Bilirubin Albumin Binding and Unbound Unconjugated Hyperbilirubinemia in Premature Infants

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Objective To evaluate the associations between unbound bilirubin (UB) and total serum bilirubin (TSB), bilirubin:albumin molar ratio (BAMR), and bilirubin albumin binding affinity (Ka) as a function of gestational age (GA) in infants born at 24-33 weeks GA.

Study design In a prospective observational study, TSB and UB were measured twice daily at least 8 hours apart during the first postnatal week. Serum albumin was measured to calculate BAMR on each day. The highest UB on each day, corresponding TSB, and serum albumin were used to calculate the Ka on each day.

Results For the 166 infants studied, peak UB significantly correlated with concomitant Ka ($r = -0.44$, $P = .001$) but not with concomitant TSB or BAMR after adjusting for GA. On multiple regression analyses, there was a significant association of concomitant Ka (–0.06, 95% CI –0.08 to –0.04, $P = .0001$), but not concomitant TSB or BAMR with peak UB after controlling for GA, birth weight, race, and sex. GA group was a significant effect modifier for the association between Ka and peak UB (0.03, 95% CI 0.02-0.04, $P < .001$). Interaction analyses showed the association between concomitant Ka and peak UB was significant for the 24-30 weeks GA group infants, but not for the 30[1/7]-33 weeks GA group infants.

Conclusions Peak UB was primarily associated with a decrease in binding affinity in infants ≤30 weeks GA. Interventions aimed at improving binding affinity may be important in decreasing the risk of bilirubin-induced neurotoxicity. *(J Pediatr 2018;192:47-52).*

During the first postnatal week, premature infants are at increased risk of bilirubin-induced neurotoxicity at lower concentrations of total serum bilirubin (TSB) than term infants.[1,2] The increased risk for bilirubin-induced neurotoxicity is due to increased susceptibility of premature neuronal cells to bilirubin injury when unbound bilirubin (UB, bilirubin not bound to protein),[3-5] but not bilirubin bound to albumin, crosses the intact blood brain barrier and the neuronal cell membrane.[6] The critical role of UB in the pathogenesis of bilirubin-induced neurotoxicity has been corroborated by several studies demonstrating that peak UB is a better predictor of abnormal neurologic outcomes than TSB in premature and term infants.[5,7-13] Therefore, interventions targeted to prevent or resolve unbound unconjugated hyperbilirubinemia will be important in reducing bilirubin-induced neurotoxicity.[14]

Serum or plasma UB concentrations depend on TSB concentrations, the bilirubin:albumin molar ratio (BAMR, a measure of bilirubin binding capacity of albumin), and the bilirubin-albumin binding affinity (Ka, strength of bilirubin binding to albumin).[3] Although serum albumin levels and bilirubin binding capacities are lower in preterm compared with term infants, there is little information on bilirubin-albumin binding during the first postnatal week as a function of gestational age (GA).[13,15-17] More importantly, little is known about the primary underlying mechanism for unbound unconjugated hyperbilirubinemia as a function of GA in premature infants. Such information is necessary to provide interventions to prevent or resolve unbound hyperbilirubinemia and to decrease the risk of bilirubin-induced neurotoxicity in premature infants. The objective of this study was to evaluate the underlying mechanism of unbound hyperbilirubinemia in infants ≤33 weeks GA and to determine if unbound hyperbilirubinemia during the first postnatal week is associated with an increase in TSB, increase in BAMR, or a decrease in Ka and if these associations are modified by GA.

Methods

This was a prospective observational study involving infants born at 24-33 weeks GA. The study was approved by the local institutional research review board. Informed consent was obtained from the parents of each subject.

AUC	Area under the curve
BAMR	Bilirubin:albumin molar ratio
GA	Gestational age
Ka	Bilirubin albumin binding affinity
TSB	Total serum bilirubin
UB	Unbound bilirubin

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Premature infants born at 24-33 weeks GA and admitted to the neonatal intensive care unit within 12 hours after birth were eligible. GA was assessed by obstetrical dating criteria, or when obstetrical data was inadequate, by Ballard examination. Infants with craniofacial malformations, chromosomal disorders, TORCH infections (toxoplasmosis, rubella, cytomegalovirus, syphilis, herpes, or HIV infections), family history of congenital hearing loss, or conjugated hyperbilirubinemia (direct bilirubin ≥2 mg/dL) during the first postnatal week were excluded. In addition, infants who died within 3 days after birth were excluded.

Unbound Bilirubin and Bilirubin-Albumin Binding

Blood samples to measure TSB (mg/dL) and UB (μg/dL) were drawn twice daily at least 8 hours apart starting at 24 hours after birth and continuing through the first postnatal week. The blood samples were collected in special amber colored serum separator tubes to protect them from light exposure. TSB was measured by the colorimetric method in the clinical chemistry laboratory. The same aliquot of blood was also used to measure UB. Each sample was centrifuged and analyzed immediately for UB or stored in a −80°C freezer for not more than a month before analysis of UB.[18] The validated modified peroxidase test was performed to measure UB using the Food and Drug Administration approved Arrows UB analyzer UA-1 (Arrows Company, Ltd, Osaka Japan) with 2 enzyme concentrations of precalibrated peroxidase (Arrows Reagent Kit, Arrows Company, Ltd).[19] The first order rate constant for the oxidation reaction or the peroxidase enzyme activity of 1 was confirmed using bilirubin standard (0.67 mg/mL) and albumin-free solution (ie, total bilirubin = UB) with each batch of peroxidase enzyme before UB measurement. The samples were analyzed for UB by the same investigator blinded to GA. Serum albumin concentrations (g/dL, multiply by 151 to convert to μmol/L) were measured using the same aliquot of blood using the Bromocresol Green method and used to calculate BAMR. The highest UB on each day, the corresponding TSB, and serum albumin were used to calculate the bilirubin binding affinity (Ka, L/μmol) on each day using the law of mass action equation described in the literature and shown below.[3]

$$Ka = \frac{TSB - UB}{UB\,(Albumin - TSB + UB)}$$

Phototherapy was used according to the institutional guidelines for the management of unconjugated hyperbilirubinemia in premature infants that was based on TSB concentrations, birth weight, and presence of clinical factors that increase the risk for bilirubin-induced neurotoxicity.

Subjects were subgrouped into GA groups based on GA at birth: (1) 24-26 weeks GA; (2) $26^{1/7}$-28 weeks GA; (3) $28^{1/7}$-30 weeks GA; (4) $30^{1/7}$-32 weeks GA, and (5) $32^{1/7}$-33 weeks GA.

Statistical Analyses

Statistical analyses were performed with SAS (v 9.4; SAS Institute Inc, Cary, North Carolina). Longitudinal analyses were performed to evaluate the association between each bilirubin-albumin binding measure (TSB, BAMR, and Ka) and UB across the time points using a linear mixed model. Because peak UB has been associated with bilirubin-induced neurotoxicity, peak UB was used as an outcome variable for further analyses. Partial Pearson correlation coefficients between peak UB and the other bilirubin-albumin binding measures (TSB, BAMR, and Ka) were calculated. Multiple linear regression analyses were carried out to evaluate the association between each bilirubin-albumin binding measure (TSB, BAMR, and Ka) and peak UB after controlling for GA, birth weight, sex, and race. Furthermore, the interaction effects with GA were tested and the associations were examined by each GA group. In addition, receiver operating characteristic curve analyses were used to compare the area under the curves (AUCs) of TSB, BAMR, and Ka for predicting the elevated UB level (upper 75th percentile of peak UB).

Results

A total of 345 infants born at 24-33 weeks GA were admitted to the neonatal intensive care unit over 3 years. Twelve infants (5 with major congenital malformations, 2 with TORCH infections, and 5 infants who died within the first 5 days) met exclusion criteria. A total of 167 infants ≤33 weeks GA were enrolled after obtaining consent. One infant subsequently withdrew from the study. A total of 166 infants ≤33 weeks GA were studied, with the following GA distribution: 24-26 weeks (n = 27); $26^{1/7}$-28 weeks (n = 37); $28^{1/7}$-30 weeks (n = 46); $30^{1/7}$-32 weeks (n = 28); $32^{1/7}$ -33 weeks (n = 28). The mean GA of study subjects was $28^{6/7}$ weeks (range, 24-33). The mean birth weight was 1211 g (range, 450-2595g). The majority of infants were male (n = 85, 51%), Caucasian (n = 105, 63%), and received phototherapy (n = 161, 97%) as per institutional guidelines for the management of unconjugated hyperbilirubinemia.

The daily UB and corresponding bilirubin albumin binding measures: TSB, BAMR, and Ka as a function of GA subgroups during the first postnatal week are shown in **Figure 1**. GA was negatively correlated to UB, but positively correlated to TSB, BAMR, and Ka. For infants 24-30 weeks GA, UB peaked on days 5 and 6 when TSB and BAMR were decreasing. For infants >30 weeks GA, UB peaked on days 3 to 4 and then gradually decreased with decrease in TSB and BAMR by the end of the first postnatal week. The Ka (binding affinity) was variable but decreased during the first postnatal week in infants ≤32 weeks GA, and for infants >32 weeks GA, Ka gradually improved over the first postnatal week. Using longitudinal regression analyses controlling for GA, birth weight, race, and sex, there was a significant association of TSB, BAMR, and Ka with UB across the time points during the first postnatal week ($P = .0001$).

The mean ± SD of peak TSB (mg/dL), peak BAMR, and peak UB (μg/dL) during the study period were 7.6 ± 2.6, 0.29 ± 0.09, and 1.9 ± 2.2, respectively. Peak UB correlated negatively with concomitant Ka (r = −0.44, $P = .001$), controlling for concomitant TSB, BAMR, and GA using a Pearson partial correlation analysis. There was no correlation between peak UB and concomitant TSB (r = 0.016, $P = .84$), controlling for concomi-

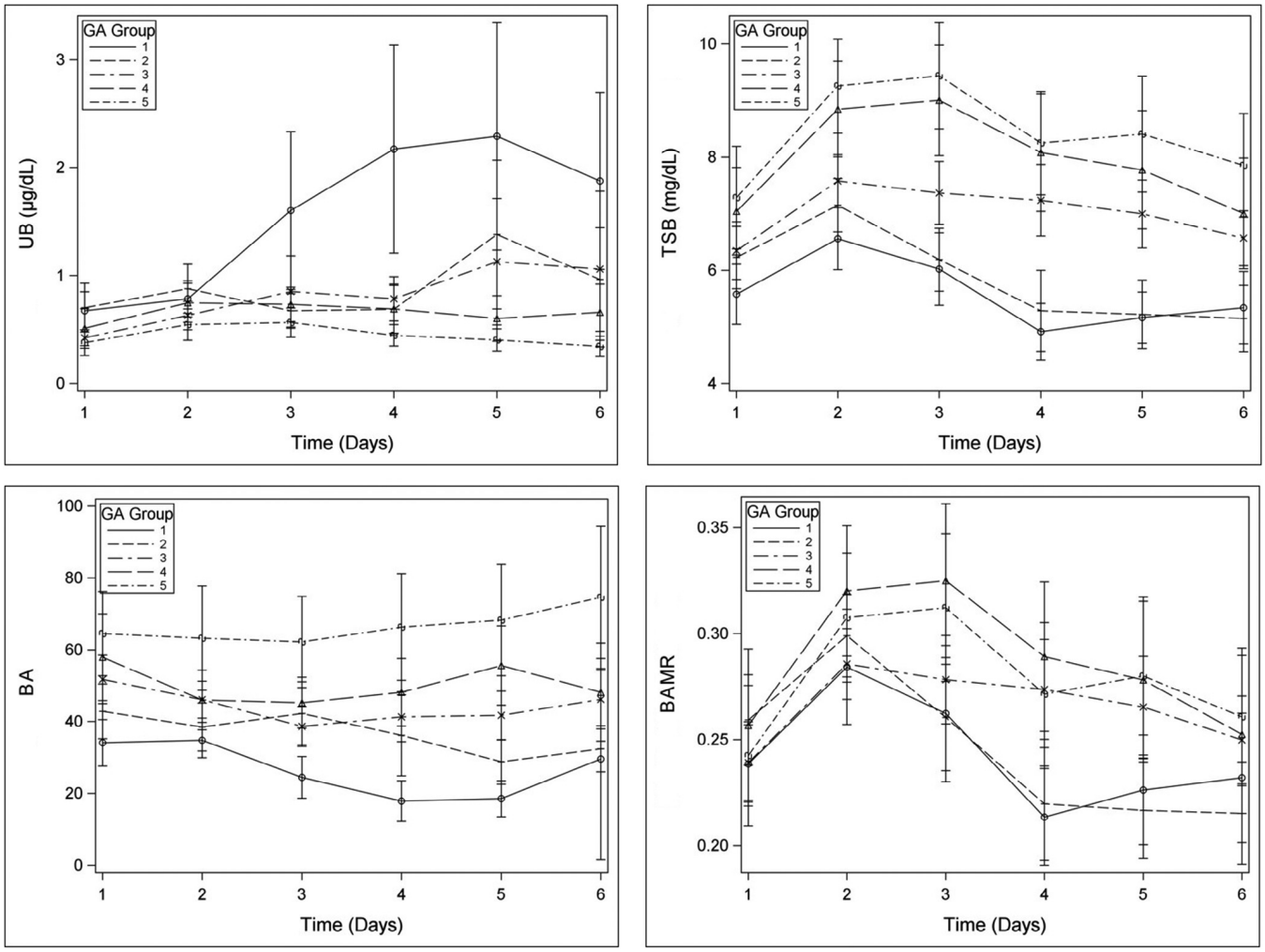

Figure 1. Bilirubin albumin binding and unbound bilirubin during the first postnatal week as a function of gestational age groups. The highest UB, TSB, BAMR, and binding affinity on each day during the first postnatal week as a function of GA groups are shown on separate graphs. The x-axis on each graph represents postnatal days starting at 24 hours after birth. The GA group 24-26 weeks (n = 27) is represented by line 1, GA group $26^{1/7}$-28 weeks (n = 37) is represented by line 2; GA group $28^{1/7}$-30 weeks (n = 46) is represented by line 3; GA group $30^{1/7}$-32 weeks (n = 28) is represented by line 4, and GA group $32^{1/7}$-33 weeks (n = 28) is represented by line 5. There was significant correlation of peak UB with concomitant binding affinity, but not with concomitant TSB or BAMR.

tant Ka, BAMR, and GA. Similarly, peak UB and concomitant BAMR were not correlated (r = 0.051, $P = .52$), controlling for concomitant Ka, TSB, and GA.

On multiple linear regression analysis, GA correlated with peak UB (−0.24, 95% CI −0.49 to −0.007, $P = .04$), controlling for birth weight, race, and sex. Peak UB decreased by 0.24 µg/dL with an increase in GA by 1 week. Peak UB was not associated with birth weight, race, or sex.

TSB was not correlated with peak UB (−0.01, 95% CI −0.18 to 0.15, $P = .88$), controlling for GA, birth weight, race, and sex. Similarly, peak UB was not associated with concomitant BAMR (1.47, 95% CI −2.7 to 5.6, $P = .49$), controlling for GA, birth weight, race, and sex. However, peak UB and concomitant Ka were correlated (−0.06, 95% CI −0.08 to −0.04, $P = .0001$), controlling for GA, birth weight, race, and sex. There was an increase in UB of 0.06 µg/dL with each unit decrease in Ka.

On further analysis by GA subgroups, GA group correlated with peak UB (−0.84, 95% CI −1.29 to −0.39, $P = .0003$), controlling for birth weight, sex, and race. In addition, GA group was evaluated as an effect modifier, and the associations between Ka and peak UB were examined by each GA group. GA group was a significant effect modifier between the association between concomitant Ka and peak UB (0.03, 95% CI 0.02-0.04, $P < .001$) after controlling for birth weight, sex, and race. The association between concomitant Ka and peak UB was significant for the 24-26 weeks GA group (estimate −0.21, $P = .0001$), $26^{1/7}$-28 weeks GA group (estimate −0.11, $P = .0001$) and the $28^{1/7}$-30 weeks GA group (estimate −0.10, $P = .0001$). There was no significant association between concomitant Ka

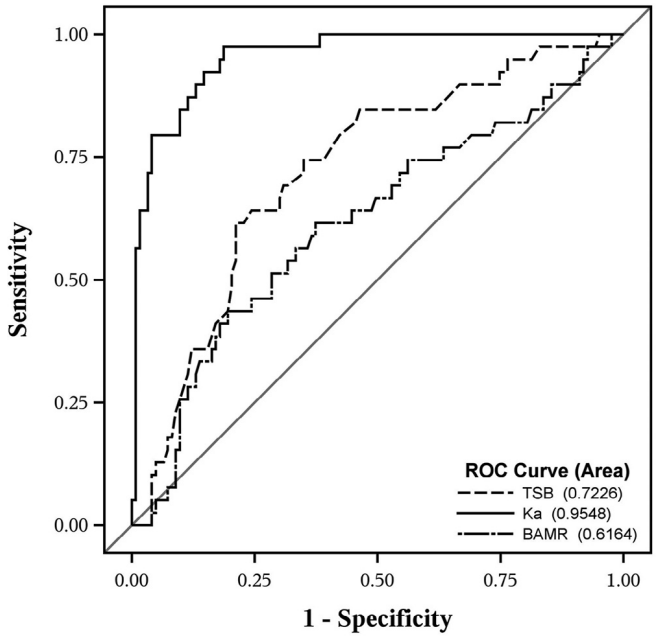

Figure 2. Bilirubin-albumin binding variables as predictors of elevated unbound bilirubin (upper 75% of peak unbound bilirubin) in premature infants. The straight line is the expected curve (unity) if the variable has no predictive value (area under unity curve 0.5). Area under the curve for Ka is significantly greater than the area under the curves for TSB and BAMR. *ROC*, receiver operating characteristic.

and peak UB for the $30^{1/7}$-32 weeks GA group (estimate –0.015, $P = .36$) or for the $32^{1/7}$-33 weeks GA group (estimate –0.01, $P = .59$). GA group did not modify the effects of the association between BAMR and peak UB (1.35, 95% CI –1.58 to 4.29, $P = .36$) or between TSB and peak UB (0.087, 95% CI –0.018 to 0.193, $P = .11$), after controlling for birth weight, sex, and race.

On a receiver operating characteristic curve analysis (**Figure 2**), there was a significant difference in the AUCs between TSB (0.722, 95% CI 0.632-0.813), BAMR (0.616, 95% CI 0.510-0.722), and Ka (0.954, 95% CI: 0.923-0.985) for predicting elevated UB (upper 75th percentile of peak UB). The AUC of Ka was significantly greater than the AUCs for TSB or BAMR ($P = .0001$).

Discussion

According to the "unbound bilirubin theory," UB crosses an intact blood brain barrier and causes neuronal injury.[6] There is now ample supportive evidence for the UB theory from clinical studies that have demonstrated that unbound unconjugated hyperbilirubinemia is associated with bilirubin-induced neurotoxicity in premature and term infants.[5,7-14,20] Furthermore, several of these studies have demonstrated that peak UB is a more specific and sensitive predictor of bilirubin-induced neurotoxicity than peak TSB and peak BAMR in premature and term infants.[7-10,20] The UB concentrations in individual infants depend on bilirubin-albumin binding, which is a function of the concentration of TSB and albumin, and the binding affinity for bilirubin.[3,6] Despite growing evidence for the critical role of UB in the pathogenesis of bilirubin-induced neurotoxicity, the natural course and the underlying mechanism of unbound unconjugated hyperbilirubinemia during the first postnatal week as a function of GA has not been well studied in a large cohort of premature infants.[3,14] Our findings suggest that unbound hyperbilirubinemia during the first postnatal week in infants ≤33 weeks GA is influenced by each of the bilirubin binding measures, including TSB, BAMR, and bilirubin binding affinity. However, more importantly, our findings suggest that the peak UB, the primary determinant of neurotoxicity, is associated primarily with a concomitant decrease in binding affinity and that this association is modified by the degree of prematurity and is seen mainly in premature infants ≤30 weeks GA.

In premature infants, variability in bilirubin binding affinity has been reported previously using the Sephadex gel filtration method.[13] Our study findings also suggest variability in bilirubin binding affinity among premature infants but also improvement in bilirubin binding affinity with increasing GA, indicating a maturational process for binding affinity. Secondly, our findings suggest that bilirubin binding affinity is lower during the first postnatal week for infants ≤30 weeks GA compared with infants >30 weeks GA. Our findings indicate that this reduced bilirubin binding affinity is primarily responsible for peak UB in premature infants ≤30 weeks GA. This is further evident with the uncoupling of the relationship between UB and TSB, specifically, despite decrease in TSB with phototherapy, UB increases in premature infants ≤30 weeks GA. Although the specific reasons for this uncoupling effect or decreased bilirubin binding affinity are unknown, it is likely that this is related to either the presence of risk factors such as sepsis, acidosis, and hypoxia or bilirubin displacers such as free fatty acids.[3,21] UB may increase at the same time that TSB decreases and this may lead to a false sense of security. In addition, other clinical risk factors, both known and unknown, can contribute to an increased risk of bilirubin-induced neurotoxicity in premature infants.[3,15]

Compared with infants ≤30 weeks GA, there was no significant association between binding affinity and peak UB among infants >30 weeks GA. The binding affinity gradually improved with postnatal age during the first week in infants >30 weeks GA. Our findings in older premature infants are in agreement with a previous report on term infants that demonstrated that binding affinity (measured indirectly using the Sephadex gel filtration method) increased as early as the third day and continued to increase, reaching adult serum levels by 5 months of age.[22] The improvement in binding affinity could be due to maturational changes in the ability of albumin to bind bilirubin with postnatal age.

Our findings on the underlying mechanism for unbound unconjugated hyperbilirubinemia may be useful to prevent bilirubin-induced neurotoxicity in premature infants. For most infants >30 weeks GA, interventions such as phototherapy or

heme-oxygenase inhibitors that are aimed to decrease TSB or bilirubin load may suffice to prevent or reduce the degree of UHB and related bilirubin-induced neurotoxicity. However, for infants ≤30 weeks GA, the factors decreasing bilirubin binding affinity need to be investigated further because it is possible that phototherapy as a sole intervention may be insufficient in preventing or reducing bilirubin-induced neurotoxicity. The findings of the only large National Institutes of Child Health and Human Development collaborative randomized trial of phototherapy conducted to date comparing phototherapy treated infants with controls given no phototherapy lend support to our speculation.[23] Phototherapy failed to demonstrate any beneficial effect on long-term neurodevelopmental outcomes despite a substantially lower mean peak TSB among phototherapy treated infants compared with control infants who did not receive phototherapy.[23]

The BAMR is often used as a surrogate measure of bilirubin binding capacity, however, we found no significant correlation or association between peak UB and concomitant BAMR. Our findings are supported by the recent multicenter randomized trial that demonstrated no additional benefit of using BAMR in combination with TSB for the management of hyperbilirubinemia in infants ≤32 weeks GA.[24] Most previous studies also have failed to show significant associations between BAMR and bilirubin-induced neurotoxicity in premature infants.[7,25,26] This lack of association in premature infants reported by previous studies may be due to low prevalence of elevated BAMR or significant hypoalbuminemia (albumin <2 g/dL) in study populations. The importance of significant hypoalbuminemia or elevated BAMR as a risk factor for neurotoxicity in premature infants was shown in a case series of low bilirubin kernicterus based on magnetic resonance imaging findings.[27] All premature infants with low bilirubin kernicterus had modestly elevated TSB (8.7-11.9 mg/dL) in addition to limited bilirubin binding capacity as reflected by significant hypoalbuminemia (serum albumin 1.3-1.9 g/dL) or elevated BAMR >0.47).[27]

The strength of this study is that it was a prospective assessment of serial UB and other bilirubin-biochemical measures evaluated twice daily during the first postnatal week in a large cohort of premature infants. UB was measured by the validated modified peroxidase test using the Food and Drug Administration-approved UB analyzer to minimize underestimation of UB.[28] Although photoisomers resulting from phototherapy may overestimate UB measurement by the peroxidase method, this has not been proven.[3] More importantly no significant change in binding affinity with phototherapy has been reported by prior studies in premature infants.[29]

Our findings suggest that a decrease in binding affinity is a more important contributor to high UB concentration than TSB or BAMR among infants ≤30 weeks GA receiving phototherapy. Therefore, interventions that will prevent or minimize the decrease in binding affinity such as the judicious use of intravenous lipid may be necessary in addition to the use of phototherapy to manage unbound unconjugated hyperbilirubinemia in premature infants ≤30 weeks GA. This also underscores the need for consideration of new guidelines for the management of unbound unconjugated hyperbilirubinemia in premature infants ≤30 weeks GA. The causal association and the significance of our findings need to be confirmed by appropriate interventional trials in order to reduce the risk of bilirubin-induced neurotoxicity in high-risk premature infants. ■

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50 Years Ago in *The Journal of Pediatrics*

Isoniazid Prophylaxis in Tuberculin Reactors

Blattner RJ. J Pediatr 1968;72:131-3

In 1967, the United States had 45 647 cases of tuberculosis, a case rate of 23.1 per 100 000, and <25% of cases occurred among the foreign-born. By 2016 there were 9287 cases and a case rate of 2.9 per 100 000, representing decreases in number and rate of 80% and 87%, respectively[1]; 67.9% occurred among the foreign-born. The epidemiology of tuberculosis in the US has changed dramatically in the past 50 years, and strategies to combat it have evolved.

In the 1960s, the high case rate of tuberculosis infection warranted universal testing of children, often through school-based programs. However, as tuberculosis rates declined, the benefit of universal testing was questioned. Furthermore, the lack of specificity of the tuberculin skin test resulted in many false-positive results caused by exposure to environmental nontuberculous mycobacteria, previous bacille Calmette-Guerin vaccination, and nonspecific reactivity. By the mid-1990s, studies showed that school-based universal testing was no longer efficient or cost-effective.[2,3] The Centers for Disease Control and Prevention and the American Academy of Pediatrics instead promoted targeted testing of high-risk children, the largest group being children who had spent extensive time in countries with a high tuberculosis burden.

Unfortunately, the abandonment of universal school-based testing has left many high-risk children without medical homes vulnerable to developing tuberculosis disease. Recently, a new school-based program used the interferon-γ release assay in combination with education, risk factor assessment, and treatment by a school nurse. This school-based study demonstrated feasibility of targeted testing and treatment in a high-risk population.[4] Maybe we are about to come full circle!

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