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Beyond brain injury biomarkers: chemoattractants and circulating progenitor cells as biomarkers of endogenous rehabilitation effort in preterm neonates with encephalopathy

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Introduction: Preclinical work and studies in adults have shown that endogenous regeneration efforts that involve mobilization of progenitor cells take place after brain injury. However, kinetics of endogenous circulating progenitor cells (CPCs) in preterm neonates is not well described, particularly their possible role regarding brain injury and regeneration. We aimed to assess the kinetics of CPCs in neonates with encephalopathy of prematurity in relation to brain injury biomarkers, chemoattractants and relevant antenatal and postanal clinical factors, in an effort to outline the related pathophysiology.

Materials and methods: 47 preterm neonates (of 28–33 weeks GA) were enrolled: 31 newborns with no or minimal brain injury (grade I IVH) and 16 prematures with encephalopathy (grade III or IV IVH, PVL or infarct). Peripheral blood samples obtained on days 1, 3, 9, 18 and 45 after birth were analyzed using flow cytometry, focusing on EPCs (early and late Endothelial Progenitor Cells), HSCs (Hematopoietic Stem Cells) and VSELs (Very Small Embryonic-Like Stem Cells). At the same time-points serum levels of S100B, Neuron-specific Enolase (NSE), Erythropoietin (EPO), Insulin-like growth factor-1 (IGF-1) and SDF-1 were also measured. Neonates were assessed postnatally with brain MRI, and with Bayley III developmental test at 2 years of corrected age.

Results: Preterms with brain injury proved to have significant increase of S100B and NSE, followed by increase of EPO and enhanced mobilization mainly of HSCs, eEPCs and lEPCs. IGF-1 was rather decreased in this group of neonates. IGF-1 and most CPCs were intense decreased in cases of antenatal or postnatal inflammation. S100B and NSE correlated with neuroimaging and language scale in Bayley III test, providing good prognostic ability.

Conclusion: The observed pattern of CPCs' mobilization and its association with neurotrophic factors following preterm brain injury indicate the existence of an endogenous brain regeneration process. Kinetics of different biomarkers and associations with clinical factors contribute to the understanding of the related pathophysiology and might help to early discriminate neonates with adverse outcome. Timely appropriate enhancement of the endogenous regeneration effort, when it is suppressed and insufficient, using neurotrophic factors and exogenous progenitor cells might be a powerful therapeutic strategy in the future to restore brain damage and improve the neurodevelopmental outcome in premature infants with brain injury.

KEYWORDS

neonate, preterm, encephalopathy, biomarkers, S100B, NSE—neuron-specific enolase, progenitor cells, stem cells

1. Introduction

Premature birth remains a leading cause of childhood mortality and morbidity worldwide (1). Advances in therapeutic protocols in neonatal intensive care unit (NICU) led to increased survival of even more immature infants, increasing the susceptibility of the immature brain to damage and the clinical challenge for neuroprotection (2). Currently, no specific therapeutic strategies are applied in routine clinical care to actively repair brain injury. The principal forms of encephalopathy in prematures are periventricular leukomalacia (PVL) and intraventricular hemorrhage (IVH) (3, 4). PVL is a white matter injury defined as a focal periventricular necrosis and a more diffuse reactive gliosis and microglial activation (5). Ischemia (mainly) and infection/inflammation lead to degeneration of late oligodendrocyte progenitors (preOLs) in the acute phase (3). It seems that early oligodendrocyte progenitors are capable of regenerating preOLs, but the latter fail to maturate and progress to normal myelination at the end (6). IVH is the most common variety of intracranial hemorrhage in preterms, starting from the subependymal germinal matrix and expanding till the periventricular space (3). The main pathogenetic mechanisms involve intravascular, vascular and extravascular factors that are affected from multiple other parameters of a critical ill preterm in the NICU (7).

*B*rain injury is often biochemically defined in preclinical and clinical studies with the use of brain biomarkers (8). Among them, S100B and Neuron Specific Enolase (NSE) have proved to be more hopeful (8). **S100B**, a calcium-binding protein that is produced primary in astrocytes, is detectable in cerebrospinal fluid (CSF), in blood circulation and in urine after brain injury (9, 10). Likewise, **NSE**, a glucose metabolism isoenzyme, that is produced mainly in neurons and neuroendocrine cells, is detectable in CSF and blood after brain damage (10).

A plethora of neuroprotective strategies have been studied in neonatal brain injury, but most of them did not obtain results that can be used in a clinical setting (9). Lately, the possible therapeutic role of neurotrophic factors and progenitor cells have won a great part of scientific interest. **Erythropoietin (EPO)** is a 30.4 kDa endogenous glycoprotein with known pleiotropic properties (11). In addition to its role in erythropoiesis, EPO is known for its neuroprotective role after hypoxia (12). Specifically, a few hours after a hypoxic-ischemic event, the expression of EPO and its receptor is increased in neurons and endothelial cells, and after days in astrocytes (13–15). Its beneficial mechanisms of action include antiexcitotoxic, antioxidant, antiapoptotic effects, as well as promotion of angiogenesis and neurogenesis (12). Caspase-3 inhibition and resultant antiapoptotic actions and thus cell survival begins with adaption of EPO to its receptor in cell surfaces (6). Most types of neural cells express EPO receptor (11). It seems that after brain injury EPO receptors are upregulated, but EPO ligand increase is insufficient and thus cell apoptosis is not prevented (11). But EPO has also neurorestorative properties in the subacute phase, promoting differentiation of pre-OL, and thus normal myelination (6). Finally, EPO act as a chemotactic factor and mobilizes endogenous progenitor cells (16). In the central nervous system, EPO is basically secreted by astrocytes (11). Multiple studies in animals (16–18) and in neonates (6, 11, 17, 19–24) obtained positive results, mostly histological and less functional, but inconsistency among researchers is remarkable and thus clinical usefulness is hampered (6). Dose, dosage timing and repeated doses are critical parameters that influence the outcome and there is a need to be further studied. **Insulin-like Growth Factor-1 (IGF-1)** is a growth factor that is essential for maturation of the fetal brain and differentiation of pre-oligodendrocytes. It has antiapoptotic actions and enhances survival of neural cells (25). It seems that its effect is beneficial in low doses and cytotoxic in higher ones (7).

Progenitor cells are multipotent cells of primitive origin that take part in the process of organogenesis intrauterine, and in tissue repair in the extrauterine life. They have the ability of self-renewal and differentiation into multiple cell lines (plasticity) when appropriate growth factors exist (26, 27). They are found in blood (**circulating progenitor cells- CPCs**), in tissues and in the bone marrow, and they are mobilized in a chemotactic way to the target-organ via chemoattractants which are released from the latter (26, 28–34). Their main mechanisms of action are anti-inflammatory and trophic action, neoangiogenesis and neurogenesis (35–37). Preclinical models (38–40) and data from clinical studies (27, 41–42) have shown that progenitor cells derived both topically, from the opposite brain hemisphere and from periphery can be mobilized toward the place of brain injury, especially considering

that the blood-brain barrier is more permeable in cases of brain damage. The most promising progenitor cell lines studied are the Hematopoietic Stem Cells (HSCs), the Very Small Embryonic-like Stem Cells (VSELs) and the Endothelial progenitor cells (EPCs). **HSCs** are mainly found in the bone marrow. They take part in hematopoiesis (43), but also in regeneration after tissue injury (44) via the chemoattractant SDF-1 and its receptor CD184 (45–48). Preclinical (49) and clinical studies in adults (50–52) after ischemic stroke have shown CD34+ cells to be mobilized and correlate with improved outcome. **Stromal cell-derived factor-1 (SDF-1)** is a chemoattractant factor for cells that have CD184 (CXCR4) receptor in their surface. It guides circulating cells to the injured tissue, or homing back to the bone marrow (53, 54). In uterus, SDF-1 has a basic role in guiding neuroblasts and in organogenesis (54), procedures that are abruptly interrupted after premature delivery. **VSELs** are cells of non-haemopoietic origin, that are lately found mainly in the cord blood and bone marrow, and take part in organogenesis and tissue regeneration (55). They are mobilized via SDF-1/CD184 axis and Hepatocyte Growth Factor (55, 56). Preclinical (56) and clinical studies in adults (53, 57) have shown mobilization and regeneration effort after stroke. **EPCs** are mainly found in bone marrow and migrate via cytokines [as erythropoietin and vascular endothelial growth factor (VEGF)] to the periphery for angiogenesis and neoangiogenesis purposes (58–60). Late (lEPCs) and early (eEPCs) endothelial progenitor cells are the two main cell lines lately described. Studies in animals (61) and adults (62–65) have shown that EPCs are increased in acute tissue injuries and decreased in chronic vascular diseases. Low oxygen enhances EPO, IGF-1 and SDF-1 release via Hypoxia-inducible factor 1-alpha (HIF-1a) (54, 66). Methodological differences in the design of these studies make comparisons difficult and impede the extraction of certain conclusions. Histochemical improvement is usually observed, but clinical long-term outcome is mostly disappointing.

This great inconsistency in the results of both preclinical models and clinical trials has hampered their clinical usefulness so far. Even more, studying limited parameters in each study and at limited time-points provides a restricted knowledge and not the whole picture, the whole biochemical frame of pathophysiological events. Awaiting of new trials with different dosages and timing of administration would provide more useful data and a possible clinical utility in the future. In this study, we speculated another way for extracting useful clinical data. We assumed that for substances and cell lines that are endogenously present in the neonate, studying the related pathophysiology (i.e., how the human body is using these molecules and cells after a deleterious event) would provide more clues for their clinical importance. To be more specific, we hypothesized that if endogenous progenitor cells are not mobilized at all after neonatal brain injury (i.e., if the human body -in its relative perfection after centuries of evolution- does not use them), it would be rather impossible that exogenous administration of them be beneficial. Similarly, if EPO and IGF-1 levels in serum are not changed after brain injury, it would be rather impossible that systematically administration of them to be of value. Evolution through centuries has probably done thousands of "trials" itself, promoting only the biochemical ways that enhance survival. We believe that mimicking biochemical and cellular patterns that promote recovery (as it is shown in cases of favorable outcome) or enhancing pathways that are malfunctioning (in cases of adverse outcome) is of great clinical value.

In this single-center pilot translational study, we intend to illustrate related pathophysiology and endogenous regeneration biochemical pathways directly in human preterm neonates with encephalopathy, aiming to confirm if preclinical observations are valid in real clinical situations. The primary aim is to explore the timeframe of brain injury using brain injury biomarkers (S100B and NSE) as a baseline for the biochemical correlations (hypothesis A, **Figure 1**) in preterms with brain injury, in preterms without obvious brain injury and in healthy full-term neonates. Similarly, we intend to investigate kinetics of possible neurotrophic and chemotactic factors (EPO, IGF-1 and SDF-1) (hypothesis B), and kinetics of some of the most promising progenitor cell lines (HSCs, VSELs, eEPCs, lEPCs)

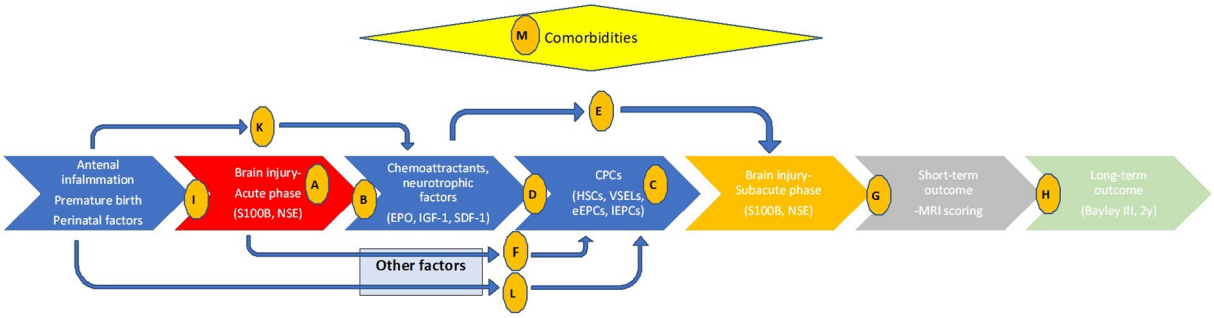

FIGURE 1
Timeline of events related to preterm brain injury, relations between clinical and biochemical parameters, and hypothesis investigated in the current study. Letters in circles indicate the hypothesis studied. Hypothesis A-M: (A) the timeframe of brain injury (S100B and NSE). (B) kinetics of neurotrophic and chemotactic factors (EPO, IGF-1 and SDF-1). (C) kinetics of CPCs (HSCs, VSELs, eEPCs, lEPCs). (D) Do neurotrophic factors/chemoattractants of interest correlate with CPCs? (E) Do neurotrophic factors of interest have neuroprotective role themselves? (F) Does brain injury alters CPCs mobilization via other biochemical factors? (G) Is there a correlation of parameters studied to MRI scoring? (H) Is there a correlation of parameters studied to Bayley III scoring? (I) Do antenatal clinical factors relate to the brain injury themselves? (K) Do antenatal clinical factors affect neurotrophic factors/chemoattractants? (L) Do antenatal clinical factors affect CPCs? (M) Do comorbidities affect parameters studied?

(hypothesis C) in these infants. Secondary aims are to explore if the neurotrophic factors we chose to investigate act as chemoattractants for CPCs in the current study (hypothesis D), or if they have neuroprotective role themselves (independently of CPCs' mobilization) (hypothesis E). Additionally, we aim to investigate if brain injury alters CPCs' mobilization via other biochemical factors that are not studied here (hypothesis F) and mostly if there is a correlation of all these parameters to neuroimaging (hypothesis G) and long-term neurodevelopmental outcome (hypothesis H). Finally, we explore if prenatal clinical factors -that are known to implicate premature birth and its complications- are related to the extent of brain injury themselves (hypothesis I), or affect neurotrophic factors/chemoattractants (hypothesis K) or progenitor cells (hypothesis L) mediating to the neurorestoration process. Interference of comorbidities are also investigated (hypothesis M, **Figure 1**). Even knowing that biochemical pathways are very complicated, even more in the critical setting of a premature in the NICU with multiple comorbidities and prenatal etiologies, in this study we provide a translational starting point that includes all these parameters simultaneously in multiple time-points, with the additional neuroimaging, short- and long-term outcome as the final point of possible clinical utility.

2. Materials and methods

2.1. Patients

Preterm newborns with a gestational age of less than 33 weeks who were born or admitted to our tertiary neonatal intensive care unit within the first 24 h of life were enrolled and studied prospectively from the first day of life. Newborns that developed IVH ≥ II grade, PVL or infarct were compared to prematures without obvious or minimal (IVH I grade) brain damage, who were considered as preterm controls. IVH was diagnosed based on serial cranial ultrasounds. PVL and infarct were diagnosed using both cranial ultrasounds and magnetic resonance imaging techniques (MRI). A group of healthy full-term neonates also participated for age-related correlations between newborns without brain injury. Factors that could influence biomarkers levels were considered as reasons for *exclusion* from the study, namely intrauterine growth retardation, major congenital or metabolic disorders, and twins with death or major abnormalities in their siblings (67–70). Hemolyzed samples were also excluded. Data on demographic, perinatal characteristics, and prematurity-associated complications were collected prospectively for all patients. The study was approved by the Ethical Committee of the Faculty of Medicine, Aristotle University of Thessaloniki, and the Scientific Committee of Hippokration Hospital. Written consent was obtained from the parents of the newborns.

Peripheral blood samples were obtained on days (d) 1 (12–24h of life), 3, 9, 18 and 45 of life and analyzed using flow cytometry, focusing on HSCs, VSELs, eEPCs and lEPCs. From the same blood samples serum and plasma was collected and stored at -80^{o} C until measurement of biochemical factors (brain injury biomarkers and chemoattractants).

2.2. Flow cytometry

Blood samples were incubated with monoclonal antibodies [PEantiCD184/CXCR4, FITCH-antiCD34, PECy5-antiCD45 (BD Biosciences, San Jose, CA, USA)] and APC-antiCD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed with a BD FACSCalibur instrument (BD FACSDiva Software, version 6, BD Biosciences, San Jose, CA, USA). 100,000 events were acquired. Levels of each cell population were expressed as a percentage of the total events. CPCs of interest were populations enriched in HSCs ($CD34^+/CD184^+/CD45^+$), VSELs ($CD34^+/CD184^+/CD45^-$), eEPCs ($CD34^+/CD45^{dim/-}/CD184^+/CD133^+$) and lECPs ($CD34^+/CD45^{dim/-}/CD184^+/CD133^-$). A detailed description of flow cytometry methodology and gating strategy has been described earlier (27).

2.3. Biochemical assessment

S100B and NSE were assessed using Immunochemiluminometric assay (ICMA) (Liaison, DiaSorin-SpA, Saluggia, Italy) according to the manufacturer's instructions. Serum levels of EPO and IGF-1 were measured using immunochemiluminometric assay (Immulite 2000XPi, Siemens, Llanberis, United Kingdom), and plasma levels of SDF-1 were assessed using ELISA (Quantikine ELISA, R & D Systems, Minneapolis, USA).

2.4. Neurodevelopmental assessment

Neurodevelopmental assessment was performed after the second year of corrected age by an experienced investigator who was unaware of the patients' medical history. Baley III test and its scales were used to examine cognitive, language (receptive and expressive) and motor (fine and gross) domains (71). Neurodevelopmental impairment (NDI) was defined as *mild* when Bayley scores were between −1 and −2 standard deviations (SD) of the mean provided by the test, and as *moderate/severe* when scores were lower than −2 SDs (72).

Brain MRI was performed in preterms with encephalopathy at term-equivalent postmenstrual age. MRI abnormalities in white matter (cystic degeneration, focal signal abnormalities, delayed myelination, thinning of the corpus callosum, dilated lateral ventricles, reduction of white matter volume), in cortical grey matter (signal abnormality, delayed gyration, dilated extracerebral CSF space), in deep grey matter and in cerebellum, as well as a total MRI brain injury score, were evaluated as Kidokoro et al. have proposed (73).

2.5. Statistical analysis

Data were presented as mean (±SD) or median (range) depending on distribution, and t-test or the Mann-Whitney U-test was used for quantitative parameters respectively. Likewise, Pearson's correlation or Spearman's rank correlation were used for correlations accordingly to distribution, and

Pearson Chi-Square or Fisher's exact test were used for qualitative variables. Receiver operating characteristics (ROC) curves were assessed using the areas under the curves as indicated. Sample size analysis was based in previous exploratory study (27) and was used to indicate the lowest sample size required to detect statistical significance. Alpha was set at 0.05% and 80% power was accepted. Calculating for an additional 10% drop-out rate, estimated sample size was defined as 11 neonates per arm. SPSS statistical package was used (SPSS statistics, IBM Corporation, version 20, Chicago, IL, USA). *P* value of less than 0.05 was considered as statistically significant. As this is a pilot study, when rational causalities exist, borderline *p* values ($0.05 > p > 0.08$) were also reported to indicate trends.

3. Results

3.1. Neonatal characteristics

47 preterm neonates were included in the study and were divided into two groups based on neuroimaging findings: patients (group 1, $n = 16$) included neonates with IVH of >II grade, PVL or infarct, whereas preterm controls (group 2, $n = 31$) included prematures with no ($n = 28$) or minimal (IVH/I grade, $n = 3$) findings of brain injury. An additional group of healthy full-term newborns was studied for comparison reasons (group 3, $n = 11$). Neonatal characteristics are presented on **Table 1**.

3.2. S100B

3.2.1. Kinetics

Preterms with encephalopathy presented higher S100B levels than preterm controls on day 1, mainly the ones with severe IVH (grade III/IV). A cut-off point of 2.51 μg/L on that day provided good prognostic ability with regards to the development of severe IVH (**Table 2**). Infants with PVL had borderline increased S100B levels on day 18. Moreover, S100B was increased in the preterm controls compared to the full-term controls on days 1 and 9. Kinetics of S100B are presented in **Figure 2** and **Table 2**.

3.2.2. Correlations

Levels of S100B in the first hours of life were correlated with the adverse event of seizures, late sepsis and death, and negatively correlated with Apgar score (1 min) and acidosis (SBE, first hours of life) (**Table 2**). Severe RDS (grade III/IV) was correlated with

TABLE 1 Demographic and perinatal characteristics of the three group of neonates studied.

Groups	1 Preterm Patients	2 Preterm Controls	3 Full-term Controls	Comparisons (*p*)
n	16	31	11	
Chorioamnionitis [*n* (%)]	3 (18.8%)	4 (12.9%)	0	-
Antenatal Steroids [*n* (%)]	7 (46.6%)	30 (96.8%)		1–2 (<0.0005)
PRoM [*n* (%)]	3 (20%)	9 (29%)	0	-
Caesarean section [*n* (%)]	9 (56%)	23 (74%)	6 (54%)	-
Multiple gestation [*n* (%)]	4 (26.6%)	15 (48.3%)	2 (18%)	-
Gestational Age (weeks) [Mean (SD)]	27.3 (2.9)	29.8 (2.1)	38 (1.2)	1–2 (<0.005)
				2–3 (<0.0005)
Birth Weight (gr) [Mean (SD)]	1,182 (495)	1,526 (396)	2,988 (330)	1–2 (<0.01)
				2–3 (<0.0005)
Male gender [*n* (%)]	7 (43.8%)	16 (51.6%)	5 (45%)	-
Apgar Score 1′ [median(range)]	3 (7)	8 (7)	8 (1)	1–2 (<0.0005)
Apgar Score 5′ [median(range)]	5 (9)	9 (5)	9 (1)	1–2 (<0.0005)
SBE (first hours of life, mmol/L) [Mean (SD)]	−12.30 (8.65)	−5.46 (3.52)	−6.79 (3.1)	1–2 (<0.01)
IVH (I/) [*n* (%)]	0	3 (9.6%)	0	-
IVH (II) [*n* (%)]	2 (12.5%)	0		
IVH (III/IV) [*n* (%)]	10 (62.5%)	0	0	
PVL [*n* (%)]	6 (37.5%)	0	0	
Infarct [*n* (%)]	1 (6.2%)	0	0	
RDS [*n* (%)]	7 (46.7%)	15 (48.4%)	2 (16.7%)	-
BPD [*n* (%)]	5 (33.3%)	5 (16.1%)		-
NEC [*n* (%)]	2 (12.5%)	9 (29%)	0	-
ROP (>3rd grade) [*n* (%)]	2 (13.3%)	1 (3.2%)	0	-
Sepsis (late) [*n* (%)]	2 (12.5%)	2 (6.5%)	0	-
Hospitalization duration (in days) (Mean/SD)	66.5 (29.6)	45.1 (25.6)		-
Death [*n*] [day of death: median (range)]	9 11 (53)	2 31.5 (51)	0	1–2 (<0.0005)

RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia; IVH, Intraventricular hemorrhage; NEC, necrotizing enterocolitis; PRoM, premature rupture of membranes (>24 h); PVL, periventricular leukomalacia; ROP, retinopathy of prematurity; SBE, standard base excess.

TABLE 2 Results of brain injury markers, chemoattractants and CPCs in preterms with encephalopathy (when compared to preterms controls).

Biomarker	Prenatal/ Perinatal Factors	d1	d3	d9	IVH (grade)	d18	d45	Comorbidities	MRI	2y-Bayley	Sens %	Spec %	AUC	r/ rho	p	Hypothesis tested
S100B (μg/l)		**7.3[8.4]** ^{*ac} **(vs 1.4[0.8])**	1.7[1.5] ^{*a} (vs 1.1(0.6]	1.5[1.3] ^{*a} (vs 1.4[0.4]		1.3[0.9] ^{*a} (vs 1.5[0.5]	1.4[0.3] ^{*a} (vs 1.2[0.4]								d1:<0.005 d3-D45: ns	A
		↑ **2.51**			III/IV						71.4	90.3		0.756	<0.05 <0.05	G
						↑			PVL						0.08	G
		2.19 1.32								RS<1SD	60 80	94/ 64.7	0.835		<0.05	H
	CS			↑											0.06	I
	↓ApSc1'	↑												-0.33	<0.05	I
	SBE	↑												-0.42	<0.01	I
		↑						seizures							<0.005	M
		↑						Late sepsis							<0.05	M
		↑^{c}						death							0.071	H
		↑						RDS III/IV							0.069	M
NSE (μg/l)		72.8 [67.5] (vs 34.9[19]) ^{*a}	38.1 [30] (vs 25.5[26]) ^{*a}	23.3 [30.1] (vs 20.9[21]) ^{*a}		35.9 [26] (vs 16.6[9]) ^{*a}	**25.1 [8.6]** (vs **15[5.7]**) ^{*a}								d18:0.078 d45: <0.05 d1/3/9:ns	A
			↑		III/IV	↑	↑								<0.05 <0.01	G
							22.6		>8^{i}		100	93.3	0.967		<0.05	G
		57.8								LS<1SD RS<1SD	75 80	88.9 100	0.889 0.953		<0.05	H
				↑				RDS III/IV							0.07	M
EPO (mIU/ml)		**14.8 [24.7]** (vs 3.6[21]) ^{*b}	**13.4[12]** (vs 6.6[29.3] ^{*b}	**4.7[6.6]** (vs 2.4[8.8]) ^{*b}		3.5[13.7] (vs 4[41.4]) ^{*b}	10.3[15.4] (vs 11.2[28]) ^{*b}								d1: <0.001 d3/9: <0.05 d18/45:ns	B
			↑^{c}	↑^{c}	III/IV										<0.05	G
		↑^{c}						PVL							0.059	G
	MultPreg	↓													0.052	K
	PreSter	↑													0.08	K
	↓ApSc1/5'	↑												-0.55	<0.001	K
	ApSc1'(<4)		↑												0.064	K
	↓pH	↑	↑											-0.5	<0.05	K
			↑S100B ^{c}	S100B<>↑^{c}		↑								0.67 0.47	0.07 <0.05	B
		↑ ^{c} ↑ ^{c}	**↓S100B**	↓S100B										-0.5 -0.39	<0.05 0.075	E
			↑NSE <>↑ ^{c}											0.43	<0.05	B
		↑<>**lEPCs**^{d}												0.59	<0.05	D
		↑	↑			↑eEPCs^{d} **↑eEPCs**^{d}								0.77 0.75	0.07 <0.05	D
			↑			**↑HSCs**^{d}								0.42	<0.05	D
		↑	↑					RDS (III/IV)							0.07 <0.05	M
IGF-1	ApSc1'(<4)					↓	↓								<0.05	K
					III/IV	↓									0.08	G
		↑S100B^{c} ↑S100B				↓^{c}	↓							-0.35 -0.51	0.077 <0.05	B
		↑NSE ↑NSE	↑NSE	↑NSE		↓ ↑NSE<>↓	↓ ↓ ↓									B

(*Continued*)

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TABLE 2 (Continued)

Biomarker	Prenatal/ Perinatal Factors	d1	d3	d9	IVH (grade)	d18	d45	Comorbidities	MRI	2y-Bayley	Sens %	Spec %	AUC	r/ rho	p	Hypothesis tested
														-0.36 -0.68 -0.37 -0.44 -0.39	<0.05 <0.0005 0.072 <0.05 <0.05	
				↓		↓	↓	Late sepsis							<0.05 0.08	M
						↓		RDS (III/IV)							<0.05	M
						↓	↓	BPD							0.079 <0.05	M
						↓ [c]	↓	durHosp						-0.45	<0.05	M
			↓			↓		death							<0.05 0.08	H
SDF-1			↓		III/IV III/IV		↓								<0.05 0.07	G
		↓						PVL							0.059	G
	PreSter	↓													0.055	K
	Chorioam	↓													0.08	K
		↑S100B		↓ ↑S100B<>↑ ↑S100B		↑ ↑S100B<>↑	↑S100B<>↑							-0.41 0.33 0.46 0.52 0.5	<0.05 0.08 <0.05 <0.05 0.08	B
		↑NSE		↓ ↑NSE		↑								-0.45 0.36	<0.01 0.067	B
			↑<>↑VSELs											0.42	<0.05	D
				↑		↑HSCs ↑<>↑HSCs								0.32 0.39	0.08 <0.05	D
						↑<>↑eEPCs								0.39	<0.05	D
		↑						**RDS (III/IV)**							<0.05	M
							↓	**BPD**							0.077	M
HSCs			↑* [g,h]	↑* [g,h]											<0.05 <0.05	C
	MultPreg	↓	↓												<0.0005 <0.05	L
	Chorioam	↓		↓		↓									<0.05 0.08 0.06	L
	↓ApSc1' ApSc1' <4		↑	↑										-0.38	<0.05 0.07	L
		↑S100B ↑S100B	↓	↓										-0.35 -0.31	<0.05 0.07	F
			↑NSE			↑ ↑NSE>>↑								0.33 0.54	0.068 <0.005	F
		↓ <0.017‰ [e]	↓	↓		↓	↓	Late sepsis			100	66.7	0.811		<0.05 0.071 <0.005 <0.05 <0.01 <0.05	M
			↓ <0.015‰ [e]					NEC			66.7	66.7	0.739		<0.05 <0.05	M
VSELs				↑				**PVL**							0.056	G
	Chorioam		↓												<0.05	L
	ApSc1' (<4)	↓				↓	↓								0.055 <0.05	L
		S100B<>↓ [c,g] ↑S100B [c,g] ↑S100B [c,g]	↓				↓							-0.49 -0.35 -0.67	<0.01 0.078 <0.005	F
			↑NSE			↑ ↑NSE>>↑								0.44 0.42	<0.05 <0.05	F
		↓ <0.014‰ [e]	↓	↓		↓	↓	Late sepsis			100	79.5	0.897		<0.01 <0.01 0.08 <0.0005 <0.05	M
						↓		RDS (III/IV)							<0.05	M
						↓		BPD							<0.05	M

(*Continued*)

10.3389/fped.2023.1151787

TABLE 2 (Continued)

Biomarker	Prenatal/ Perinatal Factors	d1	d3	d9	IVH (grade)	d18	d45	Comorbidities	MRI	2y-Bayley	Sens %	Spec %	AUC	r/ rho	p	Hypothesis tested
eEPCs			↑*	↑*											<0.005 <0.0005	C
			↑	↑	III/IV										<0.01 <0.005	G
						↑		PVL							0.08	G
			↑NSE>>↑			↑NSE>>↑								0.33 0.6	0.065 <0.001	F
	MultPreg	↓	↓	↓											<0.005 0.06 <0.05	L
	↓ApSc1/5'		↑	↑										-0.48	<0.05	L
							↑	durHosp						0.36	0.08	M
				↑				death							<0.05	H
		↓	↓			↓		Late sepsis							0.075 <0.05	M
			↓ <0.0095‰[e]					NEC			66.7	70	0.724		<0.05 <0.05	M
lEPCs		↑*	↑*	↑*											<0.05 0.056	C
		↑S100B ↑S100B	↓			↓[g]								-0.31 -0.37	0.075 0.059	F
			↑NSE			↑ ↑NSE<> ↑ ↑NSE	↑ ↑NSE<> ↑							0.47 0.58 0.56 0.45	<0.01 <0.001 <0.05 0.08	F
	↓ApSc5'	↑												-0.32	<0.05	L
	Chorioam		↓			↓	↓								<0.05 0.08	L
	MultPreg	↓													<0.005	L
						↓	↓	Late sepsis							<0.05	M
							↑	BPD							<0.05	M

ApSc1', 1 min Apgar score; ApSc5', 5 min Apgar score; BPD, bronchopulmonary dysplasia; NEC, Necrotizing enterocolitis; Chorioam, chorioamnionitis; CS, caesarean section; durHosp, duration of hospitalization; IVH, Intraventricular hemorrhage; LS, Language scale (Bayley III test); MultPreg, multiple pregnancy; ns, non-statistical significant; PreSter, prenatal steroid administration; PVL, periventricular leukomalacia; RDS, respiratory distress syndrome; RS, Receptive subscale of Language scale (Bayley III test); SBE, standard base excess.

Blue font indicates the antecedent factor in time, where red font indicates the subsequent correlated incident.

Bold font indicates statistically significant correlation.

[a]Mean [SD].

[b]Median [range].

[c]Adjusted for severe RDS.

[d]Correlation observed only in the group of prematures with encephalopathy.

[e]Percentage of the total events seen in flow cytometry (27).

[f]Not statistical significant after adjustment for severe RDS.

[g]Adjusted for late sepsis.

[h]Adjusted for NEC.

[i]Total MRI injury score (moderate or severe injury, score > 8) (73).

*Preterms with encephalopathy (vs. preterm controls).

10.3389/fped.2023.1151787

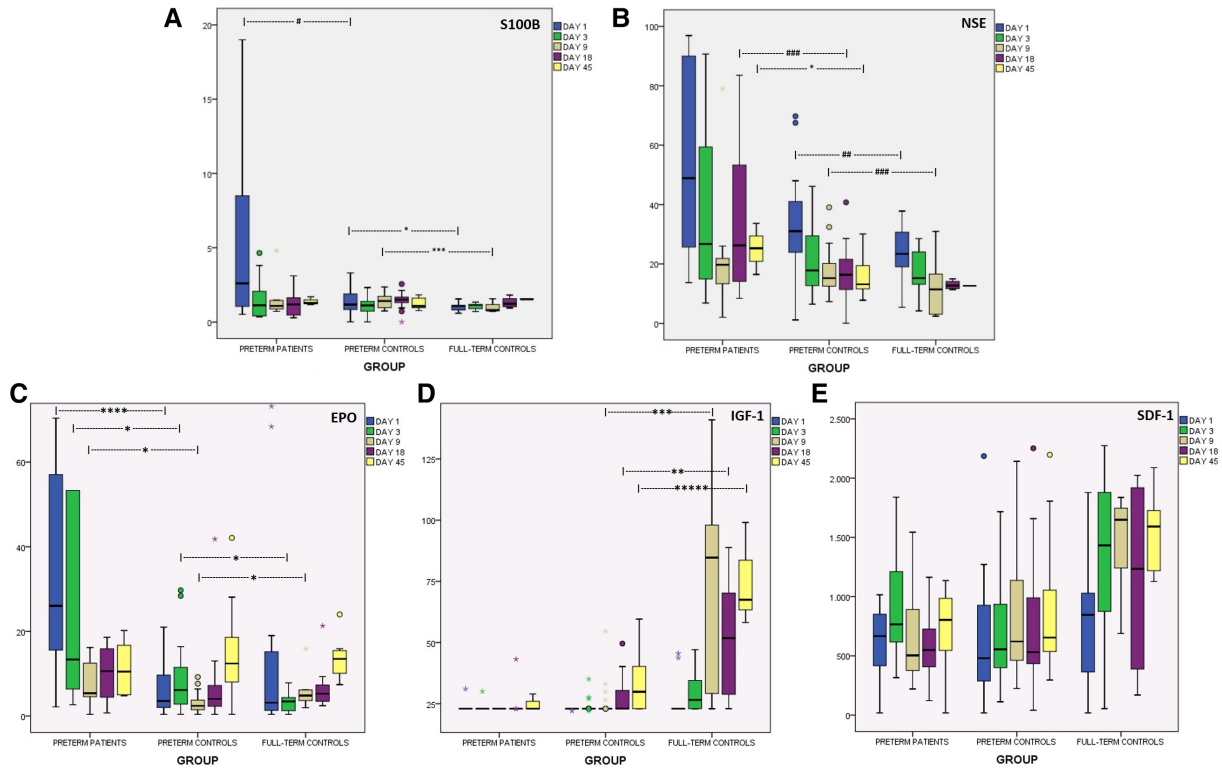

FIGURE 2
Kinetics of brain injury markers and chemoattractants in the first 45 days of life, in preterms with encephalopathy, in preterm controls and in full-term controls. (**A**) S100B (μg/L), (**B**) NSE (μg/L), (**C**) EPO (mIU/ml), (**D**) IGF-1 (ng/ml), (**E**) SDF-1 (pg/ml). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$, $^{*****}p < 0.0005$. $^{\#}p = 0.06$, $^{\#\#}p = 0.068$, $^{\#\#\#}p = 0.08$.

increased S100B on day 1, and thus correlations of S100B on that day with brain injury parameters were adjusted for severe RDS. Interestingly, there was trend to lower S100B levels on days 3 and 9 in neonates with mild RDS (I/II grade) compared to neonates with no RDS at all ($p = 0.08$). Caesarian section was correlated with marginally increased S100B levels on day 9. No correlation was observed between S100B levels on day 1 with gestational age, birth weight, gender, chorioamnionitis, premature rupture of membranes or prenatal steroid administration.

3.3. NSE

3.3.1. Kinetics

NSE levels were higher in preterms with brain damage than in healthy ones at almost all time-points, but statistical significance was reached only on day 45. The subgroup of prematures that developed severe IVH (grade III/IV) had higher levels of NSE on days 3, 18 and 45. Notably, preterms with IVH/grade I had similar levels with preterms without obvious brain injury. Additionally, preterm controls had borderline higher NSE levels on days 1 and 9 compared to full-term controls. Both healthy neonate groups presented gradually lower levels over time, in contrast to preterms with encephalopathy that had elevated levels on days 18 and 45. Kinetics of NSE levels in the first 45 days of life are presented in Figure 2 and Table 2.

3.3.2. Correlations

NSE levels on day 9 were borderline higher in cases of severe RDS compared to no RDS, and even more to mild RDS ($p = 0.07$). No correlation was found between NSE levels on the 1st day of life and gestational age, birth weight, gender, type of delivery, chorioamnionitis, premature rupture of membranes or prenatal steroid administration.

3.4. Erythropoietin

3.4.1. Kinetics

EPO levels in preterms with encephalopathy were higher on days 1 to 9 compared to preterm controls, an increase attributed mainly to PVL for day 1 and mainly to severe IVH (grade III/IV) for days 3 and 9. Additionally, healthy preterms had higher EPO levels than full-term neonates on day 3, while on day 9 the opposite correlation was observed. Levels of EPO are presented in Table 2 and Figure 2.

3.4.2. Correlations

S100B levels on days 3 and 9 were positively correlated with EPO levels of the same or the following days. Similarly, NSE levels on day 3 were positively correlated with EPO of the same day. Adversely, when seeking how EPO levels correlate with

brain injury magnitude of the following days, the opposite correlation was found: EPO on day 1 was adverse correlated with S100B of the following days (Table 2).

Additionally, EPO levels were correlated with **CPCs** levels of the same or the following days, an observation found only in the group of prematures with encephalopathy (Table 2).

Severe RDS was correlated with increased EPO levels of days 1 and 3 compared to cases of mild RDS, and thus correlations of EPO on those days with brain injury were adjusted for that parameter.

Between other clinical factors, multiple pregnancy was correlated with lower EPO levels on day 1. Stress markers at birth (namely, Apgar Score (1 and 5 min) and lower pH (first hours of life)) were correlated with higher EPO on days 1 and 3. Finally, prenatal corticosteroids were borderline correlated with higher EPO levels on day 1 (adjusted for brain injury event) (Table 2).

3.5. Insulin-like growth factor-1

3.5.1. Kinetics

Preterms with brain injury presented similar levels of IGF-1 as preterm controls, but subanalysis showed a borderline decrease in IGF-1 levels on day 18 in cases of severe IVH. Full-term neonates and healthy preterms presented gradually higher levels over time (Friedman test, $p < 0.05$), whereas preterms with encephalopathy did not. Full-term neonates had significantly higher IGF-1 levels than healthy preterms from day 9 to day 45. Levels of IGF-1 in all groups are shown in Figure 2.

3.5.2. Correlations

Higher **S100B** levels (d1) and **NSE** levels (d1-d18) were correlated with lower IGF-1 levels on days 18 and 45 (Table 2). IGF-1 generally was not correlated with **CPCs** levels in any group.

Among other parameters, Apgar score (1st min) <4 was correlated with lower IGF-1 levels on days 18 and 45. Late sepsis was correlated with lower IGF-1 levels on days 9, 18 and 45. Severe RDS was correlated with lower IGF-1 levels on day 18. Lower IGF-1 levels on days 18 and 45 was correlated with the outcome of BPD as well.

Finally, low IGF-1 levels (d18, d45) were correlated with the duration of hospitalization, and low levels on day 3 and 18 with the outcome of death (Table 2). No correlation was found between IGF-1 levels of the 1st day of life and gestational age or birth weight.

3.6. Stromal cell-derived factor-1 (SDF-1)

3.6.1. Kinetics

In the present study, SDF-1 levels were similar in all groups (Figure 2). Only the subgroup of prematures with severe IVH (III/IV grade) presented lower SDF-1 levels on day 3 and 45. Likewise, prematures with PVL had borderline lower levels on day 1 (Table 2).

3.6.2. Correlations

S100B and **NSE** levels on day 1 were adverse correlated with SDF-1 levels on day 9. On the contrary, levels of brain injury markers of day 9 and 18 were positively correlated with SDF-1 levels of the same or the following days (Table 2). Regarding **CPCs**, SDF-1 levels on day 3 were correlated with VSELs on the same day, as were SDF-1 levels (d9, d18) with HSCs and eEPCs of day 18. Among other clinical factors, prenatal corticosteroids and chorioamnionitis were marginally correlated with lower SDF-1 on day 1. Severe RDS was correlated with increased SDF-1 levels on day 1, while SDF-1 levels on day 45 were borderline lower in cases of BPD (Table 2). No correlation was found for SDF-1 levels on day 1 and birth weight or gestational age.

3.7. Haematopoietic stem cells

3.7.1. Kinetics

In prematures with encephalopathy levels of HSCs were significantly elevated on days 3 and 9 compared to their age-controls (adjusted for NEC and late sepsis) (Figure 3, Table 2).

3.7.2. Correlations

Higher **S100B** levels on day 1 were correlated with lower HSCs levels on days 3 and 9 (Table 2).

NSE levels on days 3 and 18 were correlated with higher HSCs levels on day 18. Among prenatal factors, chorioamnionitis was correlated with lower HSCs on days 1, 9 and 18, as well as multiple pregnancy was correlated with lower levels on days 1 and 3. Low Apgar score was correlated with higher HSCs on days 3 and 9. Interesting, low HSCs from birth to the 45th day of life were constantly associated with late sepsis, while levels on day 1 also proved to have also good prognostic ability. Additionally, low HSCs on day 3 were correlated with necrotising enterocolitis (NEC) (Table 2). Gestational age and birth weight were not correlated with HSCs (d1).

3.8. Very small embryonic-like stem cells

3.8.1. Kinetics

VSELs' levels were found to be similar in all groups (Figure 3). The subgroup of neonates with PVL presented borderline higher VSELs on day 9 (Table 2).

3.8.2. Correlations

S100B levels on day 1 were adverse correlated with VSELs on days 1, 3 and 45 (adjusted for late sepsis and severe RDS), whereas NSE levels on days 3 and 18 were positively correlated with VSELs of day 18 (Table 2).

Apgar score (1st min) <4 was correlated with lower VSELs on days 1, 18 and 45. A tendency for higher levels of VSELs on day 3 was found when 1st min Apgar score was between 4 and 6, compared to Apgar score higher than 6 ($p = 0.08$). VSELs levels

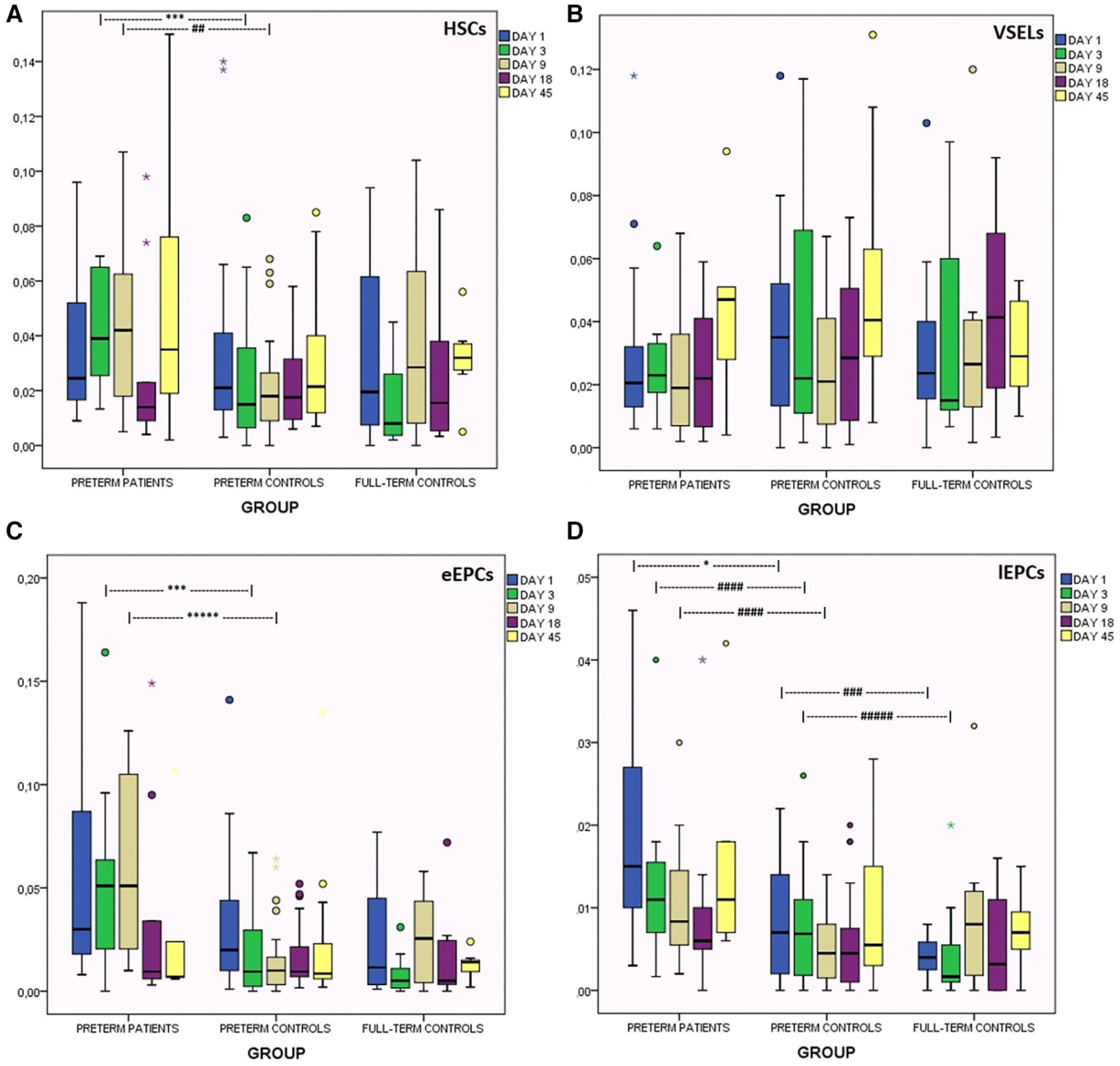

FIGURE 3
Kinetics of circulating progenitor cells in the first 45 days of life in preterms with encephalopathy, in preterm controls and in full-term controls. (A) HCS, (B) VSELs, (C) early EPCs, (D) late EPCs. *$p < 0.05$, ***$p < 0.005$, *****$p < 0.0005$. $^{\#\#}p = 0.068$, $^{\#\#\#}p = 0.08$, $^{\#\#\#\#}p = 0.056$, $^{\#\#\#\#}p = 0.074$.

on day 18 were higher in mild RDS, lower in neonates with no RDS at all, and much lower in severe RDS. On the same day (18th), VSELs levels were lower in cases of BPD as well. In cases of late sepsis, VSELs levels were severely depressed the whole one and a half month of life, whereas levels on day 1 presented good prognostic ability (Table 2). Finally, VSELs were not correlated with birth weight or gestational age.

3.9. Early endothelial progenitor cells

3.9.1. Kinetics

Regarding eEPCs, prematures with brain injury had elevated levels compared to their age controls on days 3 and 9 of life (Figure 3). Subanalysis has shown that this elevation was mainly observed in neonates with severe IVH (III/IV grade), while neonates with PVL only had marginally increased levels on day 18 (Table 2).

3.9.2. Correlations

NSE levels on days 3 and 18 were correlated with increased eEPCs of the same days. Multiple gestation was correlated with lower eEPCs levels on days 1, 3 and 9. Low Apgar score (1 and 5 min) was correlated with significantly higher eEPCs levels on days 3 and 9. Duration of hospitalization and occurrence of death was positively correlated with eEPCs. In cases of late sepsis, eEPCs levels were very low on days 1, 3 and 18. Levels on day 3 were also lower in cases of NEC (presenting moderate prognostic ability) (Table 2). eEPCs were not correlated with gestational age or birth weight.

3.10. Late endothelial progenitor cells

3.10.1. Kinetics

lEPCs levels were increased in the brain injury group on days of life 1, 3 and 9, compared to the healthy preterm group. Additionally, the latter group had borderline higher levels than full-term newborns on days 1 and 3 (Figure 3).

3.10.2. Correlations

S100B on day 1 was adversely correlated with lEPCs levels of the following days (3 and 18), whereas **NSE** was constantly positively correlated with lEPCs of the same or the following days. Low 5 min Apgar score was associated with increased lEPCs levels on day 1. Multiple gestation was correlated with lower lEPCs levels on day 1, where chorioamnionitis with lower levels on days 3, 18 and 45. Late sepsis was correlated with lower lEPCs levels on days 18 and 45, and BPD higher levels on day 45 (Table 2). lEPCs were not correlated with gestational age or birth weight.

3.11. Long-term neurodevelopmental outcome

25 preterms were assessed between 24 and 30 months of corrected age with Bayley III test. The neurodevelopmental outcome is shown in Table 3. Prematures with brain damage presented lower scores mostly on motor scale and expressive language subscale. S100B and NSE levels on the first day of life could prognosticate with high sensitivity and specificity retardation in the language domain (Table 2).

3.12. MRI imaging

Among preterms with encephalopathy that were alive at term-equivalent postmenstrual age, 6/7 were evaluated with MRI imaging. Two of them proved to have no significant detectable brain injury (total score <4), 1 had mild total brain injury score (5), 1 preterm had moderate score (8) and 2 of them had severe brain injury (total score ≥12). NSE levels on day 45 could prognosticate total brain injury score seen in MRI (Table 2).

Grade of white matter injury in MRI scoring was marginally correlated with lower scores in cognitive scale ($r = -0.825/p = 0.08$) and in motor scale ($r = -0.392/p = 0.058$), especially in gross motor subscale ($r = -0.86/p = 0.06$). Additionally, lower scores in motor scale were found in thinning of corpus callosum (gross motor subscale, $r = -0.91$, $p < 0.05$), in delayed myelination ($r = -0.395/p = 0.056$), in enlargement of subarachnoid space ($r = -0.404/p < 0.05$) and in cerebellum injury ($r = -0.36/p = 0.08$).

TABLE 3 Bayley scores in preterms with encephalopathy and in preterm controls.

Groups	Preterm Patients (Group 1)	Preterm Controls (Group 2)	Comparisons Groups (*p*)
n	5	20	
Cognitive scale	88 (16)	99.5 (12.1)	
Language scale	88.8 (20.7)	101.8 (16.3)	
Receptive	6.8 (4.2)	10.1 (3.3)	1–2 (0.073)
Expressive	8.8 (4.2)	10.4 (2.9)	
Motor scale	82.4 (16.4)	99 (13.6)	1–2 (<0.05)
Gross	8 (3.7)	10.8 (3.5)	1–2 (0.07)
Fine	5.6 (3.5)	8.8 (2.3)	
Cerebral palsy	1	0	

4. Discussion

Identifying new biomarkers which can help the clinician to make early proper therapeutic decisions is of major importance. It is desirable that these biomarkers have specific properties: they must allow accurate, instant and early detection of brain injury, they must be low-cost and minimally invasive, and finally they must be able to be applied repeatedly for longitudinal follow-up (8). Detecting and implementing certain protocols for biomarkers in clinical use may allow personalized delineation of underlining pathophysiology and instant therapeutic approaching.

4.1. S100B

An increase of S100B in prematures with encephalopathy was observed on the first day of life compared to preterms without detectable brain damage, a constant observation in previous studies (74–78). In accordance with our study, in most previous studies S100B levels in preterm controls were stable over time, whereas levels in preterms with encephalopathy were decreasing over time (74, 76, 78, 79). On the contrary, there were reports of cases of severe asphyxia (79) or PVL where S100B levels presented an increasing trend over time (74, 78). S100B has a very small half-life of 30–100 min (8) and thus protracted increased levels depict protracted cell necrosis or apoptosis. Additionally, we found that a cut-off point of 2.51 μg/L on the first day of life was capable of early discrimination of preterms at risk for severe IVH, whereas Metallinou et al. report a much higher cut-off point of 17.74 μg/L (75). In the current study, preterms without encephalopathy also had higher levels of brain injury biomarkers compared to full-term controls on days 1 and 9 of life. Other researchers have observed increased levels of S100B in healthy full-term neonates compared to adults, and have interpreted this phenomenon as a result of the immature brain-blood barrier in neonates (80) or the increased metabolism of neuroglial cells in this age group (81). Our finding could have a similar age-based explanation, but the possible neurotoxic implications of the relatively increased oxidative stress in preterms (although without obvious brain damage) cannot be ignored.

Regarding increased S100B levels in severe RDS, it is known that severe hypoxia could have a neurotoxic effect (9). Additionally, a ventilated premature with severe RDS could have

significant fluctuations in cerebral blood flow (with the latter being a critical issue leading to IVH) (82). Moreover, in the current study S100B levels were observed to be higher in neonates with no RDS compared to ones with mild RDS (I/II grade), indicating the existence of a possible preconditioning model (mediated by mild hypercapnia and hypoxia-inducible factor-1a (HIF-1a) and its target genes, a well described model of endogenous response in the literature (3)). Similarly, after caesarian section the increased S100B levels we found could be attributed to the need of an emergency delivery (in cases of fetal distress) if the increase was observed on days 1 and 3. But having in mind the small half-life of S100B, lower S100B levels on day 9 after vaginal birth might indicate the existence of an endogenous neuroprotective preconditioning model of "mild stress" during vaginal delivery, as it has been described (83).

Moreover, the influence of preceding infection/inflammation on hypoxic-ischemic brain injury is known to be critical in preterms (4), and prenatal inflammation is known to be associated with increased maternal and fetal S100B levels (84). In the current study, we did not find correlation between prenatal inflammation and serum S100B levels in the infant, possibly due to the fact that the half-life of the marker is indeed small and the time of the blood sampling on day 1 (12–24 h of life). Additionally, there were limited cases of chorioamnionitis in the preterm patient group to establish a certain result. Also, correlation of S100B with Apgar score and acidosis was expected. Similarly, correlation of S100B on day 1 with seizures probably reflects the magnitude of brain damage and indeed the correlation of both with severe IVH. Finally, correlation with the adverse outcome of death possibly reflects the severe life-threatening medical condition. On the other hand, correlation of early S100B on day1 with late sepsis is intriguing. As S100B did not correlate with antenatal inflammation in this study (to be noted, the latter is known to correlate with postnatal inflammation (85–87), it is possible that brain injury itself affects the immune response of the newborn, becoming more vulnerable to infections.

4.2. NSE

We found that preterms with encephalopathy had increased levels of NSE in almost all time points, mainly attributed to the subgroup of neonates with severe IVH. A more gradual decrease of NSE over time is seen virtually in all groups, which is a reflection of the longer half-time ($T_{1/2}$) of NSE (about 24 h) (88). But in preterms with brain injury a secondary increase is observed on days 18 and 45, indicating either enhanced prolonged apoptosis or sustained (necrotic) neural damage that is protracted for several weeks (secondary or tertiary phase). These findings are in accordance with the related pathophysiology and reflect the ongoing nature of preterm encephalopathy (3). A third presumption could be that this secondary increase is the effect of multiple comorbidities of a critical ill preterm neonate in the brain metabolism. Additionally, Giuseppe et al. reported increased NSE levels on days 1, 2 and 7 of life in preterms with severe asphyxia, compared to mild asphyxia and controls (79). Finally, as observed for S100B, preterm controls had increased NSE levels compared to healthy full-term neonates, and correlation of NSE levels with RDS was again revealed (i.e., increased NSE levels in severe RDS and a preconditioning model for mild RDS).

4.3. EPO

Prematures with severe IVH presented higher levels of EPO on days 3 and 9, while ones with PVL higher EPO levels on day 1 (adjusted for severe RDS). In the current study EPO was correlated with Apgar score, acidosis and RDS. It is well known that EPO is released in cases of hypoxia (via HIF-1a) (66), but constant and prolonged (till the 18th day) correlation with brain injury markers (adjusted for RDS) indicates that EPO is possibly increased also as a neurotrophic factor (independently of the presence of severe hypoxia/RDS), in the frame of an endogenous regeneration effort after CNS injury, as has been also shown in the past (24, 89). Interestingly, the higher the EPO levels on day 1, the lower the S100B levels in the following days (3 and 9), indicating once more the neuroprotective role of EPO itself, a well-established role in the literature (7, 11).This observation is also in accordance to Li et al., who found decreased serum S100B levels after EPO treatment (90).

Moreover, serum EPO levels were not associated with CPCs in preterm controls (in accordance with Bui et al. (91), but they were only elevated in the group of preterms with encephalopathy, indicating its neuroprotective action and its role as a chemoattractant for CPCs. Specifically, EPO was correlated with higher levels of HSCs (reflecting its hematopoietic role) and even more with EPCs. The latter is also observed in adults (92, 93), and it is possibly related to the release of EPO in the penumbra around the necrotic/injured cell zone and the chemotaxis of EPCs in an attempt for neoangiogenesis.

4.4. IGF-1

IGF-1 is a known trophic factor with additional neuroprotective and antiapoptotic properties (94, 95). It is shown to be protective to pre-OLs and promotes oligodendroglial proliferation (94). The degree of brain injury (S100B and NSE) was constantly correlated with lower IGF-1 levels in the following days, in accordance with previous observations (96–98). This means that both injury in neurons and in their microenvironment (astrocytes) lead to lower levels of a trophic and neuroprotective factor, impeding regeneration of brain injury itself and especially mediating in the pathogenesis of PVL (inability of pre-OLs' maturation). Additionally, IGF-1 is a general trophic factor (99), and decreased IGF-1 is related to inhibition of recovery of any other organ damaged in a premature neonate with multiple comorbidities in the neonatal intensive care unit (NICU). It is not surprising that, low IGF-1 was correlated with duration of hospitalization and death. A

general pattern is illustrated in **Figure 2**: a rapid gradual increase of IGF-1 was observed mainly in healthy full-term neonates over time during the first 45 days of life, in a much lesser degree an IGF increase in preterms without encephalopathy, whereas preterm with brain injury had just measurable IGF-1 levels only in day 45 [in accordance to Hansen-Pupp et al. (100)]. Inability of preterms patients to increase IGF-1 levels over time might mirror the most severe clinical condition of these neonates in the NICU that inhibits the release of a trophic factor. Similarly, IGF-1 increase was inhibited by other stress or inflammation conditions as well: very low Apgar score, severe RDS and late sepsis. IGF-1 was not proved to be a chemoattractant for the cell lines of interest in the current study. IGF-1 is known to be gradually increasing during pregnancy (101), which explains its lower levels observed in prematures compared to full-term neonates in the current study.

4.5. SDF-1

Although studies in adults constantly showed increased serum SDF-1 levels after tissue injury (57, 102), studies in neonates are not so conclusive (103–105). In adults, the main role of SDF-1 is chemotaxis in cases of tissue injury (57, 102, 106). In utero, SDF-1 is implicated in mobilization of progenitor cells for organogenesis (54), a procedure that is abruptly interrupted after premature delivery. It is possible that premature transition from intrauterine to extrauterine life implicates signals from tissue injury, especially in the first days of life. Initial adverse correlation of S100B and NSE levels on day 1 with latter SDF-1 levels might depict the abrupt inhibition of normal SDF-1 activity *in utero* after premature birth. In our study, brain injury was correlated positively with SDF-1 [i.e., presenting a role of chemotaxis after injury, as seen in adult models (57, 102)] in full-term neonates (data not shown) and in preterms only after day 9 of life. More studies are needed for a conclusive theory (103, 104, 107). Additionally, in this study, SDF-1 levels were increased in full-term newborns compared to preterms, but this has not reached statistical significance, in accordance to other researchers (103, 104). Machalinska et al. (107) have found increased levels in full-term neonates at the 10th week of life. We also observed positive (but not constant) correlations of SDF-1 levels with CPCs, that are indicative of its role as a chemoattractant. It is certain, that in neonates more factors are implicated, especially in a neonate in the setting of critical illness and therefore more studies are required.

4.6. CPCs

Correlations between brain injury markers and CPCs are of great interest. S100B was constantly related to low CPCs' levels of the same or the following days, whereas NSE was correlated to increased CPCs levels over time. As this is the first study to our knowledge that CPCs are studied together with brain injury markers, we can only make assumptions about this observation. In an ideal model, in cases that only neuronal injury is taking place (i.e., NSE increasement), the intact micro-environment of the neurons (i.e., astrocytes and endothelium) will release chemoattractants in an effort to recruit neuroprotective factors, as CPCs from the periphery. But when cytotoxic damage includes the micro-environment as well (i.e., astrocyte injury, with S100B increasement), signals via chemoattractants cannot be released and CPCs are not mobilized from periphery towards the brain. Another explanation might issue at the same time. As it is known that neurons are more vulnerable than astrocytes to hypoxia (7), NSE is a more sensitive brain injury marker and it is increased in an milder hypoxic event, where only neurons are damaged and astrocytes are spared. When S100B is increased there is an indication of a more severe brain damage and a more severe general clinical condition, in which both chemotaxis chain and peripheral mobilization of CPCs are affected. It seems that in more severe clinical conditions (hypoxic or oxidative), neither the "signal" is released, nor the recipient (CPCs) is capable to respond.

Another general observation in the current study is the inhibited mobilization of many cell lines in cases of inflammation, both antenatally (chorioamnionitis) and postnatally (late sepsis and NEC). It is well known that prenatal inflammation can predispose to postnatal inflammation (108–110), the latter being the second main mechanism (together with ischemia) that lead to preterm white matter injury (3, 7). If reduction of CPCs is the mediated step between antenatal and postnatal inflammation (i.e., antenatal inflammation leads to decreased CPCs, and decreased CPCs predispose to postnatal infection), or reduced CPCs are the 3rd hit in the pathway to white matter injury [taking into account their trophic actions in cases organogenesis and in cases of tissue injury as well (35–37)] this needs to be clarified in the future. Moreover, we observed that especially late sepsis was constantly correlated with decreased CPCs levels at almost any time point during the first one and a half month of life. At the end of the day, inflammation of any origin results in reduced CPCs, i.e., reduced regenerative ability for tissue injuries of any origin.

It is essential to be aware that different studies in the literature characterize cell lines in a different way, and this is a major problem for comparisons and for certain conclusions to be drawn. The less antibodies are used to identify cell lines, the less specific is the cell line characterization. E.g., $CD34^+$ cells are found in HSCs but also in VSELs and EPCs. Below reports of different studies will be presented, but absolute comparisons are not possible.

4.6.1. HSCs

In the current study, HSCs were found to be elevated in preterms with encephalopathy on days 3 and 9, but no differences were found on day 1. That means that HSCs' levels probably do not play a preventive role for the future appearance of brain damage, but rather HSCs are elevated secondarily as a response to the occurrence of brain injury.

In agreement with the present study, Borghesi et al. found no correlation of $CD34^+/CD45^+$ cells of day 1 with IVH, but correlation on day 3 with PVL (111). Likewise, Paviotti et al. did not find correlation on day 1 for $CD34^+$ cells (112). On the contrary, Kotowski et al. report decreased levels of HSCs (as

$CD184^+/lin^-/CD45^+$) in the umbilical cord in newborns that had later IVH or any complication of prematurity, indicating a possible protective role of these cells (104).

We found no correlation of HSCs with gestational age and birth weight, as Kotowski et al. (measuring $CD184^+lin^-CD45^+$ cells) (104) and Paviotti et al.($CD34^+$) (112) did, but most researchers that measure $CD34^+$ (91, 105, 113–115) or ($CD34^+CD45^+$) (111) cells did find an inverse correlation with gestational age.

4.6.2. VSELs

In the current study VSELs kinetics was similar in all groups, with a trend to gradually increase from day 9 to day 45. Only subgroup of preterms with IVH had higher levels on day 9. Kotowski et al. also report steady levels in the first 6 weeks of life ($CD184^+lin^-CD45^-$) and a trend to higher levels in prematures compared to full-term babies (104). Machalinska et al. report increased levels in preterms at the 10th week of life (107). We found no correlation of levels on day 1 with birth weight, but Kotowski et al. reported higher levels in low birth weight prematures. Regarding correlation with Apgar score and RDS, the common preconditioning pattern was observed once again: higher VSELs levels in mild stress (Apgar score between 4 and 6) compared to VSELs levels when Apgar score was >6, and much lower VSELs levels in severe stress (Apgar score <4). Similarly, higher VSELS levels were observed in mild RDS compared to no RDS at all (where severe RDS presents the lower VSELS levels). It seems that in mild stress the endogenous regeneration mechanisms are mobilized, but in severe stress these mechanisms are assaulted and are unable to help endogenous regeneration. Identifying neonates with suppressed endogenous mechanisms might be a target for exogenous administration of neuroprotective substances or progenitor cells in the future.

4.6.3. eEPCs

EPCs are novel markers of angiogenesis, playing a vital role in vascular growth and repair (59). eEPCs were found increased in preterms with encephalopathy (mainly with IVH), on days 3 and 9 of life. Strauss et al. found elevated endothelial RNA markers after the 2nd week of life (116), where Safranow et al. found elevated EPCs in cord blood in those neonates (103). eEPCs were also elevated in cases of low Apgar score in the current study, as also in Safranow et al. The above observation indicates that increasement of EPCs levels is probably a secondary process as a response to stress/brain injury, whereas increased levels in cord blood that were reported by Safranow et al. need to be further studied. Other researchers could not establish a correlation of eEPCs with brain damage (111, 112). Correlation with duration of hospitalization and death probably depicts the severity of the clinical condition, in which the eEPCs are elevated. We found no correlation of eEPCs on day 1 with birth weight and gestational age, whereas Safranow et al. found inverse correlation (103).

4.6.4. lEPCs

lEPCs were found increased in preterms with encephalopathy and in low Apgar score [in accordance with Safranow et al. (103)], indicating that perinatal stress and brain injury mobilize pluripotent progenitor cells with neoangiogenetic and regeneration abilities. Elevated lEPCs in healthy preterms compared to healthy full-term neonates depicts the ongoing process of organogenesis and angiogenesis in the fetus, disrupted suddenly by the preterm birth [in accordance with Safranow et al. (103)]. Similarly, increased lEPCs on day 45 in neonates with BPD might depict a trend for enhanced neoangiogenesis.

The main limitation of the current study is the relatively small number of patients, especially after the second week of life (due to the adverse event of death), that did not allow multiple regression analysis for confounding factors. This was a single-center study, with long follow up, and thus a relatively small number of patients could be enrolled. Nevertheless, absence of correlation between biomarkers and several important perinatal characteristics (as gestational age, birth weight, type of delivery or premature rupture of membranes) decreases importantly the number of possible confounders. Additionally, when confounders were observed (e.g., sepsis, RDS, NEC), confirmation of observed correlations after exclusion of neonates with these parameters did establish the results. Moreover, some observations were reported having only borderline significance when there was a meaningful rationale or when constant patterns were observed, because the scope of this pilot study was to highlight unknown pathophysiological pathways and correlations, that would be clarified by future studies, and not to come to final conclusions.

In conclusion, in this study we aimed to investigate if various preclinical observations are valid in preterm neonates, measuring multiple parameters in a single study and in multiple time-points. S100B and NSE proved to be very sensitive markers of brain injury, illustrating the time and extent of brain injury (hypothesis A). Additionally, they proved to have good prognostic ability for neuroimaging (S100B could prognosticate severe IVH, and NSE MRI scoring—hypothesis G) and language deficits at 2 years of corrected age (hypothesis H). These biomarkers are well studied, relatively low-cost and minimally invasive, and it seems that their clinical utility would be of great value. Regarding chemoattractants and neurotrophic factors, EPO was intensively increased in preterm brain injury, whereas IGF-1 and SDF-1 were rather decreased (hypothesis B). Similarly, CPCs were intense mobilized in those neonates, mainly HSCs and EPCs (hypothesis C) (**Figure 1**, **Table 2**). EPO and SDF-1 proved to act as chemoattractants for CPCs, but IGF-1 did not (hypothesis D). EPO also was observed to act as a neuroprotective factor itself, while IGF-1 indicated its trophic role as well (hypothesis E). Mobilization of CPCs was intense and not fully attributed to the chemoattractants we studied, indicating that other factors mediated as well (hypothesis F). Additionally, antenatal factors did not correlate with brain injury markers (hypothesis I), but multiple pregnancy, prenatal steroid administration and chorioamnionitis had moderate impact on EPO and SDF-1 levels (hypothesis K) and more intense impact on levels of CPCs (hypothesis L). Finally, correlation of chemoattractants and CPCs with comorbidities (namely RDS, sepsis and NEC) was rather impressive (hypothesis M), delineating relation of inflammation with brain injury and related

outcome. It is of special interest that a repeated preconditioning model was observed, with clinical importance that could be very valuable for future therapeutical strategies.

Increased chemoattractants and CPCs after brain injury, as well as decreased trophic factors and inability of CPCs mobilization in severe clinical conditions, delineate the endogenous regeneration effort or its suppression respectively. Individualized identification of the critical ill neonate whose endogenous regeneration mechanisms are suppressed could be a target for exogenous administration of neurotrophic factors or progenitor/stem cells in the near future.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee of the Faculty of Medicine, Aristotle University of Thessaloniki. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

NE: planning, implementation, supervision, data analysis, article writing, VS: total supervision, GK: flow cytometry, KK: neurodevelopmental assessment, GK: flow cytometry, SA: biochemical measurements' suprevisor, VD: MRI scoring, IM: neurodevelopmental assessment, VD: total supervisor. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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