Pediatric RESEARCH

REVIEW ARTICLE

Translation Investigation

Bilirubin binding in jaundiced newborns: from bench to bedside?

Charles E. Ahlfors[1], Vinod K. Bhutani[1], Ronald J. Wong[1] and David K. Stevenson[1]

Background: Bilirubin-induced neurologic dysfunction (BIND) is a spectrum of preventable neurological sequelae in jaundiced newborns. Current total plasma bilirubin (B_T) concentration thresholds for phototherapy and/or exchange transfusion poorly predict BIND. Methods: The unbound (free) bilirubin (B_f) measured at these B_T thresholds provides additional information about the risk for BIND. B_f can be readily adapted to clinical use by determining B_f population parameters at current B_T thresholds. These parameters can be established using a plasma bilirubin binding panel (BBP) consisting of B_T, B_f, and two empiric constants, the maximum B_T (B_{Tmax}) and the corresponding equilibrium association bilirubin constant (K). *Results:* B_{Tmax} and K provide the variables needed to accurately estimate B_f at $B_T < B_{Tmax}$ to obtain B_f at threshold B_T in patient samples. Once B_f population parameters are known, the BBP in a newborn can be used to identify poor bilirubin binding (higher B_f at the threshold B_T compared with the population) and increased risk of BIND. Conclusion: The BBP can also be used in jaundice screening to better identify the actual B_T at which intervention would be prudent. The BBP is used with current B_T thresholds to better identify the risk of BIND and whether and when to intervene.

Pediatric Research (2018) 84:494–498; https://doi.org/10.1038/s41390-018-0010-3

Clinicians have known for decades that newborns with hyperbilirubinemia and excessive bilirubin production or poor plasma bilirubin binding have an increased risk of bilirubin-induced neurologic dysfunction (BIND, kernicterus).[1–5] Both conditions increase brain bilirubin exposure, excessive production by increasing the bilirubin load,[4, 6–8] and poor plasma bilirubin binding by increasing the plasma levels of unbound (free) bilirubin (B_f), which is the bilirubin species moving freely between the vascular and extravascular spaces.[1–3, 9–14]

The elevated B_f resulting from poor bilirubin binding forces a greater percentage of the accumulating bilirubin load (bilirubin produced – bilirubin excreted) into the extravascular space, exposing the brain to more bilirubin.[2, 5, 9, 10, 12, 14] The vascular bilirubin level and plasma total bilirubin concentration (B_T) will be correspondingly lower.[1, 3, 9, 10] If Andersen et al.[1] had measured B_f in their premature newborns given daily sulfisoxazole or tetracycline, they would have found it to be about twice as high in those receiving sulfisoxazole versus those receiving tetracycline at any B_T.[9] A greater load of bilirubin was therefore needed in the sulfisoxazole cohort to reach a given B_T e.g., >12 mg/dL,[9] and the increased extravascular bilirubin lead to BIND occurring at B_T well below that at which BIND would ordinarily occur in this population.[9, 15] Clearly, both poor bilirubin binding and the size of the bilirubin load were important factors in determining whether newborns receiving sulfisoxazole actually developed BIND.[3, 9, 14, 16]

The axiom (not hypothesis) emanating from these elderly data is: both B_T and B_f contribute important information about the risk of BIND in hyperbilirubinemic newborns. B_T reflects the size of the bilirubin load, and B_f provides information about the percentage of the bilirubin load to which the brain is exposed at that B_T. Bilirubin binding varies considerably in newborn and adult populations, making it impossible to gauge the B_f from the magnitude of B_T.[9, 10, 16–18]

Currently, the hour-specific B_T is used to assess the size of the bilirubin load,[19] and if coupled with B_f measurements, clinicians would have even more information about the brain bilirubin exposure and the risk of BIND.[16, 20] Recent studies suggest the risk of BIND in term newborns with $B_T \geq 30$ mg/dL is about 10%.[21–23] Since there is considerable uncertainty as to when an exchange transfusion should be initiated in these newborns,[21–25] measuring bilirubin binding would be more helpful in identifying those needing the procedure.

Remarkably, despite the accumulating studies documenting the importance of B_f in assessing the risk of BIND,[26–34] bilirubin binding has not found its way to the bedside except in Japan,[35] where B_T and B_f are used routinely and successfully for many years.[30–33] Elsewhere, B_T alone guides treatment with phototherapy and/or exchange transfusion using B_T thresholds derived from ever-evolving clinical experience and expert consensus.[36–38] Unfortunately, this approach has resulted in excessive treatment, erratic clinician compliance, and considerable confusion without eliminating BIND.[39–53] Adding bilirubin binding to the clinical armamentarium is unlikely to resolve all these issues, but it will certainly offer some improvements for the current situation.

[1]Department of Pediatrics Division of Neonatal and Developmental Medicine, Stanford University School of Medicine, Stanford, CA, USA
Correspondence: Charles E. Ahlfors (ligand@centurytel.net)

Received: 14 October 2017 Revised: 23 February 2018 Accepted: 22 March 2018
Published online: 2 July 2018

Table 1. Bilirubin binding variables[54, 55, 64]

Variable, units	Parameter	Comments, relationships	Test
B_T, mg/dL	Total plasma bilirubin (≅bound bilirubin)	$B_T - B_f \approx B_T$	CL HRP
B_f, μg/dL	Unbound (free) bilirubin		HRP FP
A_T, g/dL	Plasma albumin	1 g of albumin = 8.8 mg bilirubin (mole/mole)	CL
BBC, mg/dL	B_T at which ALL plasma bilirubin binding sites are occupied	Determined by titrating sample with bilirubin[69]	No Test
HMF 'BBC', mg/dL	Concentration of binding sites where bound bilirubin fluoresces		HMF
B_{Tmax}, mg/dL	Empiric mass action binding constant	Used with K to calculate B_f at $B_T < B_{Tmax}$	HRP
K, dL/μg	Equilibrium association binding constant for B_{Tmax}	$K = \frac{B_T}{B_f(B_{Tmax} - B_T)}$	Calculated

CL clinical laboratory, *HRP* peroxidase test, *HMF* hematofluorometry jematofluorometry, *FP* fluorescent probe

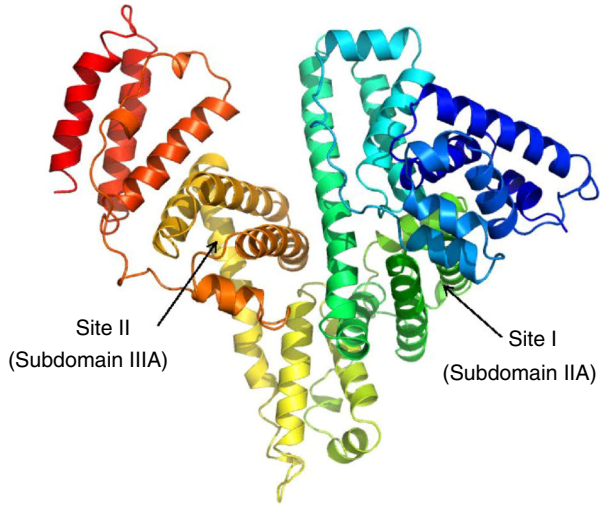

Fig. 1 Schematic of the albumin molecule showing two bilirubin binding sites. Bilirubin bound to site I fluoresces and its equilibrium association constant (K_{hi}) is about 10-fold more tightly than site II[69]

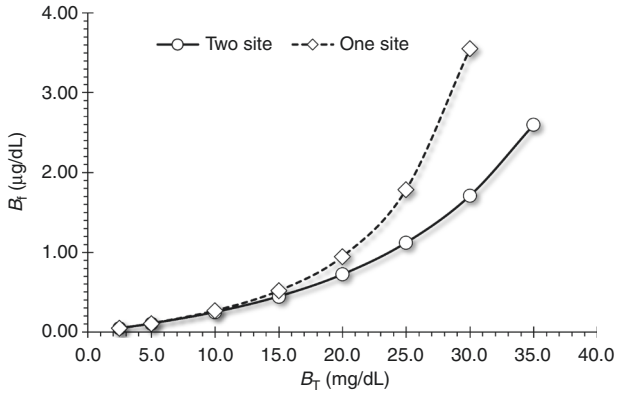

Fig. 2 The change in B_f as B_T increases at an albumin concentration of 3.5 g/dL and assuming a single binding site with $K_{hi} = 1.71$ dL/μg (100 L/μmol) or two binding sites with $K_{hi} = 1.71$ dL/μg and $K_{lo} = 0.171$ dL/μg (see Fig. 1). The individual points were calculated using standard binding equations.[62, 63] The lower affinity binding site significantly impacts the change in B_f over the clinically relevant range of B_T

The clinical use of bilirubin binding has been effectively blocked because clinicians have been asking the wrong questions. B_T was the original and only clinical laboratory (CL) test available for quantifying newborn jaundice, and it was several years before clinically suitable 'bilirubin binding tests' were developed.[5, 54–56] Clinicians then asked for proof that the 'binding tests' were superior to B_T as predictors of BIND, which was not forthcoming.[57–59] The better question would have been 'How well does a jaundiced newborn who has reached a threshold B_T bind bilirubin?' That question provides a natural pathway for bringing bilirubin binding to the bedside that is not dependent on problematic prospective studies comparing how well 'binding tests' and B_T predict BIND.

In this Review, we argue that the key to bringing bilirubin binding safely and expeditiously to the bedside is to determine the population parameters for B_f at current B_T thresholds. The B_f population data will serve as the reference metric[60] for quantifying how well a jaundiced newborn binds bilirubin as an additional factor in determining their risk of BIND, irrespective of whether the incidence of BIND is known. B_f population parameters at threshold B_T levels can be obtained by using a bilirubin binding panel (BBP) to measure bilirubin binding in about 400 patients from the population of interest.[20, 61] In our paradigm, both B_T and B_f are included in the BBP.

Two additional variables are needed in the BBP, the maximum B_T (B_{Tmax}) and its corresponding equilibrium association constant (K). B_{Tmax} is an empirical mass action upper limit of B_T in a sample that is used with K to calculate B_f at any $B_T < B_{Tmax}$ (Equation 1). B_{Tmax} and K are necessary to calculate B_f at the threshold B_T level since the B_T of a sample will rarely be the threshold B_T. It is important to note that B_{Tmax} is not the plasma bilirubin binding capacity (BBC), i.e., the B_T level at which ALL the plasma bilirubin binding sites are occupied. There are no tests for determining BBC although the term continues to be mistakenly applied to 'bilirubin binding test' endpoints.[17, 55] The BBP variables and other binding measurements are summarized in Table. 1.

The mass action chemical and mathematical relationships hematofluometry, not jematofluometry, could not get system to eliminate J and add H between the BBP components (B_T, B_f, B_{Tmax}, and K) are shown below.[62, 63] B_T is the bound bilirubin concentration, since B_f is orders of magnitude less than B_T (μg/dL versus mg/dL) and bound bilirubin = $B_T - B_f \cong B_T$. The unoccupied binding sites in the derivation of Equation 1 are $B_{Tmax} - B_T$.[63]

$$(B_{Tmax} - B_T) + B_f \underset{\leftrightarrows}{K} B_T$$
$$K = \frac{BT}{B_f(B_{Tmax} - B_T)} \quad (1)$$

A practical issue is how the BBP variables are obtained. B_T has long been measured by the clinical laboratory (CL), and clinically suitable methods for measuring B_f are available.[35, 54, 64] K is calculated from B_T, B_f, and B_{Tmax} using Equation 1. B_{Tmax}, however, is a 'new' variable introduced herein to avoid the long-standing misconception that albumin has only one bilirubin binding site that is clinically relevant.[17, 55, 64–68] The advantage of using the 'single-site' model is that the simplest mass action equations apply (e.g., Equation 1), and measured variables such as the concentrations of albumin or albumin-bound bilirubin that fluoresces[55, 68]

Table 2. Summary of bilirubin binding measurements before and after sample bilirubin enrichment in 110 samples from 72 newborns 24 to 34 weeks' GA (median 30 weeks) with birthweights 0.483–2.490 kg (median 1.305 kg). The B/A_T molar ratios varied from 0.10 to 0.76 and 0.31 to 1.45 before and after sample bilirubin enrichment, respectively

	B_T[a] (mg/dL)	CL A_T (g/dL)	HMF[b] 'BBC' (mg/dL)	HRP B_f (μg/dL)	Enriched (HRP)	
					B_T (mg/dL)	B_f (μg/dL)
Mean	7.4	2.4	24.5	0.46	14.3	1.79
SD	2.6	0.3	5.8	0.28	4.0	1.55
Range	1.7−15.7	1.5−3.2	11.7−42.5	0.06−1.66	6.3−23.2	0.42−10.6
Median	7.4	2.4	24.1	0.38	13.7	1.28
25th	5.8	2.2	20.7	0.28	11.2	0.93
75th	9.0	2.7	27.5	0.59	17.3	1.95

[a]Average of CL B_T and HRP B_T before bilirubin enrichment.
[b]The instrument readout is 'BBC' referring to the maximum concentration of bound bilirubin that fluoresces, not the plasma BBC

The inaccuracy of the 'single-site' model in predicting B_f at non-sample B_T is illustrated using bilirubin binding data from 72 newborns < 35 weeks' GA.[70] Table 2 contains a summary of the data before and after sample bilirubin enrichment. The concentration of binding sites where the bound bilirubin fluoresces (HMF 'BBC') was measured using hematofluorometry (HMF).[55] Table 3 shows the B_f predicted using the CL albumin (A_T) or the HMF 'BBC' as B_{Tmax} in Equation 1, with the corresponding K's calculated using the sample B_f before bilirubin enrichment. As expected from Fig. 2, both predicted higher average B_f at the enriched B_T compared with the measured B_f, and B_f could not be calculated in several samples because the enriched B_T exceeded the B_{Tmax} determined using the CL A_T or the HMF test.

An empirical B_{Tmax} that does not require assumptions about the actual bilirubin binding sites and will provide accurate estimates of B_f at non-sample $B_T < B_{Tmax}$ can be readily obtained from the B_T and B_f measured before and after sample bilirubin enrichment (B_{T1}, B_{f1} and B_{T2}, B_{f2}). B_{Tmax} and K in Equation 1 are both considered unknowns, and Equation 1 is solved for B_{Tmax} using Equation 2 (70). Enriching samples with bilirubin to B_T above the relevant B_T thresholds and re-measuring B_T and B_f to obtain B_{Tmax} and K for accurate estimates of B_f at threshold $B_T < B_{Tmax}$ will not require substantially more sample volume or time for testing.[17, 54, 71]

Table 3. B_f calculated at the enriched B_T for the 110 samples in Table 2 using Equation 1 and B_{Tmax} estimated using either the CL A_T ($8.8 \times A_T$ in g/dL) or the HMF 'BBC' and their corresponding K's determined using Equation 1

	CL B_{Tmax} mg/dL	CL K dL/μg	CL B_f μg/dL	[a]HMF 'BBC' mg/dL	HMF KdL/μg	HMF B_f μg/dL
n	97	97	97	100	100	100
Mean	22.0	1.47	2.54[b]	24.9	1.25	1.97[c]
SD	2.4	0.60	5.63	5.7	0.54	3.29
Range	16.7–28.2	0.38–3.02	0.19–3.77	11.7–42.5	0.33–3.12	0.19–25.8

[a]BBC = B_{Tmax} = concentration of binding sites where bound bilirubin fluoresces
[b]Paired *t*-test $p = 0.035$ with corresponding 97 measured B_f (mean = 1.45 ± 0.98 μg/dL)
[c]Paired *t*-test $p = 0.13$ with corresponding 100 measured B_f (mean = 1.58 ± 1.13 μg/dL)

Table 4. Hyperbilirubinemia treatment guidelines for newborns <35 weeks' GA[38]

Gestational age (weeks)	B_T (mg/dL)	
	Phototherapy	Exchange transfusion
$<28^{0/7}$	5–6	11–14
$28–29^{6/7}$	6–8	12–14
$30–31^{6/7}$	8–10	13–16
$32–33^{6/7}$	10–12	15–18
$34–34^{6/7}$	12–14	17–19

$$B_{Tmax} = \frac{B_{T1}B_{T2}(B_{f2}-B_{f1})}{B_{T1}B_{f2} - B_{T2}-B_{f1}} \quad (2)$$

Once B_f population parameters are available, the BBP can be used to help determine how best to treat a newborn that has reached or exceeded a B_T threshold and also as a screening tool[72] to individualize the threshold B_T as warranted. Table 4 shows current B_T threshold guidelines for phototherapy and exchange transfusion in newborns <35 weeks' GA. Consider a clinician (armed with bilirubin binding) confronting a <28-week GA newborn that has reached the phototherapy B_T threshold of 5 mg/dL. Assume the median B_f for the population at $B_T = 5$ mg/dL is 0.30 μg/dL with the 25th percentile 0.15 μg/dL and the 75th percentile 0.45 μg/dL. If the newborn's B_f is >0.30 μg/dL phototherapy is indicated and if it is >0.45 μg/dL there is considerable cause for concern as the binding is 'poor' relative to the population. If the median B_f for the population at the threshold exchange transfusion B_T were 1.0 μg/dL,[34] B_{Tmax} and K from the newborn's BBP and the B_f of 1.0 μg/dL can be used in Equation 1 to calculate the actual B_T at which the B_f would reach 1.0 μg/dL. This approach provides considerably more information about the risk of BIND even though the actual risk is unknown and individualizes care.

It is important to note that B_f varies with the local environment, temperature, sample dilution, GA, illness, and the presence of endogenous or exogenous ligands competing for bilirubin

can be designated as the 'BBC' and substituted for B_{Tmax} in Equation 1(17,55,64-68). Unfortunately, Equation 1 will not provide accurate estimates of B_f at non-sample B_T using the 'BBC' and corresponding K provided by these other methods.

Albumin has at least two bilirubin binding sites as shown in Fig. 1 .[69] The ~10-fold difference in their affinity for bilirubin as quantified by their respective association constants, $K_{hi} > 10{\cdot}K_{lo}$[62, 63, 69] is used to justify the assumption that only the high affinity site where bound bilirubin also fluoresces is clinically relevant.[18, 54, 67] Fig. 2 shows that both sites will significantly impact the change in B_f as B_T increases, invalidating the assumption that only the high affinity site is clinically relevant.

binding sites.[9, 10, 14, 17, 73–80] Therefore, comparisons of outcome and B_f across institutions will require measuring B_f under standard conditions. B_{Tmax} and K, although empirical with respect to specific bilirubin binding sites, are critical binding components required for reliable determination of B_f at relevant threshold B_T. The use of the BBP will individualize care and as patient data accumulates may help better clarify the relationships between neonatal bilirubin exposure and BIND. In the meantime, the BBP can provide additional guidance for the clinicians caring for jaundiced newborns.

SUMMARY

BIND is a spectrum of neurological disorders affecting jaundiced newborns and unpredictably related to the magnitude of B_T. The BBP (B_T, B_f, B_{Tmax}, and K) can provide important additional information about the risk of developing BIND and the need for treatment at threshold B_T, irrespective of whether the actual incidence of BIND at or above that B_T is known. The BBP components can be obtained using minimal modification of well described, clinically suitable methods, and when configured as described in this Review, provide accurate estimations of B_f at $B_T < B_{Tmax}$. The BBP can be used to establish B_f population parameters at current threshold B_T that are the metric for quantifying how well a newborn binds bilirubin, which provides important additional information the risk of BIND and need for intervention The BBP is useful as a screening tool as well as for determining the urgency of treatment in newborns reaching or exceeding threshold B_T. This approach to the clinical of use of bilirubin binding augments current clinical practice within the context of established B_T thresholds and helps individualize care.

ACKNOWLEDGEMENTS

We would like to thank our clinical research coordinators, Martin E. Castillo Cuadrado and Cynthia Montiel, for all their efforts in enrolling subjects and for their technical support. This work was supported, in part, by the Mary L Johnson Research Fund and the Christopher Hess Research Fund.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. Andersen, D. H., Blanc, W. A., Crozier, D. N. & Silverman, W. A. A difference in mortality rate and incidence of kernicterus among premature infants allotted to two prophylactic antibacterial regimens. *Pediatrics* **18**, 614–625 (1956).
2. Blanc, W. A. & Johnson, L. Studies on kernicterus; relationship with sulfonamide intoxication, report on kernicterus in rats with glucuronyl transferase deficiency and review of pathogenesis. *J. Neuropathol. Exp. Neurol.* **18**, 165–187 (1959).
3. Harris, R. C., Lucey, J. F. & Maclean, J. R. Kernicterus in premature infants associated with low concentrations of bilirubin in the plasma. *Pediatrics* **21**, 875–884 (1958).
4. Hsia, D. Y., Allen, F. H. Jr., Gellis, S. S. & Diamond, L. K. Erythroblastosis fetalis. VIII. Studies of serum bilirubin in relation to Kernicterus. *N. Engl. J. Med* **247**, 668–671 (1952).
5. Odell, G. B. Studies in kernicterus. I. The protein binding of bilirubin. *J. Clin. Invest* **38**, 823–833 (1959).
6. Maisels, M. J., Pathak, A., Nelson, N. M., Nathan, D. G. & Smith, C. A. Endogenous production of carbon monoxide in normal and erythroblastotic newborn infants. *J. Clin. Invest* **50**, 1–8 (1971).
7. Maisels, M. J., Pathak, A. & Nelson, N. M. The effect of exchange transfusion on endogenous carbon monoxide production in erythroblastotic infants. *J. Pediatr.* **81**, 705–709 (1972).
8. Stevenson, D. K. et al. Prediction of hyperbilirubinemia in near-term and term infants. *Pediatrics* **108**, 31–39 (2001).
9. Ahlfors, C. E. Unbound bilirubin associated with kernicterus: a historical approach. *J. Pediatr.* **137**, 540–544 (2000).
10. Ahlfors, C. E. Benzyl alcohol, kernicterus, and unbound bilirubin. *J. Pediatr.* **139**, 317–319 (2001).
11. Bratlid, D. How bilirubin gets into the brain. *Clin. Perinatol.* **17**, 449–465 (1990).
12. Davis, D. R. & Yeary, R. A. Effects of sulfadimethoxine on tissue distribution of (14C)bilirubin in the newborn and adult hyperbilirubinemic Gunn rats. *Pediatr. Res.* **9**, 846–850 (1975).
13. Diamond, I. & Schmid, R. Experimental bilirubin encephalopathy. The mode of entry of bilirubin-14C into the central nervous system. *J. Clin. Invest* **45**, 678–689 (1966).
14. Oie, S. & Levy, G. Effect of sulfisoxazole on pharmacokinetics of free and plasma protein-bound bilirubin in experimental unconjugated hyperbilirubinemia. *J. Pharm. Sci.* **68**, 6–9 (1979).
15. Crosse, V. M., Meyer, T. C. & Gerrard, J. W. Kernicterus and prematurity. *Arch. Dis. Child* **30**, 501–508 (1955).
16. Ahlfors, C. E. & Parker, A. E. Bilirubin binding contributes to the increase in total bilirubin concentration in newborns with jaundice. *Pediatrics* **126**, e639–e643 (2010).
17. Bender, G. J., Cashore, W. J. & Oh, W. Ontogeny of bilirubin-binding capacity and the effect of clinical status in premature infants born at less than 1300 grams. *Pediatrics* **120**, 1067–1073 (2007).
18. Lorey, F. W., Ahlfors, C. E., Smith, D. G. & Neel, J. V. Bilirubin binding by variant albumins in Yanomama Indians. *Am. J. Hum. Genet* **36**, 1112–1120 (1984).
19. Bhutani, V. K., Johnson, L. & Sivieri, E. M. Predictive ability of a predischarge hour-specific serum bilirubin for subsequent significant hyperbilirubinemia in healthy term and near-term newborns. *Pediatrics* **103**, 6–14 (1999).
20. Ahlfors C. E. The bilirubin binding panel: a Henderson–Hasselbalch approach to neonatal hyperbilirubinemia. Pediatrics **138**, e20154378 (2016).
21. Kuzniewicz, M. W. et al. Incidence, etiology, and outcomes of hazardous hyperbilirubinemia in newborns. *Pediatrics* **134**, 504–509 (2014).
22. Wickremasinghe, A. C. et al. Risk of sensorineural hearing loss and bilirubin exchange transfusion thresholds. *Pediatrics* **136**, 505–512 (2015).
23. Wu, Y. W. et al. Risk for cerebral palsy in infants with total serum bilirubin levels at or above the exchange transfusion threshold: a population-based study. *JAMA Pediatr.* **169**, 239–246 (2015).
24. Hansen, T. W. Acute management of extreme neonatal jaundice—the potential benefits of intensified phototherapy and interruption of enterohepatic bilirubin circulation. *Acta Paediatr.* **86**, 843–846 (1997).
25. Hansen, T. W. The role of phototherapy in the crash-cart approach to extreme neonatal jaundice. *Semin. Perinatol.* **35**, 171–174 (2011).
26. Ahlfors, C. E., Amin, S. B. & Parker, A. E. Unbound bilirubin predicts abnormal automated auditory brainstem response in a diverse newborn population. *J. Perinatol.* **29**, 305–309 (2009).
27. Amin, S. B. et al. Auditory toxicity in late preterm and term neonates with severe jaundice. *Dev. Med. Child Neurol.* **59**, 297–303 (2017).
28. Amin S. B., et al. Chronic auditory toxicity in late preterm and term infants with significant hyperbilirubinemia. Pediatrics **140**, e20164009 (2017).
29. Cashore, W. J. & Oh, W. Unbound bilirubin and kernicterus in low-birth-weight infants. *Pediatrics* **69**, 481–485 (1982).
30. Funato, M., Tamai, H., Shimada, S. & Nakamura, H. Vigintiphobia, unbound bilirubin, and auditory brainstem responses. *Pediatrics* **93**, 50–53 (1994).
31. Ichinomiya, K. et al. Problems with using total serum bilirubin as a criterion for phototherapy in extremely low-birthweight infants. *Pediatr. Int.* **56**, 731–734 (2014).
32. Morioka, I. et al. Serum unbound bilirubin as a predictor for clinical kernicterus in extremely low birth weight infants at a late age in the neonatal intensive care unit. *Brain Dev.* **37**, 753–757 (2015).
33. Nakamura, H., Yonetani, M., Uetani, Y., Funato, M. & Lee, Y. Determination of serum unbound bilirubin for prediction of kernicterus in low birthweight infants. *Acta Paediatr. Jpn.* **34**, 642–647 (1992).
34. Ritter, D. A., Kenny, J. D., Norton, H. J. & Rudolph, A. J. A prospective study of free bilirubin and other risk factors in the development of kernicterus in premature infants. *Pediatrics* **69**, 260–266 (1982).
35. Shimabuku, R. & Nakamura, H. Total and unbound bilirubin determination using an automated peroxidase micromethod. *Kobe J. Med. Sci.* **28**, 91–104 (1982).
36. American Academy of Pediatrics. Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics* **114**, 297–316 (2004).
37. Maisels, M. J. et al. Hyperbilirubinemia in the newborn infant or =35 weeks' gestation: an update with clarifications. *Pediatrics* **124**, 1193–1198 (2009).
38. Maisels, M. J., Watchko, J. F., Bhutani, V. K. & Stevenson, D. K. An approach to the management of hyperbilirubinemia in the preterm infant less than 35 weeks of gestation. *J. Perinatol.* **32**, 660–664 (2012).
39. Bhutani, V. K. et al. Kernicterus: epidemiological strategies for its prevention through systems-based approaches. *J. Perinatol.* **24**, 650–662 (2004).

40. Bhutani, V. K., Johnson, L. H. & Shapiro, S. M. Kernicterus in sick and preterm infants (1999-2002): a need for an effective preventive approach. *Semin. Perinatol.* **28**, 319–325 (2004).
41. Holtzman, N. A. Management of hyperbilirubinemia: quality of evidence and cost. *Pediatrics* **114**, 1086–1088 (2004).
42. Ip, S. et al. An evidence-based review of important issues concerning neonatal hyperbilirubinemia. *Pediatrics* **114**, e130–e153 (2004).
43. Johnson, L., Bhutani, V. K., Karp, K., Sivieri, E. M. & Shapiro, S. M. Clinical report from the pilot USA Kernicterus Registry (1992 to 2004). *J. Perinatol.* **29**(Suppl 1), S25–S45 (2009).
44. Johnson, L. H., Bhutani, V. K. & Brown, A. K. System-based approach to management of neonatal jaundice and prevention of kernicterus. *J. Pediatr.* **140**, 396–403 (2002).
45. Maisels, M. J. & Newman, T. B. Kernicterus in otherwise healthy, breast-fed term newborns. *Pediatrics* **96**, 730–733 (1995).
46. Newman, T. B. & Maisels, M. J. Does hyperbilirubinemia damage the brain of healthy full-term infants? *Clin. Perinatol.* **17**, 331–358 (1990).
47. Newman, T. B. & Maisels, M. J. Evaluation and treatment of jaundice in the term newborn: a kinder, gentler approach. *Pediatrics* **89**, 809–818 (1992).
48. Watchko, J. F. & Maisels, M. J. The enigma of low bilirubin kernicterus in premature infants: why does it still occur, and is it preventable? *Semin. Perinatol.* **38**, 397–406 (2014).
49. Newman, T. B. & Klebanoff, M. A. Neonatal hyperbilirubinemia and long-term outcome: another look at the Collaborative Perinatal Project. *Pediatrics* **92**, 651–657 (1993).
50. Newman, T. B. & Maisels, M. J. Less aggressive treatment of neonatal jaundice and reports of kernicterus: lessons about practice guidelines. *Pediatrics* **105**, 242–245 (2000).
51. Maisels, M. J. & Newman, T. B. Bilirubin and neurological dysfunction—do we need to change what we are doing? *Pediatr. Res.* **50**, 677–678 (2001).
52. Atkinson, L. R., Escobar, G. J., Takayama, J. I. & Newman, T. B. Phototherapy use in jaundiced newborns in a large managed care organization: do clinicians adhere to the guideline? *Pediatrics* **111**, e555–e561 (2003).
53. Chang, P. W., Newman, T. B. & Maisels, M. J. Update on predicting severe hyperbilirubinemia and bilirubin neurotoxicity risks in neonates. *Curr. Pediatr. Rev.* **13**, 181–187 (2017).
54. Jacobsen, J. & Wennberg, R. P. Determination of unbound bilirubin in the serum of newborns. *Clin. Chem.* **20**, 783 (1974).
55. Lamola, A. A., Eisinger, J., Blumberg, W. E., Patel, S. C. & Flores, J. Fluorometric study of the partition of bilirubin among blood components: basis for rapid microassays of bilirubin and bilirubin binding capacity in whole blood. *Anal. Biochem.* **100**, 25–42 (1979).
56. Porter, E. G. & Waters, W. J. A rapid micromethod for measuring the reserve albumin bindingn capacity in serum from newborn infants with hyperbilirubinemia. *J. Lab. Clin. Med.* **67**, 660–668 (1966).
57. Gitzelmann-Cumarasamy, N. & Kuenzle, C. C. Bilirubin binding tests: living up to expectations? *Pediatrics* **64**, 375–378 (1979).
58. Levine, R. L. Bilirubin: worked out years ago? *Pediatrics* **64**, 380–385 (1979).
59. McDonagh, A. F. & Maisels, M. J. Bilirubin unbound: deja vu all over again? *Pediatrics* **117**, 523–525 (2006).
60. Sunderman, F. W. Jr. Current concepts of "normal values," "reference values," and "discrimination values," in clinical chemistry. *Clin. Chem.* **21**, 1873–1877 (1975).
61. Lott, J. A., Mitchell, L. C., Moeschberger, M. L. & Sutherland, D. E. Estimation of reference ranges: how many subjects are needed? *Clin. Chem.* **38**, 648–650 (1992).
62. Honore, B. & Brodersen, R. Albumin binding of anti-inflammatory drugs. Utility of a site-oriented versus a stoichiometric analysis. *Mol. Pharmacol.* **25**, 137–150 (1984).
63. Klotz, I. M. & Hunston, D. L. Protein affinities for small molecules: conceptions and misconceptions. *Arch. Biochem. Biophys.* **193**, 314–328 (1979).
64. Huber, A. H. et al. Fluorescence sensor for the quantification of unbound bilirubin concentrations. *Clin. Chem.* **58**, 869–876 (2012).
65. Iskander, I. et al. Serum bilirubin and bilirubin/albumin ratio as predictors of bilirubin encephalopathy. *Pediatrics* **134**, e1330–e1339 (2014).
66. Hulzebos, C. V. & Dijk, P. H. Bilirubin-albumin binding, bilirubin/albumin ratios, and free bilirubin levels: where do we stand? *Semin. Perinatol.* **38**, 412–421 (2014).
67. Hulzebos, C. V. et al. The bilirubin albumin ratio in the management of hyperbilirubinemia in preterm infants to improve neurodevelopmental outcome: a randomized controlled trial—BARTrial. *PLoS ONE* **9**, e99466 (2014).
68. Lamola, A. A. et al. Neonatal bilirubin binding capacity discerns risk of neurological dysfunction. *Pediatr. Res.* **77**, 334–339 (2015).
69. Jacobsen, J. Binding of bilirubin to human serum albumin—determination of the dissociation constants. *FEBS Lett.* **5**, 112–114 (1969).
70. Ahlfors, C. E. et al. Bilirubin binding capacity and bilirubin binding in neonatal plasma. *Pediatr. Res.* **2718**, 5 (2017).
71. Wennberg, R. P., Rasmussen, L. F., Ahlfors, C. E. & Valaes, T. Mechanized determination of the apparent unbound unconjugated bilirubin concentration in serum. *Clin. Chem.* **25**, 1444–1447 (1979).
72. Khurshid, F. & Medves, J. Effectiveness of universal hyperbilirubinemia screening on newborn health: a systematic review protocol. *JBI Database Syst. Rev. Implement. Rep.* **16**, 287–290 (2018).
73. Ahlfors, C. E. et al. Effects of sample dilution, peroxidase concentration, and chloride ion on the measurement of unbound bilirubin in premature newborns. *Clin. Biochem* **40**, 261–267 (2007).
74. Amin, S. B. Effect of free fatty acids on bilirubin-albumin binding affinity and unbound bilirubin in premature infants. *JPEN J. Parenter. Enter. Nutr.* **34**, 414–420 (2010).
75. Amin, S. B., Maisels, M. J. & Watchko, J. F. Early lipid infusions and unbound bilirubin in preterm neonates: a cause for concern? *J. Pediatr.* **184**, 6–7 (2017).
76. Hegyi, T., Kathiravan, S., Stahl, G. E., Huber, A. H. & Kleinfeld, A. Unbound free fatty acids from preterm infants treated with intralipid decouples unbound from total bilirubin potentially making phototherapy ineffective. *Neonatology* **104**, 184–187 (2013).
77. Ebbesen, F., Foged, N. & Brodersen, R. Reduced albumin binding of MADDS—a measure for bilirubin binding—in sick children. *Acta Paediatr. Scand.* **75**, 550–554 (1986).
78. Ahlfors, C. E. Effect of serum dilution on apparent unbound bilirubin concentration as measured by the peroxidase method. *Clin. Chem.* **27**, 692–696 (1981).
79. Ahlfors, C. E., Marshall, G. D., Wolcott, D. K., Olson, D. C. & Van Overmeire, B. Measurement of unbound bilirubin by the peroxidase test using Zone Fluidics. *Clin. Chim. Acta* **365**, 78–85 (2006).
80. Weisiger, R. A. et al. Affinity of human serum albumin for bilirubin varies with albumin concentration and buffer composition: results of a novel ultrafiltration method. *J. Biol. Chem.* **276**, 29953–29960 (2001).