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# Absolute lymphocyte and neutrophil counts in neonatal ischemic brain injury

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## Abstract

**Objectives:** This study aimed to identify differences in absolute neutrophils, lymphocytes, and neutrophil-to-lymphocyte ratio between neonates with two forms of ischemic brain injury, hypoxic-ischemic encephalopathy, and acute ischemic stroke, compared to controls. We also aimed to determine whether this neutrophil/lymphocyte response pattern is associated with disease severity or is a consequence of the effects of total-body cooling, an approved treatment for moderate-to-severe hypoxic-ischemic encephalopathy.

**Methods:** A retrospective chart review of 101 neonates with hypoxic-ischemic encephalopathy + total-body cooling (n = 26), hypoxic-ischemic encephalopathy (n = 12), acute ischemic stroke (n = 15), and transient tachypnea of the newborn (n = 48) was conducted; transient tachypnea of the newborn neonates were used as the control group. Absolute neutrophil count and absolute lymphocyte count at three time-intervals (0–12, 12–36, and 36–60 h after birth) were collected, and neutrophil-to-lymphocyte ratio was calculated.

**Results:** Hypoxic-ischemic encephalopathy + total-body cooling neonates demonstrated significant time-interval-dependent changes in absolute lymphocyte count and neutrophil-to-lymphocyte ratio levels compared to transient tachypnea of the newborn and acute ischemic stroke patients. Pooled analysis of absolute lymphocyte count for neonates with acute ischemic stroke and hypoxic-ischemic encephalopathy (not hypoxic-ischemic encephalopathy + total-body cooling) revealed that absolute lymphocyte count changes occurring at 0–12 h are likely due to disease progression, rather than total-body cooling treatment.

**Conclusion:** These data suggest that the neutrophil/lymphocyte response is modulated following neonatal ischemic brain injury, representing a possible target for therapeutic intervention. However, initial severity of hypoxic-ischemic encephalopathy among these patients could also account for the observed changes in the immune response to injury. Thus, additional work to clarify the contributions of cooling therapy and disease severity to neutrophil/lymphocyte response following hypoxic-ischemic encephalopathy in neonates is warranted.

## Keywords

Neonate, brain ischemia, absolute lymphocytes, severity, hypothermia treatment

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## Introduction

Hypoxic-ischemic encephalopathy (HIE) and acute ischemic stroke (AIS), both neonatal ischemic brain injury (IBI), have yearly incidences of 1–6/1000 and 1/4000 live births, respectively.<sup>1,2</sup> Although the two conditions reflect different pathophysiological processes initially (HIE is a global ischemic insult, whereas AIS is a focal brain injury),<sup>1,3,4</sup> their post-insult pathways yield many similarities: approximately 12–36 h post-injury, peak impairment of brain perfusion triggers a secondary cascade of inflammatory events irreversibly

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injuring neurons<sup>1</sup> and leading to lifelong disabilities associated with motor deficits and intellectual impairment, or even death.<sup>1–4</sup> Understanding the pathological mechanisms of neonatal IBI could optimize interventions that limit neuronal damage.

Changes in immune response could be a crucial factor in IBI pathology. Following IBI, soluble pro-inflammatory molecules, such as cytokines, chemokines, oxidants, and proteases, signal for neutrophils and lymphocytes to migrate to the injured brain region.<sup>5</sup> Currently, individual subsets of leukocytes (e.g. neutrophils and lymphocytes) have been studied within the context of neonatal IBI.<sup>6–8</sup> Trends of increased neutrophil counts post-injury (approximately 12h) have been reported, followed by a noted decline (between 24 and 72h after insult) of these cells.<sup>6,7</sup> Lymphocyte counts are also reported as elevated among neonates with IBI within a similar time window of, but generally after, neutrophils.<sup>6,8</sup> Approximately 36h post-HIE-injury, the effects of hypothermia treatment are apparent on both neutrophil and lymphocyte profiles, which both exhibit suppressed phenotypes.<sup>6</sup> Induced hypothermia, or total-body cooling (TBC), is a known treatment for moderate-to-severe HIE, with well-documented beneficial impacts on outcome.<sup>1,9,10</sup>

Among adult patients, a higher neutrophil-to-lymphocyte ratio (NLR) has been linked to stroke and has shown to be predictive of poorer outcome.<sup>11,12</sup> Yet, fundamental differences in mature versus immature brains may impact the IBI-induced immune response in neonates;<sup>13</sup> therefore, this represents an important area of study in this population. Although changes in individual leukocyte (e.g. neutrophil and lymphocyte) profiles and their related cytokines have been reported,<sup>6–8</sup> NLR profiles have not been systematically investigated in neonates with IBI. Here, we retrospectively examined differences in absolute neutrophil count (ANC) and absolute lymphocyte count (ALC) and their possible influence on NLR in the neonatal population to determine whether these measures support earlier diagnosis or quantify disease severity.

## Methods

### Patients

This retrospective analysis of clinical data existing in electronic medical records at West Virginia University Hospitals was approved by the Institutional Review Board. A search using the Epic Electronic Health Record system was performed to identify neonates with HIE (who did or did not receive therapeutic hypothermia), AIS, and transient tachypnea of the newborn (TTN) admitted between 2004 and 2014. TTN patients served as the comparison group given that they are admitted for respiratory distress not associated with ischemic pathology.

### TBC protocol

Qualifying criteria for hypothermia according to a published protocol included the presence of acute perinatal event, low blood gas or cord pH, and moderate to severe encephalopathy confirmed by two independent neurological examinations. Therapeutic hypothermia was initiated within 6h of birth to achieve and maintain an esophageal temperature of 33.5°C for a period of 72h followed by gradual re-warming over a period of 6h (see Appendix 1 for the full protocol).<sup>9</sup>

### Inclusion and exclusion criteria

Neonates with gestational ages of less than 36 weeks, major congenital malformations, metabolic conditions, or any infections were excluded. Mothers with infections or those treated with immunosuppressive or other immune system-altering drugs were also excluded.

### Data collection

A total of 101 patients were identified via International Classification of Diseases, Ninth Revision (ICD-9) codes, charts were reviewed, and HIE severity was determined.<sup>14</sup> ALC and ANC from the complete blood count with differential from birth to 60h after were collected, and NLR was calculated for each patient. The post-birth values of ALC, ANC, and NLR were divided into three time-intervals (0–12, 12–36, and 36–60h) chosen based on documented alterations of these immune cells following birth.<sup>15,16</sup> If a patient had two+ blood draws within one 12-h interval, the average of those values was recorded.

### Statistical analysis

Data were analyzed using SPSS (Version 19.0). Group differences ( $p < 0.05$ ) among diagnosis (HIE+TBC, HIE, AIS, and TTN) groups at each time-interval (0–12, 12–36, and 36–60h following birth) were tested using analysis of variance (ANOVA) for each dependent variable. Significant diagnosis main effects were followed up with post-hoc analyses using the Bonferroni correction (significant at  $p < 0.0083$ ).

## Results

### Neonatal demographics

Table 1 contains information regarding resuscitation rates, APGAR (appearance, pulse, grimace, activity, respiration) scores, and blood pH levels; Ns for each time-interval are listed in Table 2. HIE+TBC neonates tended to score worse than other groups on each of these measures. Histograms of disease severity indicate that HIE+TBC patients (Figure 1(a)) were generally more severe than HIE patients (Figure 1(b)).

**Table 1.** Neonatal patient demographics.

Groups	GA (weeks)	Sex	Res (%)	APGAR: 1 min	APGAR: 5 min	Delivery (%)	pH	Sepsis work-up (%)	Temperature (°C)
TTN	38.4	28M: 20F	14.6	6.7	8.0	47.9 SVD: 52.1 C/S	7.3	0	37.0
AIS	38.8	9M: 6F	13.3	6.6	8.8	46.7 SVD: 53.3 C/S	7.3	0	36.9
HIE	39.1	8M: 4F	58.3	2.9	5.0	50.0 SVD: 50.0 C/S	7.3	0	36.7
HIE+TBC	38.8	15M: 11F	80.8	1.8	3.2	57.7 SVD: 42.3 C/S	7.1	11.5	35.1

HIE: hypoxic-ischemic encephalopathy; TBC: total-body cooling; AIS: acute ischemic stroke; TTN: transient tachypnea of the newborn; GA: gestational age; Res: resuscitation; APGAR: appearance, pulse, grimace, activity, respiration; SVD: spontaneous vaginal delivery; C/S: caesarean-section; pH: arterial and/or venous or cord blood gas.

**Table 2.** Subject numbers for each of the three time-intervals.

Groups	0–12 h	12–36 h	36–60 h
TTN; n = 48	28	26	11
AIS; n = 15	7	7	9
HIE; n = 12	7	7	4
HIE+TBC; n = 26	25	20	13

HIE: hypoxic-ischemic encephalopathy; TBC: total-body cooling; AIS: acute ischemic stroke; TTN: transient tachypnea of the newborn.

### Neutrophil/lymphocyte/NLR response among neonatal patient populations

Immune cells were altered in distinct patient populations in a time-interval-dependent manner across the acute injury period. Specifically, during the 0–12 h time-interval, there was a significant diagnosis effect for ALC ( $F(3, 63)=4.80$ ,  $p<0.005$ ) (Figure 2(a)), but not ANC (Figure 2(c)) nor NLR (Figure 2(e)). Post-hoc analyses revealed elevated ALC among HIE+TBC neonates relative to TTN patients.

This trend was also observed during the 12–36 h time-interval. During this time, there was a significant diagnosis effect for ALC ( $F(3, 56)=7.37$ ,  $p<0.001$ ) (Figure 2(a)) but not ANC nor NLR. Post-hoc analyses revealed lowered ALC among HIE+TBC neonates relative to the TTN and AIS groups.

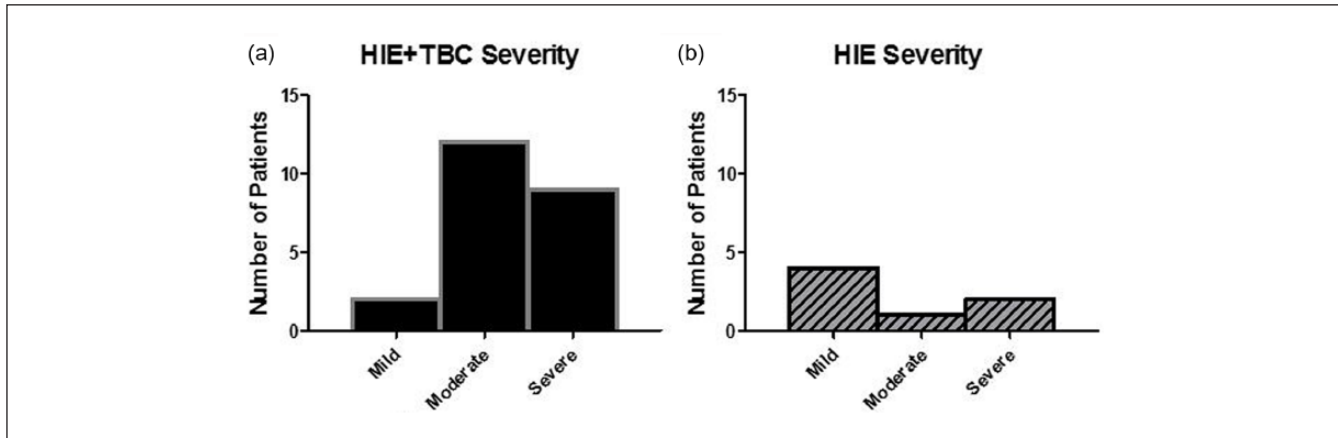
During the 36–60 h time-interval, there was a significant diagnosis effect for ALC ( $F(3, 33)=5.04$ ,  $p<0.005$ ) (Figure 2(a)). Post-hoc analyses revealed lowered ALC among HIE+TBC neonates relative to TTN patients. As well, there was a diagnosis effect for NLR ( $F(3, 33)=3.66$ ,  $p<0.05$ ) (Figure 2(e)). Post-hoc analyses revealed elevated NLR among HIE+TBC neonates relative to TTN patients. There were no effects of diagnosis on ANC.

That the HIE+TBC group had elevated immune cell counts at the early time-point initially could suggest that TBC exacerbates the immune response in neonatal HIE patients. This was a surprising finding considering that TBC is a known beneficial therapeutic intervention for HIE.<sup>1,9,10</sup> Therefore, although mechanisms of injury differ in the two brain injury conditions,<sup>1,3,4</sup> post-hoc we pooled data from patients from our sample who had sustained any kind of

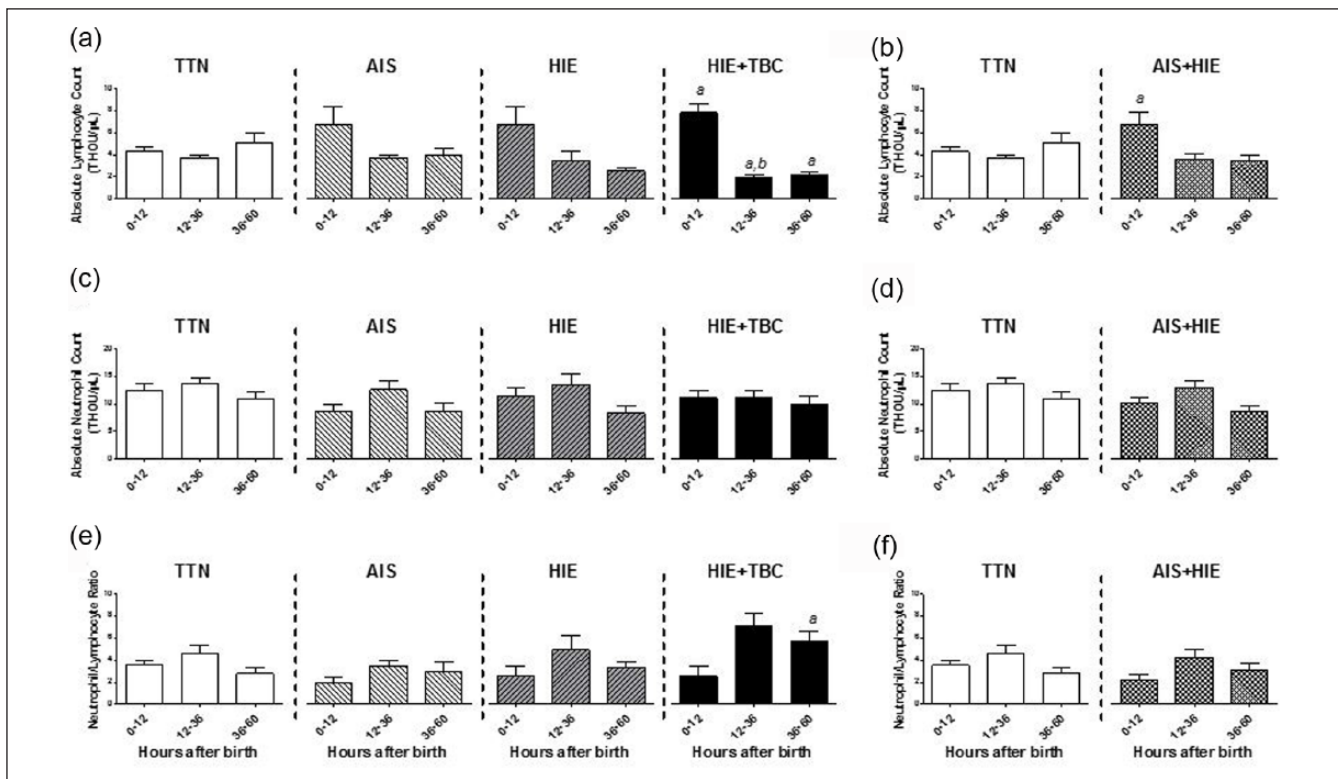
cerebral ischemia but did not undergo cooling therapy (AIS and HIE groups not including HIE+TBC) and assessed changes in ALC, ANC, and NLR relative to TTN patients at each time-interval to dissociate the impact of ischemic injury (of any kind) versus the addition of TBC treatment in ischemic brain injured neonates on immune markers. Between 0–12 h, there was a significant diagnosis effect for ALC ( $t(40)=2.68$ ,  $p<0.05$ ) (Figure 2(b)) but not ANC (Figure 2(d)) nor NLR (Figure 2(f)).

### Discussion

These preliminary findings support that activation of the immune system is an important component of IBI. Indeed, immune modulation following stroke is documented in adults; NLR is used as a diagnostic indicator of stroke, cancer, infection, and other pathologies.<sup>11–12,17–20</sup> However, the immune response of neonatal IBI is not well characterized. Moreover, IBI-related differences between adult and neonatal populations are broadly attributed to the polarized states of immune system and brain maturity. Specifically, following IBI in both populations, pro-inflammatory molecules act as signaling molecules for subsets of leukocytes, such as neutrophils and lymphocytes, which then migrate to the injured brain region.<sup>5</sup> However, known differences in these ischemic pathways are associated with adults and neonatal populations.<sup>13</sup> For example, after neonatal ischemic insult in the form of AIS, activated, resident microglial cells, rather than peripheral invading monocytes, are the primary immune cells that initiate the pro-inflammatory cascade.<sup>20</sup> These activated microglia produce pro-inflammatory cytokines and chemokines, which, in turn, increase the magnitude of neutrophil chemotaxis to the injured area, and are a crucial component to subsequent lymphocyte activation.<sup>13,20,21</sup> Additionally, there are also differences in neutrophil and lymphocyte adherence and migration following ischemic insult between the two populations. Such an example can be observed in the neutrophil populations of post-natal day 7 rat pups, where, in contrast to adults, neutrophils are not found to migrate to the brain within the early hours of post HIE-injury, but do migrate following AIS-injury. In both populations, there is evidence that lymphocytes migrate to the brain later than neutrophils, although this migration is more



**Figure 1.** (a) Histogram of HIE+TBC severity. Among HIE+TBC patients for whom level of ischemic severity was determined ( $n=23$ ), there were 2 mild, 12 moderate, and 9 severe cases; (b) Histogram of HIE severity. Among HIE patients for whom level of ischemic severity was determined ( $n=7$ ), there were four mild, one moderate, and two severe cases.



**Figure 2.** Time-point-dependent alterations in absolute neutrophil and lymphocyte counts among patient populations. (a) Mean  $\pm$  SEM ALC in each patient population. At the 0–12h time-interval, ALC are elevated in HIE+TBC ( $M=7.81$ ,  $SD=4.21$ ) versus TTN ( $M=4.29$ ,  $SD=2.09$ ) neonates. At the 12–36h time-interval, ALC are lower in HIE+TBC ( $M=1.96$ ,  $SD=0.99$ ) versus TTN ( $M=3.73$ ,  $SD=1.4$ ) and AIS ( $M=3.73$ ,  $SD=0.66$ ) neonates. At the 36–60h time-interval, ALC are lower in HIE+TBC ( $M=2.18$ ,  $SD=1.16$ ) versus TTN ( $M=5.08$ ,  $SD=2.81$ ) neonates. (b) Mean  $\pm$  SEM ALC in AIS/HIE relative to TTN. At the 0–12h time-interval, ALC are elevated in AIS/HIE ( $M=6.80$ ,  $SD=4.01$ ) versus TTN ( $M=4.29$ ,  $SD=2.09$ ) neonates. These findings suggest that changes in ALC at the 0–12h time-interval are due to ischemic injury rather than TBC treatment. (c) Mean  $\pm$  SEM ANC in each patient population. There were no significant group differences between each patient group in ANC for any of the three time-intervals. (d) Mean  $\pm$  SEM ANC in AIS/HIE relative to TTN. There were no significant group differences between each patient group in ANC for any of the three time-intervals. (e) Mean  $\pm$  SEM NLR in each patient population. At the 36–60h time-interval, NLR are lower in HIE+TBC ( $M=5.72$ ,  $SD=3.09$ ) versus TTN ( $M=2.76$ ,  $SD=1.70$ ) neonates. (f) Mean  $\pm$  SEM NLR in AIS/HIE relative to TTN. There were no significant group differences between each patient group in NLR for any of the three time-intervals. *a*=significant (Bonferroni corrected  $p<0.0083$ ) versus TTN at that time-interval; *b*=significant (Bonferroni corrected  $p<0.0083$ ) versus AIS at that time-interval.

prominent in adults than in neonates.<sup>13</sup> Additional neonatal animal studies report that modulation of neutrophils and lymphocytes does occur within the post-ischemic injury period, and that these modulations are often associated with injury severity and long-term neurological outcome.<sup>22–24</sup> Furthermore, although one study conducted in term infants with HIE found no correlation between lymphocyte number and outcome,<sup>8</sup> findings from another noted that elevated peripheral neutrophils were associated with worsened neurological outcome.<sup>7</sup> Methodological and/or population differences between these and the current study (i.e., severity was not measured in Morkos et al.<sup>7</sup> and Shah et al.<sup>8</sup>) could account for these discrepant findings.

Here, we noted a time-interval-dependent shift in lymphocyte counts among the HIE+TBC patients relative to neonates with AIS or TTN. This effect is possibly due to TBC treatment. However, given that the beneficial effects of TBC on clinical outcome following IBI are known,<sup>25</sup> our finding is not likely attributable to the effect of TBC but rather due to either the presence of an ischemic injury and/or increased IBI severity we noted in the HIE+TBC group. Specifically, in the initial post-injury hours, we report increased ALC among HIE neonates that received TBC, suggesting an impact due to TBC treatment. However, when we assessed changes in ALC following ischemia in only untreated IBI patients (AIS and HIE groups), the increase in ALC was significant. The specificity of this significant increase at the first time-interval (0–12h) suggests that the difference noted in the TTN versus HIE+TBC groups at that time-interval was likely due to the presence of IBI; the cause (ischemic injury presence/severity or effects of cooling intervention) of the changes noted at later time-intervals in ALC (12–36 and 36–60) and NLR are not completely clear from these data. Between 12–36h, ALC was significantly lower in HIE+TBC relative to comparison groups. At the 36–60h time-interval, the NLR was altered in this group, a finding consistent with the noted increased NLR that is associated with worsened clinical outcomes in adult patients.<sup>11,12</sup> While our findings are in partial congruence with leukocyte profiles of other studies, specifically lymphocyte counts,<sup>6,8</sup> discrepancies between our work and the work of others (i.e. significantly elevated ANC at the 12–24h time-point,<sup>7</sup> a finding that we did not replicate here) regarding neutrophils could be attributed to differential time-point groupings, IBI severity distribution differences, and a lack of repeated measures due to small N as further described in the “Limitations” section. Further supporting the notion that presence/severity of IBI may be accounting for our observed group differences (at least in the early time-intervals) is that while 43% of HIE patients were considered moderate-to-severe, 92% of HIE+TBC patients met this criteria. Furthermore, HIE+TBC neonates had the highest resuscitation rates, poorest APGAR scores, and lowest blood pH levels (Table 1). Thus, severity may be a key factor in the pathological progression of hypoxic brain injury and should be a covariate in future studies aimed at evaluating the

immune profile shifts in neonatal IBI patients. Taken together with the published findings of others,<sup>6,8</sup> these data suggest that shifts in peripheral neutrophil and/or lymphocyte numbers may be important in the immune response following IBI; more work is needed to clarify this effect, explore the possibility of these markers to be used as a biomarker for HIE diagnosis/severity, and identify potential avenues for therapeutic targeting of these cells.

### Limitations

Potential limitations of these findings are lack of repeated measures for patients and small N values within particular patient groups, leading to low statistical power. This study was designed to assess changes in neutrophil/lymphocyte profiles across the acute injury period in multiple IBI diagnoses. However, repeated blood draws for the TTN groups is not standard practice given that TTN neonates are relatively healthy with the exception of minor reversible respiratory distress. Moreover, during the retrospective data collection window, there were limited numbers of AIS and HIE cases from which to gather data, limiting sample sizes in these groups at several of the time-intervals assessed. Generally, statistically significant comparisons noted here were between TTN and HIE+TBC groups with the largest sample sizes. This together with our use of a stringent and conservative post-hoc test suggests that comparisons involving other groups (i.e. AIS, HIE) may have been underpowered. This low statistical power may also explain the lack of significant alterations in ANC levels in the current study that has been shown in other clinical reports.<sup>6,7</sup> Additional work with larger patient populations from whom data regarding immune cell changes across time are gathered may reveal more pronounced effects of IBI, especially among the AIS and HIE groups. Regardless, where converging findings agree is that a profound immune response following neonatal hypoxic ischemia takes place in the acute injury period, and that this response could be used as a biomarker for HIE severity or even a possible target for novel therapeutic interventions aimed at reducing mortality and improving functional outcomes.

### Conclusion

Our results support the hypothesis that the neutrophil/lymphocyte response is altered following neonatal IBI, representing a possible target for therapeutic intervention. Future work dissociating the benefits of TBC from injury severity on neutrophil/lymphocyte profiles in IBI patients is warranted.

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### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Ethical approval

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	Moderate encephalopathy	Severe encephalopathy
Level of consciousness	Lethargic	Stupor/coma
Spontaneous activity	Decreased activity	No activity
Posture	Distal flexion Full extension	Decerebrate
Tone	Hypotonia focal, general	Flaccid
Primitive reflexes	Weak suck Incomplete Moro	Absent suck Absent Moro
Autonomic system	Constricted pupils Bradycardia Periodic breathing	Skew deviation, dilated, non-reactive to light pupils Variable heart rate Apnea

## Appendix I

### Cooling protocol

Clinical findings include a cord pH or blood gas at <1 h of age, with a pH < 7.0 or base deficit > 16 mEq/L. If a blood gas is not available or pH is between 7.01 and 7.15 or base deficit of 10–15.9, the infant should have had an acute perinatal event and either an APGAR score of <5 at 10 min or continued need for assisted ventilation for >10 min. If these criteria are met, neurological examination is performed by two independent neonatologists or pediatric neurologist is obtained, to define moderate and severe encephalopathy according to seizures or presence of one or more signs in three of the six categories as follows:

Therapeutic hypothermia is initiated in eligible infants as soon as possible within 6 h of birth to achieve and maintain an esophageal temperature of 33.5°C for a period of 72 h followed by gradual re-warming by increasing the core body temperature at the rate of 0.5°C per hour over a period of 6 h.

The FDA-approved Blanketrol II Hyper/Hypothermia System (manufactured by Cincinnati Sub-Zero) is the hypothermia device for thermoregulation. The infant is placed on a cooling blanket in an open warmer with no layers of clothing between the infant and the cooling blanket. Cooling blanket should lie flat with no kinks in the connecting hoses. Infant should lay supine on the blanket. An esophageal probe will then be used to measure the set point esophageal temperature which should be at 33.5°C. Fluctuations in this temperature are to be expected but should not be greater than 1°C from the set point. Once the set point is reached, a thin layer (such as a receiving blanket) may be placed on the infant. Temperatures from the esophageal probe, infant's skin, and the cooling blanket will be monitored every 15 min for the first 4 h of therapy. Temperatures will then be recorded hourly until 12 h of cooling and then every 2 h until the infant has completed the 72 h of cooling therapy. Upon completion of 72 h cooling period, the infant will be re-warmed gradually so that the core body temperature will increase 0.5°C each hour over a 6 h period.