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Early inflammation in the absence of overt infection in preterm neonates exposed to intensive care

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ABSTRACT

Background: Systemic inflammation, typically attributed to sepsis, has been repeatedly linked to adverse long-term outcomes in infants born prematurely. However, it is unclear whether other factors can contribute to potentially harmful systemic inflammatory responses.
Objective: To determine the timing and extent of systemic inflammation occurring in absence of infection in preterm infants exposed to intensive care.
Methods: First, we screened for inflammation biomarkers most strongly linked to infection in a large prospective cohort of 425 newborns (gestational age 24–42 weeks). Second, we longitudinally measured levels of infection-related inflammation biomarkers up to 42 days of post-natal life in a series of 58 infants born ⩽30 weeks of gestation exposed to intensive care. Ante- or post-natal infections were excluded using stringent definitions including rigorous histological placental examination. Spearman correlations were used to identify putative clinical factors potentially linked to inflammation.
Results: Three biomarkers were most strongly associated with neonatal sepsis (IL-6, IL-8 and G-CSF) in the first cohort. Using these markers, we found a predominant early high intensity systemic inflammation period within the first 72 h of preterm infants' extra-uterine life. Remarkably, this systemic inflammatory response was of magnitude comparable to that observed during sepsis in absence of ante- or post-natal signs of infection, and correlated with the amount of supplemental oxygen exposure ($r = 0.51–0.60$).
Conclusions: Non-infectious sources of systemic inflammation are significant in preterm infants exposed to intensive care and may contribute to intensive care-related organ injury.

1. Introduction

Prematurely born infants are at exceedingly high risk of serious long-term cognitive and physical disabilities due to medical complications occurring early in life [1]. Several factors underlie this unique vulnerability including infection, which has been linked with injury to the developing brain [2,3]. Recent studies indicate that the systemic inflammatory response in itself may also have damaging effects [4], including potential neurotoxic effects from local high levels of cytokines [5]. In addition, high levels of systemic inflammation produces vasodilatation and hemodynamic instability, which can result in intra-cranial hemorrhage in preterm infants [6]. Understanding of the factors involved in the generation of potentially damaging pro-inflammatory responses in preterm infants is a critical step towards directing therapeutic interventions to limit harmful consequences on developing organs [7].

Most preterm infants born below 30 weeks of gestation are dependent on invasive therapies for their early survival. Inflammatory responses may be linked to exposure to mechanical ventilation [8], supplemental oxygen [9,10], parenteral nutrition [9] and perhaps even blood transfusions [11,12]. Oxidative stress from exposure to supplemental oxygen can act synergistically with systemic inflammatory responses, contributing to end-organ injury [13]. This may be particularly true in preterm infants who have attenuated anti-oxidant defenses at birth [14,15]. However, it is

Abbreviations: Early SIP, early systemic inflammation period; NEC, necrotizing enterocolitis; G-CSF, granulocyte-colony stimulating factor.

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not clear to what extent non-infectious sources contribute to the overall systemic inflammation detectable in preterm infants and whether such responses are of a magnitude comparable to responses generated during sepsis.

In this study, we hypothesized that clinically relevant systemic inflammation occurs in absence of infection and sought to identify *when* and to *what* extent systemic inflammatory responses occur outside episodes of infection in infants exposed to intensive care therapies. To achieve this objective, we first screened for inflammation biomarkers most strongly associated with sepsis in a large neonatal cohort, using multiplex assays. Second, we repeatedly probed for these validated inflammation markers longitudinally at a high frequency, in a series of preterm infants exposed to intensive care.

2. Methods

2.1. Study population

The first cohort (for validation of infection biomarkers) consisted of 425 infants born 24–42 completed weeks of gestation, whose mother presented risk factors for early-onset neonatal sepsis (i.e. <72 h of post-natal life; defined as either a positive maternal vaginal culture for group B streptococcus, preterm labor, prolonged rupture of membranes for greater than 18 h or clinical chorioamnionitis including foul smelling amniotic fluid, maternal fever >38.0 °C or fetal tachycardia >180 beats/min) at the Royal Alexandra Hospital, the Misericordia Hospital or the Grey Nuns Hospital (Edmonton, Canada). Details of this cohort have also been published elsewhere [16]. The second longitudinal cohort (for analysis of inflammatory responses) consisted of preterm neonates born at or below 30 weeks of gestation admitted to the Neonatal Intensive Care unit at Children's & Women's Health Centre of British Columbia (Vancouver, Canada), between September 2006 and October 2007. Exclusion criteria for the second cohort were life-threatening congenital cardio-respiratory malformation or anticipation of the infant's death within the first few days of post-natal life. All infants were recruited following parental informed consent. This study was approved by the University of British Columbia Clinical Research Ethics Board.

2.2. Sample collection and inflammatory cytokine measurements

For the first cohort, blood samples were collected at 12–21 h of age. For the second cohort, longitudinal samples were obtained between 0 and 42 post-natal days. In order to limit invasive sampling, residual blood was collected at time of routine intensive care clinical monitoring. All blood samples were collected in lithium heparin, spun down immediately in BD Microtainers (Becton Dickenson) to remove the cell fraction and plasma was frozen at −80 °C within 1 h of collection to minimize protein degradation. Inflammation biomarkers were measured in a 1:2 dilution using a Procarta® multiplex particle-base assay (Panomics/Affymetrix) on a Luminex analyzer (BioRad). The variability of cytokine measurements using this assay was determined to be ⩽20% based on duplicate sample measures (not shown).

2.3. Clinical definitions and data analysis

In the first cohort, cases of infection were defined as infants with a positive blood culture with clinical signs suggestive of sepsis (e.g. excessive feeding intolerance and/or abdominal distension, temperature or cardiorespiratory instability). Controls were defined as a negative blood culture, C-reactive protein <5 mg/L, an absence of clinical signs of infection and favorable outcome off antibiotic treatment >48 h). In the second cohort, necrotizing enterocolitis (NEC) was defined based on clinical signs of acute gastrointestinal deterioration or grossly bloody stools and radiological evidence of pneumatosis, free or portal air, or signs of fixed bowel dilatation with bowel wall thickening (modified Bell's staging criteria stage II or higher) [17]. Clinical sepsis was defined according to the attending neonatologist, by clinical signs suggestive of sepsis (as above) accompanied by use of antibiotics for more than 48 h. Culture-proven sepsis was identified if the infant presented a positive blood culture. None of the infants in the second cohort presented a positive cerebrospinal culture. Histological chorioamnionitis was identified by a review of placental pathology slides scored blindly according to validated criteria [18]; infants without chorioamnionitis were classified as such only if they did not show clinical signs (maternal fever >38.5 °C during labor, fetal tachycardia), or maternal or fetal histological signs on placental examination. For anti-inflammatory drug treatment, corticosteroids and indomethacin administration were considered.

For identification of infection biomarkers in the first cohort, 26 analytes (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-12/IL-23p40, IL-13, IL-17, IFN-γ, GM-CSF, TNF-α, TNF-β, G-CSF, NIP12, EOTAXIN, VEGF, IP10, NGF, RANTES, TGF-β, MIP1β, SAA) were measured. Markers to be probed in second cohort were selected based upon detectable levels and significant associations with sepsis between cases and gestational age/birth weight-matched controls (see Supplementary Table) using a Mann–Whitney *U* test ($p < 1.9 \times 10^{-3}$ adjusted for multiple comparisons using the Bonferroni method).

For analysis in the second cohort, temporal associations between plasma cytokine levels, and clinical events were initially determined for each infant to establish the level of "clinically relevant" inflammatory responses associated with sepsis or NEC. In a cluster analysis, cytokine data from all infants were aggregated to identify the highest periods of systemic inflammation throughout the study period. When more than one blood sample was available on a single day, only one was randomly selected for inclusion in the analysis without knowledge of the cytokine measurements. This approach ensured a more uniform distribution of sample per infant over the 42-day study period (median of 13 [IQ range = 10–17] data points per day), in order to minimize selection bias. In comparisons of cytokines at baseline versus during episodes of infections, we have only included events occurring after 72 h of post-natal life in order to avoid confounding influences resulting from intra-uterine infections. Therefore, each cytokine data points could be assumed independent from each other in the analysis. Cytokine levels were non-parametrically distributed and therefore were compared between groups using a Mann–Whitney *U* test. Correlations between clinical factors (gestational age, birth weight, culture-proven sepsis, anti-inflammatory drug treatment: as above, blood transfusions, or supplemental oxygen: highest daily fraction of inspired oxygen, FiO_2, used during specifically identified periods) and cytokine levels were determined using Spearman rank's coefficients, as the latter analysis does not assume linearity of the data. To avoid repeated measures, only one randomly selected cytokine measure was included in the correlation analysis. Because the vast majority on infants in the longitudinal cohort also received mechanical ventilation, correlations with this variable could not be separately analyzed. Statistics were calculated using SPSS version 11 for windows (Lead Technologies).

3. Results

3.1. Definition of significant biomarkers for neonatal sepsis

In order to identify putative biomarkers most significantly associated with systemic inflammation, we screened 26 putative

biomarkers in a large cohort of infants ($n = 425$; mean gestational age ± SD: 33.7 ± 4.2 weeks; mean ± SD birth weight: 2326 ± 914) at risk of early-onset neonatal sepsis (EONS), at birth. Of the 26 markers analyzed, 10 showed detectable levels and were associated with infection (Supplementary Fig. 1). Within this first cohort, we conducted a nested case-control determination of all 10 detectable biomarkers and identified three: IL-6, IL-8 and G-CSF, significantly associated with infection after correcting for multiple comparisons, between neonates with infection and gestational age/birth weight-matched controls without infection.

3.2. Detection of clinically relevant systemic inflammation in study population

Next, we longitudinally measured levels of IL-6, IL-8 and G-CSF in a second cohort of preterm infants exposed to intensive care. Fifty-eight infants were sampled for a total of 552 data points per marker over the first 42 post-natal days. Clinical characteristics of infants are presented in Table 1. When results from individual infants were examined, increases in cytokine levels (compared to baseline) were detected with each episodes of clinical or culture-proven sepsis, NEC or intestinal perforation, as illustrated for two representative infants (Fig. 1). When examining all infants, we identified a total 424 (daily) data points during which infants presented no culture-proven or signs of clinical sepsis, NEC and intestinal perforation (i.e. referred to as "non-infectious periods"). The cytokine levels during episodes of bacterial culture-proven or clinical sepsis, NEC or intestinal perforation were significantly higher compared to the baseline levels observed during the "non-infectious" periods (Table 2). Cytokine levels in periods of culture-proven fungal sepsis also trended much higher, although the difference with non-infectious periods was not statistically different due to small numbers (Table 2).

Table 1
Clinical characteristics of cohort of premature neonates exposed to intensive care.

Clinical variable	Mean or proportion [95% CI]
Gestational age, mean ± SD (weeks)	26.9 ± 1.7 [26.5; 27.3]
Birth weight, mean ± SD (g)	961 ± 270 [894; 1028]
Gender, % female	43 [31; 56]
Culture-proven sepsis (⩾1 episode), % infants	51 [38; 63]
Inborn, %	90 [79; 96]
Necrotizing enterocolitis, % infants	8 [4; 17]
Histological chorioamnionitis, % infants	32 [15; 38]
Endotracheal ventilation, % infants	93 [85; 98]
Indomethacin treatment, % infants	50 [37; 63]
Post-natal corticosteroid treatment, % infants	26 [16; 39]

Histological chorioamnionitis defined as maternal stage 2 or greater with involvement of fetal membranes according to validated criteria (see text); [95% CI]: 95% confidence interval.

3.3. Early systemic inflammation without evidence of infection

A cluster analysis of all infants combined indicate that the first 72 h of extra-uterine life represented a distinct period of highest cytokine levels (referred to herein as the *early systemic inflammation period*, *or early SIP*; Fig. 2). This increase in cytokine levels within the early SIP, in most cases, could not be attributed to

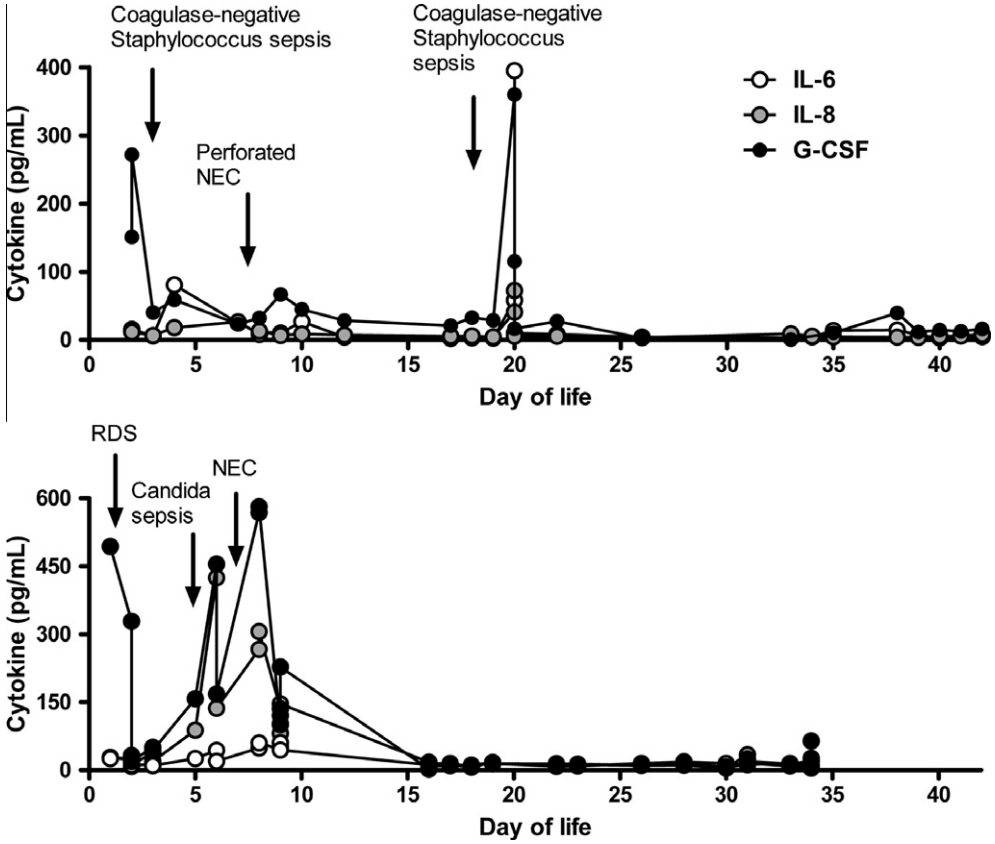

Fig. 1. Systemic inflammatory responses in two representative infants from the longitudinal study cohort. Positive detection of *clinically relevant* systemic inflammatory responses (as measured by levels of IL-6, IL-8 and G-CSF) related to the time of diagnosis (post-natal days; by 24 h periods; arrows). Of note, respiratory distress syndrome (RDS) was associated with inflammation in second infant (arrow); NEC: Necrotizing enterocolitis.

Table 2
Inflammatory responses and association with clinical event in infants from the longitudinal study cohort.

Post-natal period	Clinical event	*N*	Cytokine level (median [IQ range]) (pg/mL)		
			IL-6	IL-8	G-CSF
>72 h	No culture-proven, clinical infection, NEC or IP	N/A	7.0 [4.8–12]	4.9 [3.2–7.8]	12 [4.6–19]
	Culture-proven fungal sepsis	2	151 [94–209]	62 [56–69]	301 [167–435]
	Culture-proven bacterial sepsis	20	35 [15–70]***	12 [4.9–30]***	28 [15–120]***
	Clinical sepsis	35	11 [8.0–39]***	9.0 [4.9–18]***	32 [12–228]***
	NEC or IP[c]	4	428 [305–724]**	69 [30–135]*	165 [37–535]*
⩽72 h	All infants (for which data points were available)	32	10 [6.9–26]	5.1 [2.7–14]	29 [11–117]
	Infants without chorioamnionitis or culture-proven sepsis[a]	15	19 [7.5–53]*	11 [7.0–31]**	48 [20–379]***
	Infants without chorioamnionitis, culture-proven or clinical sepsis[a]	6	24 [8.1–72]*	10.1 [3.6–55]	47 [14–462]*

[a] Histologically defined chorioamnionitis and C-reactive protein <5 mg/dL in absence of positive blood culture.
[b] Refer to method section for definitions of culture-proven or clinical sepsis. NEC: Necrotizing enterocolitis; IP: Intestinal perforation. All statistical comparisons were made with baseline cytokine levels measured in infants with no culture-proven and clinical infection after 72 h of extra-uterine life, NEC or IP.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

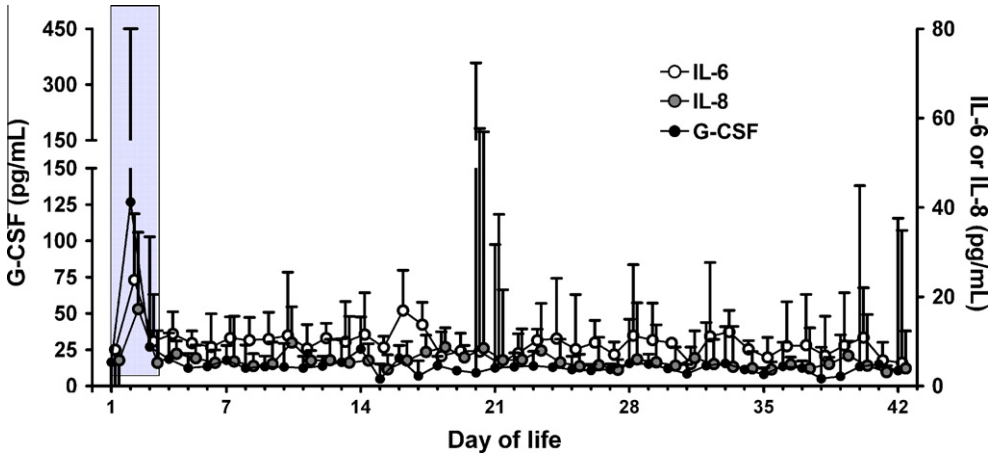

Fig. 2. Inflammatory responses over time in all infants in longitudinal study cohort. Median cytokine levels over the first 42 post-natal days. Shaded box indicates the *early systemic inflammatory period* as referred to in the text. Bars represent 75th quartile.

infectious events (for illustration, see bottom panel, Fig. 1). Indeed, sepsis, NEC and intestinal perforation are uncommon during this period [17,19]. In our series, only 2 infants developed culture-proven sepsis during the first 72 h of extra-uterine life while none of the infants developed NEC or intestinal perforation.

In order to exclude that infection is responsible for the higher systemic inflammatory response detected during the early SIP, we used stringent definitions to discard infants who were exposed to chorioamnionitis *in utero* or who had culture-proven or clinical *early-onset* sepsis (i.e. within the first 72 h of post-natal life). Chorioamnionitis and culture-proven sepsis were excluded based on an absence of maternal clinical signs, rigorous examination of placental histology, a negative blood culture at birth (performed in all infants) and at least one negative serum C-reactive protein level (<5 mg/dL). In 15 infants, *without* such signs of chorioamnionitis or culture-proven early-onset sepsis, cytokine levels in early SIP were significantly higher than baseline cytokine levels sampled at any other time during the 42-day study period (Table 2). Furthermore, when using even more stringent criteria to also exclude infants with clinical signs of sepsis, levels of IL-6 and G-CSF in the early SIP remained significantly elevated compared to baseline cytokine levels at any other time during the 42-day study period (Table 2). Notably, the systemic inflammation detected during the early SIP (Fig. 2) was of comparable magnitude to the responses measured during episodes of clinical or culture-proven sepsis (Table 2).

3.4. Clinical factors potentially responsible for the early SIP

The rise in plasma cytokine levels observed in infants in the absence of signs of infection during the early SIP period indicated that other non-infectious clinical factors may contribute to the generation of a systemic inflammatory response. Therefore, we conducted exploratory analyses to identify other potential contributing factors based on the previous literature (see Section 1). Among the clinical variables examined, we identified a moderate-to-strong correlation between peak IL-6, IL-8 or G-CSF plasma levels during this period and supplemental oxygen exposure: FiO_2 ($r = 0.51$–0.60 depending on the cytokine; $p < 0.01$). On note in regression analyses, FiO_2 was also significantly associated with either levels of IL-6 or IL-8 after adjusting for gestational age ($p < 0.01$). There were no significant correlations between cytokine responses and other variables examined, including blood transfusions. Interestingly, there was a modest, marginally significant correlation between anti-inflammatory drug treatments, and either IL-6 or IL-8 (Table 3).

4. Discussion

Inflammation has been repeatedly associated with adverse outcomes in preterm infants [4]. In this study, we determined the timing and magnitude of systemic inflammatory responses and tested

Table 3
Correlations between clinical variables and early systemic inflammation.

Clinical variable	*N*	Correlation coefficient (*r*) with cytokine level (pg/mL)[a]		
		IL-6	IL-8	G-CSF
Gestational age (weeks)	–	−0.22	−0.31	−0.27
Birth weight (g)	–	−0.16	−0.30	−0.29
Supplemental oxygen (FiO_2)	–	0.51*	0.50*	0.60*
Blood transfusion[b]	11	0.01	0.20	0.24
Anti-inflammatory treatment (indomethacin or corticosteroids)	10	0.40	0.44	0.29

[a] Spearman coefficient (*r*).
[b] Infant who received a blood transfusion within the early systemic inflammatory period.
* $p < 0.005$, all other correlations were non-significant at a $p > 0.2$ except for correlations with anti-inflammatory drug treatments, and IL-6 ($p = 0.021$) or IL-8 ($p = 0.015$).

whether inflammation occurs outside episodes of infection in infants exposed to intensive care therapies. Three biomarkers: IL-6, IL-8 and G-CSF were most significantly associated with neonatal infection and therefore were used to probe for "clinically relevant" systemic inflammatory responses in preterm infants. Levels of all three markers highly correlated with individual episodes of clinical or culture-proven sepsis, NEC or intestinal perforation in infants throughout the study period, thus confirming that our approach could detect systemic inflammatory responses produced during different types of sepsis-like illnesses. Most remarkable is our finding that "infection-like" levels of cytokines were detected in the first 72 h of extra-uterine life in the absence of clinical, bacteriological or placental histological evidence of infection.

Animal studies demonstrate that inflammation-mediated organ injury is critically modulated by critical care interventions, although data in human infants are more limited [20,21]. Previous studies have been restricted to time-focused peripheral blood or tracheal aspirate measures of inflammation, which preclude a more exhaustive temporal analysis of systemic inflammation in both ventilated and non-ventilated infants [22–25]. The recent development of flow-cytometry multiplex particle-based assays have made it possible to repeatedly and non-invasively measure longitudinally the levels of inflammatory markers from a large number of minuscule left over plasma or serum samples from routine clinical blood sampling, with high reproducibility [26]. In our study, IL-6, IL-8 and G-CSF were most strongly associated with neonatal infection (Supplementary Fig. 1). Paananen et al. independently validated markers and observed associations between IL-8 and G-CSF and bronchopulmonary dysplasia (BPD), a neonatal form of chronic lung disease [25]. Therefore, by virtue of these associations, we reasoned that IL-6, IL-8 and G-CSF would represent valid proxies for detection of potentially harmful, clinically relevant "sepsis-like" responses. Using exceedingly stringent clinical and histological definitions for ante- and post-natal infections, we were able to reasonably exclude infection as a predominant contributor of the early systemic inflammation period. Our results of increased inflammatory response in infants who show no placental evidence of inflammation versus infants with histological evidence of chorioamnionitis in the first 72 h of extra-uterine life are consistent with previous observations [25]. However, to our knowledge, this is the first study to exhaustively determine levels inflammation over time in preterm infants exposed to intensive care and to compare with levels detected during sepsis of different etiology.

The factors underlying the strong early systemic inflammatory responses are unclear. The response moderately correlated with exposure to supplemental oxygen therapy, although this result may be confounded by other associated clinical factors. Early in life, preterm neonates often require escalating respiratory support because of respiratory distress syndrome. Oxygen, more specifically reactive oxygen species (ROS) are strongly pro-inflammatory through direct activation of innate immune pathways [13]. We and others have shown that administration of supplemental oxygen to preterm infants generates significant oxidative stress resulting in detectable inflammation [9,10]. The high levels of inflammation associated with FiO_2 specifically observed in the early post-natal days might be the result of a relative deficiency in counteracting anti-oxidant mechanisms immediately following birth [15].

Limitations to our study include the small sample size and biases inherent to our non-invasive sample selection study design (i.e. use of scavenged blood samples) that limits detection of systemic inflammatory responses outside periods of greater illness or more intense daily blood sampling (mainly >12 days). Our study also lacks a causative mechanism to explain what triggers the early systemic inflammatory responses observed in absence of infection, which certainly deserves further investigation in larger cohorts. Although we have not directly measured oxidative stress, others have widely reported increases in plasma oxidant markers in infants exposed to supplemental oxygen [9,10,27,28]. The fact that anti-inflammatory medication marginally correlated with this early inflammation may reflect the tendency of clinicians to use more aggressive pharmacologic management in infants needing increased intensive care therapies, combined with a delayed anti-inflammatory effect of those drugs. Alternatively, mechanical ventilation as well as other unidentified factors linked to oxygen therapy might have contributed to the early inflammatory responses [8,29].

In conclusion, our study indicates a clinically significant systemic inflammatory response most detectable in the first 72 h of extra-uterine life in absence of overt infection, which may indicate a high risk period for the very preterm infant exposed to intensive care, amendable to preventive measures. Future investigations are necessary to elucidate relationships between inflammatory responses and non-infectious, potentially intensive care-related factors including the threshold and mode of delivery of supplemental oxygen therapy. Careful investigation of the inflammatory effects of early therapeutic interventions and their clinical consequences on developing infants may provide the opportunity to limit sustained organ injury and reduce adverse long-term neurodevelopmental consequences of early life exposures during critical transition periods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cyto.2011.08.028.

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