Faecal Calprotectin in Term and Preterm Neonates

†Nathalie Kapel, ‡Florence Campeotto, ‡Nicolas Kalach, §Mariella Baldassare, *Marie-José Butel, and ‡Christophe Dupont

ABSTRACT

Objectives: The aim of this review was to examine the characteristics of the faecal calprotectin assay in neonates and the evidence for its use as a noninvasive marker of intestinal illnesses during the neonatal period.

Methods: Bibliographic searches were performed in the MEDLINE electronic database up to February 2010 looking for the following words (all fields): ''infants'' or ''neonates'' and ''calprotectin.'' Twenty studies, in which 1180 neonates were enrolled, were selected.

Results: During the neonatal period, calprotectin levels are characterized by significantly higher values in both healthy full-term and preterm infants during their first year of life compared with reference values established for children and adults. No difference was observed according to gestational age or birth weight, whereas a higher faecal calprotectin level was detected during intestinal distress in neonates with either inflammatory or patent digestive alterations. Despite high interindividual variations, cutoff levels are proposed to identify infants with a high risk of intestinal illnesses.

Conclusions: Compared with adults and children, healthy full-term and preterm neonates have high calprotectin levels. The measurement of calprotectin levels in faeces can be a promising noninvasive clinical screening test for intestinal distress in neonates.

Key Words: digestive distress, faecal calprotectin, neonate, preterm

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The development of noninvasive biomarkers is of growing interest in clinical practice because they enable us to gain an objective evaluation of the disease activity and severity and serve as a prognostic indicator of the treatment outcome. In 2001, a National Institutes of Health working group standardized the definition of a biomarker as ''a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.'' To be of clinical value, a biomarker should be accurate, reproducible, acceptable for the patient, easy to interpret by the clinician, sensitive, and specific for the outcome it is expected to identify. Some data suggest that the knowledge of biomarker levels affects medical management (1).

Faecal inflammatory markers, which include a heterogeneous group of substances that either leak from or are generated by the inflamed intestinal mucosa, should represent a noninvasive means of measuring objectively mucosal inflammation when the latter plays a role in the assessment of disease activity. Among those markers, calprotectin, most likely originating from transepithelial migration of granulocytes, is now considered reliable for the detection of intestinal diseases and the assessment of inflammatory activity in the gastrointestinal (GI) tract, both in adults and in children (2). It would be of major interest to provide a similar marker of inflammation of the GI tract in neonates, and several studies have been performed in the neonatal population to provide cutoff levels. However, the usefulness of this marker remains controversial in the neonatal period due to high levels in this age range, more than 5 times higher than in adults and children older than 4 years of age, and to high interindividual variations in both healthy full-term and preterm infants during the first weeks of life. The purpose of this review is to examine the characteristics of faecal calprotectin in neonates and the evidence for its use as a noninvasive marker of intestinal disease during the neonatal period. Bibliographic searches were performed in the MEDLINE electronic database up to February 2010 looking for the following words (all fields): ''infants'' or ''neonates'' and ''calprotectin.''

CALPROTECTIN CHARACTERISTICS

Nature and Functions

Calprotectin was originally discovered as an antimicrobial protein, present in the cytoplasm of neutrophil granulocytes, where it constitutes about 60% of soluble cytosol proteins (3). It is also expressed on the membranes of monocytes and in some mucosal epithelial cells (4,5). It is a 36.5-kDa heterodimer composed of 1 light (MRP8) and 2 heavy (MRP14) chains (8 and 14 kDa) and belongs to the S-100 family of calcium-binding proteins (6,7). The binding of calcium induces conformational changes, the calcium-saturated state, which allows binding to other proteins (8). Calprotectin also contains histidine-based zinc-binding sequences (His-X-X-X-His motif) involved in its antibacterial activity (9). Although its exact biological function is not known, calprotectin was shown to have bactericidal and fungicidal properties (10). Various data also suggest that it may be involved in the regulation of inflammation. Calprotectin is secreted extracellularly from stimulated neutrophils (11) and monocytes (12), or is released by cell disruption or death (13). Diverse digestive diseases including inflammatory bowel diseases (IBDs) lead to a higher disposal of leukocytes in faeces (14,15). In adults, the interest in calprotectin as a marker for gut mucosal inflammation was prompted by the demonstration that 4-day faecal excretion of ^{111}indium (^{111}In) leukocytes, considered the criterion standard faecal marker of inflammation (16), positively and strongly correlated with the measurement of calprotectin in faeces. Roseth et al (17) were the first to describe such a correlation,

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From the *Ecosystème Intestinal, Probiotiques, Antibiotiques (EA 4065), Université Paris Descartes, the †Coprologie Fonctionnelle, APHP, Groupe Hospitalier Pitié-Salpêtrière, the ‡Néonatologie et Gastroentérologie Pédiatrique, APHP, Groupe Hospitalier Cochin-Saint Vincent de Paul, Université Paris Descartes, Paris, France, and the §Neonatology and Neonatal Intensive Care Unit, Department of Gynecology, Obstetrics and Neonatology, University of Bari-Policlinic Hospital, Bari, Italy.

Address correspondence and reprint requests to Christophe Dupont, Service Néonatologie, Groupe Hospitalier Cochin-St Vincent de Paul, 82 Avenue Denfert-Rochereau, 75674 Paris, France (e-mail: christophe.dupont@svp.aphp.fr).

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comparing 29 adult patients with IBD and 9 presumably healthy controls. He found a strong correlation between the average daily excretion of calprotectin or measurement of this protein in a spot stool sample obtained at day 1 and in the total 3-day excretion of ^{111}In-labelled granulocytes ($r = 0.87$, $P < 0.0001$ and $r = 0.80$, $P < 0.0001$, respectively). Tibble et al (2) confirmed that the 4-day faecal excretion of ^{111}In correlated significantly with the calprotectin concentration in a single stool sample ($r = 0.70$, $P < 0.001$) in adults with Crohn disease.

These data support calprotectin as an accurate indicator of neutrophil migration towards the GI tract. The measurement of calprotectin in faeces is therefore recognised as a reliable marker of intestinal diseases and inflammation activity in the GI tract. A recent review summarised the data about this marker in the delineation between intestinal diseases and functional conditions and its value as a noninvasive surrogate marker in IBD in both adults and children (18).

Measurement in Faeces

When bound to calcium, calprotectin is remarkably resistant to proteolysis and colonic bacterial degradation, allowing stool samples to be kept for up to 1 week at room temperature without any significant degradation (3,19–21). Moreover, calprotectin is stable at −20°C for at least 6 months. Calprotectin can thus be easily quantified in faeces. Besides, no significant differences were seen between blended and unblended stools (21). Several enzyme-linked immunosorbent assays (ELISA) using small stool samples (0.1 g) are now commercially available. Today, the reference value given by all commercially available ELISAs is 50 μg/g faeces for healthy adults and children ages from 4 to 17 years, regardless of sex (22).

Semiquantitative rapid tests for faecal calprotectin have been developed recently (23–26). These have been evaluated only in adult populations and exhibit a good performance for excluding IBD in patients with no detectable faecal calprotectin and identifying active IBD or colorectal carcinoma in patients with high calprotectin levels. In case of moderate intestinal inflammation, results seem more unpredictable (25). Using these rapid tests, the intensity of the band can be read through a digital camera to render the test semiquantitative, the intensity of the test line being appraised with an ordinal scaling spanning from 0 to 5. However, Damms and Bischoff (25) reported an evaluation of the performance of this method through receiver operator characteristic analyses showing that the conventional ELISA method was slightly better than the new rapid test, likely because of the imprecise ordinal scaling of the rapid test compared with the quantitative calprotectin measurement of the ELISA test. In the routine diagnostic, the rapid test should thus be proposed as a qualitative test, giving an indefinite direction of the severity of the GI inflammation but still requiring further quantitation and diagnostic examination when positive (23,25). No data are yet available in the neonatal population.

CALPROTECTIN MEASUREMENT IN NEONATES

All of the studies included in this review were based on the same calprotectin assay, named PhiCalTest or Calprest, manufactured by Eurospital or by Nycomed, except for Mohan et al (27) who used the Immundiagnostik test and for Golden et al (28) who used an ''in-house'' method. In all studies, the reference normal upper value was 50 μg/g (50 mg/kg), except for Carroll et al (29), who adopted a normal upper value of 30 mg/L (30 mg/kg). Samples were stored at −20°C, −70°, or −80°C and time duration between sampling and freezing was not always given, which does not seem an issue in the absence of any significant degradation of faecal calprotectin for up to 1 week at room temperature (19–21). All of the authors conducted the assay as recommended by the manufacturer, using 50 to 100 mg of faeces suspended in faecal extract buffer (weight-to-volume ratio of 1:50).

Calprotectin Reference Range in Full-term and Preterm Neonates With Apparently No GI Distress

Data regarding reference values in apparently healthy neonates were found in 16 publications (29–44), analysing populations of full-term and preterm infants (Table 1). Calprotectin is already present at high levels in the first passed meconium, that is mean (SD) 145 (79) μg/g in full-term neonates and median (range) 332 (12–9386) μg/g in very low birth weight preterms, indicating the capability of the foetal GI tract to produce and secrete this protein. The meconial levels were negatively correlated with gestational age and birth weight in term infants, a correlation lacking in preterm infants without any obvious reason for this discrepancy (36,40).

Healthy full-term and preterm infants, especially younger than 3 months old have high faecal calprotectin values, similar to those observed in children and adults with IBDs, although with large interindividual variations (Table 1). No significant differences occur between full-term and preterm neonates at the same postnatal age (31). Baldassarre et al (33) described in full-term neonates a slight but significant increase in levels at D7 compared with levels at D3, the levels then remaining similar for the first month of life. Then, a decrease occurs between 6 weeks and 6 months of life (30). Among persisting controversies, Campeotto et al (41) did not find any influence of postnatal age in preterm infants, whereas Yang et al (43) found a downward trend and Josefsson et al (40) a significant increase with postnatal age.

The mode of delivery was not found to influence faecal calprotectin in full-term neonates (32,33), whereas Josefsson et al (40) found a positive correlation with caesarean delivery in preterm infants. No relations were found with gestational age (40,41,43). Faecal calprotectin was also found to correlate negatively with antibiotic treatments in this population of very low birth weight infants (40).

The influence of the diet on calprotectin levels is not agreed on. Campeotto et al (32) and Baldassarre et al (33) found no differences between breast-fed and formula-fed infants during the first month of life in full-term infants. Golden et al (28) found faecal calprotectin concentrations to be significantly lower in breast-fed than in formula-fed infants during the ''preweaning'' period. Dorosko et al (37) and Savino et al (34) described opposite results with higher calprotectin levels in exclusively breast-fed infants compared with mixed-fed or formula-fed ones. However, the comparison between the different studies is difficult because infants were recruited at various ages. Campeotto et al (32) enrolled neonates during the first week of age, Baldassarre et al (33) followed infants during the first month of life, Golden et al (28) included infants mainly during the preweaning period, Savino et al (34) enrolled infants ages 3 months or younger, and Dorosko et al (37) monitored a wider age range. Due to calprotectin variations during the first year of age, these variations in inclusion criteria could explain the discrepancies observed between the published data. Moreover, most of these studies gave poor information about the infants' background and the composition of the formulas, which could affect calprotectin levels.

Finally, some authors investigated the link between microbiota and calprotectin levels. Oral supplementation by *Bifidobacterium lactis* Bb12, which modified the equilibrium of the gut microbiota, led to a significant decrease in calprotectin levels in the

TABLE 1. Publications providing reference values in healthy full-term and preterm neonates during the first year of life

Reference	Patients, n	Age	Faecal calprotectin, μg/g
Full-term infants		Median (range)	
Rugtveit et al (30)	20	6 wk	269 (31–2100)
	20	3 mo	264 (48–2130)
	22	6 mo	79 (9–405)
	20	1 y	67 (38–900)
Nissen et al (31)	16	3–18 d	235 (172–2880
Campeotto et al (32)	69	3–7 d	167 (22–860)
Baldassarre et al (33)	71	Day 7	245 (95–405)
		Day 12	250 (80–425)
		Day 30	255 (100–425)
Savino et al (34)	39 (breast-fed)	13–90 d	555 (123–2000)
	35 (formula fed)	23–90 d	207 (31–798)
		Mean (SD)	
Olafsdottir et al (35)	27	2–10 wk	277 (109)
Laforgia et al (36)	131	Day 3	145 (79)
Dorosko et al (37)	32	Day 3–6 mo	198 (77)
		Mean (SEM)	
Rhoads et al (38)	17	8.33 wk (0.96)	197 (46)
		Mean (95% CI)	
Baldassare et al (39)	32	1–10 mo	132 (119–145)
Preterm infants		Median (range)	
Nissen et al (31)	11	3–18 d	150 (81–221)
Josefsson et al (40)	52	1–8 wk	253 (9–1867)
Campeotto et al (41)	19	1–8 wk	160 (<15–650)
Campeotto et al (42)	95	1–4 wk	206 (16–1240)
		Mean (SD)	
Carroll et al (29)	7	<3 wk	98 (61)
Yang et al (43)	8	1–4 wk	122 (98)
Rougé et al (44)	23	1–4 wk	103 (90)

probiotic group compared with the placebo group (27). By contrast, Björkström et al (45) found no correlations with the microbiota.

As previously mentioned, all of the studies performed in either full-term or preterm neonates showed high interindividual variations. These variations may be attributed to the method of collection, in which the sample stayed in the baby's diaper. However, Olafsdottir et al (35) showed that this sampling method, in which some water is absorbed by the diaper, increases the calprotectin concentration by no more than 30%, a variation far lower than that measured in the different studies. Other hypotheses could be suggested. Because high faecal calprotectin in neonates reflects an increase in granulocytes in the intestinal lumen due to high intestinal permeability and/or development of the gut-associated lymphoid tissue, interindividual variations should be linked to environmental factors, which could individually modify this process, that is, mode of feeding, intestinal colonisation, or response to alimentary antigens (see following sections for discussion).

Why Such High Levels of Calprotectin in Apparently Healthy Neonates?

First, the high calprotectin concentrations observed in the neonatal period may be related to the unusual physiology of the neonatal gut. Indeed, a specific pattern of functioning in the first weeks of life is characterised by, among other things, an increased transmucosal leakage as shown by Walker (46). Because there is no accumulation of calprotectin-rich leucocytes in the healthy mucosa in the first few months of life, the high calprotectin concentrations observed may reflect increase in intestinal permeability leading to transepithelial migration of neutrophils, as observed in adults with IBD (47). This phenomenon, ending by the third trimester of life in a process named ''closure'' could explain the significant decrease in calprotectin levels starting from the end of the first trimester of life (30). Interestingly, preterm infants, although displaying a higher intestinal permeability, do not exhibit levels that are higher than full-term infants and no correlation is seen with the gestational age. The similarity of calprotectin levels in preterm and full-term infants is in accordance with the data of Van Elburg et al (48), showing that intestinal permeability, as measured with the lactulose/mannitol test, was higher in preterm infants compared with full-term infants only during the first 72 hours of life and then normalised to the level of full-term neonates. This can explain the higher levels of calprotectin observed in the meconium in the first days of life in preterm infants as compared with later values in faecal samples (40). Intestinal permeability remains then significantly higher during the neonatal period compared with adulthood.

Second, the gut microbiota establishment may have an effect on calprotectin release, as suggested by Baldassarre et al (33) and

Josefsson et al (40). Indeed, perinatal factors known to delay gut bacterial colonization, for example, antibiotic treatments, correlated negatively with faecal calprotectin levels, whereas factors known to favour gut bacterial colonization, for example, postnatal age, correlated positively with faecal calprotectin levels. Mohan et al (27) found a correlation with gut microbiota, whereas Björkström did not. In the latter study, the faecal microbiota was analysed by a culture technique that did not allow the growth of all of the anaerobic genera, for example, *Bacteroides* and *Clostridia* (45). The hypothesis of a relation between gut microbiota and calprotectin level is supported by the study of Splichal et al (49) observing significant increase in intestinal calprotectin release linked to the gut colonisation by *Escherichia coli* in gnotobiotic piglets. The cells containing cytoplasmic calprotectin were characterised as villous macrophages, and few positive leucocytes (macrophages and rarely neutrophils) were observed in ileal lumen. Moreover, this effect was dependent on the strain, the probiotic strain *E coli* Nissle inducing the highest calprotectin level in the intestinal lumen in this model.

Finally, the high basal calprotectin level could also reflect a minimal secretion of antimicrobial peptides in response to alimentary allergens, as suggested by Josefsson et al (40), who found a positive correlation between faecal calprotectin and volume of enteral feeds.

Because calprotectin display many biological activities, including bactericidal and fungicidal activities and immunomodulation properties, it may be postulated that this protein could exert a beneficial effect on the host defence in physiological conditions such as the intestinal ecosystem in healthy infants during the first weeks of life.

Calprotectin in Infants and/or Neonates With Digestive Distress

Some studies (29,35,38–43,50) envisioned calprotectin as a marker of intestinal diseases in full-term and preterm neonates (Table 2). Faecal calprotectin proved useful in the differential diagnosis between constitutive intestinal epithelial disorders and immuno-inflammatory disorders during severe and protracted secretory diarrhoea of the first months of life. High faecal calprotectin suggested autoimmune enteropathy or inflammatory colitis, which can often be stabilised by anti-inflammatory drugs, whereas low calprotectin levels suggested constitutive microvillus atrophy or epithelial dysplasia, which to date does not respond to any medical therapy and requires long-term parenteral nutrition or intestinal transplantation (50).

Controversial results have been found in infants with colic. A first study did not reveal any significant modifications (35), whereas a recent one reported an approximately 2-fold increase of faecal calprotectin in infants with colic (mean [SEM]: 413 [71] μg/g) compared with that of healthy infants (197 [46] μg/g, $P = 0.042$). In the last study, colicky infants were selected on the assessment of crying and fussing >3 hours/day for at least 3 days every week (38). Similar results were observed by Baldassarre et al (39), who described significantly higher faecal calprotectin levels in infants with cow's-milk allergy colitis (mean [95% confidence interval; 95% CI] 326 [269–388] μg/g) compared with that of healthy infants (132 [119–145] μg/g, $P < 0.0001$). This would imply that colic may be viewed as a variant of adolescent irritable bowel syndrome inasmuch as both conditions are characterised by low-grade GI inflammation (38).

TABLE 2. Publications providing data in full-term and preterm neonates with digestive distress

References	Patients, n	Age	Faecal calprotectin, μg/g
Full-term infants or neonates			
Severe diarrhoea (Kapel et al [50])			
Constitutive intestinal epithelial disorders	14	3–21 mo	<136
Immunoinflammatory disorders	11	2–18 mo	Median (range) 1192 (375–3095)
Infantile colic			
Olafsdottir et al (35)	76	3–9 wk	Mean (SD) 278 (105)
Rhoads et al (38)	19	Mean (SEM) 7.05 wk (0.62)	Mean (SEM) 413 (71)
Lactose intolerance			
Olafsdottir et al (35)	7	2–10 wk	Mean (SD) 300 (124)
Cow's-milk allergy colitis	30		
Baldassarre et al (39)		1–10 mo	Mean (95% CI) 326 (269–388)
Preterm infants or neonates			
Gastrointestinal illnesses			
Campeotto et al (41)	15	1–7 wk	Median (range) 468 (116–928)
Yang et al (43)	30 samples	1–4 wk	Mean (SD) 380.4 (246.3)
Campeotto et al (42)	24	1–4 wk	Median (range) 393 (52–996)
Necrotizing enterocolitis			
Carroll et al (29)*	7	Mean (SD) 12 (5) d	Mean (SD) 288.4 (49.1)
Josefsson et al (40)**	4	1.1–5.6 wk	Median (range) 8691 (685–22513)
Campeotto et al (42)	7	1–4 wk	Median (range) 832 (168–4775)
Focal intestinal perforation or covered perforation			
Josefsson et al (40)**	3	0.7–2.7 wk	Median (range) 1032 (835–9228)

* Reference value for this test: 30 mg/kg (instead of 50 mg/kg or 50 μg/g for the other studies).
** This study indicated only the maximum calprotectin levels obtained in each infant.

Intestinal distress such as enteropathy is a common condition during the first weeks of life of preterm infants, leading to the interruption of enteral feeding, and sometimes accompanied by intestinal bleeding. Clinical examination and radiology may strengthen the diagnosis, but they may also be inconclusive, particularly when differentiating necrotizing enterocolitis (NEC)—especially in the early stage of the disease—from other GI diseases. This uncertainty highlights the need for biological markers, among which calprotectin comes first, owing to its rise in children older than 4 years with IBD conditions. In preterm infants with digestive symptoms such as abdominal distension or GI bleeding, leading to interruption in enteral feeding, a transient rise of faecal calprotectin levels was seen at the onset of intestinal symptoms, followed by a decrease concomitant with illness treatment and disappearance (41,43). Yang et al (43) observed higher faecal calprotectin levels (>350 μg/g stool) among those with NEC-like symptoms or direct GI injury/stress (eg, bloody stool, bowel perforation and surgery, GI contrast study) or with other conditions (eg, severe hypotension, acidosis) that could significantly affect the permeability of the GI mucosa. We happened to suggest a similar cutoff value (>363 μg/g stool) for mild GI illnesses such as GI bleeding or abdominal distension (42). In this study, levels >363 μg/g seemed to have a sufficiently high specificity, 0.82, to justify the suspicion of intestinal inflammation, which puts the child at risk in case of prolongation of enteral feeding.

The most serious intestinal complication in preterm infants is NEC, especially in very low birth weight infants. Few studies have been performed in those infants. In the first one, Carroll et al (29) showed significantly higher levels of faecal calprotectin in 7 preterm neonates with proven NEC compared with gestational age–matched healthy infants. However, the levels observed in those NEC infants remained within values currently observed in healthy preterm infants of the same age. The threshold they proposed (<200 μg/g) was therefore low for its use in clinical practice. This could reflect the sensitivity of the assay used for this study, for which the reference value was 30 mg/L. More recently, Josefsson et al (40) measured calprotectin levels in very low birth weight infants, among them 7 with severe abdominal diseases: 4 cases of NEC, 2 cases of focal intestinal perforation, and 1 case of intestinal obstruction. They suggested a cutoff level of 2000 μg/g for identifying a severe intestinal inflammatory condition because no reference infant of their cohort had a calprotectin level above this level. However, only 3 cases of NEC and 1 case of intestinal perforation reached this cutoff level and, as a result, 3 of 7 infants with severe intestinal disease enrolled in that study never reached this cutoff. A lower cutoff level (>636 μg/g; sensitivity 0.72 and specificity 0.95) was proposed by Campeotto et al (42) to identify neonates with a high risk of severe distress, such as NEC. Using the cutoff of 2000 μg/g, the specificity would have been raised to 1 in this study, but the sensitivity would have decreased to 0.3, not usable for clinical purpose.

These studies strongly suggest that calprotectin dosage can be used as a biological marker of severe GI diseases, in particular NEC. However, studies including more cases of GI diseases with various degrees of severity should be done to determine the best threshold for a use in clinical practice in neonatology units.

CONCLUSIONS

Compared with those for adults and children, data on faecal calprotectin are poor during the neonatal period. However, all of the studies show the same trend, with description of high levels in healthy full-term and preterm neonates, accompanied by high interindividual variability. This may be a physiological response of the gut mucosa for the establishment of intestinal epithelial homeostasis and oral tolerance.

Few studies have addressed neonatal digestive distress. Despite the small sample size of the population enrolled and the big differences between the expression of study results (mean, SD, SEM, or 95% CI in some studies and median, range in others), pooling the data is not easily feasible. However, in the absence of a meta-analysis of the published articles, a remarkable concordance is seen in the faecal calprotectin values depicted, suggesting that it could become a noninvasive clinical screening test for intestinal distress in neonates. In neonates, a value above a cutoff of approximately 350 μg/g may indicate a digestive disease. To validate this cutoff value and its sensitivity, specificity, and predictive value, faecal calprotectin should now be studied in larger cohorts of neonates, analysing precisely the periods surrounding digestive distress and NEC episodes.

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