

1 **Key words:** Bronchopulmonary dysplasia, neuron-specific enolase, microtubule-
2 associated proteins, glial fibrillary acidic protein, S100 proteins

3 **1. Introduction**

4 Bronchopulmonary dysplasia (BPD) is a chronic lung disease that affects more than
5 40% of preterm newborns [1]. Postnatal corticosteroids (CS) are generally used to
6 prevent or treat BPD in preterm infants [2,3]. Dexamethasone is the most commonly
7 used agent for this purpose [4]. Postnatal CS use, particularly dexamethasone, has been
8 associated with adverse neurodevelopmental outcomes, particularly cerebral palsy
9 [5,6,7]. The DART (Dexamethasone: A Randomized Trial) study showed that low-dose
10 dexamethasone may provide short term advantages without significantly increasing the
11 risk of long-term neurological problems in preterm newborns [8].

12 Neurobiomarkers (NBs) seem to provide physicians with promising helpful data for the
13 early detection of brain injury in daily clinical practice. Some studies have shown that
14 multiple biomarkers, rather than a single biomarker, may provide additional precision in
15 the early evaluation of cases at risk of neonatal neuronal damage [9]. Therefore, we
16 studied four NBs indicating brain damage, namely S100 calcium-binding B (S100B)
17 proteins, neuron-specific enolase (NSE), Tau protein or microtubule-associated protein
18 tau (MAPT), and glial fibrillary acid protein (GFAP). Although several studies shown
19 long term neurodevelopmental outcomes of postnatal CS use for the treatment of
20 preterm infants, no study has yet shown acute neurological injury occurring during that
21 treatment. In this study, we aimed to show whether NSE, S100B, MAPT, and GFAP
22 levels would provide evidence of early neurological damage in premature infants
23 receiving postnatal low-dose dexamethasone therapy to treat BPD.

1 **2. Materials and methods**

2 **2.1. Study design**

3 This cohort study was carried out between January 2018 and June 2019 in Dicle
4 University Medical Faculty Neonatal Intensive Care Unit. The study protocol was
5 approved by the Dicle University Faculty of Medicine Ethics Committee (21.12.2017-
6 03) and the study was conducted in compliance with the universal principles of the
7 Helsinki Declaration. Written and informed consent was obtained from the parents of all
8 participants. Clinical data and blood samples were collected prospectively.

9 **2.2. Study groups**

10 Group 1 (patient group): Patients who were born at ≤ 32 weeks of gestation, diagnosed
11 with BPD, in need of respiratory support on the postnatal 28th day, and administered
12 low dose dexamethasone treatment.

13 Group 2 (control group): Patients who were born at ≤ 32 weeks of gestation, and who
14 received no respiratory support on the postnatal 28th day.

15 **2.3. Inclusion criteria**

16 1- Preterm babies born at ≤ 32 (22 0/7-32 6/7) gestational week.

17 2- Patient group; On the postnatal 28th day, patients who needed respiratory support (in
18 the form of conventional mechanical ventilation, nasal continuous positive airway
19 pressure, or oxygen therapy in the incubator) but had no evidence of infection were
20 diagnosed with BPD.

21 3- Control group; Patients who had no need for respiratory support on the postnatal 28th
22 day.

1 **2.4. Exclusion criteria**

- 2 1- Having chromosomal disorders (such as trisomy 13, 18, 21)
- 3 2- Having major congenital malformations
- 4 3- Receiving corticosteroid therapy for any reason between birth and the 28th day of life
- 5 4- Having a history of maternal chorioamnionitis
- 6 5- Having patent ductus arteriosus
- 7 6- Having intraventricular bleeding
- 8 7- Having any proven infection

9 **2.5. Exclusion criteria for discontinuation of glucocorticoid therapy**

- 10 1- Suspected infection
- 11 2- Nutritional intolerance
- 12 3- Clinical deterioration
- 13 4- Necrotizing enterocolitis
- 14 5- Gastrointestinal bleeding
- 15 6- Intracranial bleeding
- 16 7- Dexamethasone use for extubation

17 **2.6. Dexamethasone therapy**

18 In accordance with the DART study protocol [8], patients with BPD were administered

19 intravenous low-dose dexamethasone therapy at a cumulative dose of 0.89 mg/kg for 10

20 days.

1 **2.7. Collection of samples**

2 In the patient group participating in the study, the first venous blood sample was taken
3 immediately before low-dose dexamethasone treatment was administered on the
4 postnatal 28th day, and then the treatment was started. A second venous blood sample
5 was taken 12 hours after the last dose of treatment was administered. Control venous
6 blood sample was taken on the postnatal 28th day in the control group. Blood samples
7 were taken from the median cubital vein in the antecubital fossa. Serum samples were
8 then stored at -80 °C until analysis.

9 **2.8. Laboratory analysis**

10 A commercial enzyme-linked immunosorbent assay (ELISA) was used to measure
11 NSE, S100B, GFAP, and MAPT (Shanghai Sunredbio Technology Co., Ltd, Shanghai,
12 China). Biomarkers were measured in ng/mL with the ELISA method. The catalog
13 numbers of NSE, S100B, GFAP, and MAPT ELISA kits used in the study are 210-12-
14 0938, 201-12-4851, 201-12-2095, and 20112-4259, respectively.

15 As stated below in the manufacturer's instructions, each of the four ELISA kits were
16 studied separately. The kit uses a double-antibody sandwich ELISA to assay the level of
17 human kit in samples. Add kit to monoclonal antibody Enzyme well which is pre-coated
18 with human kit monoclonal antibody, incubation; then, add (kit) antibodies labeled with
19 biotin, and combined with Streptavidin-HRP to form immune complex; then carry out
20 incubation and washing again to remove the uncombined enzyme. Then add
21 Chromogen Solution A, B, the color of the liquid changes into the blue, and at the effect
22 of acid, the color finally becomes yellow. The chroma of color and the concentration of
23 the human Substance kit of sample were positively correlated.

1 **2.9. Statistical analysis**

2 The Statistical Package for Social Science (IBM SPSS software, USA) version 22 was
3 used for statistical analysis. Normality of the distribution of numerical data was
4 examined using Shapiro–Wilk test. Inter-group differences were compared using
5 unpaired Student t-test. Normally distributed numerical variables were presented as
6 mean (SD). Skewed numerical variables were presented as median (interquartile range).
7 Inter-group differences were compared using Mann Whitney-U test. Ordinal variables
8 were compared using χ^2 test for trend; comparison between two paired non-parametric
9 variables was performed with Wilcoxon rank test. Correlations were examined with
10 Pearson product-moment correlation for parametric numerical variables, and Spearman
11 rank correlation for non-parametric numerical variables, as appropriate.
12 Receiver-operating characteristic (ROC) curve analysis was used to examine the value
13 of continuous variables. To assess the overall accuracy of the ROC analyses, the area
14 under the curves (AUCs) were measured. Tests with a p value of less than 0.05 were
15 considered statistically significant.

16 **3. Results**

17 In this study, the BPD group consisted of 73 (53.7%) boys and 63 (46.3%) girls, making
18 a total of 136 patients; the control group consisted of 37 (57.8) boys and 27 (42.2%)
19 girls, making a total of 64 preterm infants. There was no statistically significant
20 difference between the groups regarding gender distribution ($p= 0.347$). In the BPD
21 group, 88.5% ($n=108$) of the patients had a history of antenatal steroid use, while the
22 corresponding figure in the control group was 63% ($n=34$) ($P= <0.001$). The mean
23 gestational age (weeks) was 27.62 ± 2.75 in the BPD group and 28.00 ± 1.45 in the control

1 group; the mean birth weight was 1008 ± 307 g in the BPD group and 1068 ± 179 g in
2 the control group ($P= 0.299$, $P= 0.155$ respectively).

3 In the BPD group, no significant difference was found between patients with a history
4 antenatal steroid use and those without with regard to the 28-day GFAP, MAPT, NSE,
5 and S100B values. In addition, no significant difference was found between the BPD
6 and control groups for the same parameters ($P = 0.215$, $P = 0.835$, $P = 0.618$, $P = 0.653$,
7 respectively).

8 There was a significant difference between the BPD group and the control group with
9 respect to the GFAP, MAPT, and NSE values measured on the 28th day whereas no
10 significant difference was found between the S100B values of both groups (Table 1). A
11 significant difference was found between the NSE values, but not GFAP, MAPT, and
12 S100B values, measured on the 28th day and after the treatment in the BPD group
13 (Table 2).

14 There was a significant correlation between the GFAP, MAPT, NSE, and S100B values
15 measured on the 28th day and other parameters in the BPD group (Table 3).

16 ROC analysis results of GFAP, MAPT, NSE and S100B values measured on the 28th
17 day in the BPD group are given in Table 4. See Figure for the ROC curve graph.

18 **4. Discussion**

19 Detection of perinatal brain damage in the acute phase in term and preterm newborns is
20 still an unsolved problem. Currently, studies to find a reliable biomarker that can predict
21 perinatal brain damage in high-risk newborns are still ongoing [9]. While severe
22 neurodevelopmental deficiencies (17% to 59%) occur in short-term follow-up in
23 premature neonates between 22 and 25 weeks of gestation, it is generally related with a

1 mortality rate as high as 50%. Long-term negative consequences include cerebral palsy
2 (9% to 18%), mental disability (5% to 36%), deafness (2% to 4%), and blindness (0.7%
3 to 9%) [10].

4 NSE is a glycolytic enzyme, which can be detected in high concentrations in the neuron
5 cell cytoplasm and is released into the extracellular space in cell death [9]. It is an early
6 biochemical predictor of brain damage in newborns and can also help us understand the
7 extent of neuronal damage and assess its prognosis [11]. The S100B protein is highly
8 specific for the nervous system, and is secreted in large amounts from astrocytes into
9 the blood and cerebrospinal fluid (CSF) in neuronal tissue damage [9]. GFAP is an
10 intermediate filament protein of the cytoskeleton in astroglia in the nervous system and
11 is rapidly released into the blood in brain injury [12,13]. MAPT is a microtubule-related
12 structural protein that is released from neurons into CSF or blood when brain damage
13 occurs [14].

14 Antenatal CS has a proven benefit in preventing complications by ensuring rapid
15 maturation of organs that do not show the desired development in the fetal life.
16 Although these drugs have clinically proven positive effects, they can also trigger some
17 negative developmental side effects [15]. In our study, no statistically significant
18 difference was found between patients with and without a history of antenatal CS use
19 with respect to the brain damage neurobiomarkers including GFAP, MAPT, NSE, and
20 S100B measured on the 28th day ($P = 0.215$, $P = 0.835$, $P = 0.618$, $P = 0.653$,
21 respectively).

22 Bronchopulmonary dysplasia is a chronic lung disease that occurs as a result of
23 barotrauma and oxygen toxicity caused by mechanical respiratory support in extremely
24 low birth weight premature neonates. It is a disease with elevated morbidity and

1 mortality risk. CSs play a major role in the treatment of BPD resulting from lung
2 inflammation. CS treatment shows positive effects by rapidly improving lung function,
3 but studies have shown that this treatment may also be associated with several serious
4 side effects in the long term [16]. Systemically administered dexamethasone is the most
5 commonly used CS to prevent and treat BPD. It has alarming long term negative effects
6 associated with its use, the worst of which is cerebral palsy [17]. Doyle et al. [8] found
7 no strong relationship with its use and long-term morbidity, although the research failed
8 to provide conclusive evidence about the long term effects of low dose dexamethasone
9 after the first week of life in ventilator-dependent neonates. However, Qin G et al. [5]
10 showed that postnatal dexamethasone use is related with an elevated risk of
11 neurodevelopmental impairment in extremely preterm newborns.

12 A large number of studies have been conducted to show brain injury in newborn infants,
13 with most being on hypoxic-ischemic encephalopathy (HIE). It was reported that the
14 concentrations of GFAP, S-100, and NSE were significantly elevated in the
15 cerebrospinal fluid of asphyxiated newborns and significantly associated with other
16 markers of long term prognosis and brain injury at 1 years of age [17]. Chaparro-Huerta
17 et al. [18] showed that serum levels of proinflammatory cytokines, S-100B, and NSE
18 were significantly elevated in newborns with brain injury compared to controls. In
19 another study, Lu et al. [19] suggested that in preterm infants, cord blood S100B had the
20 highest sensitivity for brain injury. Several studies have shown changes in serum Tau
21 protein levels in brain injury of the newborn [14]. In our study, a statistically significant
22 difference was found between the GFAP, MAPT, and NSE values measured on the 28th
23 day of the BPD group and the control group, while there was no significant difference
24 between the S100B values. Summanen et al. [20] showed that cord blood S100B in term

1 neonates is a weak predictor of brain injury. In our study, when the correlation between
2 measured NB values and other parameters was evaluated on the 28th day of the cases,
3 GFAP, MAPT, NSE and S100B values showed significant correlations with each other.
4 Cerebrospinal fluid's NSE concentrations provide invaluable information as a clinical
5 reflection of the severity of hypoxic-ischemic neuronal damage, and this information
6 may be a harbinger of an abnormal result at two years of age [21]. In a previous study,
7 NSE levels were correlated to neonatal HIE grade and an adverse outcome. The findings
8 suggested that NSE is an accurate biochemical marker for early prediction of hypoxic
9 ischemic brain injury in asphyctic newborns [22]. Sun et al. [23] recently reported that
10 S100B and CSF-NSE levels were correlated to neurodevelopmental outcomes.
11 Catherine et al. [24] evaluated whether serum levels of neuronal biomarkers correlated
12 to neurodevelopmental outcomes in term neonates at 18 months who hypoxic ischemic
13 encephalopathy and therapeutic hypothermia. They showed that serum neuronal
14 biomarkers, S100B, and NSE levels were not correlated to long-term
15 neurodevelopmental outcomes in that population. The predictive ability of serum NSE
16 levels for poor outcomes appears to be better than predictive ability for moderate or
17 severe HIE. The sensitivity and specificity values of serum NSE (cut-off value 40.0
18 $\mu\text{g/L}$) as a predictor of moderate to severe HIE were 79% and 70%, respectively, and as
19 a predictor of poor outcomes (cut-off value 45.4 $\mu\text{g/L}$) it had a sensitivity of 84% and a
20 specificity of 70% [25].

21 A systematic review showed that NSE may be a sensitive and quantitative NB of
22 neuronal damage [13]. In our study, a significant difference was found between the NSE
23 values measured on the 28th day and after the treatment in the BPD group, while there
24 was no significant difference between the measured GFAP, MAPT, and S100B values.

1 In our study, sensitivity and specificity values for NSE, GFAP, and MAPT were 65.2%
2 and 34.4% (cut-off value 4.21 ng/mL), 61.5% and 39.1% (cut-off value 0.59 ng/mL),
3 and 61.5% and 37.5% (cut-off value 17.28 ng/mL), respectively. The sensitivity and
4 specificity values of S100B were 50.5% and 50.0% (cut-off value 234.49 ng/mL),
5 respectively.

6 No study has yet shown any evidence of neurological injury in the early period (>28
7 days) in patients with BPD. In this study, we found that the BPD group had higher
8 levels of neuronal injury NBs than the control group. This finding indicates that
9 respiratory distress experienced by BPD patients may cause neuronal injury in the early
10 period. It is also unknown how much exposure to CS given for treatment contributes to
11 neurological injury early in life. In our study, we aimed to show whether the lowest
12 cumulative dose of postnatal dexamethasone exposure contributes to this brain injury in
13 premature babies diagnosed with BPD. Statistically, we observed that there was a slight
14 increase in NSE levels after treatment discontinuation in the patient group. This finding
15 raises the question that CS can be associated with brain injury, even at low doses. What
16 will be the long term clinical consequences of this increase in premature babies is an
17 issue to be investigated.

18 This study has some limitations. Firstly, the diagnosis of BPD is made on the 28th day
19 in premature patients who need respiratory support. Secondly, the long-term clinical
20 results of the data obtained in a short term in this study are unknown. However, our
21 study was aimed at obtaining evidence of early brain injury, and it has already planned
22 to follow up these patients and evaluate their future clinical results in the future.

23 Another issue worth mentioning which is the NBs used for demonstrating brain injury,
24 was also studied mostly in CSF in earlier studies but in blood in our study. This is

1 because in our study, an invasive attempt was not made to collect CSF samples from
2 premature newborns due to ethical limitations.

3 This study showed that the levels of neurobiomarkers indicating early brain injury are
4 increased in preterm babies with BPD. Although our study cannot provide conclusive
5 evidence about the long term effects of low-dose dexamethasone after the first four
6 weeks of life in premature neonates with BPD, it still suggest that there may be a
7 relationship between NSE levels and neurodevelopmental outcome. However, well-
8 designed studies with a greater number of patients using multiple neurobiomarkers are
9 needed.

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1 **Table 1.** Comparison of measured values of GFAP (ng/mL), MAPT (ng/mL), NSE
2 (ng/mL), and S100B (ng/mL) on 28 days of BPD group, and control group cases.

	BPD group (n=136)	Control group (n=64)	
Parameters*	Mean±SD	Mean±SD	P
GFAP	1.530±4.372	0.91± 1.019	0.019
MAPT	24.22±31.01	24.56±27.34	0.012
NSE	8.86±14.48±	5.19±5.094	0.000
S100B	361.16±396.40	392.12±389.5	0.465

3 * Parameters measured on the 28th day after birth. BPD: bronchopulmonary dysplasia,
4 SD: standard deviation

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1 **Table 2.** Comparison of GFAP (ng/mL), MAPT (ng/mL), NSE (ng/mL), and S100B
 2 (ng/mL) values of BPD group on 28th day and after treatment.

	Negative Ranks	Positive Ranks	
Parameters	N (Mean Rank)- (Sum of Ranks)	N (Mean Rank)-(Sum of Ranks)	P*
GFAP	64 (57.63)-(3688)	59 (66.75)-(3938)	0.752
MAPT	136 (55 ^d)-(60,29)	66 (61.59)- (4065)	0.333
NSE	46 (59.11)-(2719)	75 (62.16)-(4662)	0.012
S100B	61 (58.20)-(3550)	62 (65.74)-(4076)	0.507

3 * Wilcoxon Signed Ranks Test

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1 **Table 3.** Correlation between GFAP (ng/mL), MAPT (ng/mL), NSE (ng/mL), and S100B
 2 (ng/mL) values and other parameters of the cases measured on the 28th day.

Parameters	GFAP		MAPT		NSE		S100B	
	r	P**	r	P**	r	P**	r	P**
GFAP*			0.484	<0.001	0.435	<0.001	0.618	<0.001
MAPT*	0.484	<0.001			0.538	<0.001	0.568	<0.001
NSE*	0.435	<0.001	0.538	<0.001			0.458	<0.001
S100B*	0.618	<0.001	0.568	<0.001	0.458	<0.001		

3 *28 days measured value, **Spearman's rho.

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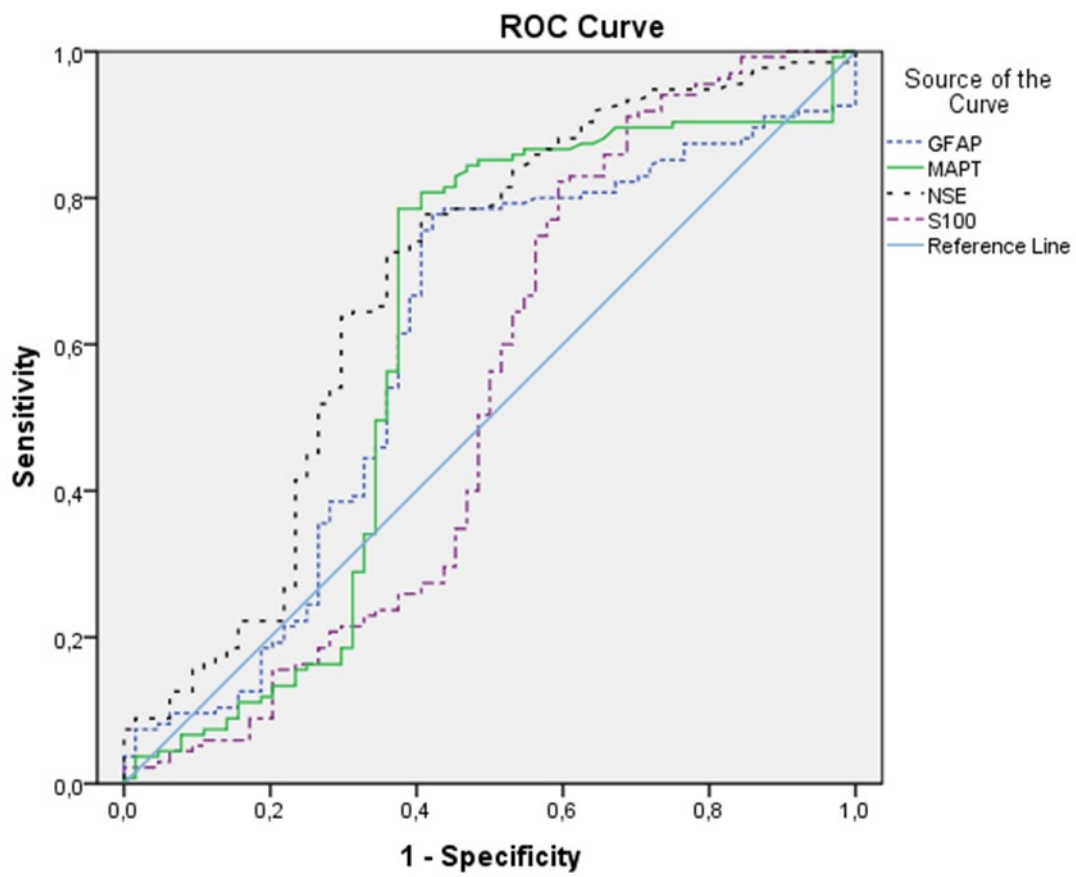
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- 1 **Table 4.** Sensitivity and specificity at cut-off points of GFAP (ng/mL), MAPT (ng/mL),
 2 NSE (ng/mL) and S100B (ng/mL) values measured on the 28th day for BPD diagnosis.

Indicators	Area under the ROV curve	(%95 CI)		Cutt-of point (ng/mL)	p	Sensitivity (%)	Specificity (%)
GFAP	0.600	0.510	0.690	0.588594	0.023	61.5	39.1
MAPT	0.607	0.510	0.704	17.28005	0.015	61.5	37.5
NSE	0.680	0.594	0.766	4.216890	<0.001	65.2	34.4
S100B	0.529	0.430	0.627	234.4936	0.513	50.5	50.0

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Diagonal segments are produced by ties.

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2 **Figure.** ROC curve graph of GFAP, MAPT, NSE and S100B values.

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