High Urinary Concentrations of Activin A in Asphyxiated Full-Term Newborns with Moderate or Severe Hypoxic Ischemic Encephalopathy, Pasquale Florio,1 Stefano Luisi,1 Bashir Moataza,2 Michela Torricelli,1 Ibsamer Iman,2 Mofeed Hala,2 Aboulgar Hanna,2 Felice Petraglia,3 and Diego Gazzolo1,2

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Background: Hypoxic ischemic encephalopathy (HIE) is a major cause of permanent neurological disabilities in full-term newborns. We measured activin in urine collected immediately after birth in asphyxiated full-term newborns, and assessed the ability of the measurements to predict the occurrence of perinatal encephalopathy.

Methods: We studied 30 infants with perinatal asphyxia and 30 healthy term neonates at the same gestational age. We recorded routine laboratory variables, cranial assessments by standard cerebral ultrasound, and the presence or absence of neurological abnormalities during the first 7 days after birth. Urinary activin A concentrations were measured at first urination and 12, 24, 48, and 72 h after birth.

Results: Asphyxiated infants were subdivided as follows: group A (n = 18): no or mild HIE with good prognosis and group B (n = 12): moderate or severe HIE with a greater risk of neurological handicap. Activin A concentrations in urine collected at birth (median collection time at first urination <2 h) and at 12, 24, 48, and 72 h from birth were significantly (P < .0001) higher in asphyxiated newborns with moderate or severe HIE (Group B) than in those with absent or mild HIE (group A) and controls. Concentrations did not differ between group A and controls. Activin A concentrations were >0.08 µg/L at first urination in 10 of 12 patients with moderate or severe HIE but in none of 18 patients with no or mild HIE.

Conclusions: Activin A measurements in urine soon after birth may be a promising tool to identify which asphyxiated infants are at risk of neurological sequelae.

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Hypoxic ischemic encephalopathy (HIE) is an important cause of neonatal mortality, and morbidity and permanent neurological disabilities occur in ~25%–28% of asphyxiated infants (AI) (1, 2). Activin A, a glycoprotein expressed in the central nervous system (3) shows increased concentrations after hypoxic-ischemic brain injury (4). An early increase in activin A has been found in newborns with perinatal hypoxia (5) and intraventricular hemorrhage (IVH) (6) and in infants developing HIE (7). We measured activin A in urine collected immediately after birth in AI patients and assessed the potential clinical usefulness of its measurement to predict the occurrence of HIE.

We studied 30 consecutive AI patients born between April 2001 and June 2005. All were delivered by emergency cesarean section because of acute fetal distress, defined according to guidelines of the American College of Obstetricians and Gynecologists (8). Asphyxia was defined as the presence of 3 or more of the following: Apgar score <3 at the 5th minute, pH <7.0, BE ≤ -12 in cord blood, or the need for positive pressure ventilation (>3-min).

Controls were 30 healthy term neonates at the same gestational age delivered consecutively to the case patients either by elective cesarean section (n = 9) or vaginally (n = 21), discharged from the hospitals after 72 h from birth, and fulfilling all of the following criteria: no maternal illness, no signs of fetal distress, pH >7.2 in cord blood or venous blood, and Apgar scores at 1 and 5 min >7. Infants with any malformation, systemic infection, intrauterine growth retardation, or cardiac or hemolytic disease were excluded from the study. Other exclusion criteria were multiple pregnancies; congenital or perinatal infections including chorioamnionitis, and maternal drug addiction, hypertension, or diabetes. Informed consent was obtained from all parents of the patients before inclusion in the study, for which local Human Investigations Committee approval was obtained.

AI patients were mechanically ventilated and sedated with Fentanyl (Fentanest®, Pharmacia and Upjohn®) 0.5–2.5 µg/kg per hour, and Midazolam (Ipnovel®, Roche) 50–400 µg/kg per hour. All the AI patients underwent cerebral ultrasound scanning and neurological examination performed by a single examiner who did not know the results of the urine test.

Standard cerebral ultrasound (Acuson 128SP5) was performed at the time of urine sampling, at 72 h after admission, and on discharge from the hospital. In the controls, cerebral ultrasound patterns were evaluated before discharge from the hospital 72 h after birth. The radiologist who read the sonograms outcomes was blinded to the patient groups (control or asphyxiated).

In the AI patients, the presence within the first 7 days after birth of HIE was classified according to the criteria of Sarnat and Sarnat (9). Electroencephalography was performed in the AI patients within 7 days of birth.

Physical examinations, including neurological examinations, were performed daily at the same times as urine sample collection. We used the Prechtl test to classify neonatal neurological conditions (10, 11), assigning each infant to 1 of 3 diagnostic groups, normal, suspect, or abnormal, in accordance with Jurgens-van der Zee et al. (12).

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Activin A was measured at first urination (time 0, mean collection time 1 h), 12 (time 1), 24 (time 2), 48 (time 3), and 72 (time 4) h of age. In the AI patients, a catheter was inserted into the bladder for urine sampling because of their critical clinical conditions and the effects of sedative drugs. In the controls, urine samples were collected with a standard urine collector (Serenity, Artsana S.p.A) at the indicated time points and at discharge from the nursery. At each time point, 1-mL urine samples collected in the previous hour were immediately centrifuged at 900g for 10 min, and supernatants were stored at −70 °C. Activin A measurements were made by laboratorians blinded with respect to the neurological findings and the group to which the patient belonged; tests were performed in duplicate with a 2-site enzyme immunoassay from Serotec. The detection limit (limit of the blank) was <10 ng/L; intra- and interassay imprecision (CVs) were 2.5% and 3.0%, respectively.

After the Kolmogorov–Smirnov test showed gaussian distributions, data were expressed as means (SE), and the statistical significance was assessed by ANOVA with Tukey test as a post hoc test. Incidences of abnormal cerebral ultrasound and of neurological outcome in patient groups were compared with the 2-tail Fisher exact test. Cutoff points for defining high activin A for prediction of HIE were chosen by ROC analysis (13).

Weight, gestational age, and sex distribution did not differ at birth between AI and control groups (see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol53/issue3).

We divided AI patients into 2 groups according to the occurrence of HIE within the first 7 days after birth; group A (n = 18) with no or mild HIE and good prognosis and group B (n = 12) with moderate or severe HIE and a greater risk of neurological handicap. Apgar scores at the 1st and 5th minutes, pH, PCO2, base excess, and the incidence of acute respiratory distress syndrome in AI patients were significantly different from those in controls (P < 0.001, for all) regardless of the severity of HIE. Acute respiratory distress syndrome incidence did not differ between the 2 asphyxiated subgroups (group A, 7/18; group B, 5/12) (see Tables 1 and 2 in the online Data Supplement).

At birth, periventricular hypechogenicity was observed in 15 of 30 AI patients (group A, n = 8; group B, n = 7; P > 0.05). Cerebral ultrasound patterns did not differ between the 2 asphyxiated subgroups at birth or at 12, 24, and 48 h (P > 0.05 for all). At 72 h, cerebral ultrasound was negative for cerebral bleeding in all but 6 group B infants (2 with middle cerebral artery infarction, 2 with IVH, and 2 with IVH and ventricular dilation). In the controls, cerebral ultrasound patterns were negative for bleeding or other central nervous system diseases.

On admission, 12 of 30 AI patients were classified as suspect at neurological examination (7 with hypotonia or hypertonía, 5 with hyperexcitability). Neurological patterns did not change at the monitoring time points. Urea, creatinine clearance, and urine gravity in the 3 groups were superimposable at different monitoring time points (P > 0.05 for all), and urine volume and output did not differ between AI groups A and B (P > 0.05 for all).

Urine activin A was detectable in all samples and was significantly (P < 0.0001) higher in group B than in group A or controls (Fig. 1). Urine activin A was not related to the type of brain injury in group B. In infants with cerebral bleeding, mean (SE) activin A concentrations were significantly higher than in group A (P < 0.001) and controls (P < 0.001). Excluding infants with cerebral bleeding, urine mean (SE) activin A was still significantly higher in group B [0.19 (0.04) µg/L] than in group A (P < 0.001) and controls (P < 0.001). Finally, no significant difference was found between group A and controls at any collection time (Fig. 1).

Activin A > 0.08 µg/L at first urination had a sensitivity of 83% and a specificity of 100% for predicting the development of moderate or severe HIE. The sensitivity and specificity of measurements obtained between 12 and 72 h were 100% and 98%, respectively (Table 1).

In summary, we found that urine activin A was higher in full-term AI patients who progressed to HIE and that high activin A was related to the development of hypoxic-ischemic lesions. The low molecular weight of activin A (~28-kDa) (14) allowed it to be excreted in urine through a passive mechanism (15), and renal function appeared to be normal or at least not different among the groups in this study. Because activin A is of neuronal origin (3), the present data support the hypothesis that damage to the brain from hypoxia/asphyxia increases activin A in urine. These findings are consistent with the evidence of increased activin A concentrations in newborns with perinatal hypoxia (5), IVH (6), and subsequent HIE (7). Because the protein half-life is estimated to be

![Fig. 1. Activin A (µg/L; expressed as mean and SE) at first urination (time 0, mean collection time 1 h), and at 12 (time 1), 24 (time 2), 45 (time 3), and 72 (time 4) hours in asphyxiated infants with normal (●) and abnormal (○) neurological outcome and in control (□) groups. Mean activin A was significantly higher at all monitoring time-points (P < 0.001) in the asphyxiated group with abnormal neurological outcome.](Image)
∼1 h in the circulation (16), the persistently high urine concentrations of activin A in AI patients who later developed brain damage were likely attributable to the continuous release of the protein from damaged nervous tissue.

Urine activin A was already higher in those infants with adverse neurological outcome than in the asphyxiated group without adverse outcome or in healthy controls at a stage when ultrasound and other diagnostic procedures did not predict outcome. These findings provide support for monitoring activin A in urine at birth for use as a measurable marker of brain injury, offering the possibility of repeated monitoring to evaluate the efficacy of therapeutic measures. Indeed, it has not been known how long the window of opportunity for intervention remains open, but studies on perinatal animals have shown more rapid cell destruction and suggest that the earlier the intervention after perinatal asphyxia insult, the greater the possibility of success (17, 18).

### References


### Table 1. Indices of diagnostic accuracy of activin A for prediction of moderate or severe HIE.

<table>
<thead>
<tr>
<th>Location</th>
<th>AUC (95% CI)*</th>
<th>Activin A Cutoff, µg/L</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>First urination</td>
<td>0.91 (0.81–0.97)</td>
<td>&gt;0.08</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>12 h (1)</td>
<td>0.90 (0.81–0.94)</td>
<td>0.07</td>
<td>81</td>
<td>99</td>
</tr>
<tr>
<td>24 h (2)</td>
<td>0.87 (0.76–0.94)</td>
<td>&gt;0.08</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>48 h (3)</td>
<td>0.92 (0.82–0.97)</td>
<td>&gt;0.05</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>72 h (4)</td>
<td>0.99 (0.92–0.99)</td>
<td>&gt;0.09</td>
<td>92</td>
<td>100</td>
</tr>
</tbody>
</table>

* AUC, area under the receiver operator characteristic (ROC) curve; CI, coefficient interval.

A Simple Method for DNA Isolation from Clotted Blood Extricated Rapidly from Serum Separator Tubes, Steven Se Fum Wong, Jeffrey J. Kuei, Naina Prasad, Etsemaye Agonafer, Gustavo A. Mendoza, Trevor J. Pemberton, and Pragna I. Patel1,2,1 Institute for Genetic Medicine and the 2 Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA; * address correspondence to this author at: Institute for Genetic Medicine, University of Southern California, 2250 Alcazar Street, CSC-240, Los Angeles, CA 90033; fax 323-442-2764, e-mail pragna@usc.edu

**Background:** After clinical laboratory tests have been performed, it can be difficult to obtain DNA without further patient involvement. Although the blood clot remaining within the serum-separation tube after serum collection is a source of DNA, recovery of the clot from the tube is a significant challenge.

**Method:** We devised a method to efficiently remove clotted blood from the serum-separation gel and extract DNA from clotted whole blood samples, obtaining maximum yield of the DNA without DNA contamination by the separation gel. The method involved centrifugation of the sample in the inverted original 10-mL collection tube to displace the separation gel for easy isolation of the blood clot and shearing of the blood clot by centrifugation through a 20-gauge wire mesh cone at 2000g in a swinging-bucket rotor. After erythrocyte lysis and proteinase-K digestion of the fragmented clot, DNA was precipitated with isopropanol in the presence of glycogen.

**Results:** The mean amount of DNA obtained from a 4-mL clotted blood sample prepared by this method was 37.1 µg for clots processed soon after collection, with a reduction to 0.439 µg for clots stored for 1 month before extraction. The quality of the DNA was comparable to that extracted directly from whole blood, and it was found to be suitable for PCR-mediated analysis.