Increased Plasma Concentrations of Activin A Predict Intraventricular Hemorrhage in Preterm Newborns

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Background: Intraventricular hemorrhage (IVH) is a major cause of neurologic disabilities in preterm newborns. We evaluated the use of plasma activin A concentrations to predict the development of perinatal IVH.

Methods: We measured nucleated erythrocyte (NRBC) counts, plasma activin A, hypoxanthine (Hyp), and xanthine (Xan) in arterial blood samples obtained from 53 preterm infants during the first hour after birth. Cerebral ultrasound was performed within 48 h of birth and repeated at 5- or 6-day intervals until the age of 4 weeks.

Results: Grade I or II IVH was detected during the first 10 days of life in 11 of 53 patients (21%). Activin A, Hyp, and Xan concentrations and NRBC counts were higher in preterm newborns who subsequently developed IVH than in those who did not ($P < 0.0001$, except $P = 0.019$ for Xan). Neonatal activin A was correlated ($P < 0.0001$) with Hyp ($r = 0.95$), Xan ($r = 0.90$), and NRBC count ($r = 0.90$) in newborns without later IVH and in those who developed IVH (Hyp, $r = 0.89$, $P = 0.0002$; Xan, $r = 0.95$, $P < 0.0001$; NRBC count, $r = 0.90$, $P = 0.0002$). At a cutoff of 0.8 µg/L activin A, the sensitivity and specificity were 100% [11 of 11; 95% confidence interval (CI), 71%–100%] and 93% (39 of 42; 95% CI, 81%–98%), and positive and negative predictive values were 79% (95% CI, 61%–100%) and 0% (95% CI, 0%–2%), respectively. The area under the ROC curve was 0.98.

Conclusions: Activin A concentrations at birth are increased in preterm newborns who later develop IVH

and may be useful for early identification of infants with hypoxic-ischemic brain insults who are at high risk for IVH.

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Intraventricular hemorrhage (IVH),[1] the most common variety of cerebral hemorrhage, affects ~15%–20% of preterm infants and therefore is a major concern in their care *(1–3)*. Perinatal hypoxia is an important risk factor in the pathogenesis of IVH because it alters mechanisms that regulate cerebral blood flow and triggers a cascade of biochemical events that begins with a shift from oxidative to anaerobic metabolism and leads to oxidative brain damage resulting from excessive production of free radicals *(4, 5)*.

We investigated the association of IVH (detected by ultrasonographic findings) and biochemical markers of perinatal hypoxia. Because perinatal hypoxia leads to increased absolute nucleated erythrocyte (NRBC) counts *(6)* and plasma concentrations of hypoxanthine (Hyp) *(5, 7)* and xanthine (Xan) *(5, 7)*, [both indicators of oxidative stress *(4, 5)*], we chose these as potential markers. We also measured concentrations of plasma activin A. Activin A is a glycoprotein composed of 2 *β*A subunits that belong to the transforming growth factor-*β* superfamily of differentiation factors and is expressed in the central nervous system *(8)*. Several models of hypoxic–ischemic brain injury have demonstrated induction of activin A in various brain regions *(9–11)*. Moreover, intrauterine hypoxia increases activin A in the fetal circulation of preterm newborns *(12)*, and high activin A concentrations have been found in cerebrospinal fluid *(13)* and urine (unpub-

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[1] Nonstandard abbreviations: IVH, intraventricular hemorrhage; NRBC, nucleated erythrocyte; Hyp, hypoxanthine; Xan, xanthine; CI, confidence interval; and LR, likelihood ratio.

lished) of asphyxiated full-term newborns who experienced brain damage.

Materials and Methods

We evaluated 53 consecutively born preterm infants born at ≤32 weeks of gestation and admitted to Siena University Hospital at the Department of Pediatrics, Obstetrics and Reproductive Medicine between July 2002 and June 2003. We excluded from the study infants with births complicated by congenital malformations, inborn errors of metabolism, blood group incompatibility, sepsis, maternal diabetes, multiple births, or anemia. Vaginal delivery occurred in 28 women; emergency cesarean section was performed in 25 cases for indications including threatened fetal status as defined by the American College of Obstetricians and Gynecologists (bradycardia, fetal heart rate late decelerations, severe and repetitive fetal heart rate variable decelerations, reduced beat-to-beat variability) *(14)*. We administered corticosteroid therapy (intramuscular β-methasone, 12 mg/24 h for 2 days) to 27 women, and 14 of 28 women who delivered vaginally received tocolytic therapy (intravenous ritodrine, 30–50 mg/h). No newborns required delivery by forceps.

Perinatal hypoxia was defined as the presence of at least 2 of the following conditions: intrapartum distress, as indicated by fetal bradycardia (heart rate <100 beats per min, late decelerations, or an absence of heart rate variability), an Apgar score of ≤6 at 5 min, need for resuscitation for >1 min with positive-pressure ventilation and oxygen immediately after birth, and a blood pH ≤7.20 in the umbilical vein *(12)*. The degree of hypoxia was ascertained by measurement of Hyp and Xan concentrations in cord blood.

The study was approved by the Human Ethics Committee of the Medical Faculty, University of Siena, and informed written parental consent was obtained before enrollment of each infant.

LABORATORY TESTS

To measure erythrocyte count, arterial blood pH, ion concentrations, plasma glucose, and base deficit in the newborns during the first hour after birth, we collected arterial blood samples (1 mL) from the umbilical cord into heparin-containing tubes without the use of catheters.

CRANIAL ASSESSMENT

To check for IVH, we performed cerebral ultrasound scanning on newborns, within 48 h of birth, with a real-time ultrasound machine (Interspec Apogee) with a transducer frequency emission of 7.5 mHz and repeated the scan serially at 5- or 6-day intervals until the age of 4 weeks *(15)*. A single neonatologist experienced in ultrasonography and blind to patient history evaluated the neonatal scans. Clinical management and intensive care procedures were the same for all babies. The occurrence of IVH was diagnosed according to Papile et al. *(16)*.

NRBC COUNT ASSESSMENT

We performed a complete blood cell count and a total leukocyte count. NRBC count was expressed as absolute erythroblast count (NRBCs/mm^3) and calculated by light microscopic examination of May-Grunwald-Giemsa–stained blood smears. The blood was immediately centrifuged and subdivided into aliquots for chemical determinations.

MEASUREMENT OF HYP AND XAN

Hyp and Xan in plasma were measured in a blinded fashion within 2 h after blood sampling, to avoid storage effects. After centrifugation, the plasma and buffy coat were removed. Plasma Hyp and Xan concentrations were measured with a Vista 5500 HPLC equipped with a variable-wavelength ultraviolet detector (model 4290; Varian). The analytical system also included a ready-to-use prepacked Supelcosil LC-18 column by Supelco [250 × 4.6 mm (i.d.); 5 μm bead size], with a precolumn [20 × 4.6 mm (i.d.)] filled with the same packing (Supelguard; Supelco). Two solvents, 0.01 mol/L potassiumosphate buffer at pH 5.5 (A) and methanol (B), were used. The mobile phase gradient was 0% B at 0 min, 10% B at 10 min, 20% B at 20 min, and 0% B at 30 min. The next sample was injected 10 min later. The flow rate was 1 mL/min, and the wavelength was 220 nm.

ACTIVIN A ASSAY

Activin A measurements were performed blinded and in duplicate with a specific 2-site enzyme immunoassay from Serotec *(12)*. The analytic detection limit of the activin A assay was <10 ng/L; intra- and interassay CVs were 5.0% and 9.0%, respectively. Cross-reactivities with various inhibin-related proteins were <0.5%.

STATISTICAL ANALYSIS

We used the Kolmogorov–Smirnov test to confirm that the distributions of activin A, Hyp, and Xan concentrations and NRBC counts were not gaussian. We expressed the data as mean (SE) and analyzed for statistically significant differences by unpaired *t*-test. We used Pearson correlations and analyzed the differences between groups for each categorical variable with χ^2 and Fisher exact tests.

Using ROC curves *(17)*, we estimated the probability of developing IVH after having none or 1 positive test (higher than the cutoff point) and compared it with the pretest probability, defined as the prevalence of brain damage in the whole group of newborns *(18)*.

Results

NEONATAL OUTCOMES

Characteristics of preterm newborns are shown in Table 1. Of 53 newborns enrolled, 11 developed grade I or grade II IVH, according to the criteria of Papile et al. *(16)*, in the first 10 days of life. None had ultrasonographic signs of IVH at birth or within 24 h of birth. Infants with and

Table 1. Preterm newborn patient data recorded at admission to the newborn intensive care unit.[a]

	IVH (n = 11)	Controls (n = 42)	P
Gestational age, weeks	28.12 (1.5)	28.39 (0.05)	NS[b]
Birth weight, g	1120.44 (259.16)	1163.31 (109.42)	NS
5-min Apgar score	6.09 (1.44)	8.89 (1.76)	0.0001
Emergency cesarean section, n	9	16	NS
Prenatal corticosteroid therapy, n	9	18	NS
Preeclampsia, n	4	8	NS
Perinatal hypoxia, n	8	6	0.019
Blood pH	7.10 (0.18)	7.29 (0.10)	0.0004
Plasma glucose, g/L	470 (161.3)	462.1 (114.2)	NS
Base excess, mmol/L	−11.67 (9.3)	−5.86 (5.27)	NS
Na^+, mmol/L	134.27 (2.19)	134.89 (2.62)	NS
K^+, mmol/L	4.30 (0.45)	4.51 (0.41)	NS
Ca^{2+}, mmol/L	1.25 (0.12)	1.31 (0.09)	NS
Heart rate, beats/min	148.4 (10.99)	138.5 (8.16)	0.01
Systolic arterial pressure, mmHg	49.55 (6.57)	52.78 (7.08)	NS
Diastolic arterial pressure, mmHg	25.77 (4.52)	29.92 (5.32)	NS

[a] Data are the mean (SE).
[b] NS, not significant ($P > 0.05$).

without IVH differed significantly in Apgar score at 5 min ($P < 0.0001$), blood pH ($P < 0.001$), prevalence of hypoxia ($P = 0.016$), and heart rate ($P < 0.01$; Table 1).

FETAL PLASMA ACTIVIN A, HYP, AND XAN CONCENTRATIONS, AND NRBC COUNT

Activin A, Hyp, and Xan concentrations and NRBC counts were significantly higher in preterm newborns who developed IVH than in those who did not (Fig. 1). Fetal plasma activin A correlated significantly with Hyp ($r = 0.948$; $P < 0.0001$) and Xan ($r = 0.897$; $P < 0.0001$) concentrations and the NRBC count ($r = 0.895$; $P < 0.0001$) in newborns without IVH as well as in those who developed IVH at follow-up (Hyp, $r = 0.890$, $P = 0.0002$; Xan, $r = 0.950$, $P < 0.0001$; NRBC count, $r = 0.897$, $P = 0.0002$; data not shown).

PREDICTION OF IVH IN PRETERM NEWBORNS

Activin A was >0.8 μg/L in 11 of 11 patients with IVH [sensitivity, 100%; 95% confidence interval (CI), 71%–100%] and <0.8 μg/L in 39 of 42 without IVH (sensitivity, 93%; 95% CI, 81%–98%). The area under the ROC curve was 0.98, and the positive and negative likelihood ratios (LRs) were 14 and 0, respectively (Fig. 2 and Table 2).

In contrast, Hyp was >0.8 mg/L in 8 of 11 patients with IVH (sensitivity, 73%; 95% CI, 39%–94%) and <0.8 mg/L in 33 of 42 without IVH (sensitivity, 79%; 95% CI, 63%–90%), with an area under the ROC curve of 0.78, and the positive and negative LRs of 3.39 and 0.35, respectively (Fig. 2 and Table 2).

Xan was >0.99 mg/L in 9 of 11 patients with IVH (sensitivity, 82%; 95% CI, 48%–97.2%) and <0.99 mg/L in 33 of 42 newborns without IVH (sensitivity, 79%; 95% CI, 63%–90%). The area under the ROC curve was 0.76, and

the positive and negative LRs were 3.82 and 0.23, respectively (Fig. 2 and Table 2).

NRBC count at a cutoff of 1529 cells/mm³ showed a sensitivity of 73% in 8 of 11 patients (95% CI, 39%–94%) and a specificity of 83% (35 of 42; 95% CI, 69%–93%) as a single marker of IVH, with an ROC area under the curve of 0.78, and positive and negative LRs of 4.36 and 0.33, respectively (Fig. 2 and Table 2).

The areas under the ROC curve for activin A were significantly higher than those for Hyp ($P = 0.001$; difference between areas, 0.196; 95% CI, 0.1–0.3), Xan ($P = 0.015$; difference between areas, 0.221; 95% CI, 0.04−0.4), and NRBC count ($P = 0.030$; difference between areas, 0.201; 95% CI, 0.02–0.4), as evaluated by pairwise comparison of ROC curves.

Of 53 newborns, 11 had IVH, giving an overall prevalence of 21% (95% CI, 10%–32%), which was the probability of IVH in preterm newborns before measurement of activin A, Hyp, Xan, and NRBC count (pretest probability). If plasma activin A concentrations were high (i.e., above the thresholds defined by the ROC curve analysis), the probability of IVH (positive predictive value) was as high as 79% (95% CI, 61%–100%; Fig. 3), but if activin A concentrations were within the reference interval, that probability was as low as 0% (95% CI, 0%–2%). The probability of predicting IVH by a high Hyp concentration was 67% (95% CI, 36%–98%; Fig. 3), whereas the probability was 9% (95% CI, 1%–17%) for concentrations below the thresholds identified through ROC curve analysis. With respect to Xan concentration and NRBC count, the probabilities of early detection of IVH in the presence of values above the thresholds defined by the ROC curve analysis were 50% (95% CI, 27%–73%) and 53% (95% CI, 28%–78%), respectively (Fig. 3), and 6% (95% CI, 0%–14%)
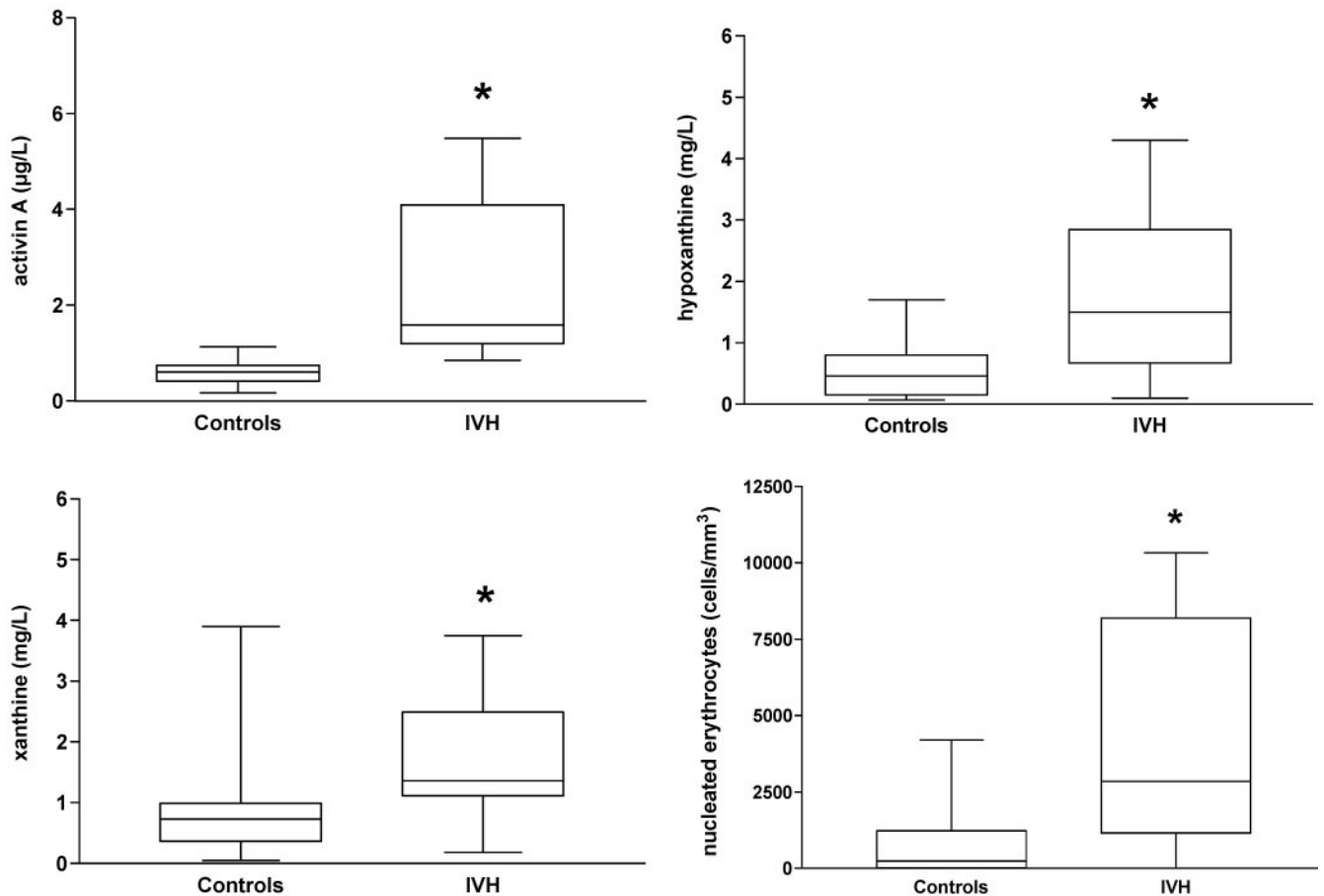
Fig. 1. Plasma concentrations of activin A, Hyp, and Xan and NRBC count in preterm babies with or without IVH at follow-up.

The *boxes* represent the medians (*line inside* each *box*) and interquartile ranges (*limits* of *boxes*). The *whiskers* represent minimum and maximum values. ∗, *P* <0.00001

and 8% (95% CI, 0%–16%), respectively, if concentrations were within the reference intervals.

Discussion

The detection of both βA mRNA and activin protein almost exclusively in neurons adjacent to lesion sites suggests that local activin A production increases in response to neuronal damage (9–11, 19). Furthermore, recent in vivo data have suggested that, in the presence of hypoxia and/or asphyxia, activin A concentrations increase in the central nervous system, as in full-term infants who have suffered asphyxia, later developed brain damage, and were found to have increased activin A concentrations in cerebrospinal fluid (13) and urine (P. Florio et al. High urinary concentrations of activin A in asphyxiated full-term newborns with moderate or severe hypoxic ischemic encephalopathy; submitted for publication). On the other hand, increased activin-A concentrations have been observed in the amniotic fluid of patients who subsequently died of intrauterine fetal hypoxia (20) and in the plasma of hypoxic preterm newborns (12).

These reports and our findings of increased plasma concentrations of activin A in newborns who developed IVH suggest that increased activin A concentrations are a direct indication of increased production in the central nervous system as a response to acute hypoxic neuronal damage. This hypothesis is further reinforced by the observation that similar activin A induction occurs in vitro after hypoxic–ischemic injury and in response to cytotoxic lesions (9–11) and by our finding that activin A, Hyp, and Xan concentrations and NRBC counts, all biochemical markers of hypoxia, were increased in newborns who developed IVH.

Recent studies have revealed the role of activin A as a critical modulator of growth and survival in cytoprotection and tissue repair (21). The neurotrophic function of activin A after hypoxic–ischemic brain injury (11, 22–25) enhances the survival of midbrain and hippocampal neurons (24, 25), decreases ischemic brain injury in infant rats (11), and shields striatal and midbrain neurons against neurotoxic damage (22, 23, 25). These findings and the evidence that activin A prevents apoptosis (26) and inhibits caspase (27), 2 important pathways involved in neuronal death (28), support the hypothesis that oversecretion of activin A in the presence of hypoxia/asphyxia may serve a neuroprotective function, reducing neuronal
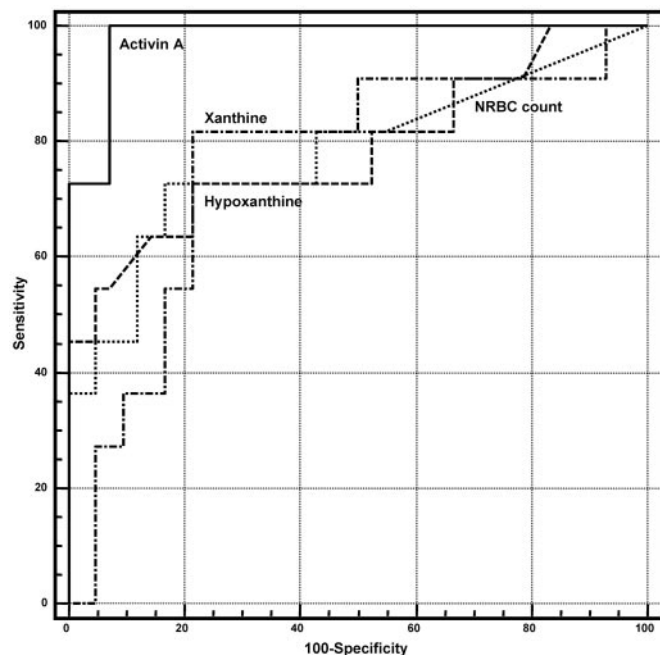
Fig. 2. ROC curve analysis of plasma concentrations of activin A, Hyp, and Xan, and of NRBC count for the prediction of IVH.

Activin A at the cutoff of 0.8 μg/L achieved the best accuracy for prediction of IVH in preterm newborns, showing a sensitivity of 100% (95% CI, 71%–100%) and a specificity of 93% (95% CI, 74%–96%; area under the ROC curve, 0.981). The area under the ROC curve for activin A was significantly higher than those for Hyp ($P = 0.036$), Xan ($P = 0.024$), and NRBC count ($P = 0.037$).

loss from brain injury. Moreover, the evidence that activin A directly potentiates the proliferation and differentiation of erythroid progenitors (29–31) and the present findings of a positive correlation between NRBC count and activin A concentrations suggest a role for activin A in activating erythropoiesis to increase the oxygen supply to the brain after hypoxia (32).

Our study results also suggest that indications of hypoxic–ischemic brain insult and high risk for IVH might be detectable at an earlier stage with activin A measurement than with ultrasound and other diagnostic procedures. We found that activin A showed 100% sensitivity and 88% specificity as a single marker for the prediction of hypoxic–ischemic brain insult in preterm
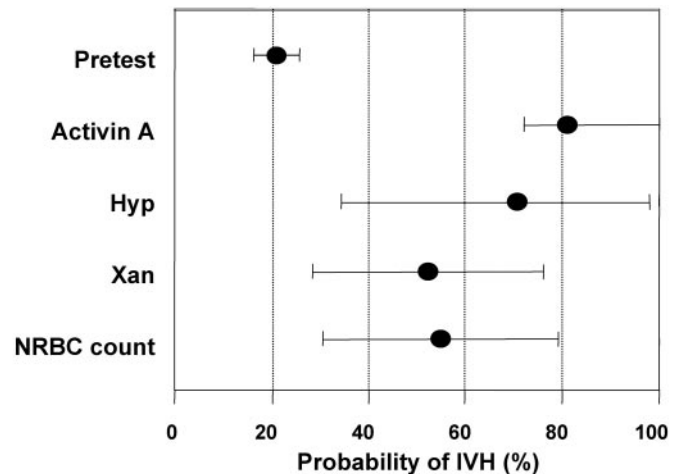

Fig. 3. Probability of developing IVH among all patients (pretest probability) and after different test results (posttest probability).

Data are reported as the percentage, and the *error bars* indicate the 95% CI for each probability.

newborns. Therefore, activin A concentrations above the threshold defined by the ROC curve analysis indicated a probability of IVH as high as 79%, but concentrations with positive and negative predictive values that differed from the pretest probability of the disease in our population (21%) indicated a probability as low as 0%. The high accuracy of activin A might be explained by the strong up-regulation of activin A in the brain within 6 h of injury (11, 19, 33), whereas a longer period is needed for the appearance of nucleated erythrocytes, Hyp, and Ha in blood when fetal Po_2 decreases and oxidative stress is activated (32, 34). In any case, the availability of indicators of subclinical lesions at a time when other monitoring indicators are unable to detect bleeding might allow early recognition of the hemorrhaged lesion, permitting the prevention and/or treatment of clinical neurologic damage. Because of the delay of hours or days between hypoxemic events and detection of the hemorrhagic lesion by conventional sonograms, any increase in activin A might be useful for identifying infants at high risk for hemorrhage before the damage becomes visible, so that

Table 2. Sensitivity, specificity, and predictive values of activin A, Hyp, and Xan concentrations and NRBC count at birth for early detection of IVH in preterm infants.

	Cutoff value	Sensitivity, %	Specificity, %	Positive LR	Negative LR	AUC[a]
Activin A	0.8 μg/L	100	93	14.0	0	0.98[b]
95% CI		71–100	81–98			0.9–1.0
Hyp	0.8 mg/L	73	79	3.39	0.35	0.78
95% CI		39–94	63–90			0.6–0.9
Xan	0.99 mg/L	82	79	3.82	0.23	0.76
95% CI		48–97	63–90			0.6–0.9
NRBC count	1529 cells/mm^3	73	83	4.36	0.33	0.78
95% CI		39–94	69–93			0.6–0.9

[a] AUC, area under the receiver operating characteristic curve.
[a] $P < 0.05$ vs other areas under the curves.

interventions to prevent or lessen the severity of hemorrhage might be initiated as soon as possible.

In conclusion, activin A concentrations are increased in preterm newborns who develop IVH. Thus, activin A measurement might be useful for identification of hypoxic–ischemic brain insult and high risk for IVH in infants before the appearance of related biophysical signs.

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