenderness, with or without abdominal cellulitis or right lower quadrant mass	Same as IIA, plus portal vein gas, with or without ascites
IIIA	Advanced NEC- severely ill, bowel intact	Same as IIB, plus hypotension bradycardia, severe apnea, combined respiratory and metabolic acidosis, disseminated intravascular coagulation, and neutropenia	Same as above, plus signs of generalised peritonitis, marked tenderness, and distension of abdomen	Same as IIB, plus definite ascites
IIIB	Advanced NEC- severely ill, bowel perforated	Same as IIIA	Same as IIIA	Same as IIB, plus pneumoperitoneum

Radiological findings of NEC

The diagnosis of NEC depends largely upon radiographic findings. According to Bell (1985), stage I (suspected NEC) is characterised by abdominal distension and ileus findings in plain abdominal radiographs. Stage II (definite NEC) includes intestinal pneumatosis and portal venous air on X-ray. Walsh and Kliegman (1986) further divided this stage into A for “mildly ill” and B for “moderately ill”. Stage III (advanced NEC) is characterised by pneumoperitoneum seen in radiographs. Walsh and Kliegman (1986) stage III type A presents with bowel intact, whereas type B is with perforated bowel.

The earliest roentgenographic finding is most commonly a non-specific intestinal dilation and edema. Intestinal pneumatosis may be either linear (subserosal) or cystic (submucosal), the accumulation of gas being produced by gas-forming bacteria in the submucosa or subserosa (Ricketts 1994). Pneumatosis intestinalis is present in 70-80% of cases (Kosloske 1979). However, this finding can appear with a significant delay, transiently, and often misinterpreted as air mixed with feces, blood or meconium. Other signs of intestinal gangrene or impending perforation are portal venous gas (PVG), a fixed distended bowel loop, free intraperitoneal fluid or pneumoperitoneum.

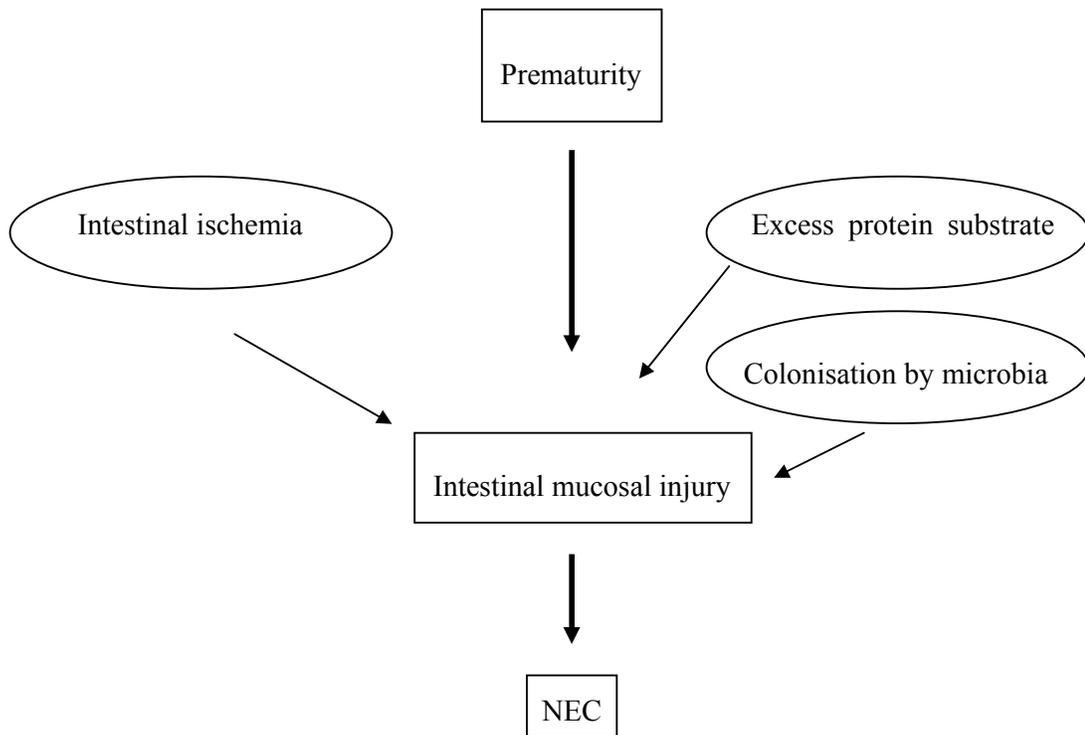
PVG is identified on the abdominal radiograph in 25% of NEC cases (Kosloske 1979) as a linear branching lucency within the portal venous system. Portal venous gas has often been reported to be associated with pan-intestinal involvement in the VLBW infant (Merrit 1984). Portal vein ultrasonography appears to be much more sensitive in the detection of PVG compared with plain radiography (Lindley et al. 1986), and as a result of its portable nature is a reliable and readily available tool for the early diagnosis of NEC in any questionable case (Merrit 1984). Ultrasonography is also used to evaluate the presence and character of ascites (particulate matter probably indicates perforation), and to identify a site for paracentesis (Chandler et al. 2000).

Air in the Morison pouch, which appears as a triangular lucency in the right upper quadrant, represents an early sign of pneumoperitoneum, and is thus significant as a clear indication for surgery (Brill et al. 1990). Rapid diagnosis of pneumoperitoneum has also been reported by transillumination of the abdomen using a cold fiber-optic light source in neonates with NEC (Dutta et al. 1998).

Pathogenesis of NEC

Although NEC is a prominent cause of neonatal morbidity and mortality, its pathogenesis remains incompletely understood. Available theories do not satisfactorily explain the spectrum of observed manifestations of the disease. NEC most likely represents a complex interaction of factors predisposing to mucosal injury and the infant's subsequent response (Neu 1996).

Figure 1. Factors involved in the pathogenesis of NEC.



NEC occurs concomitant with prematurity by the coincidence of two of three pathological events: 1) intestinal ischemia; 2) excess protein substrate in the intestinal lumen; and 3) colonisation by pathogenic bacteria (Kosloske 1984). This hypothesis derives from previous theories by Santulli and coworkers (1975), who implicated all three events, and by Lawrence and coworkers (1982), according to whom a single event, abnormal bacterial colonisation, was considered sufficient to induce NEC.

The assumption of Kosloske (1984) has also been supported by findings of pathologists reviewing specimens with NEC, which invariably showed coagulation (ischemic) necrosis, inflammation and bacterial overgrowth, all present in varying degrees of severity (Ballance 1990). Reparative tissue changes such as epithelial regeneration, granulation tissue formation, and fibrosis were also found in the majority of cases, suggesting ongoing tissue injury of at least several days' duration (Ballance 1990).

Intestinal ischemia clearly occurs in NEC, as evidenced by the histopathologic presence of inflammatory cell infiltration, mucosal edema, ulceration and coagulative necrosis (Ledbetter and Juul 2000). It is unclear, however, whether ischemia is the primary initiator or the end result of intestinal injury. Early observational studies reported a significant association between numerous ischemic events such as perinatal asphyxia, umbilical arterial catheterisation, polycythemia, exchange transfusion, RDS and cyanotic congenital heart disease (Covert et al. 1989) and the development of NEC. Medications such as indomethacin and methylxanthines, which have been shown to reduce superior mesenteric blood flow, have also been implicated. Many of these perinatal insults were believed to induce the "diving seal reflex" by which blood flow is selectively shunted away from non-vital organs such as the intestine (Kliegman 1990, Kosloske 1994). However, many infants with NEC have no history of perinatal depression at birth, and do not present with signs of NEC until several weeks of life. Recent epidemiologic studies also fail to confirm an association between these hypoxic factors and the development of NEC (Covert et al. 1989). The conflicting data suggest that ischemia may be a secondary event reflecting a culmination of the various factors.

Prematurity is the only pathogenetic factor consistently found in epidemiologic studies to be an independent determinant of NEC. The increased susceptibility is attributed to an immature mucosal barrier and barrier response (Neu 1996). In the presence of low intraluminal gastric acid and proteolytic activity, the incompletely innervated, poorly organised, relatively permeable epithelial barrier is vulnerable to bacterial colonisation and pathogenic overgrowth (Israel 1994, Van Camp et al. 1994, Neu 1996). In premature infants, the humoral and cellular response to this overgrowth is impaired.

Enteral feedings have been implicated as a significant contributor in the pathogenesis of NEC. Although NEC can occur in infants who have never been enterally fed, 90-95% of cases occur in infants with a history of recent volume advancement or re-initiation of enteral feedings (Stoll 1994). The introduction of feedings into the intestinal lumen presumably causes a disruption of mucosal integrity, blood flow and motility. A substantially higher incidence of NEC has been reported in formula-fed compared with exclusively breast-fed infants, and attributed to a lack of immuno-protective factors (Lucas and Cole 1990). The timing, initial volume and advancement of feedings are important factors in

determining the degree of the ensuing mucosal insult. Since much of the research in this area is conflicting, no consensus has been reached regarding the most effective feeding regimen.

The well-documented epidemics of NEC and the improvement in attack rate following the implementation of strict infection control measures validate the role of infection in the pathogenesis of NEC. The role of bacteria is two-fold. Fermentation of carbohydrate substrates by bacteria leads to the formation of hydrogen gas (the gas found in pneumatosis intestinalis). Furthermore, as mucosal integrity is compromised, bacteria “translocate” to regional lymph nodes and activate resident macrophages. Colonisation of the intestine with bacterial species must precede bacterial translocation (Van Camp et al. 1994). The physiological growth of intestinal microflora and the pathological modifications of this microflora have been assessed by means of fecal bacterial measurements.

A large number of inflammatory mediators as well as molecular mechanisms involved in the pathogenesis of NEC have been studied in an attempt to obtain a clear conception of this disorder and to discover novel preventive measures. Among these, platelet-activating factor (PAF)(Caplan et al. 1994, Caplan et al. 1997), defensin messenger RNA (Salzman et al. 1998), nitric oxide and inducible nitric oxide synthase (Ford et al. 1997, Nadler et al. 2000), IL-1, IL-6 and TNF- α (Viscardi et al. 1997), IL-11 (Nadler et al. 2001), magnesium and copper deficiency (Caddell 1996), oxygen-derived free radicals (Akisu et al. 2002), a lack of epidermal growth factor (Shin et al. 2000), heparin-binding hepatocyte growth factor (Srivastava et al. 1999), intestinal trefoil factor (Tan et al. 2000), hematopoietic cytokines (Ledbetter and Juul 2000), cyclo-oxygenase 2/nuclear factor kappa B pathway (Chung et al. 2001) are all potential contributors in the development of NEC.

Epidemiology of NEC

NEC mainly affects infants in NICUs, and both sporadic cases and nosocomial outbreaks have been described (Boccia et al. 2001). No seasonal pattern has been reported. In most studies, male and female infants have been found to be equally affected. Age at onset is also inversely related to gestational age. In full-term infants, NEC appears to occur at a median age of two days, with more than 40% presenting on the first day of life (Stoll 1994). Immature infants continue to be at prolonged risk of NEC; Stoll reported a mean age at diagnosis of 20.2 days for infants born at 30 or fewer weeks' gestation, 13.8 days for those born at 31 to 33 weeks, and 5.4 days for those born at 34 or more weeks (Stoll 1994). Among infants of gestational ages < 28 weeks, the median age of onset was 22 days, whereas in infants born between 28 and 32 weeks and between 33 and 36 weeks gestation, the median ages of onset were 13 and 4 days respectively (Llanos et al. 2002). The risk of NEC persists until a postconceptional age of at least 36 weeks is reached.

There are few population-based studies of the epidemiology of NEC previous to the introduction of surfactant. A study by Wilson and associates (1981) reported an incidence of NEC of 0.9 cases per 1000 live births, Ryder and colleagues (1980) a rate of 2.4 cases of NEC per 1000 live births and a group under Wiswell (1988) 1.3 per 1000 live births from 1980 to 1985 in the United States.

The study by Llanos and coworkers (2002) represents a population-based (117 892 newborns) analysis of the epidemiology of NEC in the post-surfactant era in the United States. In this study NEC annually affects 0.72 infants per 1000 live births among all neonates. The risk of developing NEC was 1.61 times higher during the period 1995-98 than 1991-94 (Llanos et al. 2002).

Full-term neonates account for only 5–25% of all cases of NEC (Ng 2001), which clearly underlines the association between preterm birth and NEC. The incidence varies inversely with birth weight and gestational age (Covert et al 1989, Stoll 1994). The group of low-birth-weight infants (LBW <2500g) had a 144.8 higher risk of developing NEC than infants with birth weights \geq 2500g (Llanos et al. 2002). Those most susceptible appear to be infants weighing less than 1000g at birth and under 28 weeks gestation (Rowe et al. 1994, Chandler and Hebra 2000).

The incidence of NEC varies from country to country as well as among different centres within the same country. NEC affects 33 infants per 1000 live births

among VLBW infants in the United States (Llanos et al. 2002). In Japan the incidence of NEC among VLBW infants has reached 1-2%, in Austria 7%, in Greece 10%, in Argentina 14%, and in Hong Kong 28% (Eibl et al. 1988, Halac et al. 1990, Siu et al. 1998). In United States the incidence of NEC in infants with birth weights <750g was 16.4% during the period 1991-94 and 17.4% during the period 1995-98. In infants with birth weights 750-999g it was 65.6% during the period 1991-94 and 28.6% during the period 1995-98. The incidence of NEC with birth weights 1000-1500g was 15.9% during the period 1991-94 and 57.2% during the period 1995-98 (Llanos et al. 2002).

Risk factors in NEC

Although extensive research has been undertaken concerning the etiology of this devastating disease, it has not yet been fully elucidated. A variety of studies have sought to identify objective risk factors in infants with NEC, several of them from the era before surfactant (Stoll et al. 1980, Uauy et al. 1991, Beeby and Jeffery 1992), and there appears to be a lack of agreement in the literature. Differences between study designs and populations may be assumed to explain the controversy.

Potential risk factors associated with the development of NEC in full-term infants might differ from the corresponding factors in preterm infants. In the former, reported risk factors include congenital heart disease such as coarctation, perinatal asphyxia, hypoglycemia, polycythemia, respiratory distress, protracted diarrhea, maternal pre-eclampsia (Goldberg et al. 1983, Wilson et al. 1983, Hasegawa et al. 1997), myelomeningocele and lipomyelomeningocele (Costello et al. 1988, Chang et al. 1997), cow's milk protein intolerance (Eggertsen and Pereira 1989) and anti-c rhesus incompatibility (Roig and Burchfield 1994). A study evaluating infants born weighing more than 2000g (Martinez-Tallo et al. 1997) considered prolonged rupture of the membranes, chorionamnionitis, an Apgar score of less than 7 at 1 and 5 min, respiratory problems, congenital heart disease, hypoglycemia and exchange transfusions as factors frequently present in this group of infants with NEC.

It seems that NEC is the product of an interaction of numerous factors, prematurity being the single parameter most frequently encountered. Earlier work has almost consistently demonstrated an increasing risk of NEC with decreasing birth weight and gestational age (Stoll et al. 1980, Uauy et al. 1991, Beeby and Jeffery 1992). Among other characteristics at admission, cesarean section, race, gender (Uauy et al. 1991), multiple birth (Samm et al. 1986), low cord pH and low 1-minute Apgar scores (Beeby and Jeffery 1992) have been described as risk factors underlying NEC.

Various additional risk factors in preterm infants before and at birth, and factors connected with postnatal problems and their treatments during the neonatal period have been implicated. Among prenatal factors, maternal indomethacin treatment for preterm labor (Norton et al. 1993), maternal cocaine use (Engum and Grosfeld 1998) maternal hemorrhage and prolonged duration of rupture of membranes (Uauy et al. 1991) have been thought to increase the risk of NEC. Maternal chorionamnionitis might cause intestinal microbial colonisation and NEC (Caplan and MacKendrick 1994). IUGR has been found to increase the risk of NEC (Hackett et al. 1987, Beeby and Jeffery 1992). Hypoxic insult to the gut, predisposing to NEC, seems to occur in growth-retarded fetuses due to placental insufficiency, especially in cases such as end-diastolic block or reversed end-diastolic flow in umbilical arterial Doppler flow velocity waveforms (Hackett et al. 1987, Craigo et al. 1996), although other studies do not show such an association (Kirsten et al. 1999, Baschat et al. 2000).

An association of respiratory disease with NEC has been rarely reported (Lucas and Cole 1990, Tapia-Rombo et al. 1993), and mostly in studies from the era before surfactant (Bunton et al. 1977, Palmer et al. 1987, Smith et al. 1980) although other work from the same era show no such association (Stoll et al. 1980). Hypoalbuminemia (Atkinson et al. 1989), symptomatic or subclinical perinatal infection (Gibbs 2001) and hypoxic ischemic encephalopathy (Tapia-Rombo et al. 1993) have been associated with NEC. Indomethacin treatment of patent ductus arteriosus (PDA) has been claimed to increase the risk of NEC, at least when prolonged courses are used (Tammela et al. 1999). Other studies (Stoll et al. 1980, Beeby and Jeffery 1992) find no association between the occurrence of PDA and NEC, regardless of the method of treatment. Controversial data can also be found concerning the role of umbilical arterial catheters (UACs) in the development of NEC, suggesting either prolonged UAC use as a predisposing factor (Palmer et al. 1987), or no correlation between UAC and NEC (Stoll et al. 1980, Beeby and Jeffery 1992).

Enteral feeding as a risk factor for the development of NEC was formerly a matter of consensus (Eyal et al. 1982, Kliegman et al. 1982), and since 1978 a careful feeding regimen has been recommended for premature infants (Brown and Sweet 1978), although 5-10% of NEC cases occur in patients who have never been enterally fed (Kliegman and Fanaroff 1981). A paper by Schanler and associates suggests that early initiation of enteral feeding is of benefit (Schanler et al. 1999). In a large randomised study on 962 preterm infants by Lucas and Cole (1990), delayed enteral feeding seemed to protect from NEC in formula-fed, but not in breast-milk-fed infants. Early enteral feeding was associated with a reduced risk of nosocomial sepsis but not with the risk of NEC in VLBW infants. These findings support the use of early enteral feeding, but needs to be confirmed in a large randomised controlled trial (Flidel-Rimon O et al. 2004). Hyperosmolarity of the feedings caused by breast milk fortifier has also been

suggested to have a role in the development of NEC in preterm infants (Book et al. 1975).

Increased duration of morphine administration as a risk factor has not either been reported. Morphine infusion has improved breathing in synchrony with ventilator and shortened the duration of oxygen supplementation in preterm infants (Dyke et al. 1995). In a study by Anand (Anand et al. 1999) the pilot trial suggests that preemptive analgesia given by continuous low-dose morphine infusion may reduce the incidence of poor neurologic outcomes in preterm neonates who require ventilatory support, but a recent study by the same investigator with 898 ventilated preterm neonates from 16 centres (Anand et al. 2004) suggests that pre-emptive morphine infusions did not reduce the frequency of severe IVH, PVL or death in ventilated preterm infants. Additional morphine doses reduced clinical signs of pain, but were associated with significant adverse effects in ventilated preterm infants. Gastrointestinal symptoms or NEC were not evaluated.

Laboratory parameters predictive of NEC

Infants affected by NEC exhibit, to varying degrees, laboratory abnormalities such as leucopenia or leucocytosis, anemia, thrombocytopenia, hypo- or hyperglycemia, electrolyte abnormalities, metabolic acidosis and clotting abnormalities.

Various investigators have sought to identify laboratory measurements predictive of NEC, among them metabolic acidosis (Buras et al. 1986), hyponatremia (O'Neill et al. 1975, O'Neill and Holcomb 1979), thrombocytopenia (O'Neill et al. 1975, Hutter et al. 1976, O'Neill and Holcomb 1979, Ververidis et al. 2001), a low absolute granulocyte count (Hutter et al. 1976), leucopenia (Gupta et al. 1994a) high ratios of immature to total leucocyte counts (I:T ratios) (Gupta et al. 1994a), platelet-neutrophil product (Ragazzi et al. 2003) and clotting abnormalities. Mainly, the purpose has been to search for indications for surgery, aiming to detect intestinal gangrene before intestinal perforation develops.

Anemia of multifactorial origin is observed concomitant with NEC. Hemolytic anemia, blood loss anemia, iatrogenic anemia and anemia of prematurity occurs in infants at highest risk of NEC (Kling and Hutter 2003). Neutrophilia with or without increased I:T ratio is seen in less severe NEC as a normal adaptive response to inflammation. However, neutropenia is seen in severe NEC (Kling and Hutter 2003) and has been associated with poor prognosis (Hutter et al. 1976). The reference values of normal neonates for the maximum I:T proportion for the first 24 hours of life is 0.16, falling then gradually to 0.13 by 60 hours and remaining at the same level until 120 hours of age. From 5 to 28

days of age it remains unchanged at a value of 0.12. An increased I:T proportion was observed to be predictive in 52 to 82% of counts associated with bacterial infection (Manroe et al. 1979). In infants of more than 34 weeks gestation, lower total neutrophil counts, higher immature neutrophil number, and greater immature: total neutrophil ratio at first presentation of NEC predicted which infants ultimately required surgical treatment of NEC, but these parameters became insignificant in the low-birth-weight group below 34 weeks (Schober and Nassiri 1994).

Infants with severe NEC develop thrombocytopenia (Hutter et al. 1976, Patel 1977, Mehta et al. 1980). Lower platelet counts have been reported to be associated with greater disease severity (Hutter et al. 1976), the prevalence of thrombocytopenia being particularly high in infants requiring laparotomy (O'Neill and Holcomb 1979). A sudden, profound drop in platelet count has been held to predict the presence of a gangrenous bowel (O'Neill et al. 1975, Ross et al. 1989) and may indicate the need for operative intervention (Foglia 1995). In infants born at 24-27 weeks gestation the lowest median platelet count and the lowest median activities of coagulation factors II, VII and X were observed. The levels increased with increasing gestational age (Salonvaara et al. 2003).

Tissue hypoperfusion and liver dysfunction induce increased lactic acid production and metabolic acidosis in critically ill preterm infants with sepsis (Fitzgerald et al. 1992). Acidosis has been regarded as an indication for surgery in NEC (Buras et al. 1986). Persistent pH less than 7.2 not responding to adequate fluid volume replacement has emerged as an ominous prognostic sign of bowel necrosis (Buras et al. 1986).

The serum glucose level may be normal, elevated or decreased in NEC, depending on the amount of stress, sepsis and vascular compromise the infant is experiencing (Foglia 1995). Hypoglycemia occurs fairly commonly among infected infants in the neonatal period, especially associated with infections caused by gram-negative bacilli. In contrast, most infected infants who remain normoglycemic have gram-positive organisms and mixed flora (Yeung et al. 1970). On the other hand, hyperglycemia and simultaneously abnormally low insulin levels, indicating inadequate insulin response, have been reported in an infant suffering from *Escherichia coli* sepsis (James and Blessa 1979). Recently Hall and coworkers studied 95 infants with confirmed NEC. Maximum glucose concentration was determined for each infant. Hyperglycemia is common in infants with NEC admitted to the NICU, 69% becoming hyperglycemic. Hyperglycemia is associated with an increase in late mortality; infants with hyperglycemia have a higher mortality rate (33%) than infants without hyperglycemia (18%). Hyperglycemia was the only independent factor significantly related to length of stay in intensive care unit (Hall et al. 2004).

Urine samples from neonates with NEC show significantly higher computed tomography attenuation coefficients than those from neonates without NEC after enteral administration of iodinated water-soluble contrast material, iohexol. In NEC infants with intestinal mucosal injury, iohexol permeates through the bowel wall to the peritoneum and is excreted by the kidneys into the bladder. Computed tomography examination of urine may allow early detection of NEC and iohexol is said to be well tolerated (Rencken et al. 1997).

Treatment of NEC

Treatment of infants with NEC includes a regimen of bowel rest (nothing-by-mouth status for up to 10-14 days), gastric decompression by orogastric drainage, systemic antibiotics and parenteral nutrition. Infants with perforation are generally operated upon; however, there has recently been interest in primary peritoneal drainage as an alternative.

Initial broad-spectrum antibiotic coverage consists according to Lee and Polin (2003) of ampicillin covering streptococcus viridans-species, enterococcus (not *Enterococcus faecium*), gram-positive anaerobes, gentamycin covering aerobic gram-negative rods and clindamycin covering aerobic gram-positive streptococcus, staphylococcus and anaerobes. With the increasing prevalence of infections from coagulase-negative staphylococcus, vancomycin may be used instead of ampicillin, vancomycin covering staphylococcus, streptococcus and enterococcus (Lee and Polin 2003). Amikacin, covering aerobic gram-negative rods and metronidazole covering anaerobes, has been substituted for gentamycin and clindamycin (Foglia 1995). However, antimicrobial choices should be guided by local resistance patterns. Enteral antibiotics are not recommended (Lee and Polin 2003).

In cases where persistent clinical deterioration or signs of impending perforation or intestinal gangrene are present, operative intervention may be considered. In the absence of pneumoperitoneum, abdominal paracentesis may be helpful in confirming the presence of intestinal gangrene (Ricketts 1994). Currently, intestinal perforation remains the only absolute indication for laparotomy. The cardinal principle in surgical management is excision of grossly necrotic segments and exteriorisation of viable ends to allow for continued bowel decompression (Foglia 1995). Recently, primary peritoneal drainage (PPD) has been proposed as an alternative to surgical treatment. Ein and colleagues first described the use of PPD for perforated NEC in VLBW infants in 1977; however, its use was limited to unstable VLBW infants (Ein et al. 1977). Although initially implemented as a temporising measure, a number of published reports documented the successful use of PPD as adjunctive therapy prior to

planned laparotomy (LAP) or as definitive therapy, particularly in extremely-low-birth-weight infants less than 1000g (Cheu et al. 1988, Takamatsu et al. 1992, Morgan et al. 1994, Azarow et al. 1997). A meta-analysis by Moss and associates also demonstrated comparable combined probability of survival for infants with perforated NEC who were treated with either procedure (67% in the LAP group vs 55% in the PPD group, $p=0.27$) even in the presence of a significant treatment assignment bias favouring the LAP group, a greater proportion of smaller infants undergoing PPD compared with LAP (931 vs. 1615g). The study did not show an advantage for either group (Moss et al. 2001). A potential extension of the use of peritoneal drains in NEC is Moore's "patch, drain and wait" laparotomy approach, which consists in limited patching of major perforations, gastrostomy tube drainage, bilateral peritoneal drains and long-term parenteral nutrition (Ricketts 1994, Moore 2000). In cases of isolated or multifocal NEC, some investigators advocate resection and primary anastomosis. When massive pneumatosis intestinalis without definite intestinal gangrene is present, proximal diversion via a high jejunostomy is recommended to minimise bacterial proliferation (Ricketts 1994).

Outcome of NEC

Although infant mortality has diminished overall, NEC-associated mortality has increased from 11.5 to 12.3/100 000 (Holman et al. 1997) or 10-30% of all NEC cases across centres (Caplan and Jilling 2001a). Approximately 20-70% of infants with NEC require surgical intervention (Engum and Grosfeld 1998, Stoll 1994), and it has been reported that those who more often require this treatment modality are the ones who belong to the lowest gestational age and birthweight group (Stanford et al. 2002). Among infants undergoing laparotomy due to NEC, the postoperative mortality has been 23% (Llanos et al. 2002) and among infants of birth weights less than 1500g 26% (Camberos et al. 2002). The majority of postoperative complications are related to the stoma or wound. NEC has been reported to recur in up to 6% of infants; however, no consistent association has been shown between recurrence and feeding regimen, anatomical site of injury, or management of initial disease (Stringer et al. 1993). Total parenteral nutrition (TPN)-related complications, including cholestasis and liver function failure may also occur. Infants with NEC also have extended hospitalisations (22 days in medical NEC and 60 days in surgical NEC longer than their healthy matched controls) (Bisquera et al. 2002). Although VLBW infants with severe disease may be at higher risk of adverse outcomes (Tobiansky et al. 1995, Sonntag et al. 2000), neurodevelopmental outcome measured at school age appears favourable in most infants (Stanford et al. 2002). Long-term sequelae, including short-gut syndrome, milder degrees of malabsorption and intestinal strictures develop in 10 to 20% of survivors (Stevenson et al. 1980, Llanos et al. 2002); 48% of

survivors are completely normal children and 15% of all survivors have moderate to severe neurologic impairment (Stevenson et al. 1980).

Short-bowel syndrome

Short-bowel syndrome (SBS) is a condition characterised by rapid intestinal transit time leading to malabsorption of nutrients and diarrhea with growth retardation in children (Fleming and Remington 1981, Shanbhogue and Molenaar 1994, Vanderhoof and Langnas 1997, Wilmore and Robinson 2000, Scolapio 2002, Buchman et al. 2003). SBS has been defined based on the expected jejunoileal length for gestational age; in neonates operated on between 27 and 35 weeks gestation, the remaining jejunoileal segment had to be < 50 cm and for neonates over 35 weeks, <72 cm (Galea et al. 1992). SBS occurs in 8-23% of operated NEC infants (Ricketts and Jerles 1990, Horwitz et al. 1995, Patel et al. 1998). The incidence of SBS associated with NEC has been seen to decrease from 23% to 6-8% after a follow-up of 10 months to 7 years, respectively (Jackman et al. 1990, Ricketts and Jerles 1990). However, in addition to being the most important cause of SBS in children, NEC also carries the poorest SBS prognosis (Galea et al. 1992, Georgeson and Breaux 1992, Bueno et al. 1999, Thompson et al. 2000). Some success with intestinal transplantation has been reported in this subset. Although experience is limited, the overall reported one- and three-year survival rates for intestinal transplantation are 60 and 54%, respectively (Vennarecci et al. 2000).

Prevention of NEC

Numerous preventative measures have been studied since the initial recognition of NEC as a distinct clinical entity. Some of these seem quite promising and are currently being further investigated, whereas others have been more or less abandoned by reason of their limited applicability or potential risks accompanying them.

NEC has been observed in the form of epidemics associated with specific infectious agents, and infection control practices have thus predictably been shown to limit such epidemics (Book et al. 1977). Prophylactic enteral antibiotic administration has also been applied, apparently to reduce bacterial overgrowth (Stiehm 1990). Avoidance of preterm birth, use of antenatal steroids (Halac et al. 1990) and breast-milk feeding (Kosloske 1994) are practices which offer the

greatest potential benefits, probable but not proven efficacy (Lee and Polin 2003).

Among trends most commonly mentioned in the literature in the context of prevention-albeit of unproven efficacy or limited data- are passive immunisation with oral IgA and IgG (Eibl et al. 1988, Siu et al. 1998), egg phospholipid-containing neonatal formula enhancing immature intestinal functions to lower the incidence of NEC (Carlson et al. 1998), elevated levels of nitric oxide produced by enterocytes in the intestinal wall of infants with NEC (Ford et al. 1997), magnesium and copper deficiency contribute to NEC (Caddell 1996), the enhancement of PAF acetylhydrolase activity with steroids or recombinant enzyme reducing the incidence of PAF-induced intestinal injury in NEC (Caplan et al. 1997), and PAF receptor antagonist WEB 2170 reducing the incidence of PAF-induced NEC (Caplan 1994).

More recently novel preventative modalities have been attempted, for example the administration of recombinant human erythropoietin to increase stimulated erythropoiesis in NEC infants (Brown and Keith 1999), recombinant human granulocyte colony-stimulating factor to increase the neutrophil count in NEC infants (Kocherlakota and La Gamma 1997), pentoxifylline affecting the synthesis of TNF and IL-6 as well as reducing the mortality rate in premature infants with sepsis (Lauterbach et al. 1999), beneficial effects of cyclosporine and rapamycin in small-bowel ischemic injury (Puglisi et al. 1996); beneficial effects of L-arginine and L-carnitine on hypoxia/reoxygenation-induced NEC may be mediated via mechanisms preventing free radical damage (Akisu et al. 2002), the acidification of feeds to prevent bacterial overgrowth (Carrion and Egan 1990), dietary polyunsaturated fatty acid reducing the incidence of NEC with associated changes in PAF metabolism and bacterial translocation (Caplan and Jilling 2001b), epidermal growth factor stimulating intestinal repair processes (Dvorak et al. 2002) and vitamin A supplementation ameliorating intestinal mucosal injury (Nafday et al. 2002). Administration of probiotics, including *Lactobacillus acidophilus* and *Bifidobacterium infantis* to neonates in intensive care has been suggested to reduce NEC and mortality associated with NEC in a study with historical control cases. (Hoyos et al. 1999). *Lactobacillus acidophilus* and *Bifidobacterium infantis* as probiotics fed enterally with breast milk reduces the incidence and severity of NEC in VLBW infants (Lin et al. 2005). These strategies to prevent NEC are likewise still of unproven efficacy or with limited data.

Development of intestinal microflora

At birth, an infant's gastrointestinal tract is sterile but rapidly becomes colonised with organisms from the mother and the local environment. Organisms are

introduced into the sterile fetal intestine by contact with maternal vaginal flora and the rooming-in ward or NICU. During the first days of life enterococci and members of the family *Enterobacteriaceae* constitute the predominant organisms in stools from full-term infants (Rotimi and Duerden 1981, Yoshioka et al. 1983). Thus Van Camp and colleagues found that species such as *Escherichia coli*, streptococci and *Bacteroides* are commonly isolated during the immediate neonatal period (Van Camp et al. 1994). Colonisation with aerobic and anaerobic flora normally occurs by 10 days of age (Kosloske 1994). Bifidobacteria then become predominant in stools from most breast-fed infants, while *Enterobacteriaceae*, *Bacteroides* species and clostridia remain at high levels in the stools of formula-fed infants (Long and Swenson 1977, Balmer et al. 1989). In the Van Camp study over the first few weeks to months, the relative concentrations of *Escherichia coli* and streptococci declined as the concentrations of lactobacilli and *Bacteroides* rise (Van Camp et al. 1994). Introduction of enteral feedings alters this pattern of intestinal colonisation. Formula is associated with an early appearance of *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella*, whereas breast feeding induces an early appearance of *Enterobacteriaceae* and *Bifidobacterium* (Van Camp et al. 1994, Claud and Walker 2001). Regardless of the choice of feeding, bifidobacteria gradually come to predominate. Fecal colonisation in full-term infants born by cesarean section is delayed, and infants born vaginally are colonised with bacteria of the *Bacteroides fragilis* group more often than those born by cesarean section (Grönlund et al. 1999).

In preterm infants fed mostly with formula, fecal flora studies from the pre-surfactant era show a delayed colonisation with predominantly gram-negative aerobic flora and few anaerobes (Goldmann et al. 1978, Blakey et al. 1982, Bell et al. 1984). In infants requiring neonatal intensive care, colonisation occurs slowly. Following the initiation of feedings in these infants, only a few species are present. If the hospitalisation remains uncomplicated, enteric colonisation continues to diversify. However, colonisation is often delayed or reversed by interruptions in the feeding regimen or by the administration of broad-spectrum antibiotics (Bennet et al. 1986, Kosloske 1994, Van Camp et al. 1994). Feeding has a significant effect on the composition of the intestinal microbial colonisation in full-term infants (Yoshioka et al. 1983, Balmer and Wharton 1989), and in extremely low birth weight infants (Gewolb et al. 1999), although results to the contrary in healthy infants have been published (Lejeune et al. 1984).

Infectious agents associated with NEC

The role of infection in the pathogenesis of NEC is not known, but there is evidence to suggest that microbes are to some degree involved in the process (Willoughby and Pickering 1994). Although many organisms have been associated with NEC, most are those frequently found to colonise the intestine. Infants who developed NEC have proved more likely to have fecal colonisation with aerobic gram-negative organisms than infants without NEC in the same NICU (Bell et al. 1978a).

Investigation by standard microbiological methods has failed to identify any single causative agent consistently associated with NEC (Gupta et al. 1994b). Bacteremia has been documented in up to 35% of NEC cases. Although no single pathogen has been consistently identified, *Escherichia coli*, *Klebsiella*, *Serratia*, *Enterobacter*, *Pseudomonas*, *Salmonella*, *Clostridium perfringens*, *Clostridium difficile*, *Clostridium butyricum*, coagulase-negative staphylococci, *Enterococcus*, coronavirus, rotavirus and enterovirus have been associated with NEC (Bell et al. 1979b, Powell et al. 1980, Cushing 1983, Kliegman and Fanaroff 1984, Kosloske et al. 1985, Scheifele 1990, Millar et al. 1992, Willoughby and Pickering 1994)(Table 2.). The type of organism recovered varies with disease severity. In modified Bell stages I and II NEC, gram-positive organisms appear to be the predominant pathogens recovered. As NEC worsens, enteric organisms are more commonly isolated (Uauy et al. 1991). The gastrointestinal flora of infants with NEC appears to differ from that of similar but uninfected infants, and temporal variations seem to occur in the resident gastrointestinal microflora of infants in an NICU environment, with concomitant variations in the incidence of NEC (Bell et al. 1979a). The same pathogenic organisms with identical antibiotic susceptibility patterns have been detected in both stool and blood samples from NEC infants with positive blood cultures (Roback et al. 1974).

Table 2. Infectious agents associated with necrotising enterocolitis.

Viruses

Coronavirus
Enterovirus
Rotavirus

Aerobic Gram-positive Bacteria

Enterococcus species
Coagulase-negative staphylococcus
Staphylococcus aureus

Aerobic Gram-negative Bacteria

Enterobacter species
Escherichia coli
Klebsiella species
Pseudomonas aeruginosa
Salmonella species
Serratia species

Anaerobic Gram-positive Bacteria

Clostridium butyricum
Clostridium difficile
Clostridium perfringens

Fungi

Candida albicans

Sporadic enteroviral and adenoviral infections have been held to be fairly common etiologic agents in newborn infants hospitalised for symptoms suggestive of systemic infection (Rosenlew et al. 1999). Rotavirus epidemics in newborn nurseries have been described in the United Kingdom (Chrystie et al. 1978), Australia (Bishop et al. 1983), Sweden (Grillner et al. 1985), the US (Rodriguez et al. 1982) and several developing countries, including India (Jayashree et al. 1988, Cicirello et al. 1994), Bangla Desh (Kilgore et al. 1996) and Venezuela (Perez-Schael et al. 1984). The source of the nursery outbreaks has in several reports been admission of infants infected with rotavirus (Van Rentgerhem et al. 1980, Rodriguez et al. 1982, Dearlove et al. 1983, Grillner et al. 1985). Nosocomial rotavirus infection has been proved to be associated with a high incidence of apneic-bradycardic spells in diarrheic premature neonates (Riedel et al. 1996) and more rarely with NEC (Rotbart et al. 1983). Sharma and colleagues reported 129 NEC cases in 2444 admissions in the NICU. Thirty-eight (29%) were rotavirus positive. In their study rotavirus-associated NEC was

a less severe disease than NEC associated with other organisms. Monitoring rotavirus infection in the community, adhering to infection control measures, human milk feedings and improving neonatal immunity against rotavirus infection may reduce the incidence of rotavirus-associated NEC (Sharma et al. 2004). Nonetheless the presence of virus in the stools seems mostly be either asymptomatic or associated with mild symptoms even in premature infants (Van Rentgerhem et al. 1980). Feeding with breast milk as well as the mother's handwashing before touching the infant has been described to offer some protection against rotavirus acquisition among neonates (Totterdell et al. 1976, Jayashree et al. 1988). Adenovirus infections can be a cause of less severe neonatal infections with respiratory symptoms (Rosenlew et al. 1999) or the clinical course of the illness can be severe, including progressive pneumonia, coagulopathy and death (Abzug and Levin 1991, Aebi et al. 1997). The correlation of rotavirus and adenovirus epidemics in the community with virus acquisition rates in hospitalised newborn infants is not known. Also, the role of the presence of virus in the stools in relation to their gastrointestinal and other types of symptoms is unknown.

AIMS OF THE STUDY

To evaluate the frequency and the most important risk factors in necrotising enterocolitis in infants born before 33 weeks gestation (I)

To identify early laboratory findings possibly predictive of the development of necrotising enterocolitis with or without intestinal perforation in infants born before 33 weeks gestation (II)

To establish the effect of mode of delivery on the fecal microbial colonisation in infants born before 33 weeks of gestation and to investigate the fecal microbial flora in infants born before 33 weeks of gestation developing or not developing necrotising enterocolitis and to seek associations between findings in dominant fecal bacterial and fungal cultures in infants born before 33 weeks of gestation and developing necrotising enterocolitis (III)

To evaluate the rate of nosocomial rotavirus and adenovirus infections in stools in newborn neonates during prolonged hospitalisation in a neonatal intensive care unit and to study the association between rotaviruses and adenoviruses detected in the stools and symptoms of necrotising enterocolitis in infants treated in a neonatal unit (IV)

PATIENTS AND METHODS

Patients and study design

The study population consisted of 140 consecutive infants born before 33 weeks gestation and admitted to the neonatal intensive care unit (NICU) of Tampere University Hospital from February 1998 to October 1999. All infants were prospectively followed up for any gastrointestinal or infectious symptoms.

In study I, 26 infants developed symptoms of NEC (the NEC group); 12 of them had NEC gr II-III (the severe NEC group). The rest of the original study population were the controls (N=114). Clinical data were obtained from obstetric and patient records concerning risk factors for NEC. The NEC group and the severe NEC group were compared to the control group.

In study II, 14 of the 26 infants who developed symptoms of NEC (the NEC group) developed NEC stage I, four stage II and eight stage III with intestinal perforation. The stage III infants underwent laparotomy. The subgroup with NEC gr II-III (N=12) and those with intestinal perforation NEC gr III (N=7) were also studied separately. For each NEC infant, two control infants matched according to birth weight, gestational and postnatal age were selected from the original study population (the control group, N=52). Results of blood tests made in the NEC group three days, two days and one day before NEC and at the onset of NEC symptoms were recorded, as well as those from blood tests of the controls at the corresponding time points as clinically indicated.

In study III, stool samples were available for dominant culturable bacterial and fungal cultures from 21 infants who developed symptoms of NEC (the NEC group). For each NEC infant, two control infants matched according to gestational age, birth weight and post conceptional age were selected from the original study population (the control group, N=42). Sufficient numbers of stool samples for GLC analysis were available from 18 of the NEC infants. For statistical comparison, two subgroups, each consisting of 18 infants, were created from the original control group, control group 1 matching better with the NEC infants than control group 2 in respect of gestational age and birth weight. Stool samples for GLC and dominant microbial cultures were collected twice weekly from birth until discharge from the NICU or up to two months of age. Upon suspicion of NEC, microbial cultures from blood and stools were obtained and in cases of intestinal perforation, biopsy samples for cultures were obtained at laparotomy.

In study IV, the study population included all infants admitted to the neonatal intensive care unit between February 1998 and April 1999, whose duration of hospitalisation exceeded one week. The duration of the screening was fifteen months in order to cover two periods of rotavirus epidemics. The epidemics were monitored by recording the rotavirus- and adenoviruspositive cases of acute gastroenteritis treated in the pediatric wards in Tampere University Hospital. Altogether 308 infants entered the study, their median duration of hospitalisation being 23 (range 7-377) days. Of this total, 210 were admitted directly from the delivery room and 60 from the rooming-in wards; 38 were referred from other hospitals. Altogether 181 infants were preterm and 127 term, 20 had birth weights less than 1000g, 35 between 1000 and 1500g, and 253 between 1500g and 5160g. About one-third of the infants were treated in the intensive care unit, of these 83 preterm and 20 term. Altogether 167 were hospitalised on account of various minor problems, including prematurity, suspected infection, hypoglycemia, hyperbilirubinemia and transient tachypnoea; 38 infants were admitted for congenital anomalies. Stool samples for rotavirus and adenovirus screening by means of enzyme-linked immunosorbent assay from study infants were collected every Tuesday until discharge from the hospital. Positive samples were further analysed by polymerase chain reaction. During this period, necrotising enterocolitis was diagnosed in sixteen infants, eight stage I and eight stage II-III with intestinal perforation. In these cases stool specimens were obtained at the onset of NEC symptoms.

Facilities of the neonatal unit and neonatal management of infants born before 33 weeks' gestation

The neonatal unit at Tampere University Hospital, a referral unit with a catchment area of about one million inhabitants, is a 25-bed ward, including a neonatal intensive care unit of eight beds. It has a common anteroom with washing facilities. Only one of the nine infant rooms in the ward is an isolation room with an airlock. Before entering the ward, visitors are instructed to wash their hands carefully. The infants' family members are allowed to visit the ward freely, provided they have no symptoms of infection.

Diagnostic criteria for NEC were clinical symptoms and signs, including feeding intolerance, abdominal distension and motility difficulties, abnormal consistency of stools or blood in the stools, and radiographic findings (e.g. pneumatosis intestinalis, a dilated loop, pneumoperitoneum and /or presence of portal venous gas), interpreted by a pediatric radiologist. The NEC infants were classified according to modified Bell's criteria (Bell et al. 1978b). All infants diagnosed as having NEC were treated in a similar manner. Oral feedings were discontinued and a nasogastric tube placed to decompress the abdomen. Fluids were administered intravenously and TPN given. The infants were treated with

intravenous antibiotics, cefotaxime, metronidazole and netilmycin, for seven to ten days. The initial empirical combination was changed, if necessary, according to the susceptibility of the causative organism.

Maternal infection was defined as symptoms of chorionamnionitis with C-reactive protein levels of more than 20 mg/l, the symptoms developing within 72 hours before delivery, and in chorionamnionitis cases the mothers received intravenous ampicillin, netilmycin and metronidazole before delivery. Ampicillin and netilmycin was the empirical antibiotic combination used in suspected neonatal early onset sepsis.

Continuous morphine infusion at a dosage of 10-30 micrograms/kg per hour was initiated in all ventilator-treated infants for pain relief and sedation. In addition, intravenous morphine boluses of 100 micrograms/kg were administered if the patient evinced pain, and prior to painful procedures. The infusion was tapered and discontinued at the discretion of those responsible, if it was felt that the general condition of the patient was improving and the patient better tolerated the treatment and handling procedures. If the infant showed hemodynamic instability requiring inotropics, enteral feeding was withheld until inotropic infusion could be stopped. In such cases and if milk was not tolerated, total parenteral nutrition (TPN) was administered from the second or third day of life onwards. A high position, the tip above the diaphragm, was used when umbilical arterial catheters (UAC) were inserted. The maximum duration of UAC use was 10 days and the catheter was removed immediately in cases with developing NEC symptoms. Breast milk administration was initiated on the first or second day of life, in cases without the contraindications mentioned, with the mothers' own breast milk and /or donated banked breast milk at a dosage of 20 ml/kg/day, and the daily dosage was increased by a maximum of 20 ml/kg/day, the proportion of TPN being reduced correspondingly. Breast milk fortifier (PreSemp®, 0.8 g protein/bag, 7 kcal energy/bag, 2.15 g/bag, 2.15g/100 ml breast milk) was administered to infants of birth weights less than 1500 g, when a full dose of breast milk was reached, up to a weight of 2000 g.

Intrauterine growth retardation (IUGR) was determined as a birth weight lower than two standard deviations from the mean weight for gestational age. Respiratory distress syndrome (RDS) was defined as typical findings in chest X-rays, need for assisted ventilation and surfactant administration, this being used as rescue therapy at the discretion of the attending neonatologist. A significant patent ductus arteriosus (PDA) was diagnosed according to clinical symptoms and confirmed by echocardiography (Tammela et al. 1999). The definition of bronchopulmonary dysplasia (BPD) included need of oxygen supplementation and typical findings in chest X-rays at a corrected age of 36 weeks' gestation (Shennan et al. 1988). Cranial ultrasound examination was performed in each case 2-3 times during the first week of life, once weekly up to the age of four weeks and once in two weeks later until hospital discharge. Intraventricular hemorrhages (IVH) were diagnosed and classified according to Papile and colleagues (1978).

Methods

Assessment of medical history (I)

Clinical data concerning risk factors for NEC were obtained from obstetric and patient records, including information on maternal history and medications, birth history, prolonged rupture of membranes, development of chorionamnionitis and abnormal findings in the umbilical arterial Doppler velocity waveforms, the infants' gestational age, birth weight, sex, Apgar scores, hospital course and medications, feeding history, gastrointestinal symptoms, clinical and radiological findings with NEC, outcome and duration of hospital stay.

Laboratory measurements (II)

Blood tests included hemoglobin, hematocrit, platelet and leucocyte counts, I:T. ratios (immature neutrophil count as a proportion of the total neutrophil count), C-reactive protein (CRP), pH, base excess, glucose, sodium and potassium levels.

Gas-liquid chromatography (III)

The stool samples were stored at -70°C and gas-liquid chromatography (GLC) analysis subsequently performed at the Department of Medical Microbiology at the University of Turku, Finland. GLC was used to evaluate the bacterial fatty acid profiles of the samples. The bacterial material was first separated from other components and free fatty acids in the material (Vaahtovuori et al. 2001). GLC of the bacterial cellular fatty acids was performed as previously described (Eerola and Lehtonen 1988). The collected bacterial mass was saponified, methylated and analysed. In brief, the harvested bacteria were incubated for 30 minutes at 100°C in 15% (wt/vol) NaOH in 50% aqueous methanol and then acidified to pH 2 with 6N aqueous HCl in CH_3OH . The methylated fatty acids were then extracted with ethyl ether and hexane. The GLC analysis was performed with an HP6890A gas chromatograph with an Ultra 2 (cross linked 5% PH ME Siloxane) 25m X 0.2 mm column (HP 19091 B) combined with HP ChemStation analysis software. Ultra-high-purity helium was used as the carrier gas.

Bacterial and fungal cultures (III)

The dominant culturable bacterial and fungal cultures were studied at the Department of Clinical Microbiology in Tampere University Hospital, Finland. Stool samples were cultured semi-quantitatively on selective and non-selective agars. One to three most dominant species detected in this semi-quantitative assessment were determined as the dominant culturable flora, and different colonies were counted and identified. For aerobic flora blood agar, chocolate agar, MacConkey agar, Staphylococcus medium number 110 agar (Oxoid, UK) and DIXO-agar for yeasts were used. For anaerobic flora egg yolk agar and Bacteroides bile esculin agar were used. Both aerobic and anaerobic bacteria were cultured for 48 h at 37°C. Anaerobic cultures were done in an anaerobic cabinet (Concept 300, Ruskinn Technology Ltd, UK). Aerobic bacteria were cultured in a 5% CO₂ atmosphere.

Blood samples for cultures were injected into Bactec® blood culture bottles. Biopsy samples obtained at laparotomy were cultured for aerobic agar and thioglycolate agar.

Enzyme-linked immunosorbent assays (IV)

Stool specimens were tested for rotavirus by means of ELISA (IDEA™ Rotavirus kit, DAKO Ltd, UK) according to the manufacturer's instructions. The test detects Group A rotaviruses in human fecal samples with virus particle counts as low as 7.8×10^5 per ml. The samples were also tested for adenovirus by ELISA (IDEA™ Adenovirus kit, DAKO Ltd, UK). The test detects adenovirus hexon antigen, utilising a monoclonal antibody, in a solid-phase sandwich enzyme immunoassay.

Polymerase chain reaction (IV)

Adenovirus-positive samples were further analysed by polymerase chain reaction (PCR) using both E 1b and hexon specific primers as described by Allard and associates (1990, 1992). The stool samples of the NEC infants were tested with PCR for adenovirus (Allardt et al. 1990, 1992) and reverse transcription- (RT-) PCR for rotavirus, using primers for VP7- antigen, as described by Gouvea and colleagues (1990). The conditions of the RT-PCR assay have been described elsewhere (Pang et al. 1999).

Statistical analyses

Mean and SD or median and range for skewed distributions are given as descriptive statistics. Differences between the NEC group, the NEC grade II-III group, the NEC grade III group and the control group were tested by Chi-square test and Fisher's exact test for categorical variables and Student's t-test and Mann-Whitney U test for continuous variables.

To evaluate the independent effect of each possible risk factor, multivariate logistic regression analysis was used in study I. The potential risk factors associated with NEC were grouped for statistical analysis in five groups: prenatal factors, characteristics at birth, ventilation, hemodynamics and nutrition and fluid therapy. First each risk factor group was entered in the model separately. Thereafter a combined logistic regression model was formed from those variables which were statistically significant in each separate model.

In study II Bonferroni correction was used, $p < 0.01$ being considered statistically significant. Analysis of variance for repeated measures was used to determine whether there were statistically significant changes in laboratory parameters and whether the changes differed between the NEC grade II-III and control group during the study period. In the case of skewed distributions, logarithmic transformation was used. CRP was tested by Friedman's test separately for NEC gr II-III group and control group, because transformations failed to normalise the distribution.

In study III the differences between the NEC and control groups, infants born by cesarean section and by vaginal delivery were tested by Chi-square test and Fisher's exact test for categorical and Student's t-test and Mann-Whitney U test for continuous variables. Kruskal-Wallis test and one-way analysis of variance were used for multiple comparisons. The analysis of GLC data was based on computerised comparisons of bacterial cellular fatty acid profiles. Each bacterial species has a typical cellular fatty acid composition and chromatogram (Fig. 2). The cellular fatty acid profile of a multi-bacterial sample consists of cellular fatty acids of all bacteria present in the sample. The analysis of the GLC chromatograms was based on the assumption that samples with similar bacterial composition would yield identical fatty acid profiles. The samples were compared to each other and similarity indices calculated for each sample pair. These indices were presented as correlation matrices. To calculate the statistical significance of a difference between two groups, the variation in cellular fatty acid profiles within each group was compared to that between the groups. The variation within a group was determined by calculating the mean \pm standard deviation (SD) for all paired comparisons within a given group. The variation between two groups was calculated by comparing each cellular fatty acid profile in one group to all profiles in the other group. The mean \pm SD was calculated for all of these comparisons (Fig. 3). The samples obtained from NEC and control groups were arranged according to group allocation. The samples were also further subdivided according to day of sampling. Finally, the variation

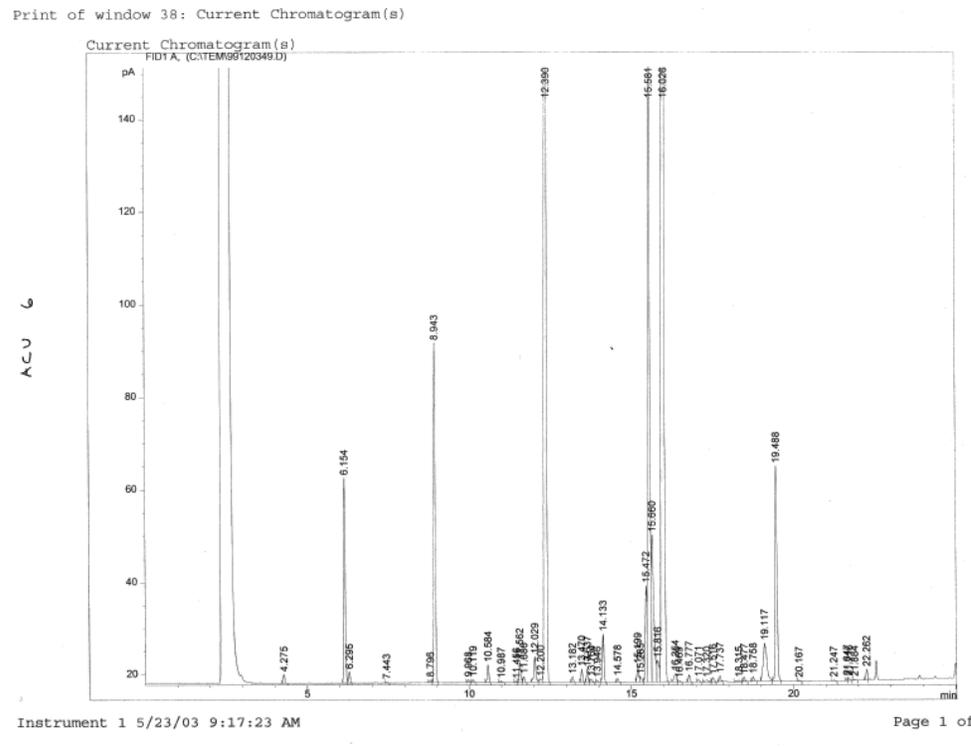
between the groups was compared to that within the groups by calculating a z-value in order to determine the P-value from the z-table (Eerola and Lehtonen 1988).

Data were analysed using SPSS for Windows version 10.0 statistical software (SPSS, Chicago, IL). P-values <0.05 were considered statistically significant.

Ethics

The Ethical Committee of Tampere University Hospital had approved the study protocol and informed written consent was obtained from the parents.

Figure 2. Chromatogram showing a fatty acid profile of a stool sample. The peaks represent the relative amount of each individual fatty acid.



RESULTS

Frequency of and risk factors in NEC

Of the 140 infants studied, 26 (18.6%) developed necrotising enterocolitis, 12 of these (8.6%) grade II-III (severe NEC). The clinical characteristics of the NEC, severe NEC and control groups are presented in Table 3 (I). The percentages of cases with maternal glucocorticoid or indomethacin medication, maternal toxemia or diabetes were similar in both NEC groups and in the controls. Intrauterine growth retardation was statistically significantly more frequent in the severe NEC group than in the controls. Premature rupture of the membranes (PROM) had occurred in about half of the cases in the NEC and control groups. The numbers of mothers receiving antibiotics within 72 hours before delivery did not differ statistically significantly between the groups. However, preterm delivery of the mothers in the whole NEC group was connected with chorionamnionitis statistically significantly more frequently than in the control group, the difference between the severe NEC cases and the controls being no longer statistically significant. Umbilical artery Doppler examination before birth had been performed in only 78 cases, 9 (34.6%) in the NEC group and 69 (60.5%) in the control group. Umbilical artery Doppler block was seen in more than half of the cases examined in the NEC group, a statistically significantly larger percentage than in the control group.

The NEC infants were born at statistically significantly earlier weeks of gestation and were of lower birth weights than the control infants. Also the mean one- and five-minute Apgar scores were statistically significantly lower in the whole NEC group, but not in the severe NEC group compared with the control group. About half of the infants in all groups were born via vaginal route and the female/male ratios in the groups were similar. Multiple birth was statistically significantly less common in both NEC groups than among the controls, the latter group also including, in addition to twins, ten triplet infants.

Respiratory problems, including the rate of respiratory distress syndrome, as well as the number of cases with apneic spells managed with theophyllin medication, were more common, and the median durations of assisted ventilation, nasal continuous positive airway pressure (CPAP) treatment and oxygen supplementation statistically significantly longer in the NEC than in the control group. To facilitate weaning from the ventilator, infants in the NEC group received dexamethasone statistically significantly more often compared with the controls.

There were more cases receiving morphine medication, and the duration of morphine administration was statistically significantly longer in the NEC group. A similar difference between the groups was found in the use of inotropics for hemodynamic instability. The occurrence of a significant PDA was statistically significantly more frequent in the severe NEC group, and the PDAs needed management for closure either by indomethacin or surgical ligation more frequently in the NEC groups than in the controls.

Obviously, as a consequence of NEC itself and its management, the median duration of TPN was statistically significantly longer in the NEC group than in the controls. Use of UACs did not differ among the groups. Enteral feeding was initiated, the full dose of breast milk was reached and breast milk fortifier started at statistically significantly later ages in the NEC group compared to controls. Breast milk fortifier was used statistically significantly more frequently in the NEC cases.

Table 3. Clinical characteristics of NEC, severe NEC and control infants (p* all NEC vs. controls and p** severe NEC vs. controls), ¹Median (range)

	NEC INFANTS N=26	p*	SEVERE NEC INFANTS N=12	p**	CONTROLS N=114
Prenatal factors					
Maternal chorioamnionitis	15 (57.7%)	0.016	6 (50.0%)	0.223	37 (32.5%)
IUGR	6 (23.1%)	0.117	4 (33.3%)	0.034	13 (11.4%)
Umbilical artery Doppler block (N=78)	5 (55.6%)	<0.001	3 (60.0%)	0.002	7 (10.1%)
Characteristics at birth					
Gestational age (weeks, mean (SD))	26 (2,0)	<0.001	27 (1,9)	<0.001	29 (2,6)
Birth weight (g, mean (SD))	952 (322,4)	<0.001	926 (217)	<0.001	1440 (497)
Apgar score 5 min (mean (SD))	5.85 (2.3)	<0.001	6.8 (2.3)	0.223	7.51 (2,02)
Ventilation					
Assisted ventilation (days) ¹	14.0 (0-62)	<0.001	10.0 (0-49)	<0.001	0.0 (0-51)
Oxygen supplementation (days) ¹	53.5 (0-104)	<0.001	41.5 (2-94)	<0.001	2.0 (0-108)
Hemodynamics					
Morphine use (days) ¹	5.0 (0-15)	<0.001	7.5 (0-15)	<0.001	0.0 (0-14)
Use of inotropics (days) ¹	3.0 (0-19)	<0.001	3.5 (0-14)	0.001	0.0 (0-9)
PDA	9 (34.6%)	0.069	7 (58.3%)	0.002	21 (18.4%)
Nutrition and fluid therapy					
TPN (days) ¹	22.5 (5-61)	<0.001	28 (5-61)	<0.001	1.5 (0-30)
Age at start of breast milk (days) ¹	4.0 (1-23)	<0.001	3.5 (1-23)	<0.001	1.0 (0-30)
- start at an age < 4 days	18 (69.2 %)	0.002	5 (41.7%)	0.001	104 (91.2 %)
Breast milk fortifier	21 (80.8%)	0.001	9 (75.0%)	0.040	50(43.9%)

In the NEC group 10 (38.5%) and in the control group 11 (9.6%) of the infants developed BPD ($p<0.001$) and 4 (15.4%) ($p=0.859$) of the infants in the groups had IVH, respectively. Three infants in the NEC group died, all due to NEC. Nineteen in the control group died (7 of severe IVH, 7 of pulmonary hypoplasia, 3 of severe RDS, and two due to pulmonary hemorrhage). The median duration of hospital stay was 83.0 days in the NEC group and 36.5 days in the control group ($p<0.001$).

The NEC symptoms started at a median of 6 (range 2-34) days after birth. The mean (SD) gestational age of those in whom NEC developed later than this was 26.5 (1.4) weeks and in the remaining NEC cases 27.4 (2.3) weeks, $p=0.08$. In four cases the symptoms developed before commencement of enteral feedings. According to the classification (Bell et al. 1978b), 14 infants had NEC grade I, 4 NEC grade II and 8 NEC grade III. Twelve infants showed pneumatosis intestinalis in abdominal X-rays. Eight had perforation of the intestine and underwent laparotomy.

According to logistic regression analysis the statistically most significant prenatal factor associated with NEC was maternal infection, maternal indomethacin medication being almost statistically significant (Table 4). Of the characteristics at birth, multiple birth seemed to be associated with low incidence of NEC statistically almost significantly. Of the ventilation variables, a long median duration of assisted ventilation was associated with an increased risk of NEC. The most statistically significant hemodynamic factor associated with NEC was increased duration of morphine administration. Use of breast milk fortifier was a risk factor, whereas age of less than four days at start of breast milk seemed to be associated with low incidence of NEC among the nutrition and fluid therapy variables.

In the combined model, according to logistic regression analysis, the most prominent factors related to NEC were increased duration of morphine administration and use of breast milk fortifier.

In the corresponding logistic regression analysis including only the severe NEC infants, IUGR emerged as a statistically significant prenatal factor (OR 4.55, 95% CI 1.00, 19.5, $p=0.041$) and increased duration of assisted ventilation (OR 1.24, 95% CI 1.03, 1.50, $p=0.023$) and morphine administration (OR 1.59, 95% CI 1.21, 2.10, $p=0.0011$) were the statistically significant postnatal factors associated with NEC.

In the combined model including only the severe NEC infants, duration of morphine administration (OR 1.91, 95% CI 1.26, 2.90, $p=0.002$) remained the only risk factor to reach statistical significance.

Table 4. Risk factors in the NEC and control groups according to logistic regression analysis.

	P	OR	95%CI for OR
Prenatal factors			
Maternal glucocorticoids	0.808	0.834	0.194-3.588
Maternal indomethacin	0.091	2.400	0.868-6.632
Maternal toxemia	0.902	1.086	0.290-4.064
Maternal infection	0.033	2.955	1.090-8.010
PROM (days)	0.293	0.999	0.996-1.001
IUGR	0.106	2.640	0.813-8.570
Characteristics at birth			
Gestational age (weeks)	0.481	0.860	0.566-1.308
Birth weight (g)	0.207	0.998	0.996-1.001
Delivery route	0.425	1.558	0.524-4.626
Sex	0.136	2.299	0.769-6.875
Multiple birth	0.094	0.345	0.099-1.200
Apgar 5 min	0.342	0.891	0.701-1.131
Ventilation			
RDS	0.377	1.813	0.485-6.781
Duration of			
- assisted ventilation (days)	0.093	1.079	0.988-1.178
- NCPAP therapy (days)	0.148	1.061	0.979-1.149
- oxygen supplementation (days)	0.991	1.000	0.961-1.041
Theophyllin medication(days)	0.477	1.015	0.974-1.058
Dexamethasone (days)	0.891	0.983	0.773-1.251
Hemodynamics			
Morphine (days)	0.002	1.302	1.105-1.534
Inotropics (days)	0.066	1.189	0.989-1.430
PDA	0.857	0.895	0.268-2.987
Nutrition and fluid therapy			
Umbilical artery catheter (days)	0.136	1.121	0.965-1.302
Age at start of			
breast milk < 4 days	0.022	0.268	0.086-0.829
Breast milk fortifier	0.016	3.846	1.287-11.494
Combined model			
Maternal infection	0.328	1.755	0.569-5.416
Maternal indomethacin	0.135	2.612	0.741-9.211
Multiple birth	0.123	0.307	0.069-1.375
Duration of			
assisted ventilation (days)	0.263	1.031	0.977-1.087
Morphine (days)	0.029	1.264	1.025-1.559
Age at start of			
breast milk <4 days	0.980	0.977	0.156- 6.124
Breast milk fortifier (days)	0.042	3.812	1.047-13.875

Laboratory parameters predictive of NEC

The mean hemoglobin value was marginally statistically significantly lower in the NEC group than in the control group on the day of the onset of NEC symptoms (Table 5). The corresponding hematocrit values and absolute leucocyte counts did not differ statistically significantly. More than half of the infants with intestinal perforation had leucocyte counts higher than $30 \times 10^9/l$ and marginally statistically significantly higher median leucocyte counts compared to the controls. Data on I:T ratios were available for only about half of the infants in all groups and comparisons between NEC and control cases did not reach statistical significance. The platelet levels were statistically significantly lower in the whole NEC group and in both subgroups than in the controls. In blood gas measurements more than half of the infants with intestinal perforation had pH less than 7.25. The pH value was marginally significantly lower in the NEC and statistically significantly in the NEC grade III group than in the control group. Metabolic acidosis occurred in the infants with grade NEC III. The base excess was marginally significantly lower in the whole NEC group and in the NEC grade II-III group, and statistically significantly lower in the NEC grade III group than in the control group. As there was no statistically significant difference between the groups in median CRP, mean sodium and median potassium values, these parameters were not useful. The median blood glucose level was statistically significantly higher in the NEC and in the NEC grade III compared to the control group. In the infants with intestinal perforation glucose levels rose within 24 hours from day 3 to day 4 by a median 1.5 mmol/l.

According to analysis of variance for repeated measures the hemoglobin ($p=0.006$), the hematocrit ($p=0.038$), the platelet ($p=0.029$) and the potassium ($p=0.006$) values were statistically significantly lower in the NEC grade II-III group than in the control group. In the NEC grade II-III group the platelet level decreased on successive days 1 to 4, whereas in the control group it was increasing, the magnitude of the change differing statistically significantly between the groups ($p=0.001$). The leucocyte counts ($p=0.016$) and the I:T ratios ($p=0.014$) were statistically significantly higher in the NEC grade II-III group than in the control group. In the NEC grade II-III group the glucose levels rose on days 1 through 4 ($p=0.048$) and were significantly higher than in the control group ($p<0.001$).

Table 5. Laboratory values on the day of onset of NEC symptoms in the NEC, the NEC gr II-III, the NEC gr III and the control groups.

	NEC N = 25	P ₁	NEC gr II-III N = 11	P ₂	NEC gr III N = 7	P ₃	Control N = 51
Hb, mean (SD) (range)	133 (18) (103-181)	0.027	129 (17) (107-158)	0.055	133 (18) (112-158)	0.171	146 (21) (117-207)
Leuc, median (range)	13.4 (3.0-58.5)	0.474	14.5 (4.5-53.1)	0.188	30.4 (9.5-53.1)	0.043	11.1 (3.3-54.6)
>30	5(19.2%)	0.065	4(33.3%)	0.006	4(57.1%)	<0.001	3(5.8%)
PLT, mean (SD) (range)	203 (84) (40-374)	<0.001	193(83) (78-371)	<0.001	172(72) (78-280)	0.001	316 (109) (62-625)
	N = 26		N = 12		N = 7		N = 40
pH, mean (SD) (range)	7.26 (0.11) (6.86-7.40)	0.011	7.28 (0.08) (7.14-7.40)	0.095	7.24 (0.06) (7.14-7.29)	0.009	7.32 (0.08) (6.99-7.47)
<7.25	10(38.5%)	0.011	5(41.7%)	0.022	4(57.1%)	0.005	6(12.8%)
BE, mean (SD) (range)	-7.9 (5.1) (-17.1-1.5)	0.011	-8.0 (5.4) (-16.8-1.5)	0.014	-11.4 (3.4) (-16.8- -6.3)	<0.001	-5.4 (3.2) (-11.9-0.6)
Gluc, median (range)	5.5 (2.4-10.2)	0.002	5.4 (2.9-10.2)	0.066	6.0 (3.7-10.2)	0.002	3.9 (1.9-11.3)
Gluc4-3, mean (range)	0.63 (-2.4-3.5)	0.498	0.82 (-2.4-3.5)	0.456	1.59 (-1.0-3.5)	0.026	0.37 (-1.6-6.2)

Effects of mode of delivery and NEC on the intestinal microflora

No statistically significant differences were found between NEC and control groups in mode of delivery or in administration of antimicrobial drugs to the mother during delivery. Almost all NEC and control infants received antibiotics after birth and the duration of use was similar.

In the analysis of the whole study population, the fecal fatty acid profiles of infants born via the vaginal route differed statistically significantly from those of infants born by cesarean section ($p = 0.001$).

Figure 3 shows the difference within the NEC group on each sampling day in comparison with the first stool sample obtained. The graph shows the difference calculated as mean \pm (SD) values of the similarity indices between individual sample pairs. In the NEC group, an increased difference from baseline prior to the onset of NEC (D1, D2) was seen in the stool fatty acid profiles on the day NEC was diagnosed (D3) and thereafter (D4, D5, D6, D7). The difference between samples obtained on the day before NEC (D2) and those collected on the day of diagnosis of NEC (D3) was statistically significant in GLC analysis, $p=0.028$. In both control groups no such alteration and no statistically significant difference was seen.

The inter-group comparisons between the NEC and control group 1, and between control groups 1 and 2, revealed no statistically significant differences in GLC analyses of stool samples, whereas the difference between the NEC group and control group 2 was highly significant, $p < 0.001$.

At the age of seven days, *Staphylococcus* species dominated both in the infants born by cesarean section and in those born via the vaginal route in the dominant bacterial and fungal cultures from the stools. In the vaginally born infants *Escherichia coli* was significantly more frequent at the same age.

In the dominant bacterial and fungal cultures from the stool samples obtained when NEC was diagnosed *Enterococcus* species and *Candida albicans* colonisation was statistically significantly more frequent in the NEC infants than in the controls.

Five NEC infants had positive blood cultures, and the same organisms cultured from blood were detected in the stool samples obtained concomitantly when NEC was diagnosed. Of these infants, three were colonised with *Candida albicans*, one with *Serratia marcescens*, and one with *Staphylococcus epidermidis*, the last two isolates having the same antibiotic sensitivity as the ones detected in the stool samples.

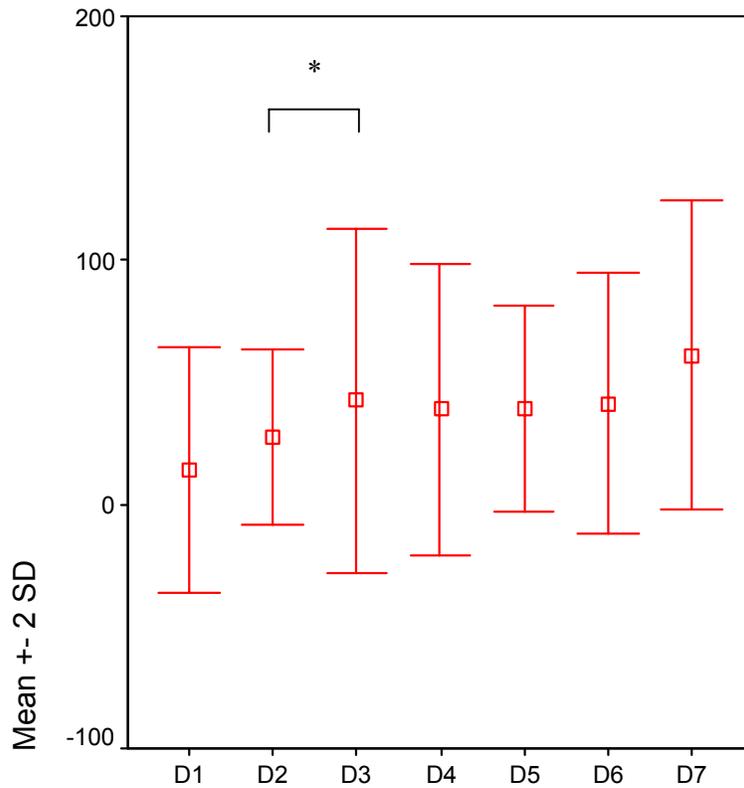


Figure 3. Graph showing the difference in fatty acid profiles of stool samples obtained on different days in the NEC group. The points represent the mean \pm SD of similarity indices. The vertical axis represents the similarity index. D1 and D2 are samples before NEC. D3 is the day NEC was diagnosed, D4, D5, D6 and D7 are samples taken after NEC. The difference between samples D2 and D3 is statistically significant, $p=0.028$ (statistical analysis, see text).

Rotavirus and adenovirus screening in hospitalised neonates

Rotavirus infections in the Tampere area occurred in a seasonal manner with peaks from March to May 1998 and from March to April 1999. During the 15-month period 174 infants were hospitalised in the pediatric wards with rotavirus gastroenteritis, and 32 of these had a nosocomial rotavirus infection. Adenovirus infections were sporadic throughout the period. A total of 12 adenovirus-positive patients were found in the hospitalised children.

Altogether 1020 samples were collected during the study period from the infants in the neonatal unit. No samples were found to be rotavirus-positive. Adenovirus antigen was detected in stool samples from five infants, of whom four had abdominal distension and watery stools and one was asymptomatic. One of the infants had severe NEC, *Serratia marcescens* septicemia and disseminated intravascular coagulopathy. Two infants were detected in May 1998, two in August 1998 and one in October 1998. The adenovirus-positive samples were further tested by means of PCR, which was negative in all infants. Thus, adenovirus positivity could not be confirmed by PCR.

During the period in question, necrotising enterocolitis was diagnosed in sixteen infants, eight of them grade I and eight grade II-III. Enteric adenovirus type 40 and 41 PCR was positive in a stool sample from a female infant born at 27+6 weeks of gestational age and weighing 1100g at birth. After birth, she had respiratory distress syndrome and *Bacteroides fragilis* septicemia. At six weeks of age, in June 1999, she developed NEC symptoms, including blood in stools and feeding intolerance. Abdominal X-rays showed dilated loops in the intestines without pneumatosis. She received conservative management, including antibiotics and intravenous fluid therapy and recovered without sequelae. Her blood cultures during the NEC period were negative. Another patient, a male twin born at 25+3 weeks of gestation with a birth weight of 885g, developed *Candida albicans* septicemia and symptoms of NEC at the age of one week during an epidemic in the surrounding community in March 1999. Rotavirus G1 PCR was positive in his stool samples obtained at the onset of symptoms. Three days after starting antibiotic treatment, intestinal perforation and peritonitis were diagnosed and the infant underwent laparotomy. He died a few hours after the operation due to hemodynamic collapse. *Candida albicans* was isolated in his blood and peritoneal fluid cultures.

DISCUSSION

Methodological aspects

The small sample sizes, especially in the NEC stage II and III groups, weaken the power of analyses and increase the risk of type II error. On the other hand, prolonging the study period in order to increase the number of recruited patients would have introduced bias caused by developing and changing treatment practices during the study. A multicentre study would have increased the number of recruited patients.

NEC was defined and classified here according to Bell's (1978b) criteria, currently used and well accepted in the literature. Stage I NEC is generally regarded as suspected and stage II-III as proven NEC (Bell et al. 1978b). Thus, stage I NEC might include cases with transient functional gastrointestinal motility disturbances without real NEC disease.

Moreover, because the smallest and most preterm infants are the most vulnerable and at highest risk of NEC, it is almost impossible to find appropriate age- and birth-weight-matched control infants, a circumstance which might make for some patient selection bias.

Frequency of and risk factors in NEC

The incidence figures for NEC might differ significantly among centres studied (Uauy et al. 1991), and also between different countries worldwide, depending on the definition of NEC and on the population studied. In the study referred to involving 2681 VLBW infants, the occurrence of suspected NEC was 17.2% and proven NEC 10.1%. National prospective studies on infants of birth weights less than 1000g from Sweden (Finnström et al. 1997) and Finland (Tommiska et al. 2001) report similar neonatal mortality rates, but strikingly different rates of NEC, in Sweden only 2% and in Finland 22%. In the Swedish report, however, no definition of NEC is given and it is possible that only the most severe NEC cases had been recorded. Our study population included infants of birth weights ranging between 505 and 3030g, and the occurrence of suspected NEC was 18.6% and proven NEC 8.6%, the proportion of the latter being comparable with our rate of severe NEC cases. Earlier reports suggest that the onset of NEC in infants of lower gestational ages occurs at later ages than in less premature cases

(Stoll et al. 1980, Uauy et al. 1991), whereas in our population no such association between gestational age and development of NEC symptoms could be found, although there is a trend. Feeding practices in the earlier studies might have been different from ours, postponing the start of enteral feedings to later ages and using more formula feedings than here.

In one population-based survey (Llanos et al. 2002) the average annual incidence of proven NEC was 0.72 cases per 1000 live births. The highest incidence occurred among infants weighing 750-1000g at birth and declined with increasing birth weight. The risk of developing NEC was 1.61 times higher during the 1995-98 period than 1991-94. The introduction of exogenous surfactant in 1990 for the treatment of preterm infants with respiratory distress syndrome (RDS) and the increasing (Lee and Polin 2003) birth rate of LBW infants have increased the number of neonates surviving the traditional diseases of prematurity (Horbar et al. 1993). As NEC is a disease of preterm infants surviving the initial critical days after birth, it will continue to be a cause of significant morbidity and mortality in LBW infants.

Among prenatal risk factors, maternal chorioamnionitis might cause intestinal microbial colonisation with invasive pathogenic bacteria, and activate inflammatory mediators involved in bowel injury and necrosis (Caplan and MacKendrick 1994). IUGR was associated with the risk of severe NEC in our cases, confirming earlier reports (Hackett et al. 1987, Beeby and Jeffery 1992). Increased duration of assisted ventilation, but not RDS, seemed to be associated with NEC in the present cases, although in the severe NEC cases the association was only almost statistically significant in logistic regression analysis. An association of respiratory disease with NEC has previously been only rarely reported (Lucas and Cole 1990), and mostly in studies from the era before surfactant (Bunton et al. 1977, Smith et al. 1980, Palmer et al. 1987), although other work from the same era shows no such association (Stoll et al. 1980). Infants who need prolonged assisted ventilation are obviously more prone to hypoxia and ischemia, which might also affect the intestinal tract. Further, survival of very immature small and sick infants, and hence the numbers of infants needing prolonged assisted ventilation, have increased.

The role of enteral feeding as a risk factor for the development of NEC was formerly a matter of consensus (Eyal et al. 1982, Kliegman et al. 1982), and since 1978 a careful feeding regimen has been recommended for premature infants (Brown and Sweet 1978), although 5-10% of NEC cases occur in infants who have never been enterally fed (Kliegman and Fanaroff 1981). A paper by Schandler and associates, on the other hand, suggests that early initiation of enteral feeding is of benefit (Schanler et al. 1999). A large randomised study of 962 preterm infants by Lucas and Cole (1990) suggested that delayed enteral feeding might protect from NEC in formula-fed, but not in breast-milk-fed infants. All our infants received breast milk and here, early initiation of milk feeding seemed to be a factor potentially protective from NEC. On the other hand, those infants with early initiation were in a better condition. Use of breast

milk fortifier emerged as a risk factor for NEC among the nutritional characteristics evaluated in the present study. Breast milk was fortified only in VLBW infants, and a high osmolarity of the feedings has also previously been suggested to be noxious for the gastrointestinal tract and to have a role in the development of NEC in these vulnerable infants (Book et al. 1975). On the other hand, no statistically significant association was seen between breast milk fortification and severe NEC. It is thus possible that administration of fortified breast milk might be related more to functional gastrointestinal disturbances, including motility disorders, than with proven NEC.

Increased duration of morphine administration emerged as the strongest predictor of NEC in this study, remaining significant in the combined model. Such an association has not been previously reported. It is clear that morphine medication is mostly used in the sickest infants, who may have on-going insults to their gastrointestinal tracts, and may therefore be at a continued risk of developing NEC. Morphine infusion has improved breathing in synchrony with the ventilator and shortened the duration of oxygen supplementation in preterm infants (Dyke et al. 1995). Recently, in a pilot trial on 67 ventilator-treated neonates of less than 33 weeks' gestation (Anand et al. 1999), the infants were randomised within the first three days after birth to receive continuous infusions of either midazolam, morphine or placebo, and a more favorable neurologic outcome during the neonatal period was suggested in the morphine group compared with the remainder. No differences either in neurobehavioral or in secondary clinical outcome measures at 36 weeks' corrected age were found between the three groups. The group size in the study in question was small and the authors did not report whether any NEC cases occurred among the infants studied. Thus, no trials are so far on record to demonstrate the benefits over hazards of sedation or analgesia in neonates receiving mechanical ventilation (Alexander and Todres 1998). Newborn infants have an increased susceptibility to the side-effects of morphine, including decreased intestinal motility and constipation (Chay et al. 1992, Anand et al. 2004), this possibly inducing feeding problems and in some cases even a process developing into NEC. Whether morphine could be a causative factor in NEC needs to be evaluated in further studies.

Laboratory parameters predictive of NEC

A complete blood count is included in the standard diagnostic blood tests in all neonates in whom an infection, including NEC, is suspected. Anemia of multifactorial origin is observed with NEC. Infants with NEC or sepsis commonly receive erythrocyte transfusions (Kling et al. 1997, Ringer et al. 1998). Hemolytic anemia is common with NEC; 25 out of 40 infants with NEC have yielded evidence of red blood cell fragmentation in the peripheral blood

(Hutter et al. 1976). Likewise blood loss anemia is seen; 12 out of 40 infants with NEC had clinically significant bleeding associated with thrombocytopenia, with 4/40 experiencing severe bleeding which contributed to death (Hutter et al. 1976). Sites of massive bleeding include bloody stools, peritoneal hemorrhage, pulmonary hemorrhage, hemopericardium plus myocardial hemorrhage, and intracranial hemorrhage. Iatrogenic anemia may contribute to the problem, since frequent blood sampling is mandatory. Anemia of prematurity occurs in infants at highest risk of NEC (Kling et al. 2003). In our NEC infants, the hemoglobin values were on every day before and at the onset of NEC significantly lower than those in the control infants.

Neutrophilia with or without increased I:T ratio is seen in less severe NEC as a normal adaptative response to inflammation. However, neutropenia is seen in severe NEC. Neutropenia has been reported in NEC, with 14 out of 40 infants exhibiting neutropenia, defined as a neutrophil count less than $1500/\text{mm}^3$. The mean neutrophil count in survivors was higher than in those who died, suggesting that neutropenia was associated with a poorer prognosis (Hutter et al. 1976). Only one of our NEC infants was granulocytopenic and leucocyte counts of more than $30 \times 10^9/\text{l}$ seemed to be especially pathognomonic in infants with a present or developing intestinal perforation.

An increased I:T ratio was observed to be predictive in 52 to 82% of counts associated with bacterial infection, including NEC (Manroe et al. 1979). In infants of more than 34 weeks of gestation, lower total neutrophil counts, higher immature neutrophil numbers, and greater immature: total neutrophil ratio at first presentation of NEC predicted which infants ultimately required surgical treatment of NEC (Schober and Nassiri 1994). In our NEC infants, I:T ratios at the onset of NEC were not different between either of the NEC groups and controls. In our study only infants of less than 33 weeks of gestational ages were included, and those with grade III NEC were the smallest and most immature. It can therefore be speculated that the I:T ratio might not be a particularly valuable diagnostic tool in the case of very premature infants with a suspicion of NEC.

Infants with severe NEC develop thrombocytopenia (Hutter et al. 1976, Patel 1977, Mehta et al. 1980). Lower platelet counts have been reported to be associated with greater disease severity. The incidence of thrombocytopenia in NEC is 65% to 90% (Sola et al. 2000). In infants of more than 34 weeks of gestation lower platelet counts at the presentation of NEC predict which infants will ultimately require surgical treatment of NEC (Schober and Nassiri 1994). In other studies, platelet counts falling over the first 24 hours have been associated with those who ultimately require surgery (Ross et al. 1989, Gupta et al. 1994a, Schober and Nassiri 1994). Platelet counts of less than $100 \times 10^9/\text{l}$ or a fall in serial platelet counts were indicators of intestinal gangrene with poor outcome (Ververidis et al. 2001). Nadir platelet counts were lower in non-survivors, compared to survivors, as well as lower in those with pan-intestinal disease, compared to less severe disease (Ververidis et al. 2001). In our study, the platelet counts at the onset of symptoms were significantly lower in the NEC infants than those in the control infants at the corresponding postnatal ages, but the mean

platelet levels did not drop below reference levels and no clear threshold level could be identified. In our infants there was no significant rapid drop in platelet counts at the onset of NEC.

Persistently elevated serum lactic acid concentrations and metabolic acidosis may be an early marker of preterm neonatal sepsis, including NEC. Lactic acidosis is frequently seen in critically ill infants. The causes of elevated lactic acid concentrations include tissue hypoperfusion and liver dysfunction (Fitzgerald et al. 1992). Metabolic acidosis is an early marker of gangrened bowel and is an indication for surgery in NEC. Persistent acidosis, i.e. pH less than 7.2 despite adequate volume resuscitation, has been identified as a sign of poor prognosis and a pressing indicator of need for surgical treatment (O'Neill et al. 1975). In our NEC infants, mean base excess every day before and at the onset of NEC in the NEC grade III group were lower than in the control group. Before symptoms manifest themselves, progressively increasing metabolic acidosis might indicate a developing NEC process. PH levels less than 7.25 seemed to be associated with a present or developing intestinal perforation at the onset of NEC.

Hypoglycemia occurs fairly commonly among infected infants in the neonatal period. The bacteria most often implicated as the cause of infections associated with neonatal hypoglycemia are gram-negative bacilli and normoglycemia with gram-positive organisms and mixed flora (Yeung et al. 1970). Also, hyperglycemia and *Escherichia coli* sepsis have been reported in one infant (James and Blessa 1979). Hyperglycemia is common in infants with NEC and is associated with an increase in late mortality and longer intensive care stay. Aggressive glycemic control may improve the outcome in NEC infants (Hall et al. 2004). In our NEC infants during 24 hours preceding the onset of NEC symptoms, a rise in blood glucose level by at least 1.5 mmol/l seemed to be associated with the development of grade II-III NEC. The occurrence of hypoglycemia in our study was not associated with NEC, because hypoglycemic blood glucose levels were detected in the NEC infants and in the controls equally often.

Effects of mode of delivery and NEC on the intestinal microflora

In infants born before 33 weeks of gestation GLC analysis of fecal bacterial cellular fatty acids to assess differences in the intestinal microbial flora has not to our knowledge been studied. Our results by this method show that the fecal fatty-acid profiles of infants born via the vaginal route differ statistically significantly from those of infants born by cesarean section. Gut colonisation is delayed in infants born by cesarean section, and intestinal colonisation is consequently

abnormal for several weeks (Long and Swenson 1977, Bennet and Nord 1987). The primary gut flora in full-term infants born by cesarean section may be disturbed for up to 6 months after birth when cultured on nonselective and selective media (Grönlund et al. 1999).

Bacterial overgrowth of a limited number of virulent species in the intestine is more likely to occur in preterm infants, as these are colonised by few aerobic bacterial strains compared with term infants (Gewolb et al. 1999). Infants with NEC have shown a statistically significant increase in their breath hydrogen levels during the 24 hours before the onset of NEC (Cheu et al. 1989). In our NEC infants the fatty-acid profiles in stools had changed statistically significantly at the onset of NEC compared with the corresponding fatty-acid profiles in the same infants when they were free of symptoms. It can be speculated that the change seen in the bacterial composition reflects bacterial overgrowth occurring in the intestine concomitant with NEC symptoms.

The GLC profiles of the NEC group differed significantly from the profiles in the control group 2, but not from control group 1. The infants in the NEC group as well as in the control group 1 were of lower gestational ages and birth weights than in the control group 2. In view of lower birth weights, enteral feeding was initiated later in the NEC and in the control group 1 and in these groups the use of breast-milk fortifier was more frequent. The smallest and sickest infants tolerate milk poorly and NEC causes an interruption in enteral feedings. It may be assumed that differences in feeding are the most probable causes of the difference found between the NEC group and control group 2.

Investigation by standard microbiological methods has not identified any specific bacterial pathogen in the pathogenesis of NEC, but it would appear that certain species, including members of the family *Enterobacteriaceae*, *Clostridia* species and coagulase-negative staphylococci are closely associated with the pathogenesis of NEC (Bell et al. 1979b, Powell et al. 1980, Kosloske et al. 1985, Scheifele 1990, Millar et al. 1992, Cushing 1983). The gastrointestinal flora of infants with NEC differs from that of similar but uninfected infants, and temporal variations occur in the resident gastrointestinal microflora of infants in NICUs. There is variation in the incidence of NEC and this may be associated with the organisms prevalent in the NICU (Bell et al. 1979a). In our NEC infants the most dominant bacterial and fungal microbes cultured from stools at the onset of symptoms were *Staphylococcus* species, *Enterococcus faecalis*, *Enterococcus* species and *Candida albicans*. Temporal variation, previous and present antibiotic therapy, and exposure of the infants to various devices, as well as the antibiotic policy adopted in the unit might all have had an effect on the dominance of the microbes found in the present population.

In both stool and blood samples from our infants with NEC with positive blood cultures, the same pathogenic bacteria with identical antibiotic susceptibility patterns were detected. In a previous study (Roback et al. 1974) positive blood cultures also matched bacteria found in stools from NEC infants. It seems that in NEC infants the causative organism might be among those organisms detected in their stool samples, and results of identification of a given organism and of

antibiotic susceptibility testing of the dominant bacterial and fungal organisms in fecal cultures can thus be in stool culture utilised when an empirical antibiotic combination is considered. The identification of a dominant organism resistant to the usual empirical antibiotic therapy may be a stimulus to broaden the antimicrobial coverage. Whether continuous twice-weekly surveillance of fecal bacterial colonisation would be cost-beneficial in predicting the causative agents in NEC and would be useful for follow-up of temporal variations in resident gastrointestinal microflora in the NICU needs to be established in future studies.

Screening for rotavirus and adenovirus infections in a neonatal unit

Most neonatal rotavirus infections are asymptomatic and out of the about 8-19.2% of the symptomatic cases only few develop dehydration. Feeding with breast milk as well as the mother's handwashing before touching the infant has been described to offer some protection against rotavirus acquisition among neonates (Totterdell et al. 1976, Jayashree et al. 1988). All of our screened infants received either breast milk or intravenous fluids and handwashing guidelines were carefully followed by personnel and mothers, which might partly explain the very low adenovirus and rotavirus detection rate in our population.

In a one-year survey carried out in a ward for premature infants where strict isolation measures were followed and visitors were allowed only to view the infants (Van Rentgerhem et al. 1980), stools from 199 premature infants were examined for viruses at 14 days' intervals and only 6.6% of the samples were rotavirus-positive in one winter epidemic, the personnel being the obvious source of virus acquisition. In another report from a tertiary care neonatal nursery (Rodriguez et al. 1982), rotavirus was detected in 22% and 19% of hospitalised infants during two epidemics, when rotavirus was also prevalent in the community. Our screening detected only one rotavirus-positive case in our neonatal unit during an epidemic, but no outbreak developed in the unit. The present hygienic measures would thus seem to have been sufficient.

The reservoir of rotavirus in newborns treated in obstetric wards has also caused an introduction of the virus in the neonatal intensive care unit, where 15 out of 52 infants were found to excrete the virus during a three-week period. Examination of the stools of mothers and staff has excluded the possibility of rotavirus excretion in the adults with closest contact to the infants (Grillner et al. 1985). In the several reports the source of the nursery outbreaks has been admission of infants infected with rotavirus (Van Rentgerhem et al. 1980, Rodriguez et al. 1982, Dearlove et al. 1983, Grillner et al. 1985), but not in our study. This might be partly explained by the fact that no outpatients were admitted and the majority of admissions occurred directly from the delivery room or from other hospitals. Rotavirus infections in the newborns treated in the

rooming-in wards in our hospital were not monitored and thus it can only be speculated that no epidemic had existed there. Stool samples from our NICU visitors were also not collected, but it can be assumed that the source of the only rotavirus infection diagnosed in the population unit studied was an adult. In agreement with previous observations, we can suggest that the introduction of rotavirus into the existing kinds of neonatal units seems to be relatively rare and routine screening for the virus is not necessary. Once an outbreak in rooming-in wards is found, however, rapid screening of new admissions into the ward might be indicated (Rodriguez et al. 1982).

Previous literature reports sporadic cases of neonatal adenovirus infections, where acquisition of the virus has occurred from an infected mother during the perinatal period and where the clinical course of the illness has been severe including progressive pneumonia, coagulopathy and death (Abzug and Levin 1991, Aebi et al. 1997). Adenovirus has, however, also been a cause of less severe neonatal infections with respiratory symptoms (Rosenlew et al. 1999). Our survey, where only one adenovirus-positive case, having gastrointestinal symptoms, was diagnosed, supports the conclusion that screening of asymptomatic neonates for adenovirus is not indicated.

Some evidence of the implication of viruses in the pathogenesis of necrotising enterocolitis is to hand, although their specific etiologic role is not known. Symptoms in neonatal rotavirus infections have ranged from mild watery stools to bloody diarrhea, abdominal distension, intestinal pneumatosis and ileal perforation (Dearlove et al. 1983), and rotavirus has caused an outbreak of necrotising enterocolitis (Rothbart et al. 1983). NEC has also been associated with coronavirus (Chany et al. 1982) and in a survey of 165 NEC cases from six centres in 1981-1982, rotavirus was isolated in stool samples from one infant and echovirus type 11 in stools and cerebrospinal fluid from another (British Association for Perinatal Paediatrics and the Public Health Laboratory Service Communicable Disease Surveillance Centre 1983). In our sixteen NEC infants, rotavirus PCR was positive in the stool of one infant and enteric adenovirus in another. Although the role of rotavirus in the disease severity in the former case remains undetermined, our results suggest that viruses might be significant etiologic factors underlying NEC and screening for viruses should be included in the microbiological work-ups of NEC infants.

CONCLUSIONS

1. Among infants born before 33 weeks of gestation, 18.6% of the infants had clinical signs and symptoms, as well as diagnostic results of radiographs with NEC grade I, II and III, and 8.6% evinced clinical signs and symptoms and radiological findings indicating grade II-III NEC.
2. The most important risk factors for NEC in general included administration of breast milk fortifier and increased duration of morphine infusion, prolonged morphine infusion being the only statistically significant factor associated with severe grade II-III NEC in preterm infants. The role of morphine as a causative factor in NEC needs to be established in future studies.
3. Persistent metabolic acidosis, decreasing platelet count and increasing blood glucose level on several successive days might predict developing NEC. Leucocyte values more than $30 \times 10^9/l$, pH less than 7.25 and a rapid elevation within 24 hours of glucose levels by 1.5 mmol/l or more seem to predict the development of severe NEC with intestinal perforation in infants born before 33 weeks of gestation. It can be suggested that such laboratory findings should alert the physician to look for possible signs and symptoms of NEC in a preterm infant.
4. As in full-term infants, the intestinal microbial colonisation in preterm infants born by cesarean section differs from the fecal colonisation of those born via the vaginal route. A significant change in fecal microbial colonisation, suggestive of a bacterial overgrowth, seems to occur in the gut of preterm infants at the onset of NEC. Pathogens detected in the stool samples obtained at the onset of the symptoms might have a causative role in the development of the disease.
5. Routine rotavirus and adenovirus screening by means of enzyme-linked immunosorbent assay in hospitalised asymptomatic neonates did not detect any positive cases and seems therefore not to be necessary. Nosocomial adenovirus and rotavirus infections in the presence of sufficient hygienic measures in the neonatal ward seem to be very rare. Viral etiology should be considered in infants who develop symptoms of gastro-intestinal infection or necrotising enterocolitis.

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