

# Severe neonatal hyperbilirubinemia and the brain: the old but still evolving story

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**Abstract:** The immature hepatic metabolism of bilirubin at birth is responsible for neonatal hyperbilirubinemia, present in more than 60% of otherwise healthy infants. Icterus (or jaundice), the most apparent features of the increased bilirubin level in the serum, testifies the entry of the pigment in the tissues and organs, brain included. The sensitivity of the central nervous system (CNS) to bilirubin toxicity is responsible for the potential neurologic damage, and even death. The symptoms in affected neonates suggest that selected brain areas are more specifically targeted by bilirubin, a hypothesis longer explained by the deposition of bilirubin in those areas, the “kern-icterus”. Most recently, a more complex picture and alternative explanations to the variability of the symptoms recapped by the terms bilirubin induced neurological dysfunction (BIND) or kernicterus spectrum disorder (KSD) are emerging, with pre-term neonates representing a new challenge. Here we will review what is known of the disease, from the dogma of the “kern-icterus” to the most recent findings bringing into play the stage of brain development at the time of bilirubin insult. Special emphasis will be given to the emerging population of pre-term neonates, especially sensitive to bilirubin toxicity.

**Keywords:** Pre-term, blood brain barriers; transporters; central nervous system development (CNS development); neurons

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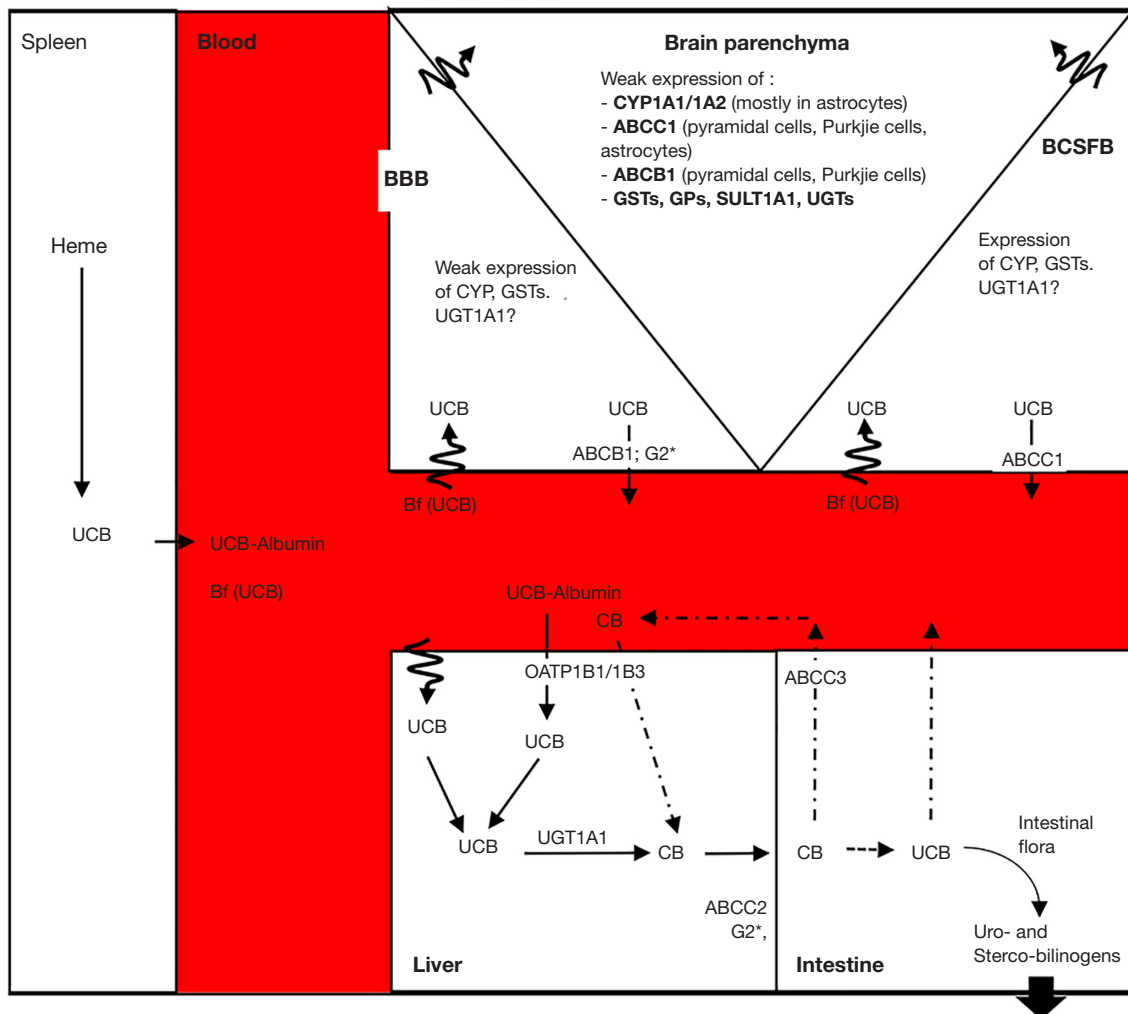
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## A review on the bilirubin catabolism with special emphasis on the neonatal age

Unconjugated bilirubin (UCB) is the yellow pigment of heme catabolism responsible for icterus. Daily formed mainly from the degradation of senescent red blood cells in the spleen, UCB flows to the liver through the blood

bound to serum albumin (see *Figure 1*). After entering the liver mainly by the active transport operated by the organic anion transport polypeptide (OATP) 1B1 and 1B3 (1,2), UCB is transformed by the uridine 5'-diphosphoglucuronosyltransferase 1A1 (UGT1A1) in the water-soluble conjugated bilirubin (CB), thereafter excreted from the body after further biotransformation powered

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**Figure 1** The liver-brain axis in bilirubin catabolism. \*, potential UCB/CB transporter. zig-zag arrows: diffusion. ABCB1: also known as multi-drug protein 1 (MRP1); ABCB2: MRP2; ABCB3: MRP3; ABCB1: also known as P glycoprotein (PGP or multidrug resistance protein 1 (MDR1); ABCG2: breast cancer resistance protein (BCRP). Heme, hemoglobin; UCB, unconjugated bilirubin; Bf, unbound, free bilirubin; OATP, organic anion transport polypeptide; UGT, uridine 5'-diphospho-glucuronosyltransferase; CB, conjugated bilirubin; ABC, ATP binding cassette transporters; CYP, cytochrome P-450 mono-oxygenase; GSTs, glutathione-S-transferases; BBB, blood brain barrier; BCSFB, blood cerebro-spinal fluid barrier.

by the intestinal flora (3). This degradation pathway balances bilirubin formation maintaining the total serum bilirubin (TSB: UCB + CB) level in a physiological range of 0.2–1 mg/dL (3.4–17.4  $\mu\text{mol/L}$ ), with UCB being the predominant fraction. When blood UCB level exceeds the serum albumin binding capability, the unbound portion of the pigment [free bilirubin (Bf), less than 0.1% in physiological conditions (4,5)] enters the tissues leading to the yellow coloration of the skin, the so-called icterus (from the Greek = yellow, or jaundice from the French “jaune”

= yellow). Icterus is common in neonates, and mainly due to (see *Table 1*): (I) the increased red blood cell turnover occurring after birth (6,35,36); (II) the undeveloped UGT1A1 activity (6,7,9); and (III) the quite total absence of the intestinal flora in neonates (11-13).

The high hydrophobic nature of Bf makes it able to diffuse across the cellular bilayer and entering the cells, if not bound to serum albumin. Based on that, in neonates (especially in pre-terms), which have a lower serum albumin level than the adults (see later on in the review,

**Table 1** Human developmental expression of the determinants of bilirubin level in blood and brain

Variable	Pre-term	Full-term	Ref.
Heme production (80% senescent red blood cells, 20% ineffective erythropoiesis + myoglobin and cytochromes)	Increased red blood cell fragility (vs. full-term)	Increased red blood cell turnover (vs. adult)	(3,6,7)
OATP1B1/1B3 (liver)	No data	0.2% vs. adult. In 1 year-old baby still only 1% of adult expression	(8)
UGT1A1 (liver)	Increased immaturity (vs. full-term)	1% vs. adult. Reaching the adult level at 3 months of age	(6,7,9,10)
Intestinal microbiota	Reduced motility, absence of the intestinal flora	Almost absent. Reaching the adult activity at 6–12 months of age	(11-13)
Serum albumin	<2.5 g/dL	<3 g/dL (vs. adult level of 4.2–4.6 g/dL)	(14)
Bilirubin-albumin (B/A) binding	Larger inter-individual variability	Lower $\{(3-5) \times 10^7 M^{-1}\}$ than adult albumin $(6.7 \times 10^6 - 10^8 M^{-1})$	(15-17)
Blood-CNS surfaces			
BBB	MVs sprouting starts at 8 Wg. Claudin expression similar to adult-one since 18 Wg. BBB unit establishment since 20 Wg	Fully established, but the still forming basal-lamina and astrocytic end-feet surrounding the MVs, might make them more fragile to damaging stimuli than the adult	(18-20)
	MVs density. Higher in the gray matter (about $111 \pm 30$ MVs/mm <sup>2</sup> ) vs. white matter (about $50 \pm 20$ MVs/mm <sup>2</sup> ) up to 32 Gw	MVs density. Gray matter: about $250 \pm 90$ vs. white matter: about $90 \pm 22$ MVs/mm <sup>2</sup> at 40 Gw. Still increasing postnatally, especially in the gray matter	
CPs (forming the BCSFB)	Already present at 7 Wg, but still uni-stratified and not polarized until 16 Gw. Villi will develop from 17 to 28 Gw. Since 29 Gw to birth, redefinition of the morphology and shape. Claudin 3 more expressed prenatally	Claudin 2 more expressed postnatally	(21,22)
ABCC1 [marker of the basolateral side (blood facing) of the choroid plexus (CPs)]	Already strongly expressed in CPs and ependyma since 22–26 Wg. Not detectable at the BBB (at any developmental age)	CPs: all ABC family C transporters are well expressed and active at birth	(23)
	Detectable in pyramidal and Purkinje cells of the cerebellum at 26 Wg, increasing up to 30 Wg		
ABCC2 (liver)	No data	0.5% vs. adult. At 12 months, still only 1% vs. adult	(8)
ABCC3 (liver)	Protein expression is fourfold lower in the fetus (16.4–37.9 weeks) than in infants and adults	Higher in infants and adults	(24)

Table 1 (continued)

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Variable	Pre-term	Full-term	Ref.
ABCB1 [marker of the endothelial cells forming the BBB, expressed at the apical side (blood facing) of the micro vessels (MVs)]	BBB. 22–26 Wg: detectable in 33–50% of samples of the brainstem, hindbrain and thalamus. From 27 to 33 Wg, it became detectable everywhere	BBB. Detectable in MVs of all regions of the brain, but its expression is still increasing from birth to adult age	(23,25-28)
	Neurons. 22–26 Wg. Detectable in pyramidal neurons of the brain stem and thalamus (20% of the samples), and Purkinje cells of the cerebellum (20% of the samples)	Neurons. 30 Wg: detectable in pyramidal neurons and h-Purkinje cells of the cerebellum (100% of the samples)	
ABCG2 (potential UCB transporter)	Liver: 1.5 folds higher in fetus (16.4–37.9 weeks) than adult	Liver: lower in infants and adults	(24-28)
	BBB: since 22 Wg detectable	No changes in expression both at the BBB and CPs (and until adult age). Possibly, its expression in CPs relies on the MVs inside the stroma	
	CPs: no/barely detectable		
Detoxifying enzymes [CYP1A1/1A2; GSTs, GPs, SULT1A1, (rodent) UGTs]	Detoxifying enzymes activity: all higher in CPs than in other CNS structures	Mainly expressed in CPs (vs. brain parenchyma or BBB). Higher postnatal activity (more than in adult age)	(28-34)
	SULT1A1 detectable since 15–20 Gw	NB: Gsts (rat samples) still high at birth in the LV CP, decreasing with development in the 4V CP, with the exception of GST $\alpha$ (ligandin) that is lower at birth than in adult life	
	NB: Gsts (rat samples) high since P2—representing approximately human 23–32 Wg)		

ABCC1: also known as multidrug protein 1 (MRP1); ABCC2: MRP2; ABCC3: MRP3; ABCB1: also known as P glycoprotein (PGP) or multidrug resistance protein 1 (MDR1); ABCG2: breast cancer resistance protein (BCRP). Wg, weeks of gestation; OATP, organic anion transporters; UGT, uridine 5' diphospho-glucuronosyltransferase; BBB, blood brain barrier; MVs, micro-vessels; CPs, choroid plexuses, forming the BCSFB, blood cerebro-spinal fluid barrier; Wg, weeks of gestation; ABC, ATP binding cassette transporters; CYP, cytochrome P450 oxygenase; GSTs, glutathione-S-transferase; GPs, glutathione peroxidase; SULT, sulfotransferase; UGT, uridine glucuronosyl-transferase; P, post-natal age in days; LV/4V CP, lateral and forth ventricle choroid plexuses.

as well as *Table 1*), serum albumin has to be considered as an additional determinant controlling the Bf entrance into the brain (37-42). The importance of serum albumin is well demonstrated also by animal models, where the brain damage in hyperbilirubinemic Gunn rats (43,44) crossbred with an-albuminemic rats occurs at much lower ( $\frac{1}{4}$ ) blood bilirubin level than in the (normo-albuminemic) Gunn strain (45). In addition, the affinity of neonatal albumin for bilirubin is lower than that of adult albumin (15,36), and largely variable between individuals (39,46-49).

At present and in the clinical practice, TSB is the value on which the indication of phototherapy (PT) is decided to prevent neurological damage. Nevertheless,

due to the large and partly unpredictable variables able to interfere with the bilirubin-albumin binding (see later on in the text) and altering Bf, the recent advances making easier the quantification of Bf also in clinic (50) offer the opportunity of positively impact on the management of the condition.

Neonatal hyperbilirubinemia may lead to two opposite conditions: (I) the so-called physiological neonatal hyperbilirubinemia, un-risky and self-resolving in 1 to 2 weeks (36,51), not needing medical interventions, and (II) the severe neonatal hyperbilirubinemia, potentially leading to conditions ranging from mild-temporary deficits, to permanent neurological alterations, recapped under the

definitions of bilirubin induced neurological dysfunction (BIND) and kernicterus spectrum disorder (KSD) (52-54).

### From the “kern-icterus” to the KSD

For a long time, the term kernicterus has been used to identify the most dramatic consequences of bilirubin neurotoxicity in the course of severe neonatal hyperbilirubinemia. This term was coined by Schmorl in late 1903 to describe a specific pattern of yellow brain coloration (kern=core, nuclei; icterus = yellow) restricted to specific CNS structures in neonates who died with severe jaundice. Affected regions included the basal ganglia, the hippocampus, the central part of the cerebellum, and the wall of the third and fourth ventricle [reviewed in (51)].

In 1966, Diamond demonstrated that the portion of serum UCB able to enter the CNS is limited to Bf, with albumin (MW: 66 KDa), physically retaining the pigment into the vascular lumen (55). Conditions inducing an opening of the blood-brain interfaces (BBI) (for example hypoxia, hypercarbia, hyperosmolarity) will increase UCB brain content and alter the dynamics of clearance of the pigment from the brain (36,56-58). The same is true when blood UCB is displaced from serum albumin. In this respect, a long and ever-increasing list of displacing molecules exists, including typical drugs and nutritional approaches used especially in pre-term neonatal care (59-66). Altogether this data indicates that Bf crosses rapidly the BBI in either direction.

Due to the good agreement with the main clinical symptoms, the MR/proton MR spectroscopy findings (67-71), and some extreme experimental model where UCB entry into the brain was acutely increased by the use of displacing agents (72-76), the concept that brain damage was due to the accumulation—the so-called “deposition”—of bilirubin in specific CNS structures—the “kern-icterus”—became a dogma affecting the research of the field for decades.

Extensive work has been done searching the reasons for the selective UCB accumulation in specific brain areas. Differences in (in and out) transport (23,77-79), binding (78,80), metabolism (81), ability to oxidize, thus decrease, UCB level (74,82), as well as different circulatory rates among CNS regions, have been hypothesized, ever without replicating the “kern-icterus”.

In the meantime, bilirubin was discovered preferentially binding to myelin (41,83), alter the cellular membrane permeability and functions (84,85), inducing a bioenergetics

crisis, apoptosis and necrosis, inflammation, redox and calcium imbalance, glutamate neurotoxicity, and synaptic excitability and transmission (82,85-95). It also alters the cell cycle (96), and acts on several cellular signaling pathways [reviewed in (29,71,97-99)].

Only in 2000, two reviews by TW Hansen (51,100) started criticizing the dogma. Hansen carried out a careful rereading of the history, reporting that, among the infants that died with jaundice, only marginal cases (less than 5%) presented the “kern-icterus”, and that in the majority of the autopsies the brain was diffusely yellow. Thus, bilirubin accumulation in specific brain areas is not required for developing neurological damage, although bilirubin toxicity affects specific structures of the CNS, while others areas are insensitive to bilirubin, despite the equal bilirubin level. This observation is well supported by recent *in vitro* (101), *ex vivo* (102) and *in vivo* animal studies (74,103).

One of the major alternative theories calls into play brain development, suggesting that the most affected regions of the brain are those in which key developmental processes are ongoing at the time of bilirubin challenging (104). In line with this conclusion are several experimental data.

*In vivo* (animal models), PT given to jaundiced animals at specific post-natal ages [P7 +/- 3 in Gunn rats (105)], fully protects from cerebellar hypoplasia. Supportive is the fact that the areas undergoing differentiation at the moment when bilirubin plasma concentration peaks show a higher bilirubin induced damage (73,105,106).

Recent *ex vivo* findings, obtained using organotypic brain cultures (OBCs) from rat pups at different post-natal ages exposed to UCB, demonstrated a developmental sensitivity to bilirubin toxicity (together with the regional sensitivity: hippocampus > inferior colliculi = cerebral cortex), with the OBCs from an 8-day-old rat brain showing the maximal damage (102).

It is well known that neurons are more sensitive to bilirubin toxicity than astrocytes (29,107), with less differentiated neurons [based on the day *in vitro* (DIV) (108,109)] showing a higher degree of mitochondrial damage, oxidative and energetic crisis (101,110), altered neurogenesis and synaptogenesis, in addition to increased mortality (111,112). Notably, ABCC1/MRP1 expression (ATP binding cassette C1/multidrug resistance protein 1, a UCB transporter) increases with the neuronal maturation *in vitro*, and ABCC1 inhibition (by MK571), enhances UCB cellular entry (113). This suggests a role of this transporter in maintaining intracellular bilirubin below damaging concentration. Despite that, ABCC1 looks not

to be sufficient to face the bilirubin challenge during severe neonatal hyperbilirubinemia *in vivo* (78,114), possibly due to its low expression in the brain parenchyma, moreover limited to few selected cellular populations (23,115).

An alternative explanation to the increased sensitivity in less mature cells/brain area has been hypothesized to be an undeveloped cellular defense [reviewed in (29)].

The oligodendrocytes are the second neuronal population largely sensitive to bilirubin toxicity. In primary culture of oligodendrocytes, UCB can interfere with maturation and differentiation, and then with the myelination process (29,103,116). Importantly, while neurogenesis in humans is a pre-natal event (possibly relevant in pre-term newborns), oligodendrogenesis is still present after birth in humans, with myelination occurring up to 10 years after birth (117). In agreement, altered myelination has been reported both in animal models (103,118) and at autopsy of infants died with kernicterus (114,119).

More recently bilirubin has been demonstrated to alter brain maturation by acting on the epigenetic of CNS development, including myelination, reinforcing the idea that bilirubin and CNS development are strictly interconnected (120). This hypothesis might be particularly important nowadays when the improvement of medical care allows more and more pre-term infants to survive. Thus, emerging is the need for a better understanding of the impact of CNS maturity (and immaturity) on the disease, as well as for a focus on bilirubin brain entry in pre- and full-term neonates.

### Severe neonatal hyperbilirubinemia: full-term and pre-terms as two distinct populations

Hyperbilirubinemia in neonates is a benign condition in the majority of cases. However, an uncontrolled and rapidly increased blood bilirubin level can lead to neurotoxicity with even deadly consequences. Frequently considered an event of the past (121), the mortality of neonatal jaundice in the early neonatal period (0–6 days) still accounts for 1,309.3 deaths per 100,000 subjects worldwide (122). The burden is highest in the low-middle income countries (LMIC), especially in Sub-Saharan Africa and South Asia, where neonatal hyperbilirubinemia is the 7th and 8th leading cause of mortality, respectively. In Western Europe and North America, neonatal hyperbilirubinemia accounts for the 9th and 13th leading cause of mortality, respectively (122). Severe hyperbilirubinemia has been recognized as the cause of

bilirubin-induced neurotoxicity which can manifest as acute brain encephalopathy (ABE), KSD, and death (123,124).

### *The risk threshold: TSB is not enough*

Several clinical differences exist among full [ $\geq 37$  weeks gestational age (Wg)] and pre-term neonates ( $< 37$  Wg) (see *Table 2*) exposed to hyperbilirubinemia. Neonatal hyperbilirubinemia affects 60% of full-term and 80% of pre-term neonates (10,125,126), with pre-term infants having a higher risk of severe jaundice with or without bilirubin-induced neurotoxicity than do full-term infants (148–150). The increased percentage of hyperbilirubinemia in pre-terms is mainly due to the enhanced (with respect to full term neonates) fragility of the red blood cell (bilirubin production), the increased liver (transport and conjugation) and intestinal immaturity, and postponed enteral feeding (7,10,148) (see *Table 1*).

While the TSB peak plotted as a function of the hours after birth is sufficient to estimate the risk of neurological sequelae in term infants (134,134), additional factors are required in pre-term babies (see *Table 2*). In this population, common is the so-called “low bilirubin kernicterus”, in which the neurological damage (symptoms and/or neuroimaging findings of brain damage) may be present even with peak TSB under the “safe level” (128,135–138).

In pre-term babies, usually, bilirubin induced brain damage is associated with extremely low birth weight (ELBW) and/or strikingly low serum albumin level (1.4 to 2.1 g/dL) (135,137,138). Indeed, at equal TSB, the risk of developing neurological sequela increases as the gestational age decreases, as reported for example by Bhutani and Wong (e.g., 10% below: 30 Wg, 5.5% below 31–32 Wg, and 1.2% below 33/34 Wg). Thus, rather than a specific threshold, in pre-term infants a range of TSB levels is more likely to be associated with the onset of neurotoxicity (133). Both body weight and serum albumin level might be considered additional (possibly personalized) indicators of the developmental maturity of the infant, in addition to the gestational age, all affecting the Bf. In agreement with it, the presence of neurological dysfunctions or even death in ELBW pre-term infants have been associated with a high level of free/unbound bilirubin (139,151), suggesting Bf as a more sensitive predictor in respect to TSB (141,152). Bf has also been found as a good predictor for auditory dysfunctions in neonates with severe jaundice (142,143,153,154), as well as for the risk of apnea in pre-term infants (144,155). The bilirubin neurotoxicity

**Table 2** Clinical differences between pre-term and full-term infants

Variable	Pre-term	Full term	Ref.
Gestational age	<37 weeks	≥37 weeks	
Prevalence of hyperbilirubinemia	80%	60%	(10,125,126)
Incidence in infants with severe hyperbilirubinemia (TSB >20 mg/dL)*			
ABE*	66.6% (n=20/30)	87.6% (n=80/89)	(127)
KSD*	93% (n=27/29)	86.4% (n=83/96)	
Death*	3.45% (n=1/29)	5.2% (n=5/96)	
Clinical manifestation			
ABE	More subtle, recurrent apnea and desaturation	Classic ABE signs: initial phase: stupor (lethargy), hypotonia, and poor sucking; Advanced phase: hypertonia, arching, retrocollis, opisthotonos, fever, high-pitched cry, apnea, inability to feed	(52,128-131)
KSD	Equally the same with term neonates with auditory neuropathy spectrum disorder predominant	Athetoid cerebral palsy (uncontrollable movement of the face, body, arms and legs), hearing loss, failure of upward gaze and dental enamel dysplasia	
Incidence of apnea associated with ABE	≥35 to <37 weeks: n=14/28; 50%	≥37 weeks: n=27/80; 33.8%	(132)
% of abnormal ABR in hyperbilirubinemic infants	25% (n=9/36)	14% (n=11/80)	(60)
Parameter for diagnosis	TSB, plus birth weight, serum albumin, and gestational age	TSB alone	(128,133-139)
Mean TSB	23–34 weeks: 5.4±1.4 mg/dL; ≥28–34 weeks: 8.6±1.6 mg/dL	≥37 weeks: 7.9±1.6 mg/dL; 5.2±3.2 nM	(59,60,140)
Mean Bf	23–34 weeks: 13.1±8.4 nM; ≥28–34 weeks: 17.8±9.7 nM		
Peak Bf in infants with abnormal ABR	<28 weeks: n=9/36; 19.0±15.9 nM	–	(39)
	≥28–34 weeks: n=11/80; 21.9±21.1 nM		
	28–32 weeks: n=25/45; 10.5±3.4 nM	–	(141)
	24–35 weeks: n=6/81; 30.1±22.4 nM	–	(142)
		≥34 weeks n=24/100; 57.6±49.9 nM	(143)
Peak of Bf in infants with central apnea	27–33 weeks: n=7/50; 39.3±44.4 µg/dL		(144)
Treatments			
Phototherapy	Prophylactic PT Possibly inefficient in reducing Bf and largely affected by displacing compounds	Perform as conservative mode	(59,60,145)
% of TSB/Bf decrease after PT	43%/null	29%/19%	(59)

**Table 2** (continued)

Table 2 (continued)

Variable	Pre-term	Full term	Ref.
Exchange transfusion (ET)	Based on TSB level threshold and gestational age, and the presence of ABE sign (less obvious)	Based on TSB level and the presence of ABE sign (most obvious)	(146)
Deaths within 7 days following ET	5% [37/704]	1% [5/457]	(147)

\*, the incidence of ABE, KSD, and death are among the infants with severe hyperbilirubinemia (TSB >20 mg/dL) only. Neither the healthy infants nor moderate-mild hyperbilirubinemia were included. ABE, acute brain encephalopathy; KSD, kernicterus spectrum disorder; TSB, total serum bilirubin; Bf, free bilirubin; PT, phototherapy.

affecting auditory system including brainstem auditory nuclei, vestibular nuclei, and auditory nerve, has been reproduced in both human and animal model of kernicterus (Gunn rat) (156,157). Nevertheless, the interplay between body weight, serum albumin level, Bf and the immaturity of the brain, one of the reasons for the increased susceptibility to neuronal damage in the pre-term population even at lower bilirubin levels (135), still has to be fully unravelled. Reliable experimental models are required.

### The clinical manifestations

#### Full-term infants

In mature infants, lethargy, hypotonia, and poor sucking are the early non-specific sign of acute bilirubin encephalopathy (ABE) (Table 2), with hypertonia (retrocollis and opisthotonos), fever and high-pitched cry, apnea, and inability to feed representing the signs of advanced stage of ABE.

The classical sign of the chronic and permanent clinical sequelae of bilirubin toxicity in term population includes motor symptoms, hearing loss due to auditory neuropathy spectrum disorder (ANSD) with or without hearing loss, visual impairment (visuo-oculomotor, usually manifests as paralysis of upward gaze, and visuo-cortical dysfunction), and dental enamel dysplasia (54,129,130). Those symptoms present a large variability among individuals, recently recapped by the term KSD (52). In addition, the neurodevelopmental sequelae, later described as spectrum of developmental disorder, have been considered as part of bilirubin-induced neurotoxicity disorders. Bilirubin-induced cognitive delay, attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), specific learning disorder and language disorder are neurodevelopmental disorders (NDDs) that have been associated with bilirubin

neurotoxicity (53,54).

The stage of brain development (the main biological mechanisms: e.g., division, differentiation, migration, myelination, etc.) at the time of bilirubin insult has been hypothesized as one of the possible explanations for this variability, as discussed above. However, hyperbilirubinemia in un-treated full-term neonates may persist up to 1 or 2 weeks after birth, and, this might be too short a time to represent different stages of the CNS development. An alternative explanation may reside in individual genetic susceptibility, explaining also why kernicterus may develop under the TSB risk threshold, or, *vice versa*, do not develop despite franc toxic TSB levels (158).

#### Pre-term infants

Pre-term infants less frequently exhibit the conventional bilirubin neurotoxicity signs (Table 2), likely due to incomplete maturation of neuronal circuit and organization. For this reason, pre-term infants are more at risk of late-diagnosis or even stay undiagnosed, which lead them to suffer from “silent morbidity and mortality” (130,159). Meanwhile, the auditory predominant sequelae are more common in pre-term neonates (128,130,131). Pre-term infants with abnormal auditory brain evokes responses (ABR, the common clinical test performing among pre-term infant to evaluate brainstem function related to auditory neural pathway), also present a more concurrent apneic events (155), possibly because both these conditions share the same neuronal location targeted by bilirubin. Moreover, increasing are the evidences reporting that hyperbilirubinemia in premature infants is followed by a higher number of apnea events (135,144,160).

Concerning the chronic and permanent clinical sequelae of bilirubin toxicity, pre-terms share the same burden as that of the full-term neonates (129,130). This may suggest



that, under chronic toxic stimuli, inducing the most severe molecular perturbations and cell death (71,104,161), the developmental stage of brain development is irrelevant.

Various clinical studies tried to link the higher prevalence of NDDs (global cognitive delay, ADHD, specific learning disorder, ASD) in pre-term infants with hyperbilirubinemia but none showed a significant association (53). It must be underlined that most of them used TSB or UCB, but not Bf.

Notably, the occurrence of athetoid cerebral palsy in more than 6 months old infants have been noticed among pre-term infants with a history of moderate hyperbilirubinemia (7–17.4 mg/dL) and despite PT during the neonatal period (128,136). A potential explanation might come from the recent and disturbing finding that PT may be ineffective in reducing Bf in pre-term infants, despite a significant decrease of the TSB (49,59,65). This dichotomy remains still unexplained. Certainly, pre-term infants often require in addition to PT drugs and nutritional approaches, and, among them, several can interfere with the bilirubin to albumin binding, increasing Bf [as previously discussed (48,65,66)].

### *Therapeutical approaches*

#### **Phototherapy**

PT is the standard treatment for neonatal hyperbilirubinemia to convert bilirubin into water-soluble photoisomers that can be excreted through bile and urine. Effective PT has progressively decreased the need for exchange transfusion (ET) in pre-term infants (145). Recently, double-PT, which use two light sources, has been demonstrated more effective for reducing TSB level compare to single PT among pre-term infants (162).

In addition to the emerging inefficacy of PT in pre-term babies (see above), PT is not a harmless treatment and overtreatment should be reevaluated in small pre-term infants (*Table 2*). Side effects can include retinal damage, burns, disturbed circadian rhythm, conjunctivitis, rashes, dehydration, hyper- and hypothermia, loose stools, melanotic nevus, bronze baby syndrome, and electrolyte disturbances (163).

In a large randomized control trial aggressive (prophylactic) PT was compared with conservative PT in ELBW ( $\leq 1,000$  g) infants. Aggressive PT was provided at a TSB value of 5 mg/dL or higher in the first week and 7 mg/dL or higher in the second week. Meanwhile, conservative PT was provided at a bilirubin value of 8 mg/dL

or higher for 501–750 g infants and 10 mg/dL or higher for 751–1,000 g infants. This study showed that aggressive PT in ELBW infants reduced neurodevelopmental impairment and hearing loss among surviving infants versus those receiving conservative PT. However, those results are offset by the post hoc analysis reporting a 99% probability of increased deaths among infants under 750 g birth weight with aggressive PT (164). These results suggest that moderate bilirubin levels may have clinically important oxidant benefits (165). Low concentration of bilirubin scavenges reactive oxygen species (ROS), reduces oxidant-induced cellular injury and attenuates oxidant stress. Since the physiologic jaundice has to be accepted as a protective mechanism for the newborn infant against ROS in the first days of life (163), aggressive, prophylactic PT looks to be counterproductive.

Indeed, the thin, translucent skin of ELBW infants and their high rate of serious illness and immature defense mechanisms may make them particularly vulnerable to the potential or documented adverse effects of PT, including photo-oxidative injury, lipid peroxidation, DNA damage, reduced mesenteric and cerebral blood flow, and hemolysis (163,166). Interestingly, a recent study reported that the cycled (intermittent) PT can reduce the mortality associated with continuous PT among the pre-term infant (167).

In any case, PT has significantly decreased the overall incidence of bilirubin neurotoxicity in most developed countries. Nevertheless, bilirubin neurotoxicity with lifelong neurological sequelae still occurs, and is a major problem in many areas of the world, especially in low- and middle-income countries (168,169). The access to health facilities, availability of PT, the possibility to measure TSB at the side of the newborn, and the variation in PT practices (such as the irradiance distances between infants and the light sources) have contributed to sub-optimal result of this therapeutical approach (168,170).

#### **Exchange transfusion**

ET is the standard therapy for severe hyperbilirubinemia with ABE (146). ET will be performed if the hyperbilirubinemia exceeds the specific level defined in clinical guidelines and exposes infants to the risk of bilirubin neurotoxicity (134). The incidence of severe hyperbilirubinemia adjusted according to the American Academy of Pediatrics thresholds for ET is low, involving  $\sim 1.2$  per 1,000 live births (171). The screening of hyperbilirubinemia and its underlying condition [e.g., rhesus and ABO isoimmunization, glucose 6 phosphate deficiency

(G6PD)], the treatment of pregnant women who are Rh-negative with Rh-factor therapy, and the increased use of PT, have drastically reduced the number of ET performed (147). Nevertheless, premature infants have a tenfold increased risk of eventual bilirubin level meeting or exceeding thresholds for ET compared with term neonates (171).

Furthermore, most studies suggest that sick pre-term infants experience a wide range of complications from ET more frequently than term infants (145,147,172,173). In a large cohort study of >1,200 pre-term and term infants who received ET for hyperbilirubinemia, pre-term infants especially those  $\leq 29$  weeks of gestational age, have greater odds of death following ET compared to term infants (147).

### The novel treatment strategies

Despite PT and ET have been widely accepted as standard treatments for severe hyperbilirubinemia to prevent ABE, the limitation of both therapies calls for new neuroprotective treatment.

Minocycline, the anti-inflammatory and antimicrobial drug, has been effectively demonstrated to prevent kernicterus in animal models of hyperbilirubinemia (174-176). However, the use of minocycline in neonates is prevented due to its inevitable side effects including tooth discoloration, increased skin hypersensitivity to light, skin and nail hyperpigmentation, and skin rash (146).

Other drugs under study for treating hyperbilirubinemia is tin-mesoporphyrin (SnMP), the potent competitive inhibitors of heme oxygenase, the key rate-limiting enzyme in the catabolism of heme to bilirubin (177). Reddy *et al.* have reported a very low birth weight infant with severe hyperbilirubinemia which, while awaiting an ET, underwent a SnMP single-dose treatment. After 10 hours of SnMP administration, TSB was gradually reduced (by 13%) and this eliminated the need for ET. No adverse effects were reported (178). A clinical trial of SnMP in 213 newborns has shown the early use of a single dose of SnMP decreased the duration of PT, reversed TSB trajectory (mean TSB declined by 18%), and reduced the severity of subsequent hyperbilirubinemia. However, data on long-term risk of BIND still lack in this study (179).

A possible alternative approach might be focused on counteracting directly into the brain the molecular mechanisms of damage triggered by bilirubin, irrespective of the TSB (102). A recently published *in vivo* work reported that curcumin was able to fully restore brain damage and behavioral abnormalities in the spontaneously hyperbilirubinemic Gunn rat by counteracting the

main pathological mechanisms of CNS damage (118). This approach might be useful where PT/ET are not available or efficient in (otherwise healthy) full-term hyperbilirubinemic newborns.

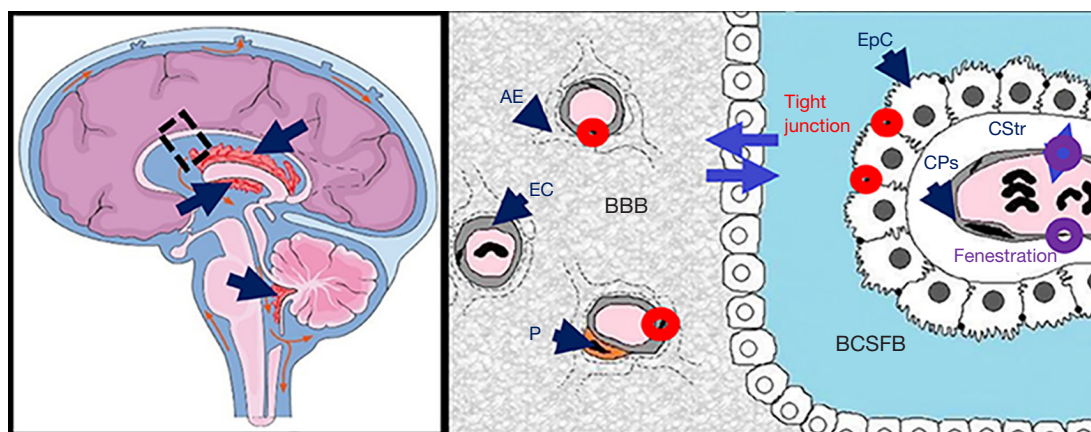
### From the serum to the brain: the BBI in brain development and KSD

To enter the brain, Bf has to cross the BBI, located at the endothelial walls of the brain vasculature [blood-brain barrier (BBB)], and at the epithelial layer of the choroid plexuses (CPs), the latter giving access to the cerebrospinal fluid (CSF) (blood-CSF barrier, BCSFB) (Figures 1,2). The barrier cells are sealed by tight junctions, and harbor multi-specific efflux transporters and detoxifying enzymes that altogether prevent the diffusion of unwanted compounds into the brain or else increase the clearance of potentially harmful metabolites from the brain (181). They also fulfill an important brain delivery function for energy substrates, micronutrients and hormones, and contribute to the specific immune privilege of the brain (21,182). Contrary to the received idea of brain barriers lacking maturity during perinatal development, both interfaces display a barrier phenotype very early during fetal life, and are functionally efficient to supply the nervous system with nutrients and biologically active molecules that match the specific needs of the developing brain.

### Development and differentiation of brain barriers

#### Brain barriers during pre- and postnatal brain development

Vascularization of the brain is an early event. It starts on embryonic day 9.5 in mice, when endothelial cells from the perineural vascular plexus invade the neuroepithelium, a process regulated by the canonical Wnt/ $\beta$ -catenin signaling. This pathway orchestrates the development of the vascular network, controls the secretion of extracellular matrix proteins, and initiates the induction in endothelial cells of their brain-specific properties such as the expression of glucose transporter 1 (GLUT-1) allowing glucose to fuel the brain (183). The endothelium of the newly formed vessels displays functional tight junctions characterized by the focal localization of tight junctions-associated proteins such as occludin and claudin 5, and reduced pinocytosis activities, both typical of the BBB phenotype. Pericytes play an important part in inducing these latter properties (184,185). Astrocytes that



**Figure 2** Neurovascular and choroid plexus networks forming the blood-brain barriers. Blood vessels penetrating the brain tissue give birth to the cerebral microvascular network whose endothelial cells (EC) are sealed by the tight junction, in both adult and fetal brain, under the influence of pericytes (P). Astrocytic endfeet (AE) surrounding the vessels, also capable of influencing some barrier properties of the brain endothelium, are mature only later during the peri and postnatal period. The choroid plexuses, to the number of four, are located in the ventricular system of the brain (dark arrows, left panel). They are formed by a highly vascularized conjunctive stroma (CStr) surrounded by an epithelium. The latter presents a tight phenotype as soon as it differentiates from the neural tube, as a result of the tight junctions sealing the cells of the epithelium (EpC), while the stromal blood vessels irrigating the choroid plexuses (CPs) present a permissive phenotype. Modified from (180). BBB, blood-brain barrier; BCSFB, blood-cerebrospinal fluid barrier.

mature essentially postnatally in rodents, play a role in the maintenance of the BBB phenotype later on (*Figure 2*). In the postnatal brain, the surface of exchange across the BBB is more limited than in the adult because the neurovascular network develops and complexifies gradually throughout development, in two waves of angiogenesis, one prenatal, the other postnatal (180).

Morphological and immunohistological studies in humans indicate a pattern a vascularization and BBB development similar to that observed in rodents, although with a different timeframe. Vascularization of the telencephalon starts around week 8 of gestation (Wg) (*Table 1*), characterized by the co-migration of endothelial sprouts and intimately associated pericytes (18). The tight junction-associated proteins occludin and claudin 5 are expressed in endothelial cells on week 12 Wg, and by week 18 Wg these proteins display an intercellular localization pattern similar to that of the adult. The blood vessel density and the percentage of blood vessel area are largest in the germinative matrix exposed to nascent CSF followed by gray matter and then the white matter in all of the gestational age between 16 and 40 weeks (19). In humans, astrocyte end-feet start to escheat endothelial cells before birth around week 20 Wg depending on the brain region (20). This is consistent with the mainly postnatal

development of end-feet in rodents, as rat neonates are considered to be equivalent to human fetuses of that stage in terms of cortical development.

Despite the early appearance of a typical morphologic BBB phenotype characterizing the developing brain vessels, the more limited thickness of the basal membrane and the gradual covering by astrocytic end-feet suggest that the BBB maybe more fragile and prone to disruption during the peri and early postnatal period than in the adult.

The CPs start developing from the dorsal part of the neural tube even before the vasculature becomes significant in fetal life, at the seventh week of gestation in humans (*Table 1*).The concurrent formation of the choroidal epithelium and vascular conjunctive stroma (*Figure 2*) is complex, interrelated, and involves bone morphogenic proteins such as *Gdf7* gene product, the homeobox protein *Otx2*, and sonic hedgehog signaling. The CPs mature early to present an “adult” phenotype at birth (*Table 1*). They keep extending after birth however to match brain growth, as a result of an active mitogenic epithelial area at the root of the CPs. A detailed description of CP development and differentiation is described in (21,22). The choroidal epithelium displays a tight phenotype as soon as it invaginate from the neural tube, and the signaling mechanism associated with this barrier phenotype has yet

to be identified. The CPs are also an important transport/secretion site of nutrients and hormones delivery to the developing brain (21,186,187). The CPs epithelial tight junctions are made of a large number of different proteins, including the transmembrane proteins occludin, claudin1, claudin 2, and claudin 3. The relative proportion of these proteins however changes with development, with claudin 2 being more expressed postnatally and in adults, and claudin 3 being more expressed prenatally, possibly in connection with the selective choroidal function linked to CSF secretion which increases after birth (30).

Efflux transporters and enzymes that participate in the neuroprotective functions of brain barriers present a variable degree of expression and function at birth, which also differ between the cerebral vasculature and the CPs (Table 1). The BCSF barrier achieves an adult phenotype earlier than the BBB. This may be linked to the sensitivity of selected efflux transporters to the signals originating from the astrocytic endfeet, the later maturing mainly during the peri- and the postnatal period (188). For instance, at the BBB, the expression of several efflux transporters such as ABCB1 (PGP; MDR1), ABCG2 (BCRP), ABCC4 (MRP4) but not of others (SLC22A8/OAT3) increases from birth to adult stage. This is especially patent for ABCB1 which is 5 times lower in the early postnatal stages than in adults (25-28). In CPs, the main efflux transporters are ABCC proteins (MRPs), and they are well expressed and active at birth. Glutathione-S-transferases and glutathione-peroxidases, the sulfotransferase SULT1A1 and at least in rodents, UDP-glucuronosyl transferases are detoxification enzymes mainly found in the CPs, that keep the concentration of various toxicants low in the brain. Most of them are especially active postnatally (31-34).

### Permeability of BBB to bilirubin

As the paracellular pathway across brain barriers is sealed by tight junctions, and non-specific pinocytosis is very low at brain barriers, protein-bound UCB has no access to the brain, except for a small fraction that could access the CSF across specific protein transporting cells from the choroidal epithelium during development (189).

In contrast, tight junctions are not a hindrance to the brain penetration of lipophilic Bf, which occurs through a transmembrane diffusional pathway (see Figure 1). Several mechanisms may however limit this diffusion of Bf in the postnatal brain.

ABCC1, expressed mainly at the BCSFB, and to some extent ABCB1, expressed mainly at the BBB, recognize

UCB as a substrate. These efflux proteins will pump part of UCB back into the blood. The process however is not expected to be very efficient in neonates as the expression of ABCB1, whose affinity for UCB is not high, is still low in brain capillaries at that developmental stage (see Table 1). UCB is also metabolized by UGT1A1 (see Figure 1). While choroidal UGT1A-dependent enzymatic activities are high during postnatal development (Table 1), whether the specific UGT1A1 isoform is active is not known. UCB can be oxidized by cytochrome P-450-dependent monooxygenases, such as CYP1A1/1A2 (Figure 1). This is however unlikely to constitute an important hindrance to the passage of UCB across the brain barriers, as CYP are not major detoxification enzymes at brain barriers [reviewed in (31,190)]. UCB transfer to the brain can also be slowed down through binding to glutathione-S-transferase (GST) subunits alpha, but the expression of these specific isoforms raises only postnatally in brain barriers, hence this mechanism is unlikely to be significant in neonates.

Altogether the evidence points to transport and metabolic mechanisms at undamaged brain barriers that may prevent or reduce the diffusion into the brain of unbound UCB circulating at a physiologically very low level in the plasma, but that should be overrun if unbound UCB reaches higher pathological levels [reviewed in (31)]. The regional differences that have been observed in brain sensitivity to UCB appear not linked to regional differences in barrier permeability to UCB. Whether localized differences in interstitial fluid movements, and CSF flow, impact the cerebral bio-disposition of UCB, and influence this regional susceptibility remains to be investigated.

### Effect of bilirubin on BBB functions

Brain interfaces are the first cerebral cells exposed to the high concentration of UCB circulating in the plasma during pathological neonatal jaundice. They may therefore be a primary target for UCB toxicity, as an alteration in the development of the neurovascular network and CPs, and of their associated barrier phenotype, would impact brain maturation.

So far, few investigations have been performed on this matter, and most are *in vitro* studies using cultured brain endothelial and epithelial cells. The integrity of the endothelial monolayers was found sensitive to UCB, while that of the choroidal epithelial monolayers was not (115,191).

Because BBB models do not yet recapitulate all features of BBI *in vivo*, more information from *in vivo* studies needs to be collected before a conclusion can be drawn

on the effect of free UCB on brain barriers integrity. A case report of a pre-term neonate with severe kernicterus indicates that signs of neurovascular network alteration was observed on autopsied brain tissue, possibly linked to an increase in vascular endothelial growth factor (VEGF) signaling (192). Given the comorbidities associated, whether UCB alone was responsible for these alterations remains to be understood. One study performed in Gunn rats, an animal model of jaundice characterized by a rapid postnatal increase in serum UCB evidenced a decrease in ABCG2 protein levels in CPs. This finding could be reproduced *in vitro* on choroidal epithelial cells chronically exposed to UCB, suggesting that ABCG2 downregulation results from a direct effect of UCB on the BCSFB (115). In a model of bile duct ligation, a decrease in ABCG2 was observed at the BBB, with evidence that UCB mediates the effect, and without apparent impairment of the integrity of the barrier (193).

These *in vivo* data suggest that UCB induces functional changes at brain barriers, rather than overt impairment of their integrity.

## Conclusions

In the last decades, a lot of bench-based work has been performed to unravel bilirubin-induced neurotoxicity. Some “dogma” fell and new knowledge raised new hypotheses that need to be experimentally and clinically explored by the “yellow researchers”. The cooperative, international, and multidisciplinary expertise will be the key to success.

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