Experimental and Clinical Necrotizing Enterocolitis

NICLAS HÖGBERG
Abstract

Necrotizing enterocolitis (NEC), a severe inflammatory disorder of the gastrointestinal tract with high morbidity and mortality, affects primarily preterm infants. The diagnosis represents a challenging task, and no biomarker has been found to aid early diagnosis with high accuracy. Microdialysis has been widely used to detect metabolites of anaerobic metabolism, enabling a local and early detection of ischemia. This thesis aims to evaluate the possibility of detecting intestinal ischemic stress in experimental and clinical NEC, by use of rectal intraluminal microdialysis.

Intraluminal rectal microdialysis was performed on rats subjected to total intestinal ischemia. Metabolites of ischemia were detectable in both ileum and rectum, with raised glycerol concentrations and lactate/pyruvate ratios. Elevated concentrations of glycerol correlated to increasing intestinal histopathological injury.

Experimental early NEC was induced in newborn rat pups, by hypoxia/re-oxygenation treatment. Development of NEC was confirmed by histopathology. Elevated glycerol concentrations were detected by rectal microdialysis.

The genetic alterations following experimental NEC in rat pups were studied with microarray. Immunohistochemistry staining was performed for tight junction proteins claudin-1 and claudin-8. Several genes were altered in experimental NEC, mainly genes regulating tight junctions and cell adhesion. Immunohistochemistry revealed reduced expression of claudin-1.

A prospective study was conducted on preterm infants with a gestational age of less than 28 weeks. The infants were admitted to a neonatal intensive care unit, and observed during a 4-week period. Rectal microdialysis was performed twice a week, and blood was drawn for analysis of I-FABP. A total of 15 infants were included in the study, whereof four infants developed NEC, and 11 served as controls. Rectal glycerol and I-FABP displayed high concentrations, which varied considerably during the observation periods, both in NEC and controls. No differences in either glycerol or I-FABP concentrations were seen in the NEC-group vs. the controls.

In conclusion, rectal microdialysis can detect metabolites of intestinal ischemia, both in experimental and clinical NEC. Rectal microdialysis is safe and could provide a valuable non-invasive aid to detect hypoxia-induced intestinal damage or ischemic stress in extremely preterm infants. In this study however, it was not possible to predict the development of clinical NEC using microdialysis or I-FABP.

Keywords: Necrotizing, Enterocolitis, Ischemia, Microdialysis, Intraluminal, I-FABP

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If you are unable to find the truth right where you are, where else do you expect to find it?
Dōgen

Till Lina, Lukas och Klara
List of Papers

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


III  Högberg, N. Stenbäck, A. Carlsson, PO. Wanders, A. Engstrand Lilja, H. *Genes regulating tight junctions and cell adhesion are altered in a rat model of experimental necrotizing enterocolitis.* Submitted manuscript.


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## Abbreviations

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<th>Description</th>
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<tr>
<td>NEC</td>
<td>Necrotizing enterocolitis</td>
</tr>
<tr>
<td>VLBW</td>
<td>Very low birth weight (&lt;1500g)</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible NO synthetase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>I-FABP</td>
<td>Intestinal fatty acid binding protein</td>
</tr>
<tr>
<td>pO₂</td>
<td>Oxygen partial tension</td>
</tr>
<tr>
<td>pCO₂</td>
<td>Carbon dioxide partial tension</td>
</tr>
<tr>
<td>BE</td>
<td>Base excess</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>r²</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>Il</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons, molecular weight</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction, cell adhesion</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie- and Adenovirus receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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Introduction

Necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency occurring in neonates. It is largely a disease affecting preterm infants, where more than 90% of the infants who develop NEC are born premature. The risk of developing NEC is directly related to decreasing gestational age and birth weight (1). Among infants with very low birth weight (VLBW <1500g), the incidence is between 10 to 15% (1-4). With mortality rates approaching 30% in VLBW infants, NEC represents a significant clinical problem. The incidence of NEC has increased in parallel with the improved survival of infants born before 24 weeks of gestation (1). An inverse relationship between the gestational age and onset of the disease has been demonstrated, and late onset NEC may occur many weeks after birth.

NEC affects the gastrointestinal tract and, in severe cases, can have profound systemic impact. NEC results in variable degrees of ischemic damage of the intestines, ranging from mild ischemia of the intestinal mucosa to transmural necrosis of the bowel wall. Both the small and large bowel can be affected, but more commonly it is only the small bowel, and the most severe lesions are often seen in the jejunum and ileum. The typical patient with NEC is a preterm infant with abdominal distention and bloody stools developing after enteral feedings are initiated. A pathological abdominal x-ray reveals bowel dilation and pneumatosis intestinalis.

Prematurity is the dominant risk factor, besides perinatal asphyxia, congenital heart disease and pulmonary disease. Initial medical treatment for NEC involves bowel rest with total parenteral nutrition, broad-spectrum antibiotics, gastric decompression through a nasogastric tube, and correction of hematologic and metabolic abnormalities. Indications for surgical intervention are intestinal perforation, or abdominal erythema. Ideally, surgery is performed when the intestine is gangrenous but not perforated. Objective staging criteria developed by Bell (1A/B to 3A/B) have been used to determine the appropriate therapy according to disease severity.

Approximately 70% of NEC patients survive. However, 50% develop long-term complications. The two most common complications are poor neurodevelopmental outcome and short bowel syndrome, a malabsorption state resulting from the removal of excessive or critical portions of the small bowel, necessary for absorption of essential nutrients. Also, intestinal stric-
tures can develop with or without a preceding perforation, and result in bowel obstruction (1, 5).

Despite intensive studies over the past 40 years, the etiology and pathophysiology of NEC remains elusive. The disease is characterized by inflammation of the bowel that can progress to intestinal necrosis, sepsis and multiple organ failure.

Although the pathophysiology of NEC still remains unclear, current evidence suggests a multifactorial cause (3). Prematurity is the main risk factor, due to immaturity of gastrointestinal motility and immune defense (6), along with an impaired intestinal barrier function (7). Other contributing factors are thought to be genetic predisposition (8-10), bacterial overgrowth and translocation (6), and enteral feeding and intestinal ischemia (11).

Intestinal ischemia is considered to be a pivotal factor in the pathogenesis of NEC (1-3, 12-14). Hypoperfusion or hypoxia results in variable degrees of necrosis of the small and large intestine, ranging from local damage of the intestinal mucosa to transmural necrosis, leading to perforation of the bowel wall. It has been noted that infants exposed to intrauterine environments marked by compromised placental blood flow (i.e. maternal hypertension, pre-eclampsia) have an increased incidence of NEC, although NEC never occurs in utero (15). Similarly, infants with post-natally diminished systemic blood flow, as occurs in patent ductus arteriosis or congenital heart diseases, also have an increased incidence of NEC (16-23), probably due to shunting blood away from the visceral organs (the Herring-Breur reflex). Furthermore, newborns respond to ischemic events with a further vasoconstriction, suggesting a diminished ability to increase oxygen uptake in response to ischemic stress. Recently, it has been shown that clinical NEC is predisposed by a mucosal compromise of blood flow (24), further aggravated when feedings are initiated leading to NEC stage 2 or 3 (definitive NEC).

Intestinal ischemia or hypoxia initially results in mucosal damage, since it is the part of the intestinal wall, which is the most vulnerable to hypoxia. This is thought to be the result of the counter-current mechanism (25), where shunting of oxygen between the afferent and efferent arterioles in the villi takes place, leading to lowered pO$_2$ at the villus tip. In infants, this effect is further aggravated due to the high affinity of oxygen to fetal hemoglobin, resulting in reduced oxygen delivery (26, 27). In newborns, the regulation of the intestinal vascular resistance, and thus blood flow, is mainly determined by the balance between vasoconstriction and vasodilation. Vasoconstriction is mediated by Endothelin-1 (ET-1), and vasodilation is mediated by the endothelial production of free radical nitric oxide (NO). In the case of endothelial cell dysfunction, such as ischemia-reperfusion injury, or inflammatory activity, the balance between vasoconstriction and dilation is altered in favor of constriction.
It is unusual for NEC to occur in the fasting neonate, where 90% of cases occur after feeding has been initiated (15). In a pig-model of NEC (28), a rapid transition from parenteral to enteral nutrition induces histological and immunological changes, with altered expression of IL-1, 6, 18, TJ-proteins (claudins), Toll-like receptors TLR-4 and TNF-alpha, suggesting a feeding-induced inflammatory activation. Although this enteral feeding can activate this inflammatory cascade, other stress factors were needed (bacteria, intestinal ischemia, hypothermia) to induce animal experimental NEC (29-31).

Introduction of feedings provide carbohydrates, which in the presence of bacterial overgrowth leads to fermentation of lactose into hydrogen gas. This in turn leads to bowel distention and gas in the intestinal wall, pneumatosis intestinalis, or gas in the vena portae. Bacterial overgrowth may primarily be a result of immature bowel movements in infants. Several common bacteria have been found in NEC, but no specific pathogenic microbe has been identified. A broad range of microbes generally found in the distal gastrointestinal tract, have been recovered from the peritoneal cavity and blood of infants with NEC. The predominant organisms include Enterobacteriaceae (i.e., Escherichia coli, Klebsiella pneumoniae), Clostridium spp., enteric pathogens (Salmonellae, Coxsackie B virus, Coronavirus, Rotavirus), and potential pathogens (Bacteroides fragilis, Cronobacter sakazakii) (32-34). Regardless of the microorganism present, infants, nevertheless, suffer from bacterial overgrowth due to immature bowel movements. Outbreaks of NEC have also been found to be caused by Cronobacter sakazakii, a contaminant of milk powder formula (35).

The intestinal permeability is central to the defense against microbes. Ischemia leading to mucosal damage or necrosis results in breach of the intestinal barrier, allowing for bacterial translocation and migration of bacterial endotoxin into the damaged tissue, which further aggravates the inflammatory cascade.

Intestinal permeability is increased in experimental NEC (36), in preterm infants (37) as well as in infants suffering from NEC (38). In human NEC, this increase in permeability could be secondary to ischemic damage to the entire bowel wall or a primary structural immaturity. Increased intestinal permeability as a primary cause leading to NEC may be considered, involving structural proteins such as tight junctions (TJ) and cell adhesion proteins. Increased permeability resulting from the "opening" of TJs may also be the result of virus-induced disruption of TJ formation (39). Other factors may contribute to disrupt the function of TJs, such as intraluminal presence of polyunsaturated fatty acids and other toxins (39, 40).

The intestinal mucosal cell layer is an important part of this barrier to intraluminal microbes. The TJs are essential components of this barrier, as they bond the adjacent mucosal cells to each other. The junctional complexes of the plasma membrane are not only epithelial barriers in paracellular
transport or barriers preventing diffusion in the plasma membrane, but also contain proteins involved in signal transduction and the maintenance of the physiological epithelial cell state. The TJ is composed of both intracellular and membrane spanning proteins. Occludin, claudin, junctional adhesion molecules (JAMs), and the Coxsackie- and Adenovirus receptor (CAR) are the major components of TJs (40). CAR is a transmembrane protein, which functions as a primary receptor for both the Coxsackie B virus and Adenovirus. CAR also functions as a marker for epithelial TJs and regulation of permeability (43). The expression of these proteins, functioning as receptors for Coxsackie B virus and Adenovirus, is related to intestinal permeability and immune response. Binding of the virus to the receptor leads to loss of TJ-function and virus entry into the cell, activating caspase-mediated apoptosis and T-cell activation.

The inflammatory reaction can be initiated by ischemia/reperfusion injury, and the introduction of feedings (11, 25, 28). The inflammatory reaction is further augmented by an immature immune system, both specific (B- and T-cell mediated) and other factors such as decreased intraluminal pH, reduced cell adhesion, and immature intestinal motility. Inflammatory components such as tumor necrosis factor α (TNF-α) and platelet activating factor (PAF) have been shown to play an important part. Elevated levels of PAF lead to further vasoconstriction and activation of TNF-α and interleukins such as IL-6, mediators of an inflammatory response. Genetic studies in a pig model of NEC has demonstrated an altered expression of innate immune defense genes such as interleukins (IL-1 alpha, IL-6, IL-18), nitric oxide synthetase, tight junction proteins (claudins), Toll-like receptors (TLR-4), and TNF-alpha (28). In human enterocytes, the expression of TLR2, TLR4, NF kappa B1, and IL-8 mRNA was increased in fetal vs. mature human enterocytes and further altered in NEC enterocytes (41). Another study suggests that TLR genetic variants can alter susceptibility to NEC in VLBW infants (8). Ischemia/reperfusion injury results in production of free oxygen radicals. Previously, genetic analysis in experimental NEC demonstrated an up-regulation in the antioxidant glutathione system (GSH) (42). The GSH antioxidant system was shown to play a crucial role in intestinal barrier protection by attenuating enterocyte death by caspase-mediated apoptosis.

From observing twins, there has been some speculation about a genetic predisposition for developing NEC, searching for a candidate gene, but evidence has been scarce. Toll-like receptors have been identified as a genetic variant in NEC (8). The difficulty with NEC is that it is a multi-factorial disease and proceeds through different stages, during which the different inflammatory mediators have different activities.
Today, NEC is diagnosed by a combination of clinical, laboratory, and radiological findings; however, these diagnostic methods lack high specificity and sensitivity for NEC. The accuracy of different biomarkers in diagnosing NEC and intestinal ischemia has been studied extensively (44-48). D-lactate, alpha glutathione S-transferase, intestinal fatty acid binding proteins, claudin-3, creatine kinase B, isoenzymes of lactate dehydrogenase (LD), and alkaline liver phosphatase (ALP) have been analyzed. D-dimer may be used as an exclusion test for intestinal ischemia; however, it lacks specificity (46) and its role in NEC is uncertain.

Intestinal fatty acid binding protein (FABP2, I-FABP) has been reported to be a useful plasma marker for early enterocyte cell death (47, 48). I-FABP is specifically present in mature enterocytes of the small and large intestine and is released as soon as the integrity of the cell membrane is compromised. I-FABP is present in very small amounts in the plasma of healthy individuals, probably representing the normal turnover of enterocytes, but levels rise rapidly after episodes of acute intestinal ischemia and inflammation, including NEC (47-51). Because of its low molecular weight, I-FABP is present in the systemic circulation and passes through the glomerular filter and can readily be detected in the urine. Thus urinary values of I-FABP as well as plasma levels provide specific information about the number of dying intestinal epithelial cells and can be used in early diagnosis of NEC or intestinal necrosis of other origin (49, 52-54).

Calprotectin is commonly used in the clinic to diagnose inflammatory bowel disease. It has been proposed as a useful marker of NEC. Patients with NEC have been shown to have a raised fecal calprotectin level at the time of diagnosis compared with controls (45, 55, 56). However, it does not seem to be useful in the early stages of the disease (55), where no difference was found between the controls and infants developing NEC at a later stage.

Claudins are part of the TJ, and have been evaluated as a marker of TJ-degradation following ischemia or hypoxia-induced intestinal damage in NEC. Decreased urinary levels of claudin-3 have been noticed in both experimentally induced intestinal damage, and in human inflammatory bowel disease (45, 57).

Experimental necrotizing enterocolitis

Experimental NEC has previously been studied by inducing NEC with hypoxia/re-oxygenation treatment in newborn rats (17, 18, 58), or a combination of hypoxia/re-oxygenation treatment, cold stress and formula gavage feeding (16-23). The induction of intestinal necrosis was verified by histopathology, revealing histopathological findings similar to what can be seen in patients with NEC. Intestinal ischemia has also been studied extensively in animal models of NEC.
Another method to induce experimental NEC in rats is to infect them with Cronobacter sakazakii (59). This method however, does not include any hypoxia/re-oxygenation treatment.

**Microdialysis, general principles**

Microdialysis is a technique to monitor the chemistry of the extracellular space in living tissue. It enables monitoring of essentially any chemical event taking place in the interstitial fluid (60).

A microdialysis probe is usually constructed as a concentric tube where the perfusion fluid enters through an inner tube, flows to its distal end, exits the tube, and enters the space between the inner tube and the outer dialysis membrane. The semi-permeable membrane at the distal end of the microdialysis catheter functions like a blood capillary and chemical substances from the extracellular fluid diffuse through the dialysis membrane into the catheter and the dialysate. Thereafter, it contains a representative proportion of the tissue fluid’s molecules. The dialysate consists of a physiological salt solution, slowly pumped through the microdialysis probe where the solution is equilibrated with the surrounding extracellular tissue fluid (Figure 1).

The gradient of a particular compound depends not only on the difference in concentration between the perfusate and the extracellular fluid but also on the velocity of flow inside the microdialysis probe. The absolute recovery (moles/time unit) of a substance from the tissue depends on the “cut-off” of the dialysis membrane, (usually defined as the molecular weight in Daltons at which 80% of the molecules are prevented from passing through the membrane), the length of the membrane, the flow rate of the perfusion fluid, and the diffusion coefficient of the compound through the extracellular fluid.

The advantage of microdialysis is the possibility to detect early signs of tissue ischemia, before any clinical signs are evident. It provides semi-continuous monitoring of tissue-specific metabolic changes, without the need for repeated blood samples. Microdialysis samples substances involved in the energy metabolism - glucose, lactate, and pyruvate - are markers for ischemia, hypoxia, and hypoglycemia in peripheral and central tissues.
Figure 1. The principle of microdialysis. A semipermeable membrane permits diffusion of extracellular substances into the tube, allowing analysis of the metabolites of interest. Reprinted with permission.

Glycerol is a marker for lipolysis or cell membrane damage (61, 62). Glutamate is a marker of cytotoxicity in brain tissue (62). Urea is a marker for urea clearance during hemodialysis. When the supply of glucose and oxygen is diminished, there is an immediate increase of lactate/pyruvate ratio, and a decrease of glucose. Obstruction of the blood flow will, thus, be detected immediately by the change in metabolites.

Microdialysis in intestinal ischemia

Acute intestinal ischemia is a significant problem in clinical practice, both in terms of treatment and diagnosis. It can manifest itself in general conditions such as sepsis, multiple organ failure, necrotizing enterocolitis in neonates, or following vascular surgery with clamping of the aorta, as well as in specific intestinal conditions such as thrombosis or embolus in the mesenteric arteries, volvulus, and strangulation. The symptoms are often not apparent until the intestinal ischemia is severe, leading to systemic responses, intestinal bleeding, or perforation. Treatment at this stage often requires bowel resections, and is associated with very high morbidity and mortality. Ischemic or anoxic events are associated with a wide range of clinical presentations both systemic and in various organ systems. Several indicators or markers have been used to study ischemia. Systemic responses to severe ischemic events and the following anaerobic metabolism such as elevated plasma lactate and acidosis are late signs. Locally detectable markers of anoxia or ischemia have the advantage of early detection of abnormalities. Local physical measurements of blood flow through laser doppler flowmetry, pH, ATP,
pO₂ and pCO₂ have all been used with varying degrees of success. Blood flow redistribution and heterogenous microcirculatory perfusion can explain maintained regional aerobic metabolism during ischemic stress, despite local signs of mesenteric hypoperfusion measured with laser doppler (superior mesenteric arterial blood flow, mucosal microcirculation), and decreased mucosal pCO₂ (63). Also, decreasing glucose concentrations suggest that substrate supply may become crucial before oxygen consumption decreases. Ischemic injury also leads to increased intestinal wall permeability, but it shows an uncertain correlation with the degree of necrosis (64).

Recent studies on intestinal ischemia with the microdialysis technique in adult animals (13, 64-67) showed a typical metabolic response to anaerobic metabolism; specifically, reduced glucose levels, production of lactate leading to an elevated lactate/pyruvate ratio, accompanied by increased glycerol levels as a result of cell-membrane phospholipid degradation caused by ischemia-induced phospholipase activation. Markers of metabolism such as glucose, lactate, pyruvate and markers of cell deterioration such as glycerol and glutamate can be measured through microdialysis, and is a direct indicator of anaerobic metabolism and cell injury.

Microdialysis has been used in various applications to monitor ischemic events. Examples include intraperitoneal monitoring after intestinal surgery and monitoring of free flaps after reconstructive surgery (67-69). Intrahepatic microdialysis has also been used as a method for early detection of postoperative complications such as liver ischemia and graft rejection after liver transplantation (70, 71). The microdialysis technique is minimally invasive and suitable for different locations such as intraparenchymatous, intraperitoneal, intravasal (72), or intestinal intraluminal approach (64, 73-76).

Different locations have been used to measure these metabolic events. In studies of intestinal ischemia, the intraperitoneal or serosal location close to the affected site has been used extensively (69, 77-79), along with the intraluminal approach. An intramural approach has also been used, but the inflammatory reactions around the probe were severe, affecting metabolism and measurements thereof (65).

The intestinal mucosa seems to be most vulnerable to ischemic events, leading to intraluminal release of the mentioned metabolites (64). The intraperitoneal (serosal) compartment reflects the metabolism of the outer layers of the intestinal wall (77). As the muscularis layer is less vulnerable to ischemic stress than the mucosa, detection of anaerobic metabolites on the serosal side reflects a later stage of the ischemic insult (64). This suggests that the intraluminal compartment is the best location for detecting biomarkers of anaerobic metabolism and cell decay at an early stage in intestinal ischemia.
Aims of the investigation

The general aim of this investigation was to evaluate the microdialysis technique on experimental and clinical NEC. We were interested in its potential use as a diagnostic tool, and to study the metabolic response following the development of experimental and clinical NEC.

Specific aims were:

1. To evaluate the possibility of measuring ischemic damage to the intestines, by placement of a rectal microdialysis catheter.
2. To set up an animal model of experimental early NEC.
3. To use the microdialysis technique on rat pups with experimental NEC.
4. To study the genetic alterations in experimental NEC in rat pups.
5. To study the potential diagnostic ability of intraluminal microdialysis compared to biomarkers and clinical routine diagnostic methods on preterm infants suffering from NEC.

In the first study, we evaluated the metabolic response in the ileum and the colon by intraluminal rectal microdialysis during total intestinal ischemia. The metabolic changes were also correlated to the histopathological findings in the mucosa.

In the second study, we studied the possibility of detecting signs of hypoxic mucosal cell damage by using intraluminal rectal microdialysis in newborn rat pups treated with hypoxia/re-oxygenation as an experimental model of early NEC.

In the third study, we examined the genetic expression in the ileum from rat pups induced with experimental NEC, compared with controls. We also used immunohistochemistry staining to study the specific expression of TJ proteins.

In the fourth study, we evaluated rectal intraluminal microdialysis and plasma I-FABP in a prospective, clinical setting. Extremely preterm infants were followed during a four-week period, during which some of them developed NEC.
Materials and methods

Animals (papers I-III)
In paper I, adult male out-bred Sprague-Dawley rats were used. Their mean weight was 360 g (range 280-425 g), and the total number of rats used was 28. The rats were purchased from Scanbur AB (Sollentuna, Sweden). They were housed in the animal department at Uppsala Biomedical Center, and kept at +22°C, with a 12-hour light/dark cycle, fed standard pellet food and water ad libitum.

In papers II and III, time-pregnant Sprague-Dawley rats were obtained on day 15 of gestation. The rats were purchased from Charles River (Charles River GmbH, Sulzfeld, Germany). They were housed in the animal department at Uppsala Biomedical Center, and kept at +22°C, with a 12-hour light/dark cycle, fed standard pellet food and water ad libitum. On day 21, the rat pups were delivered vaginally.

The studies were approved by the regional ethics committee on animal research, and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council in 1996.

Study population and setting (paper IV)
In paper IV, preterm infants of gestational age <28 weeks and weighing <1500 g were included. The study was approved by the regional committee on medical research ethics, and informed consent was obtained from the parents. No abdominal symptoms or other clinical signs of illness were present in the infants on inclusion, and there was no evidence of any infectious or otherwise complicating disease. The infants were admitted to a level III neonatal intensive care unit, and followed during a 4-week period. Any routine blood testing and x-ray scans were performed on a clinical basis. A total of 15 infants were included during this period. Four infants developed clinical NEC stage 2 or 3, confirmed by radiology or histopathology. The remaining 11 did not develop any complications or abdominal symptoms during this period, and therefore served as controls.

The diagnosis of NEC was staged according to a simplification of the Walsh and Kliegman classification (80), using three categories. Stage 1 was defined as suspected NEC, with lethargy, abdominal distention, apnea, and
bloody stools. Stage 2 was considered present when either X-rays or ultrasound revealed pneumatosis intestinalis, portal gas, intestinal perforation, or paralysis with dilated bowel loops. Stage 3 was defined when the occurrence of organ failure was present, in addition to the stage 2 criteria.

Surgical procedure (paper I)
All animals were anaesthetized with thiobutabarbital sodium (Inactin®; Sigma-Aldrich Sweden AB, Stockholm, Sweden), at a dose of 120 mg/kg body weight administered intraperitoneally. They were placed on a heated operating table, maintained at body temperature (37°C), and tracheostomized. Heparinized polyethylene catheters were inserted into the right carotid artery and jugular vein. The right carotid artery catheter was connected to a transducer in order to continuously monitor the mean arterial blood pressure. The arterial catheter was also used for blood gas sampling. The jugular vein catheter was used for continuous infusion (5 mL/h/kg body weight) with Ringer’s solution (Fresenius Kabi AS, Halden, Norway) to compensate for loss of body fluid. A laparotomy was performed and the aorta and cranial mesenteric artery were identified. The aorta was permanently clamped proximal to the cranial mesenteric artery, to create a state of total ischemia in the bowels. In the sham-operated controls, no clamping of the aorta was performed.

Induction of experimental NEC (papers II-III)
The rat pups were treated on the first day after birth. They were stressed with hypoxia/re-oxygenation treatment, breathing 100% carbon dioxide for 10 minutes, followed by re-oxygenation using 100% oxygen for another 10 minutes. The rats were then returned to their mothers' cages and allowed ad libitum nursing of maternal milk.

Microdialysis (papers I-II, IV)
In paper I, all measurements were made using a CMA 20 Elite microdialysis catheter (cut-off 20 kDa, 10 mm membrane length, CMA Microdialysis AB, Stockholm, Sweden). The microdialysis catheters were connected to a microinjection pump (CMA 102 or CMA 402, CMA Microdialysis AB) and perfused with isotonic Ringer’s solution with a flow-rate of 1µL/minute. A microdialysis catheter was placed in the sigmoid part of the colon through the rectum. Another microdialysis catheter was placed in the ileum, through a small enterotomy that was five cm proximal to the caecum, and secured with a suture. Another catheter was placed in the subcutaneous adipose tis-
sue in the upper part of the body close to the scapula. A period of 30 minutes was allowed for stabilization and in-situ perfusion before the baseline measurement, which consisted of one 30-minute period, before the aorta was clamped. Microdialysate samples were then collected every 30 minutes for a total of 210 minutes.

In paper II, all measurements were performed using a CMA 20 Elite microdialysis catheter (cut-off 20 kDa, 10 mm membrane length, CMA Microdialysis AB). The microdialysis catheter was rectally inserted 10 mm, reaching the rectosigmoid part of the colon. The microdialysis catheters were connected to the microinjection pumps (CMA 102 and CMA 402, CMA Microdialysis AB) and perfused with isotonic Ringer’s solution with a flow-rate of 0.7 µL/minute. Initially, in situ stabilization was allowed for 30 minutes. Microdialysate samples were then collected every 30 minutes for a total of 90 minutes.

In paper IV, all measurements were performed using the clinically approved CMA 70 microdialysis catheter (cut-off 20 kDa, 10 mm membrane length, Mdialysis AB, Solna, Sweden). The microdialysis catheters were connected to the microinjection pumps (CMA 107, CMA Microdialysis AB) and perfused with isotonic Ringer’s solution with a flow rate of 1.0 µL/minute. The infants were monitored during a 4-week period, with microdialysis measurements twice a week. The microdialysis catheter was rectally inserted 10 mm, and secured in position with tape. Initially, in situ stabilization was allowed for 5 minutes. Microdialysate samples were then collected every 30 minutes for a total of 90 minutes.

Samples were immediately put in a freezer at -20°C. Analyses of glucose, L-lactate, pyruvate, and glycerol were done using an enzymatic colorimetric technique on a CMA 600 Microdialysis Analyzer (CMA Microdialysis AB). The CMA 600 Analyzer was automatically calibrated at startup and recalibrated every sixth hour using standard calibration solutions from the manufacturer (CMA Microdialysis AB). Quality controls at two different concentrations for each analyte were performed every weekday. Total imprecision coefficient of variation was <10% for all analytes.

**Blood sampling (papers I, IV)**

In paper I, arterial blood gas samples were collected at baseline and at the end of the ischemic period. Measurements were made for pH, pO₂, pCO₂, base excess (BE), and hemoglobin (Hb), using an iSTAT-1 analyzer (iSTAT Corporation, East Windsor, NJ, USA).

In paper IV, blood samples were drawn for analysis of I-FABP. Routine testing of C-reactive protein was done using the standard clinical laboratory method.
Enzyme-linked immunosorbent assay (paper IV)

During a 4-week period, blood was drawn twice a week for analysis of I-FABP. A volume of 100 µL in EDTA was centrifuged, and the plasma was stored at -70°C until analysis. I-FABP was analyzed by a commercial sandwich ELISA (DY3078, R&D Systems, Minneapolis, MN, USA), in which a monoclonal antibody specific for I-FABP was coated onto microtitre plates. Standards and samples were pipetted into the wells and the peptide was bound to the immobilized antibodies. After washing, a biotinylated anti-I-FABP antibody was added. Following incubation and washing, a streptavidine-HRP conjugate was added to the wells. After incubation and washing, a substrate solution was added. The development was stopped and the absorbance was measured in a SpectraMax 250 (Molecular Devices, Sunnyvale, CA, USA). The concentrations in the samples were determined by comparing the optical density of the sample with the standard curve. The assays were calibrated against highly purified recombinant human I-FABP.

Morphology (papers I-III)

In paper I, intestinal specimens were taken from the site of the sigmoideum microdialysis catheter, at the end of the ischemic period. Intestinal specimens were also taken from sham-operated controls at the end of the experiments.

In papers II and III, intestinal specimens were taken from the ileum. The specimens were fixed in 4% formaldehyde solution, further processed, and embedded in paraffin. Sections of 5 µm were stained with hematoxylin and eosin and examined by light microscopy. The specimens were evaluated blindly with respect to the degree of ischemic injury and mucosal integrity by a pathologist.

Microarray analysis (paper III)

Twenty-four hours after induction of NEC, all animals were sacrificed by neck dislocation. A laparotomy was performed and intestinal specimens were taken from the distal part of the small bowel. The intestinal specimens were immediately placed in RNAlater solution (Qiagen GmbH, Hilden, Germany) and stored at +6°C over night. Extraction of mRNA was performed using RNeasy Mini kit (Qiagen GmbH) according to the manufacturer’s protocol.

The RNA concentration was measured with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc., Palo Alto, CA, USA). Two hundred fifty nanograms of total RNA from each sample was used to generate amplified and biotinylated sense-
strand cDNA from the entire expressed genome, according to the Ambion WT Expression Kit (P/N 4425209 Rev B 05/2009) and Affymetrix GeneChip® WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev. 1, Affymetrix Inc., Santa Clara, CA, USA). GeneChip® ST Arrays (GeneChip® Rat Gene 1.0 ST Array) were hybridized for 16 hours in a 45°C incubator, rotated at 60 rpm. According to the GeneChip® Expression Wash, Stain and Scan Manual (PN 702731 Rev. 2, Affymetrix Inc.), the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G.

Immunohistochemistry staining (paper III)

Tissue sections from the specimens were de-paraffinized in xylene, and then rehydrated in graded alcohols, according to standard procedures. Sections were heated in Tris EDTA-buffer, pH 9 (DAKO, S2367) in a microwave oven at 750 W for 10 minutes, followed by 350W for 15 minutes for antigen retrieval. The sections were allowed to cool for 20 minutes and then washed in distilled water. The tissue sections were blocked and stained by immunohistochemistry techniques with antibodies directed against the tight junction proteins claudin-1 (Invitrogen Corporation, Carlsbad, CA, USA, no. 51-9000, dilution 1:200) and claudin-8 (Invitrogen Corporation, no. 40-0700Z, dilution 1:2000). Immunohistochemistry was visualized by the use of Dako REAL Envision Peroxidase /DAB detection system (Dako Denmark A/S, Glostrup, Denmark) followed by hematoxylin counterstaining.

The epithelial cells of the intestinal villi in the immunohistochemistry sections were assessed blindly by two investigators, in a semi-quantitative manner. The claudin-1 stained cell membranes displayed only a negative or a very faint staining. Consequently, only the cytoplasmic staining was scored. Claudin-8 displayed a distinct lateral membrane staining and a cytoplasmic staining. In this case, both the staining of the membrane and the cytoplasm were scored separately. Regarding the cytoplasmic staining, the presence of small distinct granules in the cytoplasm was regarded as 1+, the presence of medium sized granules or clumps as 2+, and the presence of large sized granules or clumps was scored as 3+. Absence of granules was judged as negative. Regarding the membranes, a weak or negative staining intensity was defined as −/1+, moderately strong staining as 2+, and strong staining 3+.

Statistics (papers I-III)

For analytical statistics in paper I, the non-parametric Kruskal-Wallis test was used, and for pairwise comparison between time points, the Tukey post-hoc test was applied (Statview; Abacus Concepts, Berkeley, California,
USA). In part two of the paper, the data showed a normal distribution pattern, and one-way ANOVA was used with Holm-Sidak post-hoc test.

For analytical statistics in paper II, a Mann-Whitney U-test was performed for all comparisons (Statview; Abacus Concepts). A $p$-value <0.05 was considered statistically significant for all comparisons in papers I and II.

In paper III, subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (http://www.r-project.org) using packages available from the Bioconductor project (www.bioconductor.org). The raw data was normalized using the robust multi-array average (RMA) method first suggested by Li and Wong in 2001 (81, 82). In order to search for the genes that were expressed differentially in the NEC samples compared with the control samples, an empirical Bayes moderated t-test was then applied (83), using the “limma” package (84). To address the problem of multiple testing, the $p$-values were adjusted using the method of Benjamini and Hochberg (85). A $p$-value <0.01 was considered statistically significant for these comparisons.

For analytical statistics of the immunohistochemistry staining scores, Fischer’s exact test was performed (Statview, Abacus Concepts). A $p$-value <0.05 was considered statistically significant.
Results

Paper I

In the first series of experiments, we studied the intraluminal rectal microdialysis analyte levels of glucose, lactate, pyruvate, and glycerol in adult rats subjected to total intestinal ischemia.

On clamping the suprarenal aorta, immediate responses could be detected by microdialysis. Intraluminal glycerol started to increase immediately after clamping of the aorta, and reached a maximum at 240 minutes, with all values higher than baseline levels, $p<0.001$ (Figure 2).

Luminal lactate also started to increase directly after clamping of the aorta. Subcutaneous lactate increased gradually, approaching the luminal levels at the end of the ischemic period.

Expressing the ratio between lactate and pyruvate levels, the luminal ratio began to rise directly after clamping of the aorta, to reach the maximum at the end of the ischemic period. The luminal lactate/pyruvate ratio was also higher than at baseline, $p<0.01$ (Figure 2).

![Glycerol and Lactate/Pyruvate Ratio](image)

**Figure 2.** Intraluminal glycerol started to increase immediately after clamping the aorta, and continued to rise during the ischemic period. Luminal lactate also increased, although at a later stage. The lactate/pyruvate ratio also responded with prompt elevation.
In the sham-operated control group, subcutaneous and intraluminal glycerol and lactate values remained at baseline levels throughout the experiments.

In this study, we also compared the intraluminal glycerol levels in the ileum vs. the colon (Figure 3).

![Glycerol](image)

**Figure 3.** Intraluminal levels of glycerol in the colon and the ileum. The concentrations in the ileum were higher than the colon, and both differed from baseline levels and sham controls.

Glycerol was measured in the colon and the ileum. Both began to rise after clamping of the aorta, reaching maximum levels at 210 minutes. Glycerol in the ileum and the colon were both higher than baseline and sham operated controls at 30 minutes and throughout the ischemic period ($p<0.001$). Glycerol levels measured in the ileum were higher than glycerol levels in the colon, at 90 minutes and at the end of the ischemic period ($p<0.05$).

Luminal lactate levels also increased directly after clamping of the aorta. Colon lactate levels increased gradually, approaching the ileum lactate levels at the end of the ischemic period. Lactate in the colon and the ileum were different from baseline ($p<0.001$).

In the sham-operated control group, glycerol and lactate values in both the colon and the ileum remained at baseline levels throughout the experiments. Microscopic evaluation of the mucosal integrity was made, using sham-operated controls (N=3), and ischemic specimens (N=6). The degree of damage was classified into three degrees, and were evaluated by an independent observer. A linear regression was made on the grade of the mucosal damage and the concentrations of luminal glycerol in each animal at 240 minutes. Correlation coefficient ($r^2$) was 0.705, $p<0.01$. Increasing levels of glycerol correlated to higher degrees of mucosal damage.
Visualized as a scatter plot, the luminal glycerol concentration is expressed on the y-axis, and the mucosal integrity grade on the x-axis (Figure 4).

Figure 4. Linear regression on the grade of mucosal damage versus intraluminal concentrations of glycerol in the colon. Increasing levels of glycerol correlate to higher degrees of mucosal damage. N=9. Correlation coefficient was 0.705.

Paper II

In the following experiments, we performed all microdialysis measurements on rat pups with induced experimental early NEC. Intraluminal rectal microdialysate levels of glucose, lactate, and glycerol were detectable. Levels of pyruvate were too low to be measured in both the controls and NEC rats. Therefore, no lactate/pyruvate quotients could be calculated.

The intraluminal levels of glucose, lactate, and glycerol in controls were compared with NEC rats. Glycerol and lactate levels were higher in the NEC group compared to the controls, $p<0.05$ (Figure 5). Intraluminal levels of glucose did not differ between the two groups.
Figure 5. Intraluminal concentration of glycerol and lactate was higher in NEC compared to control rats. Asterisk denotes significant difference compared with controls.

Paper III

In this study, we analyzed the genetic transcriptome of rat pups with induced experimental early NEC, and performed immunohistochemistry staining for TJ proteins claudin 1 and 8. The results were compared to healthy rat pups.

We analyzed the transcriptional profile of genes that were either up- or downregulated at least two-fold, and with an adjusted $p$-value <0.01 for each sample. The total number of genes expressed at these levels of significance was 509. The complete dataset comprised 26,000 genes. The genes were further analyzed using the DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics software version 6.7 provided by the National Institute of Allergy and Infectious Diseases (http://david.abcc.ncifcrf.gov/).

Among the genes that were up- or downregulated, several are involved in TJ formation and cell adhesion, such as claudins (1, 8, 14, 15) and gap junction protein beta 3. Occludin was not significantly upregulated (Table 1). Fatty acid binding proteins (FABP) 5 and 6 (ileal) were upregulated, whereas FABP2 (intestinal) was downregulated. Steroid 5-alpha reductase, which controls intestinal maturation and development during embryogenesis and early development, was the most downregulated gene.
Table 1. Among the genes that were up- or downregulated, several are involved in tight junction formation and cell adhesion, as well as inflammatory response and apoptosis.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Fold change</th>
<th>Adj. p-value</th>
<th>Function</th>
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<tr>
<td>Claudin 1</td>
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</tr>
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<td>0.001948</td>
<td>Tight junction</td>
</tr>
<tr>
<td>Gap junction protein, beta 3</td>
<td>Gjb3</td>
<td>1.54</td>
<td>0.000556</td>
<td>Gap junction</td>
</tr>
</tbody>
</table>
| Fatty acid binding protein 2           | Fabp2  | -0.52       | 0.002        | Cytosolic protein, intes-
|                                        |        |             | tinal                    |
| Fatty acid binding protein 5           | Fabp5  | 1.36        | 7.85E-5      | Cytosolic protein, epi-
|                                        |        |             | dermal                    |
| Fatty acid binding protein 6           | Fabp6  | 2.14        | 7.4E-7       | Cytosolic protein, ileum |
| Interleukin 1 beta                     | Il1b   | -1.95       | 1.04E-5      | Cytokine activity         |
| Interleukin 18                         | Il18   | -1.13       | 0.001638     | Cytokine activity         |
| TNF receptor-associated factor 6       | Traf6  | 1.01        | 0.000132     | NFKB1                     |
| Caspase 3                              | Casp3  | -1.06       | 0.000566     | Apoptosis                 |
| 5-alpha reductase type 2               | Srd5a2 | -6.48       | 3.96E-5      | Steroid biosynthetic     |
|                                        |        |             | process       | Antioxidation              |
| Glutathione S-transferase A2           | Gsta2  | 3.74        | 0.005376     | MHCII                     |
| RT1 class II, locus Db1                | RT1Db1 | -1.41       | 0.000182     | MHCII                     |
| RT1 class II, locus DM beta            | RT1DMb | -1.35       | 3.04E-5      | MHCII                     |
| RT1 class II, locus Ba                 | RT1Ba  | -1.21       | 4.37E-5      | MHCII                     |
| RT1 class II, locus Da                 | RT1Da  | -1.09       | 0.000255     | MHCII                     |

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Genes regulating the inflammatory response and apoptosis were also found altered, including IL-1, IL-18, TNF alpha, caspase 3, and NF-kappa-beta 1 (TNF receptor associated factor 6).

Several downregulated genes included RT1 class II loci Ba, Da, and Db1 (major histocompatibility complex (MHC) class II family), MHC class II loci DM beta and antigen E alpha. These are involved in the formation of the MHC class II, functioning as receptors for viruses in antigen presenting cells. Glutathione S-transferase A2 was upregulated, as were several genes involved in glutathione transferase activity: glutathione S-transferase Yc2 subunit, glutathione S-transferase mu 2, 3, 4, and 7 and glutathione S-transferase theta 3.

The immunohistochemical staining for claudin-1 was scored only with regard to the intracytoplasmic components (Figure 6A, B). Regarding the staining of claudin-1, the NEC-group received a lower score than the control group ($p = 0.005$). In the NEC group, 2 rats received a moderate or strong staining score (2+ or 3+), and 10 rats received a low (1+ or negative) staining score. In the control group, 7 rats received a moderate or strong (2+ or 3+) staining score, and only one rat was scored low (1+). The immunohistochemical staining for claudin-8 was also scored, but no difference in scores could be seen between the two groups. In the NEC group, 8 rats received a moderate or strong score (2+ or 3+), and 4 rats received a low score (1+ or negative). In the control group, 2 rats were scored as moderate or strong (2+ or 3+), whereas 6 rats were scored as low (1+ or negative).

**Figure 6.** A. Moderate to strong lateral membrane staining of claudin-1 as seen mostly in the control group (original magnification x400). B. Moderate to strong staining of claudin-1 indicated by the presence of large intracytoplasmic aggregates. This was seen in many animals in the NEC group and also in a few animals in the control group (original magnification x400). A very similar picture was observed in the claudin-8 stained intestinal epithelium.
Paper IV

This paper presents a prospective, clinical pilot study on preterm infants. We included infants with a gestational age of less than 28 weeks and weighing <1500g after obtaining parental informed consent. A total of 15 infants were included during this period. Four infants developed signs of NEC, whereof three died during the observation period. The remaining 11 did not develop NEC, and therefore served as controls.

Figure 7. A-D. Glycerol, I-FABP, and CRP levels in four infants developing NEC. Glycerol values are medians with bars for maximum and minimum values.
Microdialysis catheters were placed in the rectum for intraluminal measurements of glucose, glycerol, lactate, and pyruvate. Blood samples were taken for analysis of I-FABP. Routine testing of C-reactive protein (CRP) was performed on a clinical basis.

Intraluminal microdialysate levels of glycerol were detectable; however, the concentrations of lactate, glucose, and pyruvate were too low for analysis for all infants. During the observation periods, the concentrations of glycerol, I-FABP, and CRP varied considerably in infants with NEC (Figure 7A-D) and in the controls (Figure 8 A-K). The mean levels of glycerol or I-FABP at NEC diagnosis were not higher than before clinical diagnosis, nor compared to the controls.

In control infants, both glycerol and I-FABP levels also revealed a high degree of variation, with rising and falling concentrations during the observation periods (Figure 8A-K). One infant developed symptoms of intestinal ischemia, but no signs of NEC on x-ray (Figure 8K). Laparotomy revealed expansive intestinal necrosis, as a result of mid-gut volvulus. In this patient, I-FABP clearly displayed an early elevation, accompanied by a later rise of glycerol and CRP.
Figure 8. A-J. Glycerol, I-FABP, and CRP levels in ten infants without clinical or radiological signs of NEC. Values for glycerol are medians with bars for maximum and minimum values. Glycerol and I-FABP levels varied considerably during the observation periods, with both rising and falling concentrations at different time points. Two infants (E, F) were operated on for patent ductus arteriosus. K. One infant suffered from expansive intestinal necrosis due to mid-gut volvulus.

All infants were intubated and ventilation was maintained on respirator during the observation periods as indicated (Figure 7A-D, 8A-K). Three of them suffered from pneumothorax due to ventilator trauma (Figure 7B, 8A, G). Antibiotic treatment was also initiated in all children, and maintained for different periods as indicated (Figure 7A-D, 8A-K). Two of the infants displayed a significant persistent ductus arteriosus, and were operated on thereafter (Figure 8E, F). Severe infection and sepsis was present in one patient (Figure 8C).
Discussion

Today, NEC is diagnosed by a combination of clinical, laboratory, and radiological findings. However, these diagnostic methods lack specificity and sensitivity for NEC, especially in the early phase. Any indications of NEC are evident at a later stage during the course of the disease, when intestinal damage is manifest. Early diagnosis and treatment is important to reduce the morbidity and mortality associated with NEC.

Microdialysis has previously been used to study intestinal ischemia, both in humans and animals. The advantage of the microdialysis approach is that it measures metabolites of anaerobic metabolism, locally, in the organ of interest. In early stage experimental NEC, the mucosa is primarily affected as it is the part of the intestinal wall that is most sensitive to hypoxia (86).

In humans, patients with recent abdominal surgery have been monitored with microdialysis (77, 87), and these studies have demonstrated that microdialysis is a valuable tool for detecting visceral ischemia using the intraperitoneal approach. However, no clinical studies have previously been performed using the intraluminal approach, partly because of the difficulty of placing the microdialysis catheters in the lumen. The intraluminal approach, however, seems promising, and raises the opportunity to identify anaerobic stress at an earlier stage, before systemic levels of the metabolites are reached, and before the organ of interest is severely damaged.

To our knowledge, the studies in this thesis, are the first to apply intraluminal rectal microdialysis to detect hypoxic stress in extremely preterm infants, as well as in an animal model of NEC. Although it was noted that an elevation of glycerol was seen in infants with NEC, it was not possible to detect any significant increase in glycerol concentrations prior to clinical diagnosis. In infants with complications other than NEC, intraluminal glycerol values also varied considerably, both rising and falling during the observation period. Therefore it was not possible to differentiate between NEC and the controls by observing any increase in glycerol concentrations.

We have also for the first time been able to analyze plasma levels of I-FABP in extremely preterm infants born before 28 weeks of gestation. Plasma levels of I-FABP displayed a similar pattern as glycerol, with high concentrations before the development of NEC, as well as in the control infants. In this study, elevated intraluminal glycerol levels, as well as plasma I-
FABP, were detected in infants developing NEC as well as in those who had no abdominal symptoms. These controls, however, were severely ill, with complications following extreme prematurity. Primarily, respiratory- and ventilation-associated problems dominated, resulting in long periods with low blood oxygen saturation levels. This relative hypoxic state may result in a compromised oxygenation of the intestines; particularly, the sensitive mucosal cell-layer, which could result in a hypoxia-induced mucosal cell membrane decay and release of glycerol and I-FABP into the intestinal lumen. Other complications and diseases such as sepsis, infections, persistent ductus arteriosus, and anemia were also present in the control group, which aggravates the intestinal distress.

In a previous study of I-FABP as a diagnostic marker of intestinal ischemia, the suggested cut-off point for non-reversible intestinal ischemia was 1.3 ng/ml (88). In a study on healthy preterm infants with gestational age between 28 and 33 weeks, the plasma concentrations ranged between 0.46-4.5ng/ml (50). An interesting finding in our present study is that the concentrations of I-FABP, in controls as well as infants with NEC, exceeded these levels even at an early stage. These findings may suggest that the previous suggested cut-off point or normality range of I-FABP is not relevant in this patient category of extremely preterm infants. Infants with high enterocyte turnover should theoretically display higher levels of I-FABP. Another explanation could be that the high concentrations of I-FABP in the present study reflect intestinal enterocyte damage. This fact is supported by the high intraluminal concentrations of glycerol at an early stage in controls as well as in infants later developing NEC. It is highly valuable to establish knowledge regarding the normality range of I-FABP levels in this patient category.

Our study on experimentally induced early NEC in rat pups, demonstrated that intraluminal microdialysis can detect signs of hypoxic intestinal cell damage (86). In the study, elevated levels of glycerol and lactate were measured by placement of a rectally inserted microdialysis catheter.

Experimental NEC has previously been studied by inducing NEC through a combination of asphyxia and cold stress on newborn rats or hypoxia/re-oxygenation treatment only (17, 18, 58, 86, 89). The model that we used is based on the induction of intestinal damage using hypoxia/re-oxygenation treatment on rat pups, whereas NEC in human infants is more complex in its pathogenesis. However, the histopathological findings and the genetic transcriptome in the present model indicate similarities to clinical NEC.

Using this model with newborn rats, subjected to hypoxia/re-oxygenation treatment only, histological changes of moderate ischemic damage were observed in the intestinal specimens from the ileum, and ischemic metabolic changes were detected using intraluminal microdialysis in the rectosigmoid
part of the colon. The ability to detect these metabolites by rectal microdialysis is highly valuable when clinical NEC is suspected.

Ischemic or hypoxic stress to the intestinal mucosa initially leads to release of the metabolites of anaerobic metabolism into the bowel lumen (12). We have demonstrated that these metabolites are easily detectable using microdialysis in the bowel lumen by a rectal microdialysis catheter. Increased intraluminal levels of glycerol, lactate, and raised lactate/pyruvate ratio are all markers of ischemic stress, mucosal disintegration, and cell membrane decay. Increased luminal lactate levels have a positive correlation with prolonged occlusion of the superior mesenteric artery, but whether this is due to spill-over from elevated systemic hyperlactatemia is uncertain (13, 74, 75). An increased level of intraluminal lactate also correlates with ischemic stress at an early stage, but also increases in subcutaneous adipose tissue, and is therefore unable to differentiate between local and systemic ischemia.

Intraluminal rectal lactate has also been found to be elevated during coronary artery bypass operations (90). However, intraluminal changes in lactate levels, intestinal permeability, or adenosine triphosphate levels do not correlate with the extent of histopathological intestinal damage (64), whereas elevated glycerol levels have a positive correlation to prolonged ischemic insult and severity of intestinal damage (12, 13, 64).

In our previous study on experimental intestinal ischemia (12), signs of intestinal damage in the lumen were measured by microdialysis, before systemic levels of the anaerobic metabolites were reached. Prolonged local intestinal ischemia eventually leads to systemic levels of these anaerobic metabolites. The intraluminal levels of glycerol also had a positive correlation to aggravated histological mucosal damage, in accordance with our previous work (64). This suggests that glycerol is the best marker for mucosal damage thus far.

Increased levels of glycerol are considered to originate from phospholipid degradation of the cell membrane (61, 62). Systemic glycerol can be generated through stress-induced lipolysis in response to anaerobic metabolism (61, 62). As bowel permeability increases during ischemia (13), any increased intraluminal glycerol levels could originate from spill over from the plasma. However, this has not been supported in previous studies of intestinal ischemia, where elevated intraluminal glycerol was found along with low plasma levels (64). In our first study of intestinal ischemia, clamping of the suprarenal aorta creates a state of total ischemia, so no spill-over to the intestinal lumen is therefore possible (12). Glycerol can also be generated from glucose in the bowel lumen (62); however, no difference could be found in the intraluminal levels of glucose in this study.

A potential methodological problem of microdialysis is that it only measures a relative concentration of the metabolites in the compartment of interest.
This fact makes it difficult to compare the absolute values of two different measurements performed at different time intervals. To overcome the problem of relative concentrations, ratios such as the lactate/pyruvate ratio are often used. The lactate/pyruvate ratio is considered to be independent of changes in relative recovery, making it a useful quantitative measure [18]. In our studies of both experimental and clinical NEC, intraluminal levels of lactate and pyruvate were too low to be measured. The lactate/pyruvate ratio, therefore, could not serve as an indicator of hypoxic damage in the intestines in these setups. The concentrations of glycerol, on the other hand, were much higher and easily detectable well above the detection limit of the CMA 600 analyzer. A higher relative recovery could be achieved by using microdialysis catheters with longer membranes or by using a lower perfusion flow rate. This theoretically results in higher concentrations of lactate and pyruvate, enabling calculation of the lactate/pyruvate ratio. Initially, we tried to use a 30 mm membrane, but it was not possible due to the anatomical limitations of the extremely preterm infants.

In our analysis of the transcriptome using microarray in early experimental NEC, genes regulating TJ and cell adhesion were clearly affected secondary to hypoxia. Increased intestinal permeability may be considered a major factor in NEC pathogenesis, involving these structural proteins such as TJ and other cell adhesions (20, 91-93). In human NEC, this increase in permeability could however be secondary to ischemic damage to the entire intestinal wall or a primary structural immaturity due to prematurity.

In this model of experimental NEC, we have found several TJ genes to be both up- and downregulated. The most important ones are the claudins 1, 8, 14, and 15. This was also supported by the immunohistochemical staining, where TJ protein claudin-1 was less abundant in the NEC intestine compared with the controls. Interestingly, claudin-1 was mainly located in the intracytoplasmic compartment. It could be speculated that the intracellular localization of the TJ proteins may reflect a downregulation of the TJ proteins from the outer membrane after cellular stress resulting in an increased epithelial permeability. Other junctional adhesion genes such as gap junction protein and nucleoporin were also affected.

An increased bacterial translocation is also present in NEC, which could lead to a secondary degradation of the TJs following inflammation. In addition, increased permeability resulting from the "un-lockning" of the TJs may be the result of virus-induced disruption of TJ formation (39). Occludin, claudin, junctional adhesion molecules (JAMs), and the Coxsackie- and Adenovirus receptor (CAR) are major components of TJs (40). These receptors are used by the Coxsackie- and Adenoviruses to gain access to the cells, and TJ viral receptors binding to viruses can also disrupt the paracellular barrier (39, 40). This could represent an alternative mechanism by which the intestinal
barrier function is destroyed, leading to increased permeability to both bacteria and toxins. In this context, it is of special interest that virus receptor molecules constituting part of the MCH class II were also downregulated. Although previous research has focused on intestinal bacteria as a cause of NEC, there is a possibility that viruses play a critical role in NEC pathogenesis. However, the role of viruses in NEC pathogenesis is very unclear at present.

Moreover, alterations in previously identified genes involved in the inflammatory response was confirmed, along with several genes regulating proteins used as biomarkers for NEC. Better understanding of the genes involved in the pathogenesis of NEC may lead to novel strategies for the prevention and treatment of NEC.
Conclusions

Intestinal intraluminal microdialysis can detect metabolites of ischemia or hypoxia-induced damage, and cell membrane decay in rat models of total intestinal ischemia and experimental early NEC in rat pups. A rectal microdialysis catheter can be used for intraluminal measurements.

Microdialysate intraluminal glycerol is the best marker for early intestinal cell-membrane deterioration thus far, and correlates positively to increasing mucosal damage. Glycerol is also a marker for hypoxic damage mucosal cell-membrane decay in rat experimental early NEC.

In extremely preterm infants monitored during the neonatal period, elevated intraluminal glycerol and plasma I-FABP indicates intestinal hypoxia-induced mucosal cell-membrane decay. This elevation is seen in infants developing NEC, as well as in preterm infants with other complications resulting in hypoxia, such as respiratory problems, sepsis, anemia, or persistent ductus arteriosus.

In experimental early NEC, genes regulating the TJ's and cell adhesion are altered, accompanied by a decreased protein expression of claudin-1.

In conclusion, this thesis has demonstrated that rectal intraluminal microdialysis is safe and could provide a valuable non-invasive aid to detect hypoxia-induced intestinal damage, or ischemic stress in extremely preterm infants. However, it was not possible to predict or differentiate NEC from other diagnoses with hypoxia in preterm infants, by detecting elevated levels of glycerol or I-FABP.
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