Unbound Bilirubin and Auditory Neuropathy Spectrum Disorder in Late Preterm and Term Infants with Severe Jaundice

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Objective This study evaluates whether unbound bilirubin is a better predictor of auditory neuropathy spectrum disorder (ANSD) than total serum bilirubin (TSB) or the bilirubin:albumin molar ratio (BAMR) in late preterm and term neonates with severe jaundice (TSB ≥20 mg/dL or TSB that met exchange transfusion criteria).

Study design Infants ≥34 weeks' gestation with severe jaundice during the first 2 weeks of life were eligible for the prospective observational study. A comprehensive auditory evaluation was performed within 72 hours of peak TSB. ANSD was defined as absent or abnormal auditory brainstem evoked response waveform morphology at 80-decibel click intensity in the presence of normal outer hair cell function. TSB, serum albumin, and unbound bilirubin were measured using the colorimetric, bromocresol green, and modified peroxidase method, respectively.

Results Five of 44 infants developed ANSD. By logistic regression, peak unbound bilirubin but not peak TSB or peak BAMR was associated with ANSD (OR, 4.6; 95% CI, 1.6-13.5; P = .002). On comparing receiver operating characteristic curves, the area under the curve for unbound bilirubin (0.92) was significantly greater (P = .04) compared with the area under the curve for TSB (0.50) or BAMR (0.62).

Conclusions Unbound bilirubin is a more sensitive and specific predictor of ANSD than TSB or BAMR in late preterm and term infants with severe jaundice. *(J Pediatr 2016;173:84-9)*.

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Chronic bilirubin encephalopathy (CBE), a preventable brain injury resulting from severe jaundice, is common in developing countries and has re-emerged in the US and other developed countries.[1-9] Currently, total serum bilirubin (TSB) is used primarily for the evaluation and management of severe jaundice in neonates, although previous studies have shown that TSB poorly predicts CBE in infants.[10-13] The sensitivity of TSB using a cutoff of ≥20 mg/dL for CBE is high, but its specificity is poor.[14] This poor specificity of TSB results in unnecessary costly treatment of late preterm and term infants with severe jaundice to prevent bilirubin-induced neurotoxicity. Therefore, there is a need to identify a biochemical measure with better predictability than TSB for bilirubin-induced neurotoxicity.

The auditory system is highly sensitive to overt bilirubin-induced neurotoxicity; therefore, auditory evaluation is one of the best noninvasive objective means to evaluate bilirubin-induced neurotoxicity.[15] During the early phase of infant neurodevelopment, when neurologic findings of CBE such as choreoathetoid movements and upward gaze palsy may not be evident, auditory brainstem evoked response (ABR) has been used to evaluate early subtle and transient changes in ABR latencies as a function of hyperbilirubinemia.[16,17] More recently in late preterm and term infants, severe jaundice was associated concomitantly with acute auditory neuropathy spectrum disorder (ANSD), an auditory disorder characterized by normal otoacoustic emission test (OAE), but abnormal or absent ABR.[18,19] OAEs are low-level sounds generated by outer hair cells.[20,21] The ABR is a series of electrical potentials with early waves I and II represent activity of the auditory nerve, and waves III, IV, and V represent activity of brainstem auditory structures.[22,23] In ANSD, the ABR is absent or may show a wave V, but with decreased amplitude and increased latency.[21,24] However, peak TSB concentrations have failed to discriminate infants who develop acute ANSD after severe jaundice.[18]

Emerging evidence suggests that free or unbound bilirubin (bilirubin not bound to albumin) may be a better predictor of bilirubin-induced neurotoxicity than TSB in premature and term infants.[15-17,25-29] However, the usefulness of unbound

AAP	American Academy of Pediatrics
ABR	Auditory brainstem evoked response
ANSD	Auditory neuropathy spectrum disorder
AUC	Area under the curve
BAMR	Bilirubin:albumin molar ratio
CBE	Chronic bilirubin encephalopathy
dB	Decibel
GA	Gestational age
OAE	Otoacoustic emission test
TSB	Total serum bilirubin

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Supported by the National Institutes of Health (K-23 DC006229 and R03HD61084). The authors declare no conflicts of interest.

http://dx.doi.org/10.1016/j.jpeds.2016.02.024

bilirubin as a predictor of bilirubin-induced ANSD has not been investigated using an appropriate comprehensive auditory evaluation soon after the occurrence of severe jaundice in late preterm and term infants. We hypothesized that unbound bilirubin is a more specific and sensitive predictor of bilirubin-induced ANSD than TSB or bilirubin:albumin molar ratio (BAMR). Our objective was to compare TSB, unbound bilirubin, and BAMR as predictors of auditory neuropathy spectrum disorder in late preterm and term infants with severe jaundice.

Methods

This prospective observational study included late preterm and term infants admitted to the University of Rochester Medical Center with severe jaundice. Parental consent was obtained for each subject enrolled. The study was approved by the institutional research review board.

Infants ≥34 weeks gestational age (GA) who had severe jaundice or unconjugated hyperbilirubinemia (TSB ≥20 mg/dL or TSB concentration that met exchange transfusion criteria according to American Academy of Pediatrics [AAP] guidelines) during the first 2 weeks of life were eligible for the study.[10,19,30] Our exclusion criteria included conditions often associated with sensorineural hearing loss[31]: (1) craniofacial malformations; (2) chromosomal disorders; (3) family history of congenital deafness or auditory neuropathy unrelated to prematurity or hyperbilirubinemia; (4) toxoplasmosis, other infections, rubella, cytomegalovirus infection, and herpes simplex infections; and (5) infants requiring surgical interventions at the time of severe jaundice. In addition, infants with failed newborn hearing screening test before readmission for severe jaundice were excluded. For $34^{0/7}$-$34^{6/7}$ weeks' GA infants, TSB concentration that met exchange transfusion criteria was considered the same as that for $35^{0/7}$-$37^{6/7}$ GA infants. GA was assessed by obstetric history, or if obstetric history was unreliable, by Ballard examination.

Bilirubin-Albumin Binding Variables

Blood samples for the measurement of TSB for individual subjects were drawn as clinically indicated at the discretion of the attending neonatologist in amber-colored serum separator tubes to protect from light. TSB concentration was measured (mg/dL, multiply by 17.1 to convert to μmol/L) immediately (in <2 hours) by the clinical chemistry laboratory using the standard colorimetric method. The same aliquot of blood used to measure TSB was used to measure unbound bilirubin concentration (μg/dL, multiply by 17.1 to convert to nmol/L). Unbound bilirubin was measured by the modified peroxidase method at 2 enzyme concentrations (1:25 and 1:12.5 dilutions) of precalibrated peroxidase (Arrows Co, Osaka, Japan) using a US Food and Drug Administration–approved Arrows unbound bilirubin analyzer UA-1 (Arrows Company). Serum albumin level (g/dL, multiply by 151 to convert to μmol/L) was measured for each individual subject using the bromocresol green method. The peak BAMR was calculated for each subject using the peak TSB concentration and the concurrent serum albumin concentration. The peak TSB, peak unbound bilirubin, and peak BAMR were determined for each subject.

Comprehensive Auditory Evaluation

Each subject underwent a comprehensive auditory evaluation (tympanometry, OAE test, and ABR) performed in both ears within 72 hours of peak TSB concentration and after resolution of severe jaundice by an audiologist unaware of the degree of jaundice. ABR tests were recorded with a Biological Navigator Evoked Response System (Bio-logic, Mundelein, Illinois) using 80-decibel (dB) broadband stimulus with the subjects lying supine in the crib and a skin temperature of >35.5°C. The clicks were presented at a repetition rate of 29.9/s and 3 runs of 2000 repetitions were recorded for each ear. The response was amplified (×200 000), and band-pass filtered (100-3000 Hz), and averaged over a 20-ms time epoch. The 2 most reproducible runs for each ear were averaged and used for analysis. ABRs were also performed using reverse polarity (condensation and rarefaction) to evaluate for cochlear microphonics. The OAE test was administered using DP Echoport ILO 292 (Otodynamics, London, England) on all subjects using an 80-dB click stimulus. A normal OAE was defined as a replicable response (3-dB signal to noise ratio) in the 3 highest frequency bands (2000, 3000, and 4000 Hz). Tympanometry was performed in each ear to exclude middle ear disease on all infants. Infants with abnormal ABR morphology or absent ABR waveform but normal OAE or presence of cochlear microphonics were diagnosed with acute ANSD.

Clinical Risk Factors

Clinical risk factors such as perinatal asphyxia (Apgar score of <3 at 5 minutes and/or cord pH < 7.0), congenital sepsis (culture-proven or clinical sepsis requiring ≥7 days of intravenous antibiotics), and hemolytic disorders (Rh incompatibility, ABO incompatibility, glucose-6-phosphate dehydrogenase deficiency, hereditary spherocytosis, etc) were collected prospectively. All subjects were evaluated by the physician for clinical signs and symptoms of acute bilirubin encephalopathy on admission as recommended by the AAP for consideration of immediate exchange transfusion.[30] Phototherapy and exchange transfusion were used as per AAP guidelines.[30] Intravenous gamma globulin was used as per the institutional policy for hemolytic jaundice secondary to ABO or Rh incompatibility.

Sample Size Calculation

Sample size calculation was based on the McNemar test statistic, testing the difference in specificity between the unbound bilirubin and TSB levels; sensitivity was fixed near 100%. From published data, when the empirical sensitivity is fixed at 100%, the specificities for TSB and unbound bilirubin are 0.025 and 0.275, respectively.[27,28] A prevalence of acute ANSD was expected to be ≥10% based on previously

reported studies.[18,19,32] A sample size calculation for specificity with sensitivity of >0.98, a sample size of 40 was required for an 80% power to detect the difference in specificity of 0.25 between TSB and unbound bilirubin at a 2-sided significance level.

Statistical Analyses

All statistical analyses were conducted using STATA 10 (StataCorp, College Station, Texas). Infants' characteristics were summarized with descriptive statistics and compared between those with ANSD and those without ANSD. The Fisher exact test or the χ^2 test was used for categorical variables, and the *t* test or Wilcoxon rank-sum test was used for continuous variables. All analyses were 2-sided at the .05 level of significance. Logistic regression was used to evaluate the independent association between each of the bilirubin albumin binding variables (peak TSB, peak unbound bilirubin, and peak BAMR) and acute bilirubin-induced ANSD. Variables with significant association ($P \leq .2$) to outcome or exposure variables were included in the regression model. Collinearity diagnostics was performed for continuous variables before being included in the regression model. Final model building was performed using a backward selection method. Likelihood ratio tests were performed to evaluate the inclusion of potential confounders in the final logistic regression model. Goodness of fit was evaluated using the Hosmer-Lemeshow test. Potential confounding factors were controlled in each of the final logistic regression models. The strength of associations between peak TSB, peak unbound bilirubin, and peak BAMR and ANSD was evaluated using ORs and 95% CIs.

Receiver operating characteristic curves were plotted for each of the bilirubin-albumin binding variables predicting acute bilirubin-induced ANSD and areas under the curve (AUC) were compared using the nonparametric test.[33]

Results

A total of 55 infants were admitted with severe jaundice over a 6-year period and all infants met study criteria. Of 55 infants, 44 consented and participated in the study. The mean birth weight and GA for the infants were 3287 g (SD 473) and 37.9 weeks (SD 1.8), respectively. There were 23 males (52%). The population was mostly Caucasian (n = 30 [68%]) and non-Hispanic (n = 39 [89%]).

The mean TSB and unbound bilirubin for the infants were 23.2 mg/dL (SD 4.8) and 2.0 μg/dL (SD 1.2), respectively. The mean postnatal day of peak TSB was 3.8 days (SD 2.2). Two infants had hypoalbuminemia (<3 g/dL). None of the infants had a history of in utero drug exposure, perinatal asphyxia, or culture-proven sepsis. All infants received intensive phototherapy. There were 14 infants with hemolytic disorders (7 with ABO incompatibility, 6 with Rh incompatibility, 1 with hereditary spherocytosis). Nine infants received exchange transfusion as per the AAP guidelines. None of the infants required respiratory support during the study period. Blood gases were measured in 7 infants who underwent exchange transfusion. None of these 7 infants had hypoxia (PaO_2 <45 mm Hg) or acidosis (pH <7.25). There were 4 infants with polycythemia (hematocrit >65%), 1 infant with cephalhematoma, and 1 infant with extensive bruising. The majority of infants (91%) received breast milk feeding before the occurrence of severe jaundice. None of the infants received intravenous lipid, ceftriaxone, ibuprofen, or indomethacin during the study period.

Five of 44 infants (11%) had ANSD (4 with absent ABR waveform and 1 with abnormal ABR morphology [absent wave I and III with small wave V]). Of these 5 infants, 2 infants also had clinical signs of acute bilirubin encephalopathy. The demographic and clinical characteristics of infants as a function of ANSD are shown in Table I. There was no difference in GA, birth weight, race, ethnicity, or sex between infants who developed ANSD and infants who did not develop ANSD. There was no difference in maternal chorioamnionitis, mode of delivery, Apgar score at 5 minutes, clinical sepsis, or type of enteral feeding (breast milk or formula) between the 2 groups. There was also no difference in the incidence of hemolytic disorders and polycythemia between the 2 groups. The proportion of infants who had exchange transfusion for severe jaundice was not significantly different between the 2 groups.

There was a significant difference in peak unbound bilirubin concentration, but not in peak TSB concentration and peak BAMR between infants who developed ANSD and infants who did not develop ANSD (Table II). The mean unbound bilirubin concentrations of infants with ANSD were significantly greater than those for infants without ANSD. Three of the 5 infants with ANSD had very low calculated bilirubin albumin binding affinity (<45 L/μmol), a measure of the avidity with which albumin binds

Table I. Demographic and clinical characteristics as a function of ANSD

Characteristics	Infants without ANSD (n = 39)	Infants with ANSD (n = 5)	*P*
GA (wk)*	37.9 ± 1.7	37.3 ± 2.6	.69†
Birth weight (g)*	3295 ± 472	3224 ± 531	.78†
Sex, n (% male)	19 (48)	4 (80)	.16‡
Race, n (% white)	26 (67)	4 (80)	.6‡
Ethnicity, n (% Hispanic)	5 (13)	0 (0)	1‡
Chorioamnionitis, n (%)	1 (2)	0 (0)	1‡
Mode of delivery, n (% cesarean)	7 (18)	1 (20)	1‡
Clinical sepsis, n (%)	2 (5)	1 (20)	.31‡
Apgar score at 5 min, median (IQR)	9 (9-9)	9 (7-9)	.2†
Hemolytic disorders, n (%)	12 (31)	2 (40)	.64‡
Polycythemia, n (%)	3 (7)	1 (20)	.4‡
Breast milk feeding, n (%)	35 (90)	5 (100)	.8‡
Exchange transfusion n (%)	7 (18)	2 (40)	.27

*Mean ± SD.
†Mann-Whitney *U* test.
‡Fisher exact test.

Table II. Bilirubin albumin binding variables and ANSD in late preterm and term infants with severe jaundice

	Infants without ANSD (n = 39)	Infants with ANSD (n = 5)	OR (95% CI)	*P*
Peak TSB (mg/dL)	23.1 (20.9-24.9)	22.3 (20.4-28)	1.01 (0.8-1.2)	.93
Peak BAMR	0.72 (0.64-0.78)	0.72 (0.68-0.83)	10.7 (0.02-39)	.43
Peak unbound bilirubin (μg/dL)	1.66 (1.22-2.27)	4.97 (2.74-5.21)	4.6 (1.6-13.5)	.002

Values are median (IQR).
P values were based on logistic regression analyses predicting ANSD.

bilirubin. The clinical characteristic features of 5 infants with ANSD are shown in Table III.

Because TSB, BAMR, and unbound bilirubin correlated with each other (TSB and BAMR $r = 0.90$, $P = .001$; TSB and unbound bilirubin, $r = 0.38$, $P = .01$; and unbound bilirubin and BAMR, $r = 0.39$, $P = .009$), these bilirubin exposure variables were not included in the same regression model. To account for this, 3 separate regression models were built for each bilirubin biochemical measure, specifically TSB, BAMR, and unbound bilirubin. Among the covariates (Table I), only sex and Apgar score at 5 minutes were included initially in the regression analyses. However, neither sex nor Apgar score at 5 minutes were identified as a confounder by log likelihood ratio tests. Therefore, these variables were not included in the final logistic regression models. There was a strong association between peak unbound bilirubin concentration and ANSD (OR, 4.6; 95% CI, 1.6-13.5) as shown in Table II. There was no significant association between peak TSB and ANSD as well as between peak BAMR and ANSD (Table II).

The receiver operating characteristic curves for TSB, BAMR, and unbound bilirubin as predictors of ANSD are shown in the Figure. There was a significant difference ($P = .04$) in the AUCs between the unbound bilirubin (0.92; 95% CI, 0.80-1.00) and TSB (0.50; 95% CI, 0.14-0.85) as well as between the unbound bilirubin and BAMR (0.62; 95% CI, 0.24-0.88) with unbound bilirubin as the best predictor of ANSD among all 3 exposure variables. Although the AUC for BAMR was greater than the AUC for TSB, the difference was not significant. The sensitivity and specificity of TSB ≥20 mg/dL, a cutoff used to define severe jaundice, was 0.80 and 0.15, respectively for bilirubin-induced ANSD. The unbound bilirubin concentration of ≥2.4 μg/dL was associated with 0.80 sensitivity (95% CI, 0.29-0.99) and 0.80 specificity (95% CI, 0.64-0.91) for bilirubin-induced ANSD in late preterm and term infants. The OR using this cutoff of unbound bilirubin is 15.5 (95% CI, 1.52-158.52). Four of 5 infants with acute ANSD had follow-up audiology evaluation at 2-3 months after the initial evaluation. Of these 4 infants, 2 infants showed improvement and 2 infants had persistent abnormal auditory findings.

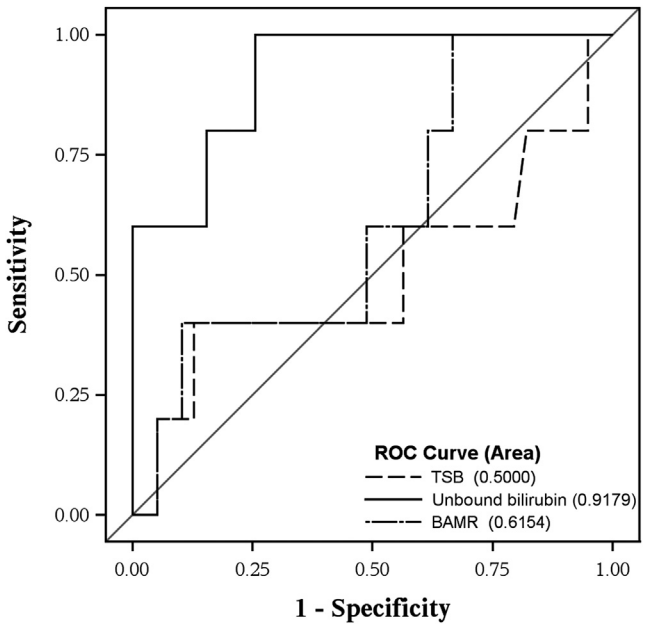

Figure. Receiver operating characteristics (ROC) curves of the peak TSB, peak unbound bilirubin, and peak BAMR as predictors of ANSD are shown. The *straight line* is the expected curve (unity) if the variable has no predictive value (area under unity curve 0.5). The AUCs are unbound bilirubin 0.92, TSB 0.50, and BAMR 0.62. The area under the unbound bilirubin curve is significantly greater than the area under the TSB and BAMR curves.

Table III. Characteristics of individual subjects with ANSD

Subject	GA (wk)	Hemolytic jaundice	Peak TSB (mg/dL)	Peak BAMR	Peak unbound bilirubin (μg/dL)
1	38	No	22.3	0.68	2.74
2	39.5	No	28	0.83	2.1
3	34	No	20.4	0.72	4.97
4	40	Yes	31.4	0.98	5.21
5	35.1	Yes	15.1	0.65	6.07

Discussion

Severe jaundice in late preterm and term infants may be associated with bilirubin-induced neurotoxicity. However, TSB, the primary biochemical measure used for the evaluation and management of severe jaundice, is a poor predictor of bilirubin-induced neurotoxicity, including ANSD. Our findings suggest that unbound bilirubin is a better predictor of ANSD than TSB or BAMR in late preterm and term infants with severe jaundice. This prospective study demonstrates the usefulness of unbound bilirubin as a predictor of acute bilirubin-induced ANSD in late preterm and term infants with severe jaundice.

CBE, characterized by choreoathetoid cerebral palsy, auditory disorders, gaze paresis, and enamel hypoplasia, is a life-long debilitating disorder caused by severe jaundice; therefore, early identification of at-risk infants during the neonatal period is paramount to facilitate intervention and improve the long-term outcome. There is ample evidence that severe jaundice may be associated with ANSD during the neonatal period.[18,19,32,34] Prospective studies have demonstrated the usefulness of comprehensive auditory evaluation during the neonatal period for identification of bilirubin-induced auditory toxicity, specifically ANSD in late preterm and term infants with severe jaundice.[18,19] However, these studies used only TSB as a biochemical measure for its association with ANSD, which failed to discriminate infants at risk for bilirubin-induced auditory toxicity.[18,19]

Our findings of the usefulness of unbound bilirubin as a predictor of bilirubin-induced neurotoxicity are in agreement with most studies in term and preterm infants that evaluated more subtle ABR changes as a function of bilirubin albumin binding variables.[16,17,27-29] In term infants, unbound bilirubin was reported to be a more sensitive and specific predictor of prolonged ABR wave latencies than TSB.[16,17] This was corroborated by Ahlfors and Parker,[28] who reported that unbound bilirubin was a better predictor than TSB of failed hearing screening tests, as evaluated by automated ABR in a retrospective case-control study. However, compared with our study, Ahlfors and Parker used proximate TSB and unbound bilirubin within 4 hours of automated ABR and failed to evaluate usefulness of BAMR. The automated ABR typically involves 35 dB and a failed screening test does not necessarily imply an auditory disorder, specifically ANSD. Compared with previous studies, we used an 80-dB ABR as recommended for the evaluation of ANSD. Similar to findings in term infants, in a prospective study involving premature infants, unbound bilirubin was found to be a more sensitive and specific predictor than TSB or the BAMR of acute abnormal changes in ABR morphology.[29] More recently, unbound bilirubin was more strongly associated than TSB or BAMR with other acute neurologic manifestations such as central apnea in premature infants.[25]

Bilirubin is bound mainly to albumin in blood; therefore, the BAMR, an index of bilirubin binding capacity, is often used in conjunction with TSB for the evaluation and management of severe jaundice.[30] However, BAMR failed to discriminate infants with ANSD and our findings of receiver operating characteristic curves suggest that BAMR does not significantly improve prediction over TSB. This finding is in agreement with a recent study that reported that BAMR does not improve prediction of bilirubin-induced neurotoxicity compared with TSB in term infants with severe jaundice.[35] Our findings of decreased calculated bilirubin albumin-binding affinity explain higher unbound bilirubin in 3 of 5 infants with ANSD and possibly explain failure of the BAMR to better predict ANSD. We did not identify acidosis, hypoxia, or sepsis among these 3 infants, which may explain the decrease in bilirubin binding affinity. We speculate the presence of unknown factors influencing bilirubin albumin binding affinity in these 3 infants who developed ANSD. Nonetheless, until the availability of the technology of unbound bilirubin measurement for clinical use, BAMR should be used in conjunction with TSB for the evaluation and management of severe jaundice as recommended by the AAP.

Despite adherence to the AAP guidelines, 5 infants developed ANSD. The AAP guidelines, which are based on limited evidence, recommend that, for infants readmitted with TSB level above the exchange level, TSB measurement should be repeated every 2-3 hours and exchange transfusion should be considered if the TSB remains greater than the levels indicated after administrating intensive phototherapy for 6 hours.[30] Three of 5 infants who met exchange transfusion criteria did not receive exchange transfusion as they responded to intensive phototherapy within 6 hours as recommended by the consensus based AAP guidelines. Our findings suggest that adherence to AAP guidelines, which are based on TSB, may not prevent bilirubin-induced brain injury completely. Larger studies involving infants with severe jaundice are required to corroborate our findings and inform evidence-based guidelines.

Because the use of hearing screening tests, such as OAE or automated ABR, alone or in combination, has limitations in identifying cases of ANSD,[24,36] all late preterm and term infants with severe jaundice should undergo a comprehensive auditory evaluation to identify infants with ANSD. Identification of children with ANSD is important because their clinical characteristics and treatment differ from other causes of hearing loss in children.[36] A typical child with ANSD presents with difficulty understanding speech that is out of proportion to the audiometric threshold impairment. These children with ANSD are at increased risk for abnormal language development.[37]

The strengths of the study are prospective measurement of biochemical measures, including unbound bilirubin measurement using the modified peroxidase method, and timely and adequate auditory evaluation of infants with severe jaundice. The findings of ANSD were observed despite immediate and appropriate treatment of severe jaundice. Our findings therefore represent more persistent and significant bilirubin-induced neurotoxicity. The weakness of the study is that very few infants had comorbid conditions other than hemolytic disorders that are known to be associated with increased risk of bilirubin-induced neurotoxicity at a lower level of TSB levels. The study also could not evaluate the influence of the duration of severe jaundice as all infants responded with rapid resolution of jaundice with aggressive treatment.

In conclusion, jaundice is associated with ANSD, which is predicted by unbound bilirubin and not TSB or BAMR in late preterm and term infants with severe jaundice. Our findings add to the growing evidence for the usefulness of unbound bilirubin in the evaluation of bilirubin-induced neurotoxicity. Future larger studies are needed to evaluate the incidence and natural course of jaundice-associated ANSD in

late preterm and term infants. A larger study may also help to evaluate the usefulness of unbound bilirubin as a predictor of bilirubin-induced neurotoxicity in the presence of clinical risk factors such as asphyxia, sepsis, and so on. Future studies are also warranted to evaluate the long-term prognostic significance of identifying infants with bilirubin-induced acute ANSD during the neonatal period. ■

We are grateful to the parents, research coordinators, nurses, and laboratory staff members for their help during the conduct of the study.

Submitted for publication Nov 21, 2015; last revision received Jan 22, 2016; accepted Feb 8, 2016.

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