C-Reactive Protein, Detected with a Highly Sensitive Assay, in Non-Infected Newborns and Those with Early Onset Infection

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Key Words

Neonate · Infection · High sensitivity C-reactive protein

Summary

Background: The aim of this study was to investigate C-reactive protein (CRP), measured by a highly sensitive method (hsCRP) in non-infected newborns and in those with suspected early onset bacterial infection (EOBI) as well as to test whether EOBI would be detectable earlier by hsCRP than by a nephelometric CRP (nsCRP) assay (thresholds > 10 mg/l) or IL-8. **Patients and Methods:** 106 neonates with signs of infection comprised the suspected EOBI group. 134 neonates with risk factors but confirmed exclusion of EOBI served as non-infected controls. **Results:** In the non-infected group, hsCRP in the first 6 h after birth was low (0.7 mg/l; SD 0.16 mg/l) but showed an increase to 4.11 mg/l (SD 3.33 mg/l) at 72 h ($p < 0.001$ vs. 6 h). The sensitivity of hsCRP (cut-off 0.3 mg/l) vs. nsCRP for EOBI was 0.46 vs. 0.23 at 6 h after clinical suspicion. Of all parameters measured, IL-8 had the highest sensitivity and specificity to detect EOBI at 6 h (0.60 and 0.90), but declined after 12 and 24 h. **Conclusion:** Lowering the CRP detection threshold by a highly sensitive assay did not improve diagnostic accuracy for EOBI.

Schlüsselwörter

Neugeborene · Infektion · Hoch-sensitives C-reaktives Protein

Zusammenfassung

Hintergrund: Ziel der Studie war es, C-reaktives Protein (CRP) mittels einer hochsensitiven Methode (hsCRP) bei Neugeborenen mit und ohne bakterieller Frühinfektion zu messen und zu ermitteln, ob sie mittels hsCRP im Vergleich zur nephelometrischen Bestimmung oder der IL-8-Konzentration früher diagnostiziert werden kann. **Patienten und Methoden:** Die Gruppe mit Infektionsverdacht bestand aus 106 Neugeborenen mit Infektionszeichen. 134 Neugeborene mit Risikofaktoren einer Infektion, bei denen eine Infektion aber ausgeschlossen werden konnte, dienten als Kontrolle. **Ergebnisse:** In der nichtinfizierten Gruppe war das hsCRP in den ersten 6 Lebensstunden niedrig (0,7 mg/l; SD 0,16mg/l) und stieg nach 72 h auf 4,11 mg/l (SD 3,33 mg/l) an ($p < 0,001$). Bei einer frühen Neugeboreneninfektion war die Sensitivität von hsCRP 6 h nach klinischem Verdacht 0,46 im Vergleich zu 0,23 mit nsCRP (Cut-off 0,3 vs. 10 mg/l). Von den getesteten Parametern wies IL-8 zu dieser Zeit die höchste Sensitivität und Spezifität auf (0,60 und 0,90). **Schlussfolgerung:** Eine niedrigere Detektionsgrenze für CRP durch eine sensitivere Nachweismethode konnte die prädiktive Wertigkeit für die frühe Neugeboreneninfektion nicht erhöhen.

Introduction

C-reactive protein (CRP) is synthesized in the liver in response to microbial infections, tissue injury, autoimmune dis-

orders, and atherosclerosis [1, 2]. Its main triggers are IL-1 β and IL-6. Therefore, its delayed increase is due to a cascade of preceding events [3]. Early onset bacterial infection (EOBI), defined as occurring within 72 h after birth, is one of the most

common challenges in neonatology [4]. As a diagnostic EOBI marker, CRP has a higher sensitivity and specificity than total neutrophil count or I/T ratio [5], considerably improving with serial measurements after 24 and 48 h [1, 6].

Considering the high mortality and serious morbidity associated with EOBI plus the fact that it may be curable when diagnosed early, CRP cannot be considered a sufficient 'early' predictor, but rather a 'late specific verifier' [4, 7, 8]. This is in contrast to alternative assays such as functional cellular analysis by flow cytometry [9, 10], but these methods require a substantial amount of technical skill. For detection of EOBI, different CRP-quantifying methods were used. Traditionally, rate nephelometry measurements for CRP (nsCRP) were performed with detection limits above 10 mg/l [9, 11–13].

Cytokines, e.g. IL-6 and IL-8, increase rapidly after exposure to bacteria and precede the increase in CRP [5, 13]. IL-6 and IL-8 have very short half-lives, and circulating concentrations drop precipitously following antimicrobial treatment, becoming almost undetectable within 24 h [5, 13]. In order to improve the sensitivity and specificity for IL-8, we developed a detergent-lysed whole blood assay, showing IL-8 concentrations to be sustained for more than 24 h and thus hopefully present neonatologists a wider window of opportunity for obtaining a blood sample [13]. This method, however, requires an additional cell lysing procedure and up to date has not evolved as a routine parameter.

Traditionally, CRP rate nephelometry measurements were performed with detection limits above 10 mg/l [11–14]. With the commercial availability of highly sensitive latex assays based on microparticle-enhanced turbidimetry [3], CRP concentrations of only 0.01 mg/l can now be quantified automatically (hsCRP) [2]. In the future, multiplexed assays could be available that simultaneously quantify CRP and cytokines from a few microliters of blood [15, 16]. Based on observations that CRP is synthesized within 6 h after exposure to an infective process [4], we tested the hypothesis that hsCRP, measured by a highly sensitive latex assay, may be superior in the detection of EOBI than nsCRP, measured by nephelometry with a cut-off value of 10 mg/l.

Patients and Methods

Patients

Neonates consecutively admitted and meeting inclusion criteria were enrolled prospectively with institutional ethics committee approval and parental consent. Blood was collected from non-infected neonates who underwent testing because of risk factors for EOBI, but had no clinical signs or subsequent laboratory changes. To exclude EOBI, blood was drawn maximally 2 times per patient at defined intervals: 6, 12, 18, and 24, 48, 60–72 h post partum. In addition patients were closely observed by experienced neonatologists at least three times per day. Blood was collected from neonates with EOBI within 6 h after clinical suspicion and 24 h later as defined below. Blood samples were obtained prior to and after antibiotic treatment. All samples were processed and analyzed within 2 h. A prerequisite was a macroscopically non-hemolytic sample.

Definition of Bacterial Infection (EOBI)

A diagnosis was based upon the presence of at least two of the following criteria within the first 72 h [11–14, 17]: One or more clinical signs compatible with EOBI, with a duration of more than 1 h, plus a consecutive elevation of CRP > 10 mg/l within 24 h after first clinical suspicion, or positive blood culture results. Based on previous studies [13, 14, 17], clinical signs were defined as follows: fever ($\geq 37.8^{\circ}\text{C}$ rectal), hypothermia ($\leq 36.5^{\circ}\text{C}$), temperature instability ($\geq 1.5^{\circ}\text{C}$), pallor, grayish skin color, poor skin perfusion (capillary refill > 2 s), tachypnea (>60 respirations/min at rest), dyspnea (grunting, nasal flaring, retractions), respiratory insufficiency, apnea, rising FiO_2 in previously stable neonate, arterial hypotension (mean arterial blood pressure < 37 mm Hg), muscular hypotonia, irritability, hyperexcitability, neck stiffness, and lethargy. The CRP cut-off of 10 mg/l has been used according to previous investigations [11–14, 17–19]. Neonates not meeting criteria for culture-proven or clinical infection were considered non-infected.

Workup Program for Suspected EOBI

An indication for clinical observation and blood screening program was one or a combination of the following criteria: history of amniotic infection, maternal leukocytosis ($>12,000$ granulocytes/ mm^3), and/or maternal CRP elevation to >10 mg/l after exclusion of infectious foci unrelated to the fetus (gastrointestinal or urinary tract infections), fetal tachycardia (>160 beats/min), prolonged rupture of membranes (≥ 12 h) in the absence of labor, maternal fever (rectal temperature $\geq 38.0^{\circ}\text{C}$), and foul smelling amniotic fluid. Growth of group B streptococci in vaginal smear was routinely screened in case of prolonged rupture of membrane.

Sample Processing and Detection of hsCRP, nsCRP, and IL-8

hsCRP serum concentrations were detected by a fully automated latex assay based on microparticle-enhanced turbidimetry. Assays were performed on an automated clinical chemistry analyzer (Hitachi 902; Hitachi Europe Ltd., Berkshire, UK). Instruments, reagents, and calibrators, which were traceable to CRM 470, were from Roche Diagnostics (Mannheim, Germany). 50 μl serum, buffered with tris(hydroxymethyl)aminomethan-hydrochloride (TRIS) buffer was well mixed with anti-CRP-coated latex microparticles. Antigen-antibody complexes were analyzed turbidimetrically. The measuring range for hsCRP was between 0.1 and 60 mg/l. nsCRP was measured by enzyme sandwich immunoassay (Vitros 250; Ortho Diagnostics, Rochester, NY, USA), with a range between 7 and 100 mg/l. For IL-8 detection, 25 μl serum were diluted with 100 μl (1:5) sample diluent (I8; DPC Biermann, Bad Nauheim, Germany). The detection limit was 2 pg/ml (standardized in accordance with the National Institute for Biological Standards and Controls Reference Preparation 89/520) and was calibrated to 7,500 pg/ml. According to an analysis using receiver operator characteristics (ROC) curves, the threshold for IL-8 plasma concentration was set to 60 pg/ml. The I/T ratio (immature neutrophils / total neutrophils) served as a neutrophil index, differentiated with microscopy by experienced technicians. An I/T ratio ≥ 0.2 was considered elevated.

Statistical Analysis

For hsCRP, nsCRP, IL-8 and I/T ratio, specificity, sensitivity, positive and negative predictive value as well as the corresponding 95% confidence intervals were calculated. ROC curves were constructed to describe the relationship between the sensitivity and the false-positive rate (1-specificity) for different parameters.

CRP and IL-8 concentrations are shown as Box-Whisker plots. Data were grouped; results obtained between 0 and 6 h are depicted as 6 h; results obtained between 6 and 12 h as 12 h; those obtained between 18 and 24 h as 24 h, etc. For the postnatal kinetics of non-infected newborns, the Wilcoxon test was applied. Correlations between CRP, Apgar score, cord blood pH were analyzed by Spearman coefficient. Comparisons between the sensitivity of hsCRP and nsCRP were performed by a two-tailed Fish-

Fig. 1. Kinetics of hsCRP in 134 non-infected full term neonates until 72 h post partum.

er's exact test. A probability of $p < 0.05$ was defined as statistically significant. All charts were created with the help of software (SigmaPlot 2000; SPSS, Chicago, IL, USA).

Results

Patients

Serological and clinical follow-up data were complete for 240 term neonates with pre-, peri-, or postnatal risk factors and/or symptoms of EOBi, comprising 134 (63.2% male) neonates without and 106 (60.4% male) neonates meeting criteria for EOBi. Positive blood cultures were found in 4 of these (3 patients *Streptococcus* group B, 1 patient *Escherichia coli*). Prenatal risk factors such as maternal fever or fetal tachycardia were seen more frequently in the EOBi group (38 vs. 21%; $p < 0.05$). 75% (80/106) of the women received at least one dose of antibiotics intrapartum. The mean time ($\pm$ SD) of clinical suspicion was 16.1 ± 6.2 h. All patients in the EOBi group received antibiotics according to our institutional standards (duration of treatment 6.7 ± 2.3 days), compared to 3.7% (5/134) in the non-infected group. Since the suspicion of EOBi did not hold ($\text{CRP} \leq 10$ mg/l, negative blood culture), treatment was discontinued after 2 days (range 1–3.4 days) in the latter group; these newborns were regarded as non-infected. None of the patients in the non-infected group was subsequently readmitted with a diagnosis of late onset bacterial infection.

CRP Kinetics in Non-Infected Neonates

hsCRP kinetics revealed an age-dependent postnatal increase from a mean of 0.70 ± 0.17 mg/l after 6 h to 2.14 ± 1.95 mg/l after 24 h ($p = 0.001$ vs. 6 h) and 4.11 ± 2.10 mg/l after 72 h ($p = 0.002$ vs. 24 h) (fig. 1). Corresponding nsCRP concentrations were < 10 mg/l in 27/28 patients (96%) after 6 h and in 29/34 patients (85%) after 24 h. hsCRP at 6 and 24 h correlated weakly with birth weight ($r^2 = 0.11$) but neither was influenced by gender nor mode of delivery nor or 5-min Apgar values (data not shown, but available on request).

Table 1. Diagnostic accuracy (exact 95% confidence interval based on binomial sampling process) of hsCRP, nsCRP, and IL-8 within 6 and 24 h after first suspicion of EOBi.

	HsCRP > 0.3 mg/l	NsCRP > 10 mg/l	IL-8 > 60 pg/ml
<i>6 h after first suspicion of EOBi</i>			
Sensitivity	0.46 (0.19–0.75)	0.23 (0.05–0.54)	0.78 (0.26–0.88)
Specificity	0.66 (0.46–0.82)	0.96 (0.82–0.99)	0.90 (0.70–0.99)
PPV	0.25 (0.15–0.65)	0.59 (0.19–0.99)	0.60 (0.35–0.97)
NPV	0.83 (0.52–0.88)	0.83 (0.56–0.86)	0.90 (0.61–0.95)
<i>24 h after first suspicion EOBi</i>			
Sensitivity	0.96 (0.79–0.99)	1.00 (0.86–1.00)	0.23 (0.05–0.54)
Specificity	0.94 (0.80–0.99)	0.88 (0.73–0.97)	1.00 (0.79–1.0)
PPV	0.80 (0.74–0.99)	0.68 (0.67–0.96)	1.00 (0.29–1.0)
NPV	0.99 (0.84–0.99)	0.94 (0.88–1.00)	0.84 (0.41–0.90)

Time Kinetics of hsCRP, nsCRP, and IL-8 in EOBi

The correlation between hsCRP and nsCRP for values > 10 mg/l was high ($r^2 = 0.93$). In the EOBi group, hsCRP concentrations were 2 mg/l (range 0.1–29.4 mg/l) at 6 h and 19.5 mg/l (range 11.0–54.0 mg/l) at 24 h after first clinical suspicion. In the course of EOBi, its diagnostic accuracy increased with highest sensitivity and specificity 24 h after first clinical suspicion (fig. 2). Sensitivity, specificity, positive and negative predictive values for hsCRP, nsCRP, IL-8 and ROC curves 6 and 24 h after first clinical suspicion of EOBi are shown in table 1 and figure 2.

In neonates with EOBi, the sensitivity of hsCRP (> 0.3 mg/l) vs. nsCRP (> 10.0 mg/dl) was 0.46 vs. 0.23 within the first 6 h; reaching a specificity of 0.66 vs. 0.96 (table 1). Both CRP values showed negative predictive values of 0.83. Of all parameters measured, IL-8 had the highest sensitivity and specificity to detect EOBi at 6 h (0.78 and 0.90), but declined after 24 h (table 1, fig. 2).

Discussion

We showed that postnatal hsCRP levels in non-infected full term neonates were well below the detection limit for nsCRP and revealed an age-dependent increase until 72 h of age (fig. 1) as also known for e.g. cellular immune markers [20, 21]. Compared to nsCRP, even very low hsCRP cut-off values within the first hours of clinical suspicion, however, could not improve sensitivity and specificity to detect suspected or culture-proven EOBi (fig. 2, table 1).

Our study is in accordance with investigations where the majority of 'apparently healthy newborns' had nsCRP values < 10.0 mg/l [22]. In our high sensitivity assay, we confirmed the previously described postnatal nsCRP increase, as detected by nephelometric methods [23]. Despite CRP not crossing the placental barrier [24], this increase occurs possibly by a

Fig. 2. ROC curves for hsCRP, nsCRP, IL-8 **a** 6 h and **b** 24 h after clinical suspicion. Sensitivity is plotted against 1-specificity for hsCRP serum level thresholds between 0.1 and 30.0 mg/l; nsCRP serum level thresholds between 10.0 and 50.0 mg/l, IL-8 serum level thresholds between 10 and 1000 pg/ml. Open circles end for reasons of cut-off value of 10 mg/l.

prostaglandin-mediated elevation of fetal or maternal CRP-inducing cytokines [25]. A 3-fold hsCRP rise occurred within the first 24 h, suggesting the influence of perinatal factors, as described with nsCRP [26]. Between 24 and 72 h, hsCRP values in our non-infected patients almost doubled, which could be attributed to physiological intestinal colonization [27], or postnatal maturation of hepatic functions [28].

As ours, the majority of published reports provide upper limits for CRP during the neonatal period from symptomatic non-infected patients [26, 29]. Although none of our patients was re-admitted with late onset infection, we are aware that hsCRP values in our non-infected group do not represent an ideal population (i.e. term infants without risk factors). The fact that for nsCRP no reference values have been established for neonates may explain the wide range of reported nsCRP sensitivities (47–100%) and specificities (6–97%) [29–31]. Although not as accurate, we previously had described the IL-8 lysate [13]; of all parameters tested in this study, IL-8 serum concentrations showed the highest sensitivity and specificity for EOBI within the first hours of clinical suspicion (table 1, fig. 2a), as already described by us and others [11–14, 17]. Our study highlights one important point: Since IL-8 plasma half-life comprises only 1–3 h, its determination may lead to false-negative results when sampling is performed later in the course of disease [13, 14], which here again is reflected by decreasing sensitivity and specificity 24 h after clinical suspicion (table 1, fig. 2b). In accordance with previous studies [13], the I/T ratio performed rather badly.

In general, our investigation reveals the problem of defining EOBI. Although the clinical course was compatible and we tried to include the duration of symptoms of more than 1 h, blood cultures were positive in only 4 patients (3.7%). Besides their low sensitivity in EOBI [32–34], this may be partly due to the implementation of periparturient treatment, with 75% of women in the EOBI group receiving antibiotics, which further decreases blood culture sensitivity [35]. 30% (32/106) of our neonates with EOBI were positive for group B streptococci. Estimates of the incidence of group B streptococci EOBI revealed that the true burden, as indicated by culture-proven cases, is underestimated since the latter can be false-negative in at least 50% of infants [36]. The high hsCRP-negative predictive values for EOBI, as with investigations on nsCRP [4, 6], may reflect a circular conclusion since our definition of clinical EOBI contained CRP as essential verifying parameter. As in various other studies [17–19, 32, 34], the majority of our patients were ‘clinically infected neonates’. Although the clinical work-up was mostly performed by experienced neonatologists, we are aware of the intrinsic restrictions of this approach.

In conclusion, the much lower threshold of CRP concentrations by a highly sensitive assay failed to improve diagnostic accuracy for detection of EOBI and cannot replace an early predictive parameter such as IL-8. Therefore, as with most diagnostic markers, hsCRP values are not specific or sensitive enough to justify provision of antibiotic treatment independent of clinical findings.

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