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Serial fecal calprotectin in the prediction of necrotizing enterocolitis in preterm neonates ^{☆☆☆☆}



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ABSTRACT

Purpose: To investigate whether serial measurements of fecal calprotectin concentrations enable us to identify infants who will develop NEC prior to development of symptoms.

Methods: Prospective matched case–control study including 100 high-risk neonates. High risk includes 1) gestational age (GA) ≤ 30 weeks, 2) birth-weight (BW) ≤ 1000 g, 3) GA 30–32 weeks and BW ≤ 1250 g, 4) born from a mother who received indomethacin for tocolysis. We matched every NEC subject with three controls for birth weight and gestational age. Fecal calprotectin was measured twice a week from day one until five weeks after birth or until NEC development. We analyzed differences in fecal calprotectin between NEC subjects and controls in the week preceding NEC onset and course of fecal calprotectin within subjects who developed NEC.

Results: Of 100 included patients, ten (median GA 27.5 weeks [24.6–29.4], BW 1010 g [775–1630]) developed NEC. The median calprotectin concentration in all samples combined was 332 $\mu\text{g/g}$ [<40 –8230] $\mu\text{g/g}$ feces. There were no differences between NEC subjects and controls, with a wide variation in both groups. In NEC subjects, there was no intraindividual rise in calprotectin before clinical symptoms occurred.

Conclusions: There are high concentrations and wide interindividual variations in calprotectin in preterm infants during the first weeks of life. Wide intraindividual variation further precludes the serial use of fecal calprotectin in the early detection or prediction of NEC in high risk infants.

Level of Evidence: III

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Necrotizing enterocolitis (NEC) is one of the most common and devastating diseases in neonates [1]. NEC mainly affects preterm infants, with a prevalence of 7% among infants with a birth-weight < 1500 g [1]. Mortality rates can reach 50%, with the highest rates among preterm infants requiring surgery [1,2].

The pathogenesis of NEC is incompletely understood but is thought to be multifactorial [1]. Factors that have been suggested to confer a predisposition to NEC include intestinal immaturity, abnormal intestinal microbial colonization and a highly immunoreactive intestinal mucosa

[1,3]. The disease entity of NEC, however, may include cases with different initial pathogenesis, ranging from an acute ischemic event to a more slowly, and subclinical developing inflammatory process [3]. Detection of this inflammatory process before the first clinical signs of NEC could enable preventive interventions and early, targeted treatment of the disease.

A marker of intestinal inflammation is fecal calprotectin [4]. Calprotectin has been used successfully in children and adults for the detection of gastrointestinal inflammation [3–5]. Calprotectin is a calcium- and zinc-binding protein (36.5-kDa) and is predominantly found in neutrophils and macrophages, where it constitutes as much as 60% of the protein in the cytosol [3–6]. Calprotectin is released upon inflammatory activation in the gut and, as a result of transepithelial migration of myeloid cells, readily detectable in feces [3,6,7]. It may therefore be a marker of NEC and would be of particular use in the prediction of the disease if it detects any subclinical inflammatory processes [3].

Previously, calprotectin has been considered as a promising diagnostic marker for NEC [8–11]. However, the high levels and wide interindividual variation in calprotectin concentration in neonates may preclude

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its use for prediction or early detection of NEC, especially when using one single measurement [8–11]. So far, studies on serial measurements in infants at risk for NEC are scarce. We hypothesize that serial measurements may be used to detect intraindividual rises in calprotectin concentrations, possibly indicating a subclinical inflammatory process preceding the first symptoms of NEC. The aim of this study was therefore to determine whether serial measurements of calprotectin concentration in high-risk neonates enable us to identify infants who develop NEC.

1. Patients and methods

1.1. Patients

We performed a case–control study of prospectively collected data. Patients and controls were derived from a prospective observational cohort study performed at the neonatal intensive care unit (NICU) of the University Medical Center Groningen (UMCG) between October 2012 and February 2014. ('CALIFORNIA' trial, trial-ID: NTR4153 in the Dutch Trial Registry) [12]. The study was approved by the local medical ethics committee. Written informed consent was obtained from both parents before inclusion into the study protocol.

In the large cohort study we included 100 infants with a high-risk for NEC consecutively. We considered an infant at high-risk if one of the following inclusion-criteria was present: 1) gestational age (GA) ≤ 30 weeks, 2) birth-weight (BW) ≤ 1000 g, 3) GA between 30 and 32 weeks and BW ≤ 1250 g, 4) born from a mother who received indomethacin for tocolysis or 5) left-sided obstructive cardiovascular disease leading to reduced splanchnic blood-flow. Infants with a postnatal age less than 48 h were eligible for recruitment into our study. Patients with congenital intestinal diseases or bowel wall defects were excluded.

For the present case–control study, we selected all infants with NEC and matched 3 controls to each infant who developed NEC. We used GA and BW as matching-criteria, allowing a maximum of $\pm 10\%$ deviation.

1.2. Clinical data

We collected maternal data including age at delivery, mode of delivery (vaginal birth or cesarean section), preterm premature rupture of membranes (PPROM) (>24 h), chorioamnionitis and preeclampsia, and neonatal data that included gender, gestational age, birth weight, antenatal treatment with steroids, reason of prematurity (spontaneous, PPRM >24 h, induced birth (maternal / fetal reason)), Apgar scores at 1, 5, and 10 min, antibiotic treatment during first 48 h postpartum, prolonged postpartum antibiotic treatment (>48 h postpartum), sepsis (defined as a presence of a positive blood culture), hemodynamically significant patent ductus arteriosus (hsPDA), ibuprofen treatment for hsPDA. In addition, we recorded the need for mechanical ventilation, treatment with inotropics, fluid resuscitation and red-blood cell transfusion during study-period.

1.3. Feeding strategies

Infants were fed own mother's milk, human donor milk, preterm formula, or a combination. Infants weighing <1200 g received enteral boluses every 2 h; infants >1200 g every 3 h. All infants were fed through nasogastric tubes. Type and volume of enteral feeding were recorded per calendar day. Start of enteral feeding was recorded in hours after birth. Time to full enteral feeding was defined as the first day without parenterally protein or fat.

1.4. NEC diagnosis

We defined NEC when, beside clinical features, pneumatosis intestinalis, portal venous gas, or both were present on abdominal X-

ray, and consistent with NEC Bell's stage 2 or 3 [13,14]. The diagnosis was made by the attending neonatologist and radiologist and subsequently confirmed by the authors (AVZ, CVH, EK, JH, AB). We defined the time of onset of NEC as the first abdominal X-ray after clinical suspicion of NEC.

1.5. Feces collection and measurement of calprotectin concentration

We collected feces within 48 h after birth and thereafter 2 times a week (every first and fourth day per week), during 5 weeks postpartum or until NICU discharge, whatever came first. If the infant did not produce feces at the scheduled day of sampling, the next feces produced was collected (up to a maximum of 2 days after the initial sampling day). Feces samples of controls were matched to the postnatal day of feces samples of infants with NEC.

Stools were collected from the diaper, stored in plastic containers and frozen at -80°C until batch analyses. Before analysis, the samples were thawed at room temperature. The calprotectin concentration was measured using the commercially available enzyme-linked immunosorbent assay kit of Bühlmann Laboratories AG (Schönenbuch Switzerland), according to manufacturer's instructions as previously described [15]. In brief, 40 to 100 mg feces was used to extract calprotectin from each feces sample. Calprotectin was assayed in a single measurement using the DS2® automated ELISA processing system (Dynex Magellan Biosciences, Chantilly, VA, USA). Samples with initial calprotectin concentrations >1800 $\mu\text{g/g}$ feces were diluted ten times in incubation buffer and remeasured. The lower detection limit was 40 $\mu\text{g/g}$ feces. Calprotectin concentrations are given in $\mu\text{g/g}$ feces.

1.6. Statistical analysis

SPSS 22.0 software for Windows (IBM SPSS Statistics 22, IBM Corp., Armonk, New York, USA) was used for statistical analyses. GraphPad prism (Graph-Pad Software, CA, USA) was used for graphics.

Variables are presented as n (%) for categorical and ordinal variables or median [range] for continuous variables, unless specified otherwise. Comparisons in medians between subgroups were performed using the chi-square test for (independent) categorical variables and Student T-test or Mann–Whitney-U test for continuous variables, as appropriate. The Wilcoxon Signed Rank test was used to compare calprotectin concentrations longitudinally within subgroups (dependent variables). All tests were two-sided and a P value $<.05$ was considered statistically significant.

2. Results

A hundred high-risk infants were consecutively enrolled in the prospective cohort. At the end of the study we excluded two subjects from analyses: in one subject it appeared that severe gastrointestinal symptoms were already present before study inclusion, one other subject developed NEC Bell's stage 2 after the study period. The final cohort consisted therefore of 98 subjects (Fig. 1). Ten infants developed NEC at a median [range] of ten days postpartum [4–30]. Bell's stage 2 occurred in 2 subjects, stage 3 in 8 subjects.

In order to perform a case–control study, we selected for each subject that developed NEC three controls from the remaining 88 infants, resulting in a total of 30 controls (Fig. 1). Patients in the control group did not develop symptoms consistent with NEC.

The ten infants that developed NEC had a median gestational age of 27.5 weeks [range 24.6–29.4] and a median birth-weight of 1010 g [range 775–1630]. Median Apgar score at 5 min was 7.0. Eight infants received antibiotics during the first 48 h after birth and in 6 infants the treatment was continued. Enteral feeding was started after a median [range] time of 5 [2–10] h after birth. Full enteral feeding was reached in 3 infants (30%) before NEC occurred. No differences were found

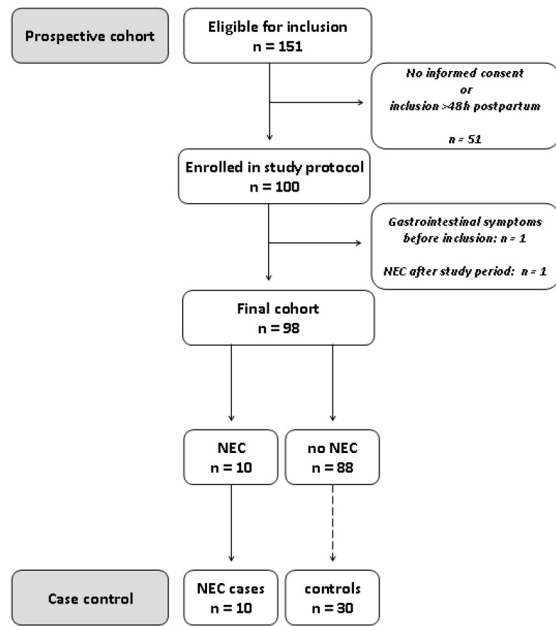


Fig. 1. Flowchart of prospective cohort trial and included subjects in present case control study.

between NEC subjects and their controls in demographic characteristics (Table 1).

A total number of 163 fecal samples were obtained, with an average of 4 samples per infant. The median calprotectin concentration calculated for all samples was 332 µg/g feces, ranging between <40 and 8230 µg/g feces.

The first postnatal feces sample was obtained at the second postnatal day [interquartile range, 1–3 days]. Calprotectin concentrations in this sample were not statistically different between subjects who ultimately developed NEC (460 µg/g [range 72–2170]) and their controls (308 [<40–8230]) ($P = .9$).

Fig. 2 displays calprotectin concentrations between NEC subjects and controls one week preceding the first clinical suspicion of NEC. No differences between NEC subjects and controls were found at 6–8 days (575 µg/g [range: 72–1180] vs. 290 µg/g [<40–2810] resp. $P = .80$), 3–5 days (312 µg/g [185–1250] vs. 340 µg/g [65–5880] resp., $P = .80$) and within 48 h before the first clinical suspicion of NEC (465 µg/g [140–620] vs. 390 µg/g [<40–2630] resp. $P = .80$) (Fig. 2).

Fig. 3 presents the courses of calprotectin concentrations within all individual NEC subjects (Fig. 3). We found no intraindividual rise in calprotectin concentrations before clinical symptoms of the disease occurred.

3. Discussion

Our study suggests that in preterm infants at high-risk for NEC, serial measurements of calprotectin concentrations do not have the ability to predict who will develop NEC and who will not. We found no differences between calprotectin concentrations in infants who ultimately developed NEC and matched controls at any time-point from birth to the first clinical suspicion of NEC. We hypothesized that we could detect an intraindividual rise in calprotectin concentrations within NEC subjects, suggesting a subclinical inflammatory process. In the present series we were not able to confirm this hypothesis.

The present study confirms the previously identified high calprotectin concentrations in infants during the first weeks of life [3,16–18]. This high concentration may reflect increased transepithelial migration of neutrophils into the intestinal lumen, as a result of higher intestinal permeability during the neonatal period [6,17,19]. Our median calprotectin concentration of 332 µg/g [range: <40–8230] was,

Table 1
Demographic characteristics of study population.

		Controls (N = 30)	P
Gestational age (wk)	27.5 [24.6–29.4]	27.6 [25.3–29.9]	.61
Birth-weight (g)	1010 [775–1630]	1025 [615–1735]	.82
Male	5 (50)	17 (56.7)	.77
Twin	3 (30)	9 (30)	.99
Antenatal steroids	9 (90)	28 (93.3)	.89
Cesarean section	3 (30)	15 (50)	.28
Maternal age (yr)	31 [19–41]	28 [24–42]	.42
Reason prematurity			
PPROM (>24 h)	4 (40)	4 (13.3)	.07
Spontaneous	3 (30)	15 (46.7)	.27
Induced birth: maternal	2 (20)	4 (16.7)	.26
Induced birth: fetal	1 (10)	7 (20)	.83
Induced birth: maternal and fetal	0	0	
APGAR-score			
1-min	4.5 [1–7]	5.0 [0–10]	.40
5-min	7.0 [1–9]	7.5 [0–10]	.59
10-min	8.0 [4–10]	8.0 [3–10]	.41
Antibiotics during first 48 h after birth	8 (80)	25 (83.8)	.89
Prolonged antibiotic treatment (>48 h after birth)	6 (60)	11 (36.7)	.29
Sepsis during study period ^a	7 (70)	12 (40)	.17
hsPDA	4 (40)	11 (36.7)	.89
Ibuprofen treatment hsPDA	4 (40)	10 (33.3)	.77
Mechanical ventilation during study inclusion	7 (70)	19 (63.3)	.77
Inotropics during study inclusion	4 (40)	3 (10)	.17
Fluid resuscitation	4 (40)	14 (46.7)	.55
RBC transfusion	8 (80)	21 (70)	.67
RBC in 48 h before NEC onset	2 (20)	Not applicable	
Feeding			
Start enteral feeding postpartum (h)	5 [2–10]	4 [1–9]	.20
Full enteral feeding before NEC onset	3 (30)	Not applicable	
Time to full enteral feeding (days)	Not applicable	12 [7–22] N = 19	
Length NICU stay (days)	40 [6–89]	27 [6–103]	.11
Death	3 (30)	4 (13.3)	.24
NEC Bell's stage 2	2 (20)		
NEC Bell's stage 3	8 (80)		
Onset NEC (days postpartum)	10 [4–30]		
Surgery	7 (70)		

Abbreviations: NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; hsPDA, hemodynamically significant patent ductus arteriosus; PPRM, preterm premature rupture of membranes; RBC, red blood cell.

Data are expressed as median [range] or number (percentage). No statistical significant differences were found between NEC cases and controls.

^a Positive blood culture.

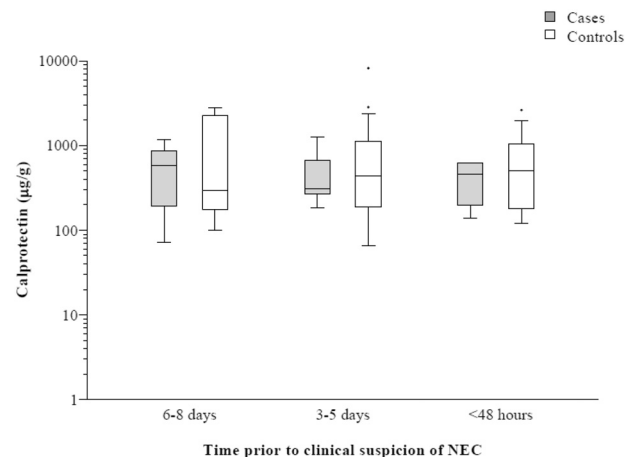


Fig. 2. Fecal calprotectin in the week before clinical suspicion of NEC.

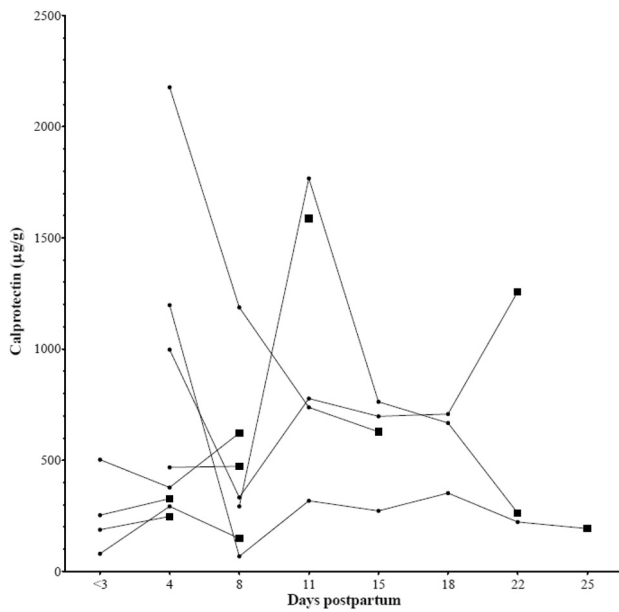


Fig. 3. Postnatal course of fecal calprotectin concentration in preterm infants with NEC.

however, even higher than concentrations reported by other authors investigating calprotectin in healthy preterm neonates during the first weeks of life (98–253 µg/g) [3,11,16–18]. Our high results may reflect a different sensitivity of the laboratory assay since we used an assay kit from a different firm (Buhlmann Laboratories AG, Schönenbuch, Switzerland) compared to all other studies (Calprest; Eurospital, Trieste, Italy) for calprotectin measurements.

In line with previous studies, samples in the present study exhibit high inter- and intraindividual ranges in calprotectin concentration [3,5,11,17,18]. Since calprotectin is stable at room temperature for various days and it resists proteolysis, the observed variations are unlikely to result from poor stability of calprotectin [17]. The method of collecting feces from the diaper may have attributed to the interindividual variations, as the absorbed water by the diaper may result in a higher concentration of calprotectin per gram of feces. However, it has been shown previously that this sampling method increases the calprotectin concentration by no more than 30%, a variation much lower than observed in the present study [20]. We therefore believe that the wide ranges in calprotectin concentrations as we found truly reflect both inter- and intraindividual variability in calprotectin excretion in our study population [17].

Fecal calprotectin concentrations have been studied in relation to NEC previously, especially in the diagnosis and prediction of severity of the disease. Several cutoff values for detection of NEC have been proposed, such as 200 µg/g by Carroll et al. [8], 286.2 µg/g by Thuijls et al. [10], 636 µg/g by Campeotto et al. [21] and the highest level of 2000 µg/g by Joseffson et al. [3]. However, all suggested cutoff values remained within the range of concentrations observed in the control infants without digestive symptoms in present study. Even the high cutoff level of 2000 µg/g to identify patients with NEC was exceeded by 9 (30%) control infants in the present study, while only one infant who ultimately developed NEC exceeded this value prior to the disease. The wide ranges and high concentrations in control infants we observed in the present study, preclude the use of suggested cutoff values of calprotectin in the prediction of NEC.

The strength of present study is the prospective design and serial feces sampling. We collected feces twice a week, starting as early as within 48 h after birth, until 5 weeks postpartum in 100 infants at a high-risk for NEC. This design enabled us to match each NEC subject to three controls based on gestational age and birth-weight. Furthermore, feces samples of NEC subjects and controls could be compared

appropriately at the same postnatal day. We included a homogenous study population and variation both in sample collection and in assay procedures was minimized.

The frequency of feces sampling (twice a week) may be considered as a limitation, as it might not have been frequent enough to detect changes in calprotectin concentrations immediately preceding the first clinical symptoms of NEC. However, in daily practice it is common that preterms produce feces with a limited frequency. A predictive fecal biomarker in preterms should therefore predict a disease preferably amply before clinical symptoms occur.

Another limitation is the relatively low number of patients, and the fact that we did not perform a formal power analysis aimed at differences in calprotectin levels prior to the study. However, performing a post-hoc power analysis using the data from Reisinger et al. and Aydemir et al. demonstrated that 10 cases and 10 controls would suffice to identify significant differences in fecal calprotectin between the groups [9,22].

To conclude, while supporting the previous findings of high concentrations and wide inter-individual variation in calprotectin in preterm infants during the first weeks of life, the present study suggests that the wide intraindividual variation further precludes the serial use of calprotectin concentrations in the early detection of NEC in high risk infants.

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