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Relevance of urinary S100B protein levels as a short-term prognostic biomarker in asphyxiated infants treated with hypothermia

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Abstract

The initial diagnosis of neonatal hypoxic-ischemic encephalopathy is based on nervous system clinical manifestations. The use of biomarkers to monitor brain injury and evaluate neuroprotective effects allows early intervention and treatment. This study was designed to determine the short-term prognostic significance of urinary S100B calcium-binding protein (S100B) in asphyxiated newborns treated with hypothermia.

An observational prospective study was conducted over a period of 5 years in 31 newborns with hypoxic-ischemic encephalopathy who received therapeutic hypothermia. The patients were divided into 2 groups: Group A (13 newborns with a normal neurological examination before discharge) and Group B (18 newborns who died during admission or had an abnormal neurologic examination before discharge). Urinary S100B was the main variable, serum S100B and neuron-specific enolase (NSE) were considered as secondary variables, and all of them were assessed on the first 3 days of life. The newborns were subsequently divided into groups with normal and abnormal electrophysiological and imaging findings.

Mean urinary S100B levels were significantly higher in group B than group A on day 1 (10.58 ± 14.82 vs 4.65 ± 9.16 μg/L, $P = .031$) and day 2 (5.16 ± 7.63 vs 0.88 ± 2.53, $P = .002$). The optimal cutoff for urinary S100B on day 1 was >1.11 μg/L of (sensitivity, 100%; specificity 60%) for the prediction of neonatal death and < 0.66 μg/L (sensitivity 83% and specificity 70%) for the prediction of a normal neurological examination before discharge. It was not possible to calculate cutoffs with a similar accuracy for serum S100B or NSE. Urinary S100B on day 1 was higher in patients with abnormal magnetic resonance imaging findings (7.89 ± 8.09 vs 4.49 ± 9.14, $P = .039$) and abnormal positron emission tomography findings (8.60 ± 9.29 vs 4.30 ± 8.28, $P = .038$). There were no significant differences in S100B levels between patients with normal and abnormal electroencephalography results.

Urinary S100B measured in the first days of life can predict neonatal death and short-term prognosis in asphyxiated newborns treated with hypothermia. The method is convenient, noninvasive, and has a higher sensitivity and specificity than measurement of serum S100B or NSE.

Abbreviations: CFM = cerebral functional monitoring, EEG = electroencephalography, HIE = hypoxic ischemic encephalopathy, MRI = magnetic resonance imaging, NSE = neuron-specific enolase, PET = positron emission tomography, S100B = S100 calcium-binding protein.

Keywords: hypoxic-ischemic encephalopathy, neuron-specific enolase, serum S-100B protein, therapeutic hypothermia, urinary S-100B protein

1. Introduction

Therapeutic hypothermia reduces cerebral injury and improves neurological outcomes secondary to hypoxic ischemic encephalopathy (HIE) in newborns. It is indicated for asphyxiated full-term or near-term newborn infants with clinical signs of HIE.[1–3] Therapeutic hypothermia must be started within 6 hours of birth.[4–6] The extent of cerebral injury following a hypoxic-ischemic insult basically depends on the balance between the causative mechanisms of irreversible injury, such as neuronal necrosis or persistent inflammation, and endogenous protection (acute phase response, recovery, and neuronal repair).[7,8] The neuroprotective strategy of therapeutic hypothermia involves the modulation of inflammatory cascade inhibition, reduced production of reactive oxygen species, and a reduction in metabolic rate.[9–12]

S100B calcium-binding protein (S100B) is an acidic calcium-binding protein of the helix-loop-helix structure family.[13–15] In the nervous system, it is mainly present in glial cells, but it can also be found in specific neuron subpopulations. As a cytokine, S100B plays a trophic role and is physiologically detectable in

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different biological fluids.[15–17] Neuron-specific enolase (NSE) is the neuronal form of intracytoplasmic glycolytic enzyme enolase and is specific to neurons and neuroectodermal cells.[18,19]

S-100B protein and NSE in the serum and cerebrospinal fluid have been used as biomarkers to determine the severity of HIE.[20] These corresponding examinations however, are invasive, and in addition, increases in levels and peaks have been found to vary across studies. Previous reports have suggested that urinary S100B might provide a better measure of nerve injury in HIE.[21,22] The objectives of this study were to assess the value of urinary S100B in the early diagnosis and short-term prognosis of HIE and to compare its performance with serum S100B and NSE in the prediction of neonatal death and electrical, imaging, and clinical alterations.

2. Materials and methods

2.1. Study design

This prospective observational study was conducted to evaluate biochemical markers in newborns with a diagnosis of moderate or severe HIE treated with hypothermia who were admitted to a tertiary university hospital over a period of 5 years (February 2011–January 2016). The study protocol was approved by the local research ethics committee (2011/434).

Early HIE was diagnosed in newborns with neonatal encephalopathy, arterial cord blood pH $\leq$7, a 5-minute Apgar score $<$ 5, and/or the need for prolonged major resuscitation.[23] Neonatal encephalopathy was evaluated using the Sarnat Grading Scale.[24] The neurological examination to grade the severity of neonatal encephalopathy was performed within 6 hours of birth, before initiation of hypothermia.

Hypothermia therapy was initiated in the first 6 hours of life and was configured to maintain the core temperature at 33.5°C for 72 hours. Infants were divided into 2 groups: those with a favorable outcome, defined as a normal clinical neurological examination before discharge from hospital (Group A) and those with an unfavorable outcome, defined as death or an abnormal clinical neurological examination before discharge (Group B). The children were subsequently classified into different groups depending on whether they had normal or abnormal electrophysiological and imaging results.

The primary variables were urinary S100B assessed on the first, second, and third days of life. The secondary variables were serum S100B, NSE, complete blood count, bleeding study, urinary evaluation, and liver enzymes, assessed all of them at the same time points. The neurological evaluation, performed before discharge, included clinical neurological examination, electroencephalography (EEG), cerebral magnetic resonance imaging (MRI), and positron emission tomography (PET).

2.2. Inclusion and exclusion criteria

All patients with early HIE and Sarnat Stage II or III during the study period were considered candidates for hypothermia and were recruited after parental consent had been obtained (Table 1). Exclusion criteria were a gestational age $<$36 weeks, weight at birth $<$1800 g, and presence of severe malformations, severe multiple organ dysfunction, or severe coagulopathy.

2.3. Population

During the 5-year study period, 31 newborns (17 males and 14 females) with moderate or severe HIE were admitted to our hospital and treated with therapeutic hypothermia. Twenty-six asphyxiated newborns were delivered by emergency cesarean section due to acute fetal distress, defined according to the American College of Obstetricians and Gynecologists as bradycardia, late deceleration of the fetal heart rate, severe and repetitive variable deceleration of the fetal heart rate, and reduced beat-to-beat variability. The other 5 children were delivered by vaginal birth due to the absence of nonreassuring fetal status. Table 1 summarizes the postnatal clinical and laboratory parameters for the 31 newborns. Six (3 males and 3 females) died in the first 3 days of life: 4 died during hypothermia (2 on the second day and 2 on the third), and 2 died after completing 3 days of treatment. Discharge time was between day 10 and 20 in 11 cases, between day 21 and 30 in 10 cases, and between day 31 and 40 in 4 cases.

Table 1

Clinical and laboratory parameters of 31 newborns studied.

Clinical and laboratory parameters	Number of newborns
Apgar score $<$5 at 5 min	31
Apgar score $\geq$5 at 5 min	0
Cord blood pH $\leq$7	31
Cord blood pH $>$7	0
Prolonged resuscitation	27
Neonatal encephalopathy	
Sarnat stage II	16
Sarnat stage III	15

2.4. Methods

Therapeutic hypothermia was applied using the Tecotherm Neo thermoregulation system (West Street, Earl Shilton, Leicester, Leicestershire, UK) (Manufacturer) device using total body modality. The newborns received whole-body cooling to an esophageal temperature of 33.5°C for 72 hours. They were slowly rewarmed to 36.5°C in steps of 0.5°C/h.

For the *neurobiomarker* analysis, serum and urine samples were collected in the first 6 hours of life and on the second and third days; they were centrifuged at 3000 rpm for at least 5 minutes. Serum samples were transferred to standard and control test tubes after diluting with 1 mL distilled water. The dilutions were kept stable at –20°C and were analyzed within 18 hours of collection. S100B levels in serum were measured using a sandwich-type electrochemiluminescence immunoassay. The results were calculated using a system-generated calibration curve based on 2-point calibration and a master curve included in the barcode of the reagent. NSE levels were determined using a Human NSE ELISA kit (DiaMetra S.r.l., cat. #: DKO073, Z.I. Paciana, Italy). S100B levels were determined using a Human S100B ELISA kit (DiaMetra S.r.l., cat. #: DKO073). Results were expressed as μg/L for S100B and as ng/mL for NSE.[25]

EEG was performed using Neuro-DMS software (Nihon Kohden, Rosbach, Germany). Abnormal EEG was defined as one of the following patterns of electrical alteration: generalized slowing, periodic patterns (e.g., burst-suppression), background suppression, electrocerebral inactivity, and less common patterns (alpha coma, beta coma, spindle coma, and triphasic waves).[26]

Brain PET scans were analyzed with the General Electric Advance NXI PET system (GE Medical Systems, Milwaukee, WI), 45 minutes after injection of 14.5 MBq of 18F-FDG, following the guidelines and recommendations of the European

Table 2

Comparison of biochemical markers in patients with a favorable outcome (normal clinical neurological examination before discharge) and an unfavorable outcome (death or abnormal clinical neurological examination before discharge).

	Favorable outcome			Unfavorable outcome			*P*		
Biochemical marker	Day 1 N=13	Day 2 N=13	Day 3 N=13	Day 1 N=18	Day 2 N=16	Day 3 N=14	Day 1	Day 2	Day 3
NSE, ng/mL	84.3 ±28.8	68.6 ±20.6	54.1±16.3	109.1±49.2	113.5±59.4	97.3±59.8	NS	.019	.37
Serum S100B, μg/L	1.77±2.01	0.86±1.31	0.48±0.38	3.71±5.21	1.84±3.88	1.21±2.14	NS	NS	NS
Urinary S-100B, μg/L	4.65±9.16	0.88±2.53	0.46±0.93	10.58±14.82	5.16±7.63	1.81±3.60	.031	.002	NS

Data are presented as mean±2SD. Significant differences between groups ($P<.05$, Wilcoxon test).
NSE=neuron-specific enolase; S100B=S100 calcium-binding protein.

Association of Nuclear Medicine for pediatric patients[27] and abnormal results were defined as focal or multifocal hypometabolism.[28]

Brain MRI was performed using a 1.5-Tesla scanner (Siemens Sonata, Malvern PA). An abnormal MRI was defined as one of the following 3 patterns of hypoxic-ischemic lesions: periventricular leukomalacia, basal ganglia and/or thalamus lesions, and multicystic encephalopathy accompanied by injury to the basal ganglia, thalamus, and/or cerebral cortex.[29]

The clinical neurological examination was performed using the Dubowitz neurological examination of the full-term newborn. Abnormal patterns included increased extensor tone in the legs, increased flexion tone in the arms, prevalent extensor tone in the neck and trunk muscles, fisting or abnormal posture of the hand or feet in absence of contractures, abnormal body movements (tremors, clonus), convulsions, abnormal eye movements, reduced or absent visual and auditory orientation, sucking abnormalities.[30]

2.5. Calculation power and statistical analysis

To calculate the sample size needed to detect differences in the primary study variable, S100B in urine, we used 1 μg/L as the cutoff for normal levels with a confidence interval (95% CI) of 85% and an error of 5%. This cutoff was taken from a study that used the same method and units to measure S100B as we did.[31] Absolute effect size was estimated to compare results between children who died during admission and those who were alive at discharge. The sample size was 30 infants for an observed power of 80%.

All statistical calculations were performed in SPSS (SPSS, v.20.0; SPSS Inc., Chicago, IL). Normality of data was analyzed using the Shapiro–Wilk test. A P value $<.05$ was considered statistically significant (2-tailed test), with a 95% 95% CI. Multiple testing correction was performed using Bonferroni correction. Qualitative variables were compared between groups using the χ^2 test and the Wilcoxon test was used to evaluate quantitative variables. Pearson correlation coefficient was used to valuate correlation between variables. S100B sensitivity and specificity were assessed using receiver operating characteristic (ROC) curves and areas under the curve (AUC).

3. Results

3.1. Urinary S100B and unfavorable outcome

Of the 31 infants with moderate or severe HIE treated with hypothermia, 13 had a favorable outcome (normal clinical neurological examination before discharge) and 18 newborns had an unfavorable outcome (death or abnormal neurological examination at discharge). Urinary S100B levels were significantly higher on the first and second days of life in Group B (unfavorable outcome). NSE levels were lower on days 2 and 3 in Group A, and no significant differences in serum S100B were noted (Table 2). There were no significant differences in complete blood count, bleeding study, urinary evaluation, or liver enzymes between the groups.

Urinary S100B levels were higher on the first, second, and third days of life in newborns who died ($P=.046$, $P=.016$, and $P=.034$ respectively). These infants also had higher serum S100B levels on day 1 and higher serum NSE levels on days 1 and 3 (Table 3).

A strong linear correlation was observed between urinary S100B and serum S100B on the first day, with a Pearson correlation coefficient of 0.76 ($P=.001$). No correlations were observed between urinary S100B and serum NSE ($r=0.29$, $P=$n.s).

3.2. Urinary S100B to predict infant death

Figure 1 shows the ROC curve for the prediction of death before discharge by urinary S100B levels on the first day of life. The optimal cutoff was 1.11 μg/L (sensitivity 100% and specificity 60%) and the AUC was 76.7% (95% CI, 58.5–94.8; $P=.046$; SE, 0.093). Figure 2 shows the ROC curve for the prediction of an unfavorable outcome (death or abnormal clinical neurologic examination); the optimal cutoff for urinary S100B was 0.66 μg/

Table 3

Comparison of biochemical markers between neonates who died during admission and those who were alive at discharge.

	Alive			Deceased			*P*		
Biochemical marker	Day 1 N=25	Day 2 N=25	Day 3 N=25	Day 1 N=6	Day 2 N=4	Day 3 N=2	Day 1	Day 2	Day 3
NSE, ng/mL	86.6±36.4	85.3±40.9	66.7±29.9	149.1±31.8	143.8±82.7	199.0±91.9	.001	NS	.006
Serum S100B, μg/L	1.97±1.99	1.34±3.20	0.81±1.61	6.75±8.14	1.77±1.65	1.49±1.41	.032	NS	NS
Urinary S-100B, μg/L	5.85±8.73	2.48±5.73	0.80±2.35	17.44±22.49	8.07±7.91	5.63±3.96	.046	.016	.034

Data are presented as mean±SD. Significant differences between groups ($P<.05$, Wilcoxon test).
NSE=neuron-specific enolase; S100B=S100 calcium-binding protein.

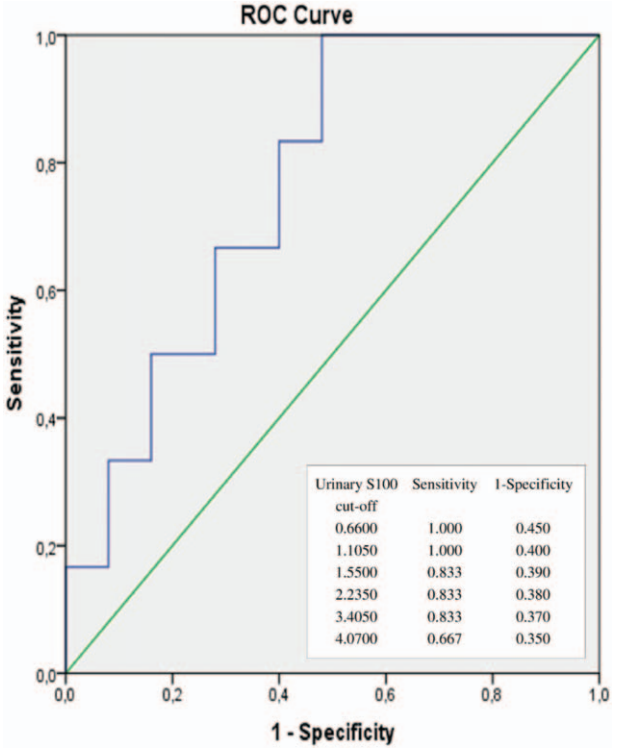

Figure 1. ROC curve for the prediction of neonatal death during hospital admission according to urinary S100B levels on the first day of life. ROC = receiver operating characteristic; S100B = S100B calcium-binding protein.

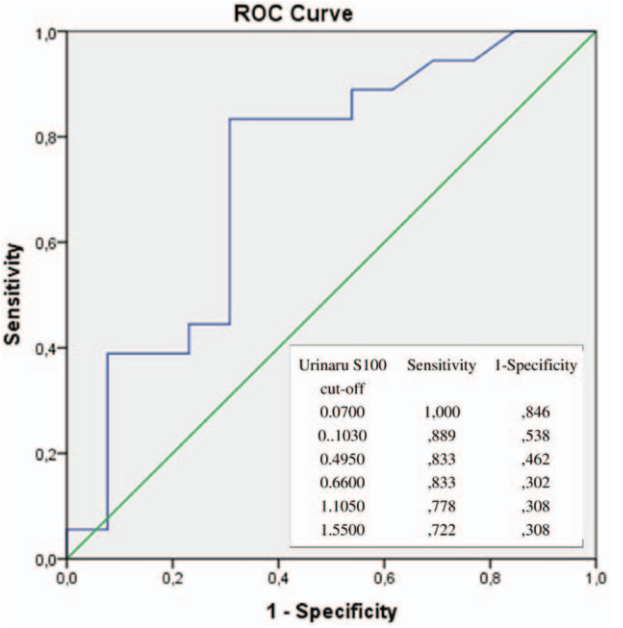

Figure 2. ROC curve for the prediction of an unfavorable outcome (neonatal death during hospital admission or an abnormal neurological examination at discharge) according to urinary S100B levels on the first day of life. ROC = receiver operating characteristic; S100B = S100B calcium-binding protein.

L (sensitivity 83% and specificity 70%) and the AUC was 73.1% (95% CI 54.1–92.1; $P = .031$; SE, 0.097). It was not possible to calculate a cutoff for serum S-100B and NSE with the required accuracy.

3.3. Urinary S100B and short-term prognosis of HIE

Of the 25 children who were still alive at discharge, 9 had abnormal EEG findings before discharge. There were no significant differences in urinary S100B, serum S100B, or NSE between children with normal and abnormal EEG findings. Ten newborns had brain MRI alterations. The group with alterations had higher mean urinary S100B levels than those without alterations on the first and second days of life. Nine newborns had abnormal cerebral PET findings, and compared with the 16 patients with normal PET findings, they had higher mean urinary S100B levels on days 1 and 2 and higher mean NSE levels on day 3 (Table 4). No significant differences were noted between the groups for complete blood count, bleeding study, urinary evaluation, or liver enzymes. Table 5 summarizes the significance of first day urinary S100B protein levels in asphyxiated infants treated with hypothermia.

4. Discussion

We have investigated the short-term prognostic relevance of urinary S100B in newborns with moderate or severe HIE treated with hypothermia. Possible correlations with serum S100B and NSE, currently the most widely used biomarkers, were investigated. Newborn infants who died during admission had imaging and clinical neurological examination alterations, and they all had higher levels of urinary S100B during the first days of life than those who did not die.

HIE is a common pathology caused by perinatal asphyxia, which is a major cause of neonatal death, neurological and behavior alterations, and long-term disability.[32] Diagnosis and prognosis of neonatal HIE is currently based on nervous clinical system manifestations and on imaging and electrophysiological techniques, such as MRI, PET, cerebral functional monitoring (CFM), and EEG. With the exception of CFM, these procedures take time, resulting in a delayed diagnosis and possibly brain injury. Newborns with severe brain injury could thus miss the critical treatment window and develop lasting neurological impairment. The use of biomarkers to monitor brain injury and evaluate neuroprotective effects could reduce mortality and complications by facilitating early intervention and treatment of neonatal HIE.[33] S100B is predominantly expressed in the nervous system and has been well established as a biochemical marker for brain injury.[34] Urinary S100B could thus prove to be an excellent biochemical marker for use in newborns with HIE.[35] In the present study, there was a statistically significant difference in mean urinary S100B levels between patients with a favorable and a nonfavorable outcome in the first 3 days of life. It is, therefore, feasible that urinary S100B could be a sensitive and reliable early indicator of severe HIE brain injury and a predictor of neonatal death. Using the ROC curve to predict the risk of neonatal death based on urinary S100B only, we estimated that a level of over 1.11 μg/L would predict death during admission, while a level of over 0.66 μg/L would predict death or an abnormal clinical neurological examination before discharge. It was not possible to calculate a cutoff with the required accuracy to predict death or abnormal clinical neurological examination with serum S-100B or NSE. To our knowledge, only one other

Table 4

Comparison of biochemical markers difference in neonates with normal and abnormal EEG, MRI, and PET findings before discharge.

	Normal EEG N=16			Abnormal EEG N=9			*P*		
Biological marker	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
NSE, ng/mL	83.7±32.0	72.7±25.4	59.3±24.4	91.6±44.8	107.7±54.3	79.8±35.5	NS	NS	NS
Serum S100B, μg/L	2.08±2.31	1.81±3.97	1.01±2.01	1.77±1.36	0.54±0.30	0.47±0.19	NS	NS	NS
Urinary S-100B, μg/L	5.18±8.93	2.39±6.62	1.11±2.91	7.05±8.73	2.62±4.01	0.26±0.35	NS	NS	NS
	Normal MRI N=15			**Abnormal MRI N=10**			***P* value**		
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
NSE, ng/mL	82.3±29.6	78.2±41.5	55.5±18.1	93.1±45.7	96.1±39.7	83.5±36.9	NS	NS	NS
Serum S100B, μg/L	1.68±1.91	0.83±1.23	0.48±0.37	2.41±2.14	2.12±4.88	1.31±2.49	NS	NS	NS
Urinary S100B, μg/L	4.49±9.14	0.85±2.35	0.41±0.87	7.89±8.09	4.92±8.23	1.38±3.59	.039	.008	NS
	Normal PET N=16			**Abnormal PET N=9**			***P***		
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
NSE, ng/mL	85.3±27.3	72.2±21.7	53.8±17.3	88.7±50.5	108.6±56.5	89.5±34.8	NS	NS	.015
Serum S100B, μg/L	1.62±1.84	0.77±1.19	0.47±0.35	2.61±2.20	2.36±5.12	1.43±2.63	NS	NS	NS
Urinary S100B, μg/L	4.30±8.28	0.92±2.29	0.38±0.84	8.60±9.29	5.24±8.66	1.55±3.77	.038	.024	NS

Data are presented as mean±SD. Significant differences between groups ($P<.05$, Wilcoxon test).
EEG=electroencephalography; MRI=magnetic resonance imaging; NSE=neuron-specific enolase; PET=positron emission tomography; S100B=S100 calcium-binding protein.

study has assessed the relationship between urinary S100B and neonatal death in HIE.[31] On the basis of a sample of 60 newborns with HIE, the authors concluded that a level of 1 μg/L or higher would predict neonatal death with an accuracy of 100%. They did not specify whether or not hypothermia was used. More studies with larger samples are needed to establish a clear and reliable baseline for relating S100B levels with neonatal death in patients with HIE.

With reference to the short-term prognostic value of urinary S100B, mean levels were higher on the first and second days of life in newborns with abnormal MRI and PET findings before discharge. This could have been due to the release of S100B from the damaged cerebral zone and its selective excretion in urine. Serum S100B was not found to have prognostic relevance, possibly because of the short biological half-life of this protein, which is about 60 minutes.[17] Serum NSE had less prognostic significance than urinary S100B in the prediction of short-term imaging alterations, and its benefit was more evident on days 2 and 3. This can be partly explained by the fact that NSE levels have been found to peak after 24 hours.[36]

Table 5

The significance of first day urinary S100B protein levels in asphyxiated infants treated with hypothermia.

Short-time prognosis	First day urinary S100B μg/L	*P*
Favorable outcome	4.65±9.16	.031
Unfavorable outcome	10.58±14.82	
Alive	5.85±8.73	.046
Deceased	17.44±22.49	
Normal MRI	4.49±9.14	.039
Abnormal MRI	7.89±8.09	
Normal PET	4.30±8.28	.038
Abnormal PET	8.60±9.29	
Cutoff to predict death before discharge	1.11	.046
Cutoff to predict abnormal clinical neurological examination	0.66	.031

Data are presented as mean±2SD. Significant differences between groups ($P<.05$, Wilcoxon test).
S100B=S100 calcium-binding protein.

Although other studies have attempted to demonstrate that urinary S100B is as an important biomarker as serum S100B and other indicators in the evaluation of asphyxiated newborns, none of them have compared urinary S100B with serum biomarkers.[21,35,37] Our study shows that urinary S100B performed significantly better than both serum S100B and NSE in evaluating outcome in newborns with moderate or severe HIE, and it was the only marker capable of predicting neonatal death. Furthermore, urine samples provide a simpler, cheaper, and less invasive method than blood or cerebrospinal fluid samples and are thus more appropriate for repeated sampling during the monitoring of neuroprotective strategies such as hypothermia. It should be also emphasized that anemia due to repeated blood sampling is common in high-risk newborns.[38] Factors that could complicate or prevent the collection of urine samples in the first hours of life include oliguria following asphyxia or the administration of sedative drugs. This complication was not observed in our study and we were able to monitor all the newborns at the predetermined time points because of the small amount of urine needed (20 μL). Other possible limitations of this study include the fact that 6 newborns died during the first days of life and that we were unable to compare our results with those of CFM, as this was not performed during the first days of life. Further studies with larger samples are needed to support the results and conclusions of this paper.

5. Conclusion

Measurement of urinary S100B during the first days of life is a useful, minimally invasive method for determining severity of brain injury and predicting short-term outcomes, including death, in asphyxiated newborns with clinical characteristics of moderate or severe HIE. Urinary S100B measurement showed higher specificity and sensitivity than serum S100B and NSE measurement and in addition is less invasive for the patient.

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