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Evaluation of Chemokine CCL18 Level in Cord and Peripheral Blood as a Predictor of Intraventricular Hemorrhage in Preterm Infants

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ABSTRACT

Background: Intraventricular hemorrhage (IVH) is a serious complication of prematurity. While early diagnosis is crucial for appropriate management, determining high-risk neonates might prompt extra preventive measures. Low levels of Chemokine (C-C motif) ligand 18 (CCL18) may predict the development of IVH in preterms.

Aim of the study: to evaluate the association between CCL18 level in cord and peripheral blood and the incidence of IVH in preterm neonates. **Patients and methods:** This prospective cohort study included, 51 preterm neonates aged (29-32 weeks). Neonates with perinatal hypoxia, brain malformations, or major congenital malformations were excluded. CCL18 was analyzed in cord blood at birth and in the peripheral blood on day 2 of life. Cranial ultrasound scans were done on day 3 and day 7.

Results: Out of the studied neonates, only 44 were enrolled,18 males (40.9%) and 26 females (59.1%). The incidence of IVH was 52.3% (23/44). No statistical differences were observed in gestational age (p=0.59), weight (p=0.192), gender (p=0.139), maternal illness (p=0.355), and Apgar score (p=0.961) in both groups. Cord and blood CCL18 were comparable in patients with and without IVH (p=0.518 & p=0.70 respectively). Impaired neurological examination and low platelet count were significantly associated with IVH (p<0.05).

Conclusion: The current work suggests that the CCL18 level in the cord or peripheral blood can't indicate preterm neonates at a higher risk of developing IVH. Through neurological examination, sequential cranial ultrasounds and platelet counts might be a more convenient tool for early detection of IVH in preterm babies.

Keywords: CCL18, Intraventricular hemorrhage, prematurity, Neonatal, Neurodevelopment.

INTRODUCTION

Intraventricular hemorrhage (IVH) is a common, and rather a serious complication of prematurity, whose incidence is inversely proportional to the gestational age. Despite all advances in neonatal medicine, the prevalence of IVH remains relatively stationary [(1)].

Hydrocephalus, blindness, cerebral palsy (CP), and even mortality are direct sequala of IVH, particularly the most severe grades (grades 3 and 4) [(2)]. Staggering reports suggest that even milder grades of IVH aren't benign as previously thought. Recent long-term follow-up studies suggest that -compared to normal neonates- those with mild IVH might be at higher risk of suffering behavioral problems, impaired cerebellar growth, white matter microstructure immaturity and dysfunction, and overall neurological impairment later in life [(3)].

In preterm babies, almost 90% of IVH occurs in the first week of life. For that reason, regular cranial ultrasound scans (up to three in the first week) are considered standard of care in most neonatal units. However, this detects the IVH after it already happened. While early diagnosis is crucial for the appropriate management and closer monitoring, determining high-risk neonates might prompt extra preventive measures, like deferring unnecessary procedures or applying IVH prevention care bundles [(4)].

The pathophysiology of IVH in preterms is complex and multifactorial. This includes frail blood vessels, fragile germinal matrix, impaired cerebral autoregulation, fluctuations in the carbon dioxide level, and other medical conditions, either maternal (e.g., sepsis) or neonatal (e.g patent ductus arteriosus PDA, coagulopathy) [(5)].

As the risk of preterm IVH increases with some inflammatory conditions (e.g., maternal chorioamnionitis), but decreases with antenatal steroids, the role of inflammatory mediators in the pathogenesis of IVH has been studied [(6)]. Thus, the discovery of biomarkers that can highlight infants at risk for injury, quantify its progression, and audit the efficacy of neuroprotective interventions is much needed [(7)].

Chemokine (C-C motif) ligand 18 **(**CCL18) -also referred to as Pulmonary and Activation Regulated Chemokine (PARC) is a member of the C-C chemokine family, whose main biologic role is mediating chemotaxis of leukocytes, mainly as a part of innate immunity [(8)]. It is present at high levels in human plasma and is located on chromosome 17q1, aside from other macrophage inflammatory proteins [(9)]. CCL18 receptors can be detected in the germinal matrix, choroid plexus, endothelium of periventricular capillaries, as well as ependymal cells [(10)].

In preterm babies, CCL18 chemokine levels in cord blood are low but notable increase from 32 weeks gestation onwards. It is well known that the incidence of IVH drops massively after 32 weeks gestation, which

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may suggest that higher levels of CCL18 are protective against IVH [11]. Behaving as an inflammatory marker, the CCL18 level was high in brain tissue biopsies of patients with traumatic brain injuries [12].

As for the neonatal population, low levels of cord CCL18 were found to be predictive of increased risk of IVH grade II-IV after adjusting for other risk factors [11].

In the longer term, investigating the possible association between CCL18 and CP revealed that preterm infants who developed cerebral palsy have lower cord blood levels of CCL18 [13]. Furthermore, a significant association was found between single nucleotide polymorphisms (SNPs) in CCL18 and susceptibility to CP, as well as the association between the SNPs and cord blood levels of CCL18, where the minor allele A was underrepresented in cases of IVH compared to controls. This association remained significant even after adjustment for other risk factors of CP [14].

The physiological role of CCL18 role and its actions are not fully understood, but it is suggested that low levels of CCL18 are an independent risk factor of IVH [11].

AIM OF THE STUDY

The study aimed to evaluate the association between CCL18 levels in cord and peripheral blood and the incidence of IVH in preterm neonates.

Study settings:

The maternity hospital of Ain Shams University is a busy, public maternity hospital, with an estimated annual rate of 18,000 deliveries. Its NICU is a tertiary specialized neonatal unit that provides specialized high-level medical and surgical services to the admitted neonates (e.g various modes of advanced neonatal ventilation, point of care ultrasound, specialized neonatal surgery, therapeutic hypothermia).

Patients and methods:

Eligibility and enrollment:

In the current prospective cohort, preterm neonates with completed gestational age between 29 and 32 weeks with birth weight between 750 and 1900 gm were eligible for enrollment. Neonates with perinatal hypoxia, a suspected inborn error of metabolism, brain malformations, or major congenital malformations were excluded. The study endpoint was reaching day 7 postnatal age.

B. History and examination:

All babies were subject to detailed maternal and perinatal history taking. This included gestational age, birth weight, mode of delivery, maternal history of chronic illness, or history suggestive of infection. A thorough neurological examination was performed at the time of enrollment and on day 7 by two senior neonatologists following the unit's protocol that was adapted from Volpe, 2018 [15].

C. Lab investigations:

I. Cord Sampling:

Parents' consent was sought before delivery. After neonatal stabilization, cord blood samples were withdrawn on EDTA by the attending neonatologist from the placental side. Samples were immediately sent to the lab where they get centrifuged and prepared for flow cytometric analysis of CCL18 expression on cord blood mononuclear cells.

II. Blood samples:

1. On day 2, venous blood samples were withdrawn f or the reassessment of CCL18 expression on peripheral blood mononuclear cells by flow cytometry.
2. On days 3 and 7: complete blood counts (CBC) using Max-M coulter and Leishman stain film.
3. All other routine labs (C-reactive protein (CRP), liver functions, electrolytes, kidney functions) were measured as per the unit's protocol or as per the medical team.

D. Radiological investigations:

For all babies <34 weeks gestation, and as per the unit's protocol, cranial ultrasonography is routinely done at least twice in the first week (1st on days 1-3 and the 2nd on day 7) for detection of IVH. For sake of standardization, all enrolled neonates had their scans done on day 3 and day 7 by two dedicated radiologists using US Scanner Samsung (HM70A) machine. The ultrasonographic grading of IVH followed the grading system suggested by ***Papile et al.,*** in **1978** [16]**.** Patients were retrospectively allocated to two groups according to the scan results. If any of the two scans was positive for any grade of IVH, the patient was allocated to the IVH group and those whose scans were free were allocated to the "no IVH group".

Statistical Analysis

Statistical package for social science (SPSS) version 23 was used for data entry, coding, and analysis. Descriptive statistics were presented by using mean, standard deviation, and range for the quantitative data while the number and percentages were used with the qualitative data.

The independent sample t-test was used to compare between two groups with parametric data while the Mann-Whitney test was used to compare between two groups with non-parametric data. The Chi-square test was used to compare groups with qualitative data and the Fisher exact test was used instead of the Chi-square test when the expected count was less than 5 in any cell of the table. The Pearson correlation coefficients were used to correlate between parameters. The confidence level was set to 95% and the margin of error accepted was set to 5%. So, the P-value was considered significant if < 0.05 and non-significant if > 0.05.

Ethical consideration:

The study protocol was approved by the local ethical committee of the Pediatric Department of Ain Shams University. Informed consent was obtained from the patient's caregivers before the delivery. Identifying information was coded and remained confidential. The study maintained the code of ethics for human studies "Declaration of Helsinki".

RESULTS

During the study period, fifty-one preterm babies were eligible for enrolment. Seven patients were excluded (three had congenital anomalies, one had difficult resuscitation and severe hypoxia and three declined to participate).

Forty-four patients were enrolled. The process of enrolment and exclusion is shown in the CONSORT chart (**Fig 1**).

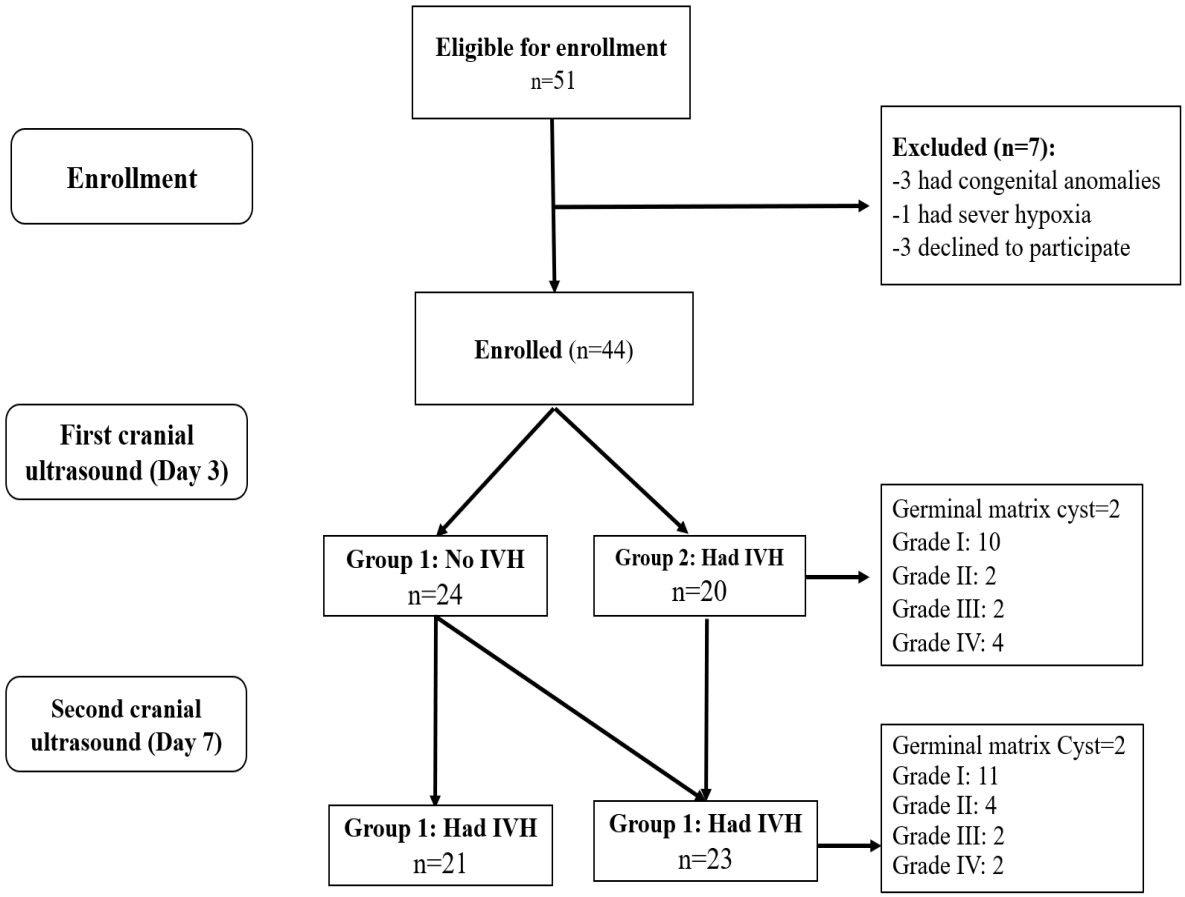

Figure (1): CONSORT flow chart

Table (1): Demographic characteristics of enrolled neonates

		No IVH (n=23)	Had IVH (n=21)	Test value	p-value
GA By date	Mean ±SD	29.57±2.77	29.09±3.18	0.537#	0.594
BW (kg)	Mean ±SD	1.35±0.32	1.22±0.35	1.326#	0.192
Gender (n/%)	Female Male	10(47.6%) 11 (52.4%)	16 (69.6%) 7 (30.4%)	2.187*	0.139
Mode of delivery	CS SVD	14 (66.7%) 7 (33.3%)	14 (60.9%) 9 (39.1%)	0.007*	0.931
Maternal illness+	n/%	3 (14.3%)	5 (21.7%)	0.8553	0.355
Multiple gestations	n/%	6 (28.6%)	11 (47.8%)	3.2011	0.0735

IVH: intraventricular hemorrhage, BW: birth weight, SD: standard deviation, CS: cesarian section, SVD, Spontaneous vaginal delivery, GA: gestational age *: Chi-square test, #Independent t-test, + Diabetes Mellitus, pre-eclampsia, chorioamnionitis,polyhydraminos, oligohydramnios.

Table 1 shows the demographic data of all enrolled neonates. There were no significant differences between the two groups regarding gestational age, gender, birth weight, mode of delivery, multiple gestations, or maternal illness (p >0.05 for all values).

Table 2: Results of cranial ultrasound scans on days 3 and 7

Results of scans	Cranial ultrasound Day 3		Cranial ultrasound Day 7	
	n	%	n	%
Normal	24	54.55	21	47.73
Germinal matrix cyst	2	4.55	2	4.55
I	10	22.73	11	25.0
II	2	4.55	4	9.09
III	2	4.55	3	6.81
IV	4	9.09	3	6.81
Total	44	100.0	44	100.0

Table 2 shows that by day 7, 23 patients had abnormal findings in their ultrasound scans. Three patients had their scans initially normal and later developed IVH. Four patients had disease progression while one patient improved (from grade 4 to 3).

Table (3): Resuscitation data of both study groups:

	no IVH n=23	IVH n=21	Test value	P-value
Apgar 1 min	5.5 (4-7)	5 (4-7)	0.048#	0.961
Apgar 5 min	7.5 (7-8)	7 (7-8.75)	0.503#	0.615
Bag and mask ventilation				
No	17 (81.0%)	13 (56.5%)	1.999*	0.157
Yes	4 (19.0%)	10 (43.5%)		
Endotracheal tube				
No	21 (100.0%)	21 (91.3%)	0.434*	0.510
Yes	0 (0%)	2 (8.7%)		

*: Chi square test#: t: Independent t-test

Table 3 shows that there were no significant differences in the resuscitation data or Apgar scores of patients with IVH or with no IVH (p>0.05 for all values).

Table (4): Findings of neurological examination in both groups

		no IVH		IVH		Test value	P-value
		n	%	n	%		
Anterior fontanelle	At level	21	100.00%	17	73.90%	6.343	0.012
	Bulge	0	0.00%	6	26.10%		
Suckling reflex	Absent	1	4.80%	5	21.70%	3.555	0.169
	Weak	4	19.00%	6	26.10%		
	Good	16	76.20%	12	52.20%		
Moro reflex	Absent/weak	1	4.80%	7	30.40%	4.864	0.027
	Good	20	95.20%	16	69.60%		
Deep tendon reflexes	Exaggerated	0	0.00%	2	8.70%	10.932	0.012
	Hyporeflexia	0	0.00%	6	26.10%		
	Not elicited	1	4.80%	3	13.00%		
	Normal	20	95.20%	12	52.20%		
Conscious	Alert	21	100.00%	12	52.20%	13.391	0.001
	Lethargy	0	0.00%	8	34.80%		
	Sedated	0	0.00%	3	13.00%		
Tone	Flaccid	0	0.00%	3	13.00%	13.396	0.003
	Hypertonic	0	0.00%	2	8.70%		
	Hypotonic	0	0.00%	6	26.10%		
	Normal	21	100.00%	12	52.20%		
	Total	21	100.00%	23	100.00%		

Table (4) shows that apart from for suckling reflex, patients with IVH had significantly abnormal neurological signs compared with those with no IVH. These included bulging anterior fontanel (p=0.012), abnormal Moro reflex (p=0.027), deep tendon reflexes (p=0.012), altered consciousness (p=0.001), and abnormal tone (p=0.003).

Table (5): Cord blood and peripheral blood CCL18 expression in both groups

		no IVH n=23	IVH n=21	Test value#	P-value
CCL18 cord (FU)	Mean ±SD	8.12 (2.37 – 16.4)	6.125 (4.4 – 14.85)	-0.652	0.518
CCL18 blood (FU)	Mean ±SD	3.05 (1.6 – 7.89)	4.4 (1.7 – 7.04)	-0.388	0.700

SD: standard deviation, FU: fluorescence unit, #Mann-Whitney test

Table (5) shows that there was a non-significant difference between the two groups regarding CCL18 expression, either in cord blood (p= 0.518) or in peripheral blood samples (p=0.70).

Table (6): Comparison between preterms with severe IVH (grades 3&4) and those with no IVH regarding CCL18 expression in cord and peripheral blood:

	Severe		Preterm without IVH		Test value#	P value
	Median	IQR	Median	IQR		
CCL18 cord	5.33	2.5 - 12.7	8.12	2.37 - 16.4	0.815	0.415
CCL18 blood	4.16	1.6 - 6.2	4.8	1.6 - 7.89	0.000	1.000

#Mann-Whitney test

Table (6) shows that on the further exclusion of milder cases of IVH (grades 1&2), there were no statistically significant differences in CCL18 expression in the cord or peripheral blood between preterm neonates with severe IVH (grades 3&4) and those with no IVH.

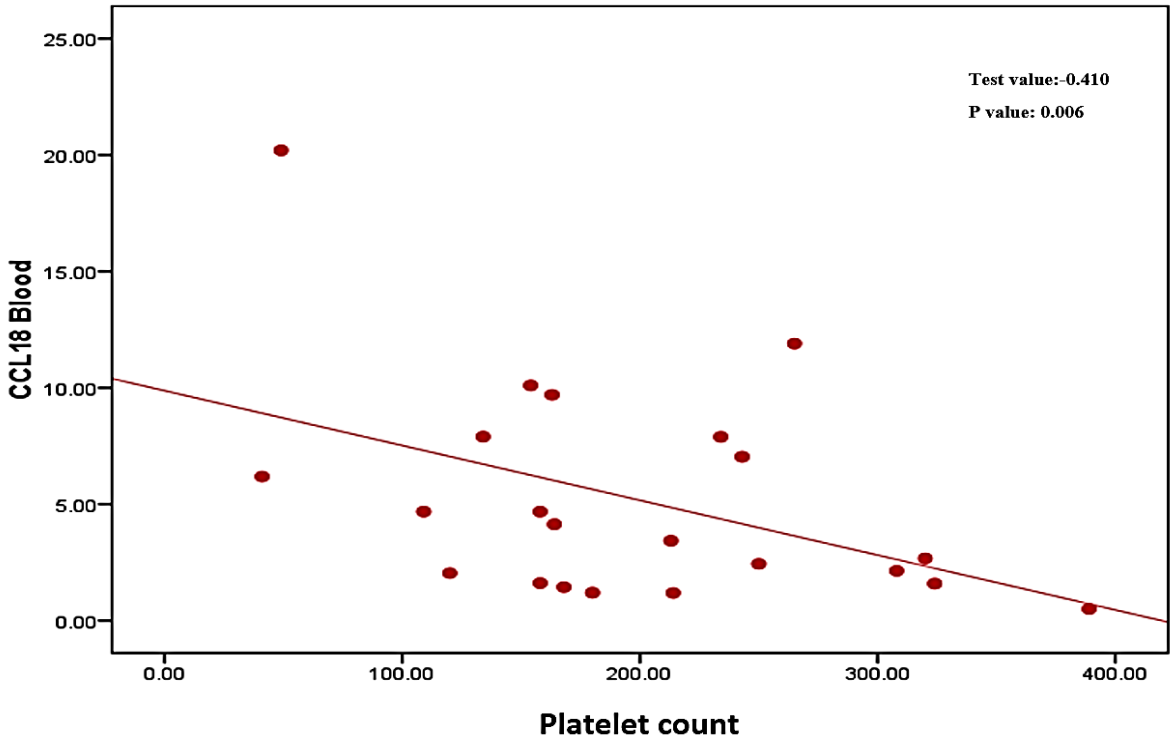

Figure (2): Pearson Correlation between CCL18 blood and platelet counts

Figure (2) shows that there was a statistically significant negative correlation between blood CCL18 expression and platelet counts (p=0.006).

DISCUSSION

The current prospective cohort was conducted to test the possible association between CCL18 levels in cord and peripheral blood and the incidence of IVH in preterm neonates.

This was done by measuring CCL18 expression in cord and peripheral blood (on day 2) and by neuroimaging using cranial ultrasound scans on days 3 and 7. Based on the results of the scans, the neonates were allocated into 2 groups (with or with no IVH).

The overall incidence of IVH in the present study was 52.3% (23/44). The incidence of IVH was not statistically correlated with patients' demographics, mode of delivery, maternal illnesses, resuscitation data, and Apgar score ($p>0.05$ for all parameters).

Contrariwise, ***Lu et al.*** [17] in their study that included 137 preterm babies <34 weeks gestation found that low gestation, maternal chorioamnionitis, low birth weight, and low Apgar scores at birth were found to be independent risk factors for IVH.

This conflict is probably because the incidence of IVH is complex and requires the presence of more than one risk factor at a time. For that reason, ***Coskun et al.*** [18] developed a clinical score to identify neonates at increased risk of IVH. The score ranges from 0-5 and can be applied to preterms of 24-34 gestation. It includes the sum of three identified risk factors for IVH (low Apgar, coagulopathy in the first week of life, and gestational age ≤ 28)**.**

The current study showed that CCL18 levels in both cord and peripheral blood were statistically comparable in both groups ($p=0.518$ & $p=0.70$ respectively). This relation didn't change after excluding patients with mild IVH from the analysis ($p=0.415$ & $p=1$ respectively).

On the contrary, ***Kallankari et al***. [11] in a prospective cohort study that included 163 preterm neonates born before 32 weeks of gestation found that CCL18 was the only marker found to be significantly associated with IVH among all 12 blood cytokines and 107 cord blood immunoproteins studied. They concluded that low cord CCL18 is an independent risk factor of IVH. In further research for the same group in 2015, researchers found that IVH together with a polymorphism of the CCL18 in preterm neonates was associated with an increased risk of CP [14].

However, there is a paucity of data regarding the possible actual effect and protective mechanism of CCL18 in the literature. In 2013, ***Douglas and Weiss*** [7] reviewed the biomarkers previously studied in premature neonates with IVH, periventricular leukomalacia, and post-hemorrhagic ventricular dilatation. They concluded that the most valuable biomarkers for neonatal IVH were Activin and S100β. They also highlighted that future studies must validate the biomarkers' results by correlating them to other clinical data, cerebral function monitoring modalities such as Near Infrared spectroscopy (NIRS) and amplitude Electroencephalogram (aEEG) and brain imaging (eg MRI), validated neurodevelopmental scores (e.g. Bayley scales). These modalities are considered references for neurological assessment, particularly, when put together [19-20].

In the present study, the IVH group had a significantly abnormal neurological examination in all studied clinical parameters ($p<0.05$) except for the suckling reflex ($p=0.169$).

Similarly, in an ancient study that examined the correlation between neurological examination to real-time cranial ultrasound in babies with IVH, ***Dubowitz et al***. [21]. found that thorough sequential neonatal neurologic examination is a comparably sensitive and less expensive tool that can be conveniently used to detect the development and follow the progression of IVH. However, this result might be due to older versions of ultrasound machines with poorer imaging quality and also can be due to the better clinical skills of neonatologists in the previous two decades compared to now.

In the present study, the IVH group had significantly lower platelet count compared to babies with no IVH ($p=0.006$).

Similarly, ***Von Lindern et al***. [22] in their large retrospective cohort that included 1569 neonates with thrombocytopenia, found that while IVH (grade 2 and above) occurred more in neonates with thrombocytopenia, this relation was independent of the severity of thrombocytopenia.

Additionally, ***Andrew et al.*** [23] in their prospective study found that the incidence of IVH in neonates <1500 grams, with and without thrombocytopenia, was 78%, and 48% respectively ($P<0.01$). Moreover, they found that severer grades of IVH occur more frequently in thrombocytopenic infants. Subsequently, serious neurologic impairment for survivors was 41% vs 7% in neonates with and without thrombocytopenic respectively.

Study limitations:

Our study has limitations in not analyzing other factors that might affect the incidence of IVH for IVH including PDA and antenatal steroids. However, in this prospective cohort study, we excluded extremely premature babies who would be more affected by these clinical conditions. Additionally, the level of studied CCL18 should not be affected in either presence or absence of these clinical conditions.

CONCLUSION

The current work suggests that CCL18 expression in the cord or peripheral blood can't be used as a biomarker for defining preterm neonates at higher risk of developing IVH. Through neurological examination, sequential cranial ultrasound and platelet count monitoring might be more feasible and convenient tools for early detection of IVH in preterm babies.

Conflict of interest:
The authors declare no conflict of interest.

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