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Time course of calprotectin, tumor necrosis factor-α and human beta-defensin type 2 excretion in stools of preterm and term newborns

Maria Elisabetta Baldassarre[1,*], Antonio Di Mauro[1], Margherita Fanelli[2], Luigi Amati[3], Luigi Corvaglia[4] and Nicola Laforgia[1]

[1]Department of Biomedical Science and Human Oncology, Neonatology and Neonatal Intensive Care Unit; [2]Medical Statistics, University of Bari; [3]Laboratory of Clinical Immunophatology, IRCCS "S. de Bellis", Castellana Grotte (BA); [4]Neonatology and Neonatal Intensive Care Unit, Policlinico "Sant'Orsola", University of Bologna, Italy.

ABSTRACT

Changes in the composition of antimicrobial peptides and antibacterial activity in the intestine during the early postnatal period, strongly influenced by external factors such as breastfeeding and the mode of delivery, have been described in humans. The aims of our study were to describe the time course of calprotectin (C) human beta defensins type 2 (hβd2) and tumor necrosis factor-α (TNF-α) excretion in stools of term newborns (TN) and preterm newborns (PN) during the first weeks of their life and to improve our understanding on the factors influencing their excretion. We longitudinally evaluated the fecal values of calprotectin, hβd2 and TNF-α in 233 stool samples from PN and TN, in relation to the mode of delivery, gestational age (GA) and type of feeding. No significant differences in the levels of any of the three factors were found in relation to the mode of delivery or feeding type. Calprotectin, hβd2 and TNF-α were influenced only by GA. Further studies are needed to get better insight into the development of the gastrointestinal mucosal immune system in newborns.

KEYWORDS: calprotectin, fecal markers, TNF-α, human-beta defensins

*Corresponding author
mariaelisabetta.baldassarre@uniba.it

INTRODUCTION

Due to a naive adaptive immune system, more than 10% of the neonates develop an infection during the first month of their life. The gastrointestinal tract is the first accessible gate for bacterial and food antigens, and the gut mucosal immune system is therefore essential in controlling antigenic responses.

Several endogenous antimicrobial peptides such as human beta defensins type 2 (hβd2) produced by the epithelial surface have been identified in the stools of newborns during the first weeks of their life [1]. They may have a pivotal role in the innate immune defense mechanism and aid in the transition from the sterile environment of the uterus to a world rich in bacteria [2].

hβd2 is an inducible peptide and the levels of this defensin increase in response to gastrointestinal inflammation [3]. In addition to antimicrobial peptides, fecal calprotectin has been studied in newborns and is thought to regulate inflammatory processes [4], and exert antimicrobial and antiproliferative properties *in vitro* [5, 6] and *in vivo* [7].

Recent studies have clearly shown that the development of the mucosal immune system may be regulated by the levels of antimicrobial peptides, calprotectin and pro-inflammatory cytokine in the

neonatal gut, which in turn may be affected by the colonization of microbiota present in the gut [2, 8].

Tumor necrosis factor-α (TNF-α) is a well-known faecal marker of inflammation [9]. This cytokine is produced by monocytes and is released after the contact with Gram-positive and Gram-negative bacteria.

Gestational age (GA), caesarean section, and type of feeding are considered to be the main factors determining gut microbiota colonization and influencing the development of the mucosal immune system of the gut [10].

This paper aims to study the time course of C, hβd2 and TNF-α in stools, in term (TN) and preterm newborns (PN) during the first weeks of their life, and the correlations among GA, days of life, and delivery and feeding type.

We believe no authors have evaluated the changes in values of these fecal markers over time in order to improve the understanding about factors that could influence their excretion in newborns.

We longitudinally evaluated the values of C, hβd2 and TNF-α in PN and TN, in relation to: 1) the way of delivery [caesarean section (CS) vs vaginal delivery (VD)], 2) GA and 3) type of feeding [breast fed (BF), formula fed (FF), breast and formula fed (BF & FF)].

MATERIALS AND METHODS

We enrolled 39 healthy PN (G1), born in Neonatology and Intensive Care Unit (NICU) of Sant'Orsola, University of Bologna, and 29 healthy TN (G2), born in NICU of Policlinico Hospital, University of Bari. Intestinal malformation, irregular passage of meconium and gastrointestinal infections were considered as exclusion criteria. Patient characteristics are summarized in table 1.

The two groups differ at baseline for GA, birth weight, type of delivery, type of feeding and antibiotic administration. Data according to GA and birth weight are expressed as mean standard deviation. Data according to type of delivery, type of feeding and antibiotic administration are expressed in percentage. At the time of collecting the sample, 33 PN needed intravenous antibiotic administration versus none in the TN group. The type of delivery was VD only in one PN. Written, informed parental consents were obtained prior to inclusion, according to the protocols approved by the local Ethics Committee.

Table 1. Characteristics of patients enrolled.

	G1 preterm newborns	**G2 term newborns**
N	39	29
GA mean (week)	30.05 ± 2.62	39.19 ± 0.96
BW mean (g)	1455.62 ± 237.2	3316.2 ± 316.3
Type of delivery		
Vaginal delivery (VD)	1 (2.6%)	17 (58.6%)
Caesarean section (CS)	38 (97.4%)	12 (41.4%)
Type of feeding		
Breast fed (BF)	4 (10.3%)	23 (79.3%)
Formula fed (FF)	13 (33.3%)	2 (6.9%)
Breast and formula fed (BF & FF)	22 (56.4%)	4 (13.8%)
Antibiotic administration		
Yes	33 (84.6%)	0
No	6 (10.4%)	29 (100%)

As it is often difficult in PN to obtain stools differing from meconium in the first week of their life, sample collection between the two groups was different. The stool samples were collected after regular passage of meconium on days 15 (d15), 30 (d30) and 60 (d60) in PN and on days 3 (d3), 7 (d7), 15 (d15) and 30 (d30) in TN.

All the stool samples were frozen at -20 °C until analysis, and were evaluated in the same laboratory at "Saverio De Bellis" Hospital, Castellana Grotte (Bari, Italy).

The concentrations of C, TNF-α and hβd2 were determined in each stool sample photometricaly using a commercially available enzyme-linked immunosorbent assay kit (C: Calprest, Eurospital, Italy; values expressed as μg/g of stool); (hβd2 and TNF-α: Immunodiagnostic, Bensheim, Germany; values expressed as ng/mg of stool).

The comparison between the two groups was performed using Students *t*-test for unpaired data on d15 and d30. A generalized linear model was built to evaluate the fecal values of calprotectin, TNF-α and hβd2, according to GA and days of life. The analysis was performed first on the whole sample and then in the two studied groups.

Analysis of variance with days of life as repeated measures was performed to evaluate the effect of the type of delivery and the type of feeding. The analysis was performed separately in the two groups because type of delivery was not evaluable in G1, given the single VD.

Data were analyzed using IBM SPSS Statistics version 20.

RESULTS

The mean fecal values of calprotectin, TNF-α and hβd2 on the different days are shown in table 2 and in figure 1. The mean values of C consistently appear to be higher in G2 than in G1 (Figure 1a), the difference being significant on d30 (Table 2). Generalized linear model of the whole sample (C = 18.837 + 0.091*days + 0.746*GA) confirms the positive effect of GA (Wald $\chi2 = 12.39$ p = 0.000) on C. The number of days of life did not affect C values (Wald $\chi2 = 0.066$ p = 0.797) (Table 3). To highlight the effect of the number of days of life on C values, a separate analysis was performed. Over time, the C values tended to decrease slightly in G1, whereas in G2 the values increased significantly (Table 3). No considerable differences in C values were found, in relation to the type of feeding, both in PN (F = 0.73 p = 0.49) and in TN (F = 0.71 p = 0.5). In G2 also the method of delivery did not affect C values (F = 2.2 p = 015).

TNF-α mean values were significantly higher in G1 than in G2 (Figure 1b) on d15 and d30 (Table 2). The generalized linear model of the whole sample (C = 83.645 - 0.062*days - 0.164*GA) confirms the negative effect of GA (Wald $\chi2 = 8.715$ p = 0.003) on C while the number of days after birth did not affect C values (Wald $\chi2 = 0.477$ p = 0.49) (Table 3). When G1 and G2 were analyzed separately, the GA effect was not significant anymore (Table 3). No significant differences in TNF-α mean values were found in association with the type of feeding both in PN (F = 0.15 p = 0.86) and in TN (F = 0.16 p = 0.85). In G2 also the method of delivery did not affect C values (F = 0.41 p = 0.53).

hβd2 mean values increase from d15 to d30 and then decrease until d60 in PN. hβd2 mean value declines from d3 to d15, followed by stabilization until d30 in TN (Figure 1c). The difference between the two groups is significant on d15 (Table 2). The generalized linear model of the whole sample (C = -70.988 + 0.857*days - 0.085*GA) confirms the negative effect of GA (Wald $\chi2 = 16.42$ p = 0.000) on C while the number of days after birth did not affect C values (Wald $\chi2 = 0.047$ p = 0.828) (Table 3). When the groups were analyzed separately the outcomes were: a significant effect of both GA and number of days after birth in G1 and a significant effect of number of days after birth in G2 (Table 3). No significant differences in hβd2 mean values were found in relation to the type of feeding both in preterm (F = 0.96 p = 0.40) and in TN (F = 3.31 p = 0.06). In G2 also the method of delivery did not affect C values (F = 0.35 p = 0.85).

DISCUSSION

It is known that the gastrointestinal mucosal immune system in newborns is not completely developed at birth and maturation continues postnatally.

Table 2. Mean values of C, TNF-α and hβd2 at different times in G1 and in G2.

	G1 preterm newborns		**G2 term newborns**			
	mean	**s.d.**	**mean**	**s.d.**	**T**	**P**
Calprotectin (µg/stool)						
d3	-	-	196.62	70.57		
d7	-	-	225.69	79.91		
d15	183.13	101.25	205.66	81.80	-0.98	0.33
d30	199.87	89.64	265.59	77.23	-3.17	**0.002**
d60	153.50	64.79	-	-		
TNF-α (ng/mg stool)						
d3	-	-	40.76	23.38		
d7	-	-	34.98	16.52		
d15	47.04	23.07	35.82	14.60	2.3	**0.025**
d30	53.60	29.49	34.59	12.34	3.62	**0.001**
d60	43.34	14.64	-	-		
hβd2 (ng/mg stool)						
d3	-	-	228.28	124.36		
d7	-	-	159.59	109.99		
d15	75.98	32.17	118.17	86.38	-2.50	**0.017**
d30	158.64	102.87	136.72	116.82	0.82	0.415
d60	116.44	90.50	-	-		

Bold characters indicate the following:
Calprotectin: the mean values are significantly higher in G2 than in G1 on d30.
TNF-α: the mean values are-significantly higher in G1 than in G2 (Figure 1b) on d15 and d30.
hβd2: the mean values are significantly higher in G1 than in G2 on d15.

It has been postulated that defensins and calprotectin contribute to host immunity by controlling the non-specific antigenic responses in the gastrointestinal tract, maintaining the balance between pathogens and normal flora, and driving the development of a healthy microbiota [11, 12].

Consistent with previous studies, the C values observed in healthy TN and PN were high, exceeding those reported in healthy adults and children. These high values could be linked to the gut microbiota establishment [13, 14, 15]: during the first weeks of life, in fact, potent chemotactic agents produced by the intestinal microbiota may stimulate the transepithelial migration of granulocytes and finally generate a high intraluminal concentration of C as a defense against invading or attacking pathogenic agents [16].

We found C values to be higher in TN than in PN in all cases studied and this difference became statistically significant on d30. This can be due to different types of microbiota establishment in TN and PN [17].

Higher GA is a factor known to favor gut bacterial colonization, which is positively related to C levels. However, factors known to delay gut bacterial colonization such as prematurity and post-natal antibiotic treatments are negatively related with C levels [12].

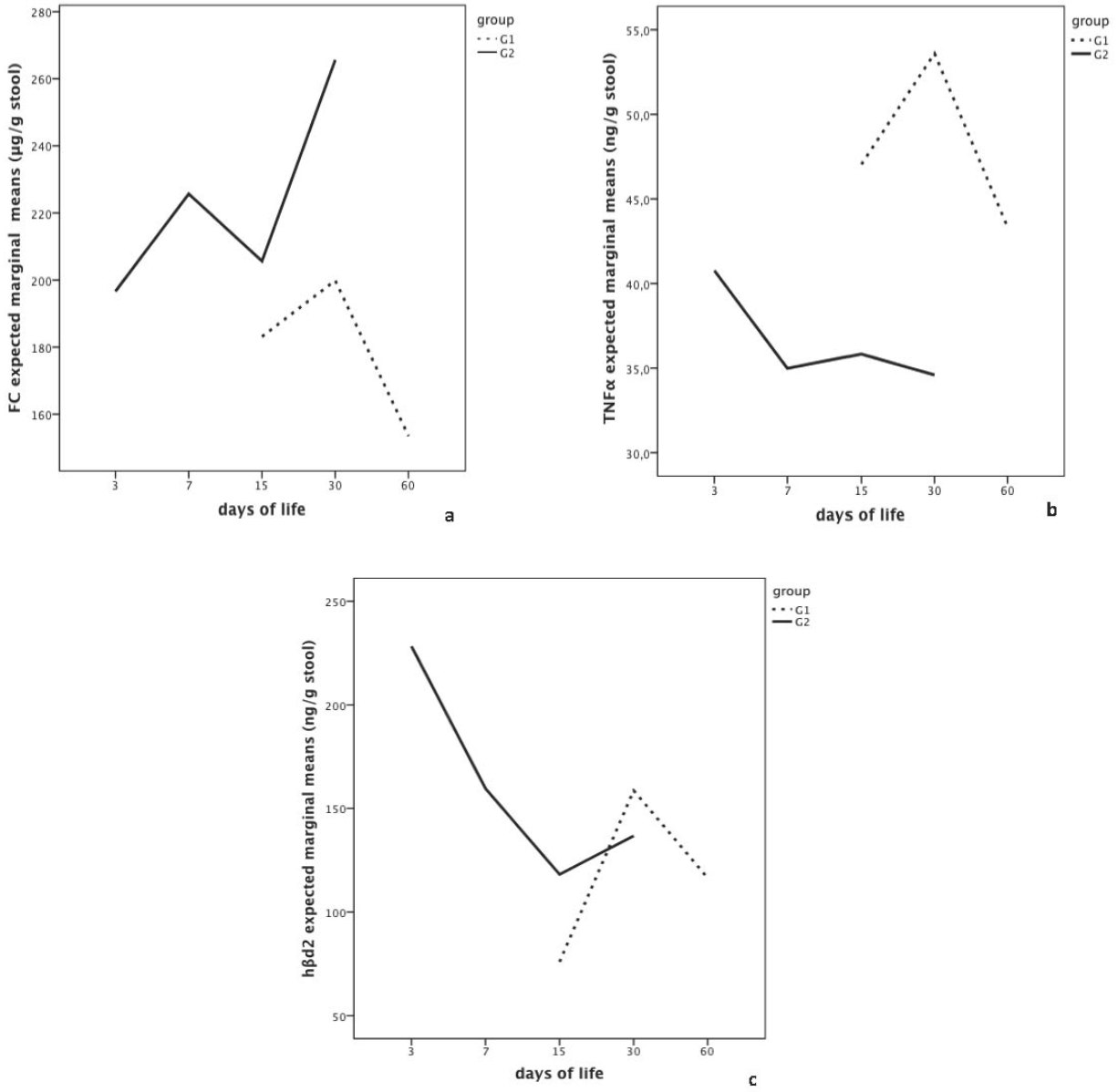

Figure 1. Time course of calprotectin (**a**), Tumor necrosis factor-alfa (**b**) and Human beta-defensins type 2 excretion (**c**) in stools of G1 and G2.

In our population, 97.4% of PN received antenatal antibiotics and 84.6% were exposed to post-natal antibiotic administration. The different types of microbiota colonization in TN and PN could be associated also to the different TNF-α values found.

Many authors found that in addition to high levels of staphylococci and clostridia, the gastrointestinal tract of pretems harbor low levels of probiotic strains such as Lactobacilli and Bifidobacteria [15]. It has been demonstrated that these probiotic strains had high capacities to reduce TNF-α concentrations in the gut [18].

Furthermore, the levels of TNF-α in PN promote an increased production of inducible hβd2, in accordance with other authors [19]. In our study, hβd2 levels in PN were lower than those reported in other studies and increased over time. This could be in response to TNF-α levels. A reduction in β-defensin production is associated with a higher risk of necrotizing enterocolitis (NEC) [20].

Table 3. Generalized linear model results.

	Whole sample	G1 preterm newborns	G2 term newborns
Calprotectin			
Intercept	18.837	60.779	-655.390
Days	0.091	**-0.755**	**2.322**
GA	**0.746**	0.688	**3.010**
TNF-α			
Intercept	83.645	47.301	-67.350
Days	-0.062	-0.111	-0.153
GA	**-0.164**	0.022	0.386
hβd2			
Intercept	-70.989	-243.845	-450.146
Days	0.085	0.648	2.428
GA	**0.857**	1.611	-2.342

Note: Bold characters indicate significant coefficients.

Very recently Campeotto *et al.* demonstrated that inducible hβd2 is up-regulated in the stools of TN and PN after microbiota conlonization and might be further increased during NEC [21]. It is important to note that none of the newborns included in this study developed NEC. We conclude that hβd2 and TNF-α are present in intestinal lumen in both TN and PN. TNF-α values were higher in PN and were inversely related to GA. hβd2 increased from d15 to 30 in PN and positively correlates to GA.

Different results obtained were then compared with Richter *et al*. [10]. This is probably related to the longitudinal collection (d3, d7, d15, d30, and d60) of stool samples in this study. It could be interesting to understand whether higher TNF-α values and increasing hβd2 values in PN are due to the effect of the inflammatory condition of the gut or due to natural defense mechanisms.

The key finding of our data is the highly significant correlation of fecal values of hβd2 with the GA and time. We found no significant effect of type of delivery and type of feeding on C, TNF-α and hβd2 values, consistent with other studies [10, 14, 15].

CONCLUSION

In conclusion, we could speculate that the mucosal gastrointestinal immune system in newborns is not completely developed at birth and maturation goes on postnatally with the enhancement of the response of defensin to inflammation. This could be a key factor for the higher risk of PN to develop gastroenteric disease. Further studies are needed to get better insight into the development of mucosal gastrointestinal immune system in newborns.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest relevant to this article.

ABBREVIATIONS

C : Calprotectin
G1 : Group 1
G2 : Group 2
GA : Gestational age
hβd2 : Human beta defensins type 2
PN : Preterm newborn
TN : Term newborn
TNF-alfa : Tumor necrosis factor alfa

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