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Unconjugated free bilirubin in preterm infants

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ABSTRACT

Background: Hyperbilirubinemia guidelines are based on total serum bilirubin (TSB), in combination with either gestational age (GA) or birth weight (BW), postnatal age and specific risk factors. However, TSB is a poor predictor of bilirubin-induced neurotoxicity (BIND). Free unconjugated bilirubin (UCBfree) and the UCBfree/TSB ratio are more directly related to BIND, but data on their postnatal courses are unknown.
Aims: To characterize the postnatal courses of UCBfree and UCBfree/TSB ratio, and assess their relationships with clinical characteristics.
Subjects: 72 preterm infants ≤ 32 weeks GA, admitted to the University Medical Center Groningen, The Netherlands.
Study design: During the first postnatal week, bilirubin plasma parameters were analyzed and their relationship with clinical parameters was analyzed. Postnatal changes were analyzed using Generalized Estimating Equations. Data are expressed as medians [ranges].
Results: Less than 10% of the cohort (GA: 29 [26–31] weeks; BW: 1165 [600–1975] g) showed hyperbilirubinemic risk factors. We observed a large variation in UCBfree (27 [1–197] nmol/L), that could partly be explained by postnatal age and gender, but not by other risk factors. Maximal UCBfree levels of 50 [13–197] nmol/L occurred at day 4 and were higher in males. In contrast to TSB, UCBfree/TSB ratios (0.19 [0.01–1.04]) were higher in infants with low GA/BW.
Conclusion: UCBfree levels vary considerably in preterm infants, despite a low incidence of hyperbilirubinemic risk factors and similar TSB-based phototherapy treatment. UCBfree could not be predicted by GA or BW, but UCBfree/TSB ratios are highest in the smallest preterms, while they have the lowest TSB levels.

1. Introduction

In preterm infants below 35 weeks of gestational age (GA), current management guidelines of unconjugated hyperbilirubinemia are mainly consensus based [1]. The treatment thresholds are based on total serum bilirubin (TSB) and mainly depend on either GA or birth weight (BW) [1–3], in combination with risk factors, such as hemolysis, hypoalbuminemia, sepsis, acidosis and respiratory problems [1,4]. The threshold TSB levels at which phototherapy is started are based on studies in which TSB levels were associated with (impaired) neurodevelopmental outcome. Consequently, TSB treatment thresholds increase with postnatal age and higher bilirubin levels are tolerated at older age. Yet, studies on bilirubin-induced neurological damage only provide limited evidence on harmful TSB levels, because most factors that increase the risk of neurodevelopmental delay (e.g. asphyxia, intracranial hemorrhage, prematurity) also increase TSB levels and bilirubin neurotoxicity susceptibility. In essence, the peak TSB level is a poor predictor of the likelihood of bilirubin-induced neurotoxicity (BIND) [1], especially in preterm infants. To illustrate this, kernicterus can occur in extremely low birth weight infants with only modestly elevated TSB levels [5,6].

A more appropriate parameter to base management guidelines on could be unconjugated non-albumin-bound bilirubin (UCBfree). UCBfree can translocate across the blood-brain barrier [7,8], where it may induce apoptosis and necrosis in specific brain areas [8,9]. Preterm infants, especially when ill, may have high UCBfree levels, partly attributable to a low bilirubin-albumin binding affinity (Ka) compared to term infants [10,11]. The UCBfree/TSB ratio, which represents the combination of magnitude (represented by TSB) and distribution (represented by UCBfree) of the accumulated bilirubin load, has been proved

Abbreviations: UCBfree, free bilirubin; TSB, total serum bilirubin; UCBfree/TSB ratio, free bilirubin/total serum bilirubin ratio; Ka, bilirubin-albumin binding affinity; GA, gestational age; BW, birth weight; HRP, horseradish peroxidase; GEE, Generalized Estimating Equations.

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to correlate better with automated auditory brain stem response than either UCBfree or TSB alone and is suggested to be the best BIND predictor [12].

UCBfree has been frequently measured using a kinetic peroxidase methodology [13], and has even resulted in UCBfree-based treatment thresholds in one country (i.e. Japan). Its accuracy, however, has been debated [13–16]. Since then, an adapted peroxidase method has been developed [16,17]. Nevertheless, an FDA approved technology to routinely measure UCBfree in the clinic is not universally available.

As a first step towards eventual application of UCBfree levels for treatment guidelines of neonatal hyperbilirubinemia, we aimed to describe the physiological postnatal course of UCBfree levels in preterm infants. Therefore, we assessed the effect of postnatal age on UCBfree and the UCBfree/TSB ratio. Furthermore, since both GA and BW are used in TSB-based treatment guidelines [1–3], we analyzed the relation between UCBfree and the UCBfree/TSB ratio with GA and BW. In addition, we analyzed the relationship between UCBfree and UCBfree/TSB ratio and gender, since gender differences in TSB levels have been previously reported in low BW infants [18]. We also determined the effects of several hyperbilirubinemic and BIND risk factors.

2. Subjects and methods

2.1. Patients

This retrospective study was carried out in 72 preterm infants ≤32 weeks GA, treated in the neonatal intensive care unit of the Beatrix Children's Hospital, University Medical Center Groningen, between April 2007 and April 2008. The patients had been included in a multicenter randomized controlled trial investigating the additional use of the Bilirubin/Albumin ratio in the treatment of preterms with hyperbilirubinemia (BARTrial; ISRCTN 74465643I). The BARTrial included 615 children, from which 72 were randomly selected for UCBfree measurements, if remaining blood sample was available. Infants were included after parental consent within 24 h after birth. Exclusion criteria were major congenital malformations, clinical syndromes, or chromosomal abnormalities. Infants were treated with phototherapy, according to the Dutch guideline (19), which is TSB-based and depends on postnatal age and BW, in combination with risk factors; asphyxia (Apgar score < 3 at 5 min), hypoxemia (PaO_2 < 5.3 kPa for >2 h), acidosis (pH < 7.15 for > 1 h), hemolysis (with positive Coombs reaction), clinical sepsis (with need for vasopressors) and intraventricular hemorrhage (>grade 2, according to Papile) [19]. No measurements could be performed in 24 patients on day 1; 7 on day 2; 6 on day 3 and 4; 2 on day 5; 5 on day 6 and 4 on day 7. Neonatal hearing was screened by measuring the automated auditory brainstem response with an ALGO® neonatal hearing screener (Natus Medical Inc., San Carlos, CA, USA) at discharge.

2.2. Bilirubin measurements

Blood samples were collected daily and plasma was stored at −80 °C under argon protected from light. TSB and UCBfree levels were determined with a Zone Fluidics system (Global Flopro, Global Fia Inc., WA), which measures UCBfree using the horseradish peroxidase (HRP) reaction [16,17]. HRP oxidizes UCB to a colorless compound, but albumin-bound UCB is protected from oxidation [17]. 3 mg HRP and 3 mg glucose oxidase were diluted in 3000 μL 0.1 M phosphate buffer and 4 and 8 μL of this solution were used for analysis. Albumin levels were determined by routine spectrophotometry on a P800 unit from Roche Diagnostics Ltd. (Basel, Switzerland) in the same week as TSB and UCBfree measurements. Phototherapy was started when indicated based on TSB measurements from the same spectrophotometer, according to the national guidelines. Ka was calculated, using the following formula: Ka = (TSB − UCBfree) / (UCBfree × (Albumin − TSB + UCBfree)) [20].

2.3. Statistics

UCBfree, TSB and albumin levels were measured, and the UCBfree/TSB ratio, Ka, and the TSB/albumin (B/A ratio) were calculated. In addition, the ranges of these parameters were assessed for every individual and for the entire cohort. Changes in UCBfree, TSB, UCBfree/TSB ratio, Ka, albumin, and the B/A ratio over time were analyzed using Generalized Estimating Equations (GEE), a technique used to assess the capacity of independent variables to explain the intra- and interindividual variation of a certain dependent variable [21]. It does not depend on complete data but includes data for all individual time points and therefore maximizes the use of available data. Demographic factors and clinical risk factors were entered in a GEE model to assess their influence on the variation in bilirubin parameters. For univariate analysis, all demographic and clinical risk factors described in Table 1 were individually entered in different GEE models to explain variation in UCBfree, TSB, UCBfree/TSB ratio, Ka, albumin, and B/A ratio, respectively. Based on univariate analysis, further GEE analyses were performed using GA, BW, gender, albumin and postnatal age. In addition to individual parameters, interaction variables (GA*postnatal day, BW*postnatal day and gender*postnatal day) were analyzed to assess differences in bilirubin course depending on GA, BW or gender. For all GEE analyses, an exchangeable working correlation matrix was used, assuming a fixed correlation between measurements within the same subject.

The relationship between bilirubin parameters, and GA and BW, was assessed at the time of maximal UCBfree levels. For this analysis, infants were divided in BW and GA cohorts. BW cohorts were based on the

Table 1
Characteristics of 72 preterm infants < 32 weeks (total X (%) infants).

Clinical characteristics	Infants (N = 72)
Gestational age in weeks, median [range]	29.1 [26.1–31.9]
Birth weight in g, median [range]	1165 [600–1975]
Male/female	38/34
Antenatal steroids (%)	
Yes	38/72 (53%)
Not completed	23/72 (32%)
No	6/72 (8%)
Unknown	5/72 (7%)
Birth trauma, total (%)	6/72 (8%)
Caput succedaneum, cephalic hematoma	1/72 (1%)
Other hematoma	5/72 (7%)
Other bruising	0/72 (0%)
Agar score < 3 at 5 min (%)	0/72 (0%)
Coombs (%)	
Positive reaction	0/72 (0%)
Negative reaction	42/72 (58%)
Unknown	30/72 (42%)
Irregular antibodies child (%)	
Positive	0/72 (0%)
Negative	61/72 (85%)
Unknown	11/72 (15%)
Irregular antibodies mother (%)	
Negative	68/68 (100%)
Sepsis (%) requiring volume expansion or vasopressants	3/72 (4%)
Hypoxemia (%)	2/72 (3%)
Acidosis (%)	0/72 (0%)
Meningitis, positive liquor culture (%)	1/72 (1%)
Intracerebral hemorrhage > grade 2 (%)	3/72 (4%)
Abnormal auditory brain stem response at discharge	3/72 (4%)
Bilirubin parameters	**Median [range]**
UCBfree (nmol/L)	27 [1–197]
TSB (μmol/L)	152 [38–304]
UCBfree/TSB ratio	0.19 [0.01–1.04]
UCBfree maximum (nmol/L)	50 [13–197]
Ka (L/μmol)	109 [23–399]
Ka minimum (L/μmol)	60 [14–279]
Albumin (g/L)	32 [17–44]
B/A ratio	4.9 [1.0–11.0]

Data are displayed as n/N (%), except for gestational age, birth weight and male/female ratio and bilirubin parameters, which are displayed as median [range].

Dutch Treatment Guideline (<1000 g, 1000–1249 g, 1250–1500 g and 1500–2000 g) [19]. GA cohorts were based on numbers and include 26–28 weeks, 29–30 weeks, and 31–32 weeks. Differences between the cohorts were assessed using Kruskal-Wallis H-test with subsequent pairwise comparison using Mann-Whitney *U* tests. Furthermore, the effect of confounding factors; antenatal steroids, phototherapy and several drugs on bilirubin parameters was assessed using Mann-Whitney *U* tests. Analyses were performed using SPSS Statistics 20 for Windows Software (SPSS Inc., Chicago, IL). Statistical significance was considered to be reached at $p < 0.05$.

3. Results

3.1. Demographic and biochemical data

This cohort consisted of 72 preterms (38 male, 34 female), with GA ranging from 26 to 32 weeks (29 ± 2; mean ± SD) and BW ranging from 600 to 1975 g (1226 ± 291). Hyperbilirubinemic risk factors were relatively scarce within the cohort (Table 1). Within this relatively healthy cohort, we observed a large variation in bilirubin parameters.

Fig. 1 shows the courses of UCBfree, TSB, UCBfree/TSB ratio, Ka, albumin, and B/A ratio during the first 7 postnatal days. Individual levels of UCBfree, TSB, UCBfree/TSB ratio, Ka, albumin, and B/A ratio varied considerably over the days. Fig. 1A shows a more or less comparable postnatal course of UCBfree levels when compared to TSB levels, except for peak UCBfree levels, which occurred slightly later (day 4 versus day 3 for TSB (Fig. 1B)), but extremely high UCBfree levels were also measured in individual infants on day 2, 3 and 5. The median variation within the same individual in UCBfree levels was 37 [range 8–155] nmol/L, and in TSB was 77 [26–168] μmol/L. Fig. 1C displays extensive variation in UCBfree/TSB ratio, suggesting that the variation in UCBfree is not simply caused by the TSB variation.

3.2. Effects of gestational age and birth weight on bilirubin parameters

All clinical characteristics displayed in Table 1 were subjected to univariate analysis. GA, BW, gender, albumin and postnatal day could significantly explain the variation in UCBfree, TSB, UCBfree/TSB ratio, Ka, albumin or the B/A ratio (Table 2). Therefore, these parameters were entered in a GEE model to assess their ability to explain the intra- and interindividual variation in UCBfree, TSB, UCBfree/TSB ratio, Ka, albumin, and B/A ratio over time (Table 2).

3.2.1. UCBfree

BW, GA, albumin and gender did not have a significant influence on the observed intra- and interindividual variation in UCBfree (Table 2). Postnatal age showed a significant explanatory capacity ($p = 0.000$) for UCBfree variation, with the highest UCBfree levels at postnatal day 4 (Fig. 1A). No significant differences in UCBfree levels were detected between different BW and different GA cohorts at the fourth postnatal day (Fig. 2A), indicating that variation in UCBfree was not explained by BW or GA.

3.2.2. TSB

In contrast to UCBfree, TSB variation during the first postnatal week could be significantly explained by BW, gender, albumin and postnatal age, independently of each other. TSB was higher upon a higher BW, higher albumin level and higher postnatal age ($p < 0.01$) (Table 2). GA and male gender explained TSB variation during the first 4 postnatal days, during which TSB was higher in males ($p = 0.034$). At day 4, TSB was significantly different between the 4 BW and 3 GA cohorts, indicating highest TSB levels in more mature preterm infants with higher birth weights at day 4 ($p = 0.000$; Fig. 2B). Infants with a higher BW and GA generally had higher TSB levels during the first postnatal week.

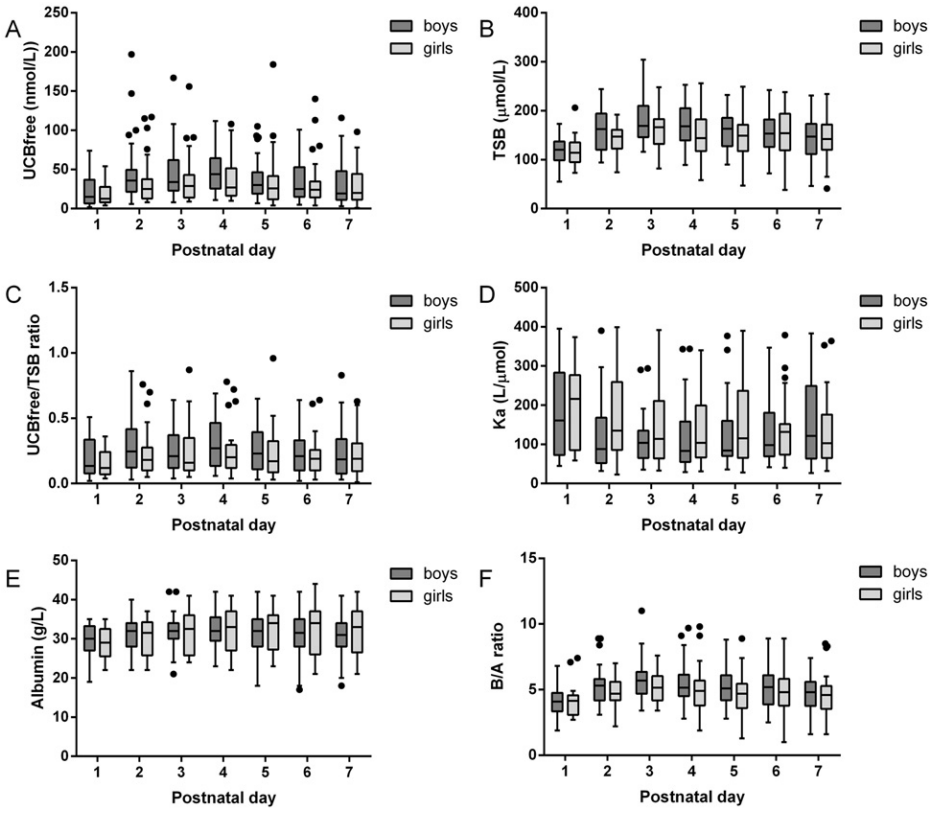

Fig. 1. Postnatal course for bilirubin parameters during the first postnatal week for boys and girls. A) Postnatal course in UCBfree. B) Postnatal course in TSB. C) Postnatal course in UCBfree/TSB ratio. D) Postnatal course in Ka. E) Postnatal course in albumin. F) Postnatal course in B/A ratio. Outliers (black dots) are depicted separately.

Table 2
Generalized Estimating Equations; univariate analysis and model.

Bilirubin parameters	Clinical parameters	Univariate analysis *p*-value	GEE model Coefficient	GEE model Confidence Interval	GEE model *p*-value
UCBfree	Gestational age	0,086			
	Birth weight	0,418			
	Gender	0,303			
	Albumin	0,375			
	Postnatal day	0,000	8,5–18,9	1,0–12,1–16,1–25,6	0,000
TSB	Gestational age	0,000	1,7	−1,9–5,3	0,358
	Birth weight	0,000	$6{,}0 \times 10^{-2}$	$4{,}0 \times 10^{-2}$–8.0×10^{-2}	0,000
	Gender[a]	0,202			
	Albumin	0,000	2,2	1,3–3,1	0,000
	Postnatal day	0,000	24,4–50,8	10,3–37,5–38,5–64,0	0,000
UCBfree/TSB ratio	Gestational age	0,001	$-2{,}0 \times 10^{-2}$	$5{,}0 \times 10^{-2}$–$1{,}0 \times 10^{-3}$	0,059
	Birth weight	0,002	$-4{,}7 \times 10^{-5}$	$0{,}0$–$7{,}3 \times 10^{-5}$	0,451
	Gender	0,442			
	Albumin	0,003	$-8{,}0 \times 10^{-3}$	$1{,}4 \times 10^{-2}$–$3{,}0 \times 10^{-3}$	0,003
	Postnatal day	0,021	$4{,}0 \times 10^{-2}$– $9{,}0 \times 10^{-2}$	$2{,}0 \times 10^{-3} - 4{,}0 \times 10^{-2}$– $9{,}0 \times 10^{-2} - 0{,}1$	0,007
Ka	Gestational age	0,000	11,3	−0,2–22,8	0,054
	Birth weight	0,001	$6{,}0 \times 10^{-2}$	$2{,}0 \times 10^{-2}$–0,1	0,132
	Gender	0,420			
	Albumin	0,513			
	Postnatal day	0,089			
Albumin	Gestational age	0,063			
	Birth weight	0,058			
	Gender	0,762			
	Postnatal day	0,000	1,6–3,0	0,8–4,0	0,000
B/A ratio	Gestational age	0,002	$9{,}0 \times 10^{-3}$	-0,1–0,2	0,907
	Birth weight	0,000	$2{,}0 \times 10^{-3}$	$1{,}0 \times 10^{-2}$–$3{,}0 \times 10^{-2}$	0,000
	Gender	0,235			
	Postnatal day	0,000	0,6–1,5	0,2–1,0–1,1–1,9	0,000

[a] When analyzed over the first 4 postnatal days, gender (p = 0,0340) and GA (p = 0.000) could significantly explain TSB variation. Clinical parameters that proved to be significant in univariate analysis were entered together in a GEE model to assess potential interactions.

3.2.3. UCBfree/TSB ratio

Both BW and GA showed a significant effect on the variation of the UCBfree/TSB ratio, but this effect vanished when corrected for postnatal age and albumin, indicating that either the effect of postnatal age ($p = 0.007$) overrides BW and GA, and/or that their effect is already captured within albumin (Table 2). The latter had a significant influence ($p = 0.003$). Interestingly, when looking at day 4, we observed a statistically significant difference between the 3 GA cohorts (Fig. 2F; $p = 0.025$), specifically between the 26–28 weeks group and the 2 older GA groups ($p < 0.05$). In addition, the UCBfree/TSB ratio was significantly different between the BW cohorts (Fig. 2E; $p = 0.014$), being higher in infants below 1500 g ($p < 0.05$), suggesting that lower GA and lower BW children may have been more at risk for BIND, even though their TSB levels were lower (Fig. 2D).

3.2.4. Ka

The observed variation in Ka during the first postnatal week could be explained by both BW and GA during univariate analysis (Table 2). However, both were not significant when simultaneously used in a GEE model. Still, when compared at day 4, infants with a BW above 1500 g showed significantly higher Ka levels than infants with a lower BW (Fig. 2G; $p = 0.019$).

3.2.5. Albumin

The observed variation in albumin could not be explained by BW, GA or gender, but slightly by postnatal age. Fig. 2I and J show a statistically significant stepwise increase in albumin with increasing BW and GA.

3.2.6. B/A ratio

The variation in B/A ratio could in univariate analysis be explained by BW, GA and postnatal day. In the GEE model, only BW and postnatal age remained significant, as was the case for TSB. However, since significance was nearly reached for GA and BW ($p = 0.063$ and $p = 0.058$, respectively), and because a positive correlation between BW and GA and albumin has been reported [22], it seems reasonable to assume that both TSB and albumin affect the B/A ratio. Figs. 2 K and L show significant differences of the B/A ratio on day 4. Like TSB, the B/A ratio is lowest in infants < 1000 g and increases with higher GA.

3.3. Gender differences in bilirubin parameters

Fig. 1 shows slightly higher UCBfree, TSB and UCBfree/TSB and B/A ratios, and slightly lower Ka values in males when compared to females. A similar pattern was predicted by the Estimated Marginal Means (EMMs), generated by the GEE model (Fig. 3). Only UCBfree levels on day 4 were significantly higher in males than in females ($p = 0.047$), indicating that males experienced higher peak UCBfree levels compared to their female postnatal age matches. We found no significant difference between males and females on day 4 regarding TSB, which could be explained by equal TSB-based treatment guidelines for both genders. There was no significant difference in UCBfree/TSB ratio on day 4, which can be explained by the fact that by calculating a ratio, the absolute magnitude of both original variables vanishes. Ka and albumin courses did not differ significantly between males and females. The B/A ratio was also not significantly different between males and females, ($p = 0.068$).

3.4. Effect of medications on bilirubin parameters

We reported the use of several drugs with known bilirubin interactions [23]; phenobarbital, ibuprofen, hydrochloride thiazide, caffeine and several antibiotics. For phenobarbital ($n = 1$), hydrochloride thiazide ($n = 0$), and ibuprofen ($n = 0$), the prescription incidence was so low that no conclusions could be drawn. Virtually all infants received

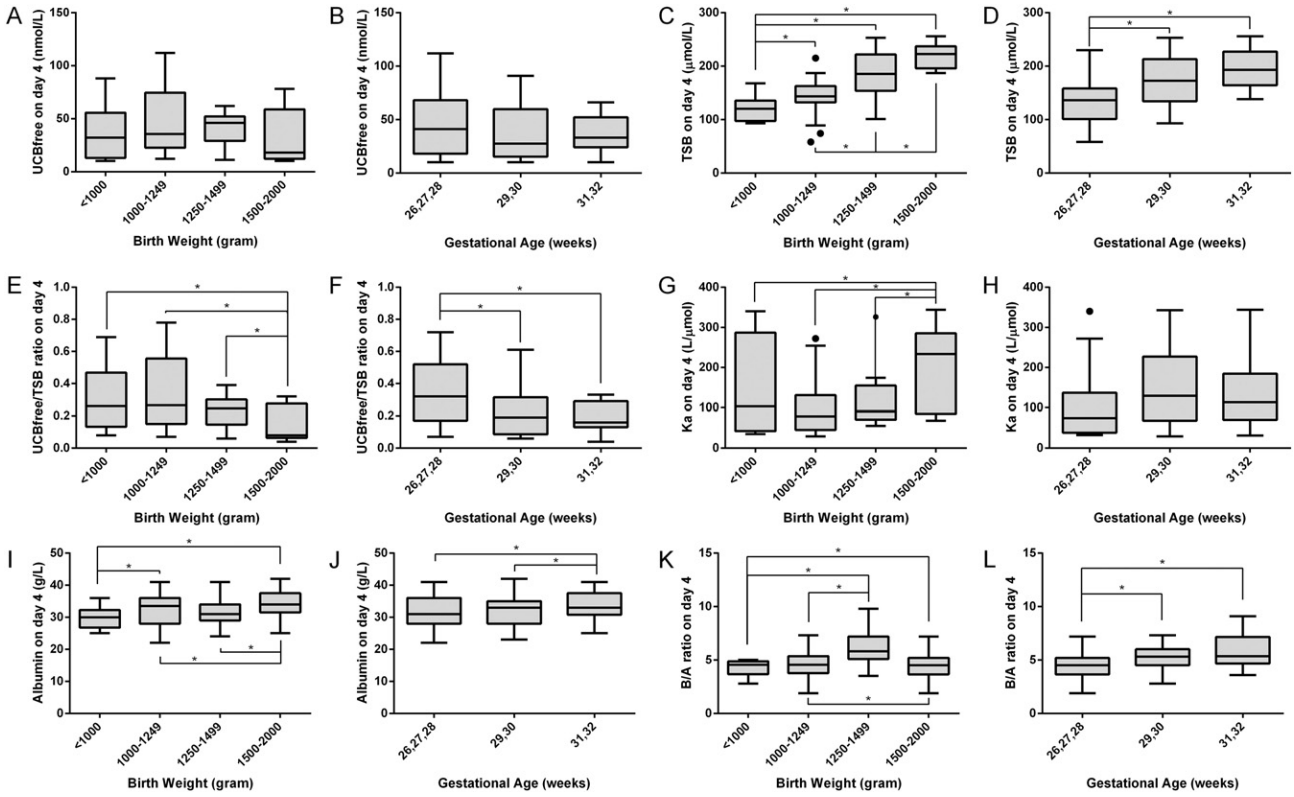

Fig. 2. Bilirubin parameters on day 4, divided in 4 BW or 3 GA cohorts. A) UCBfree in 4 BW cohorts, no significant differences. B) UCBfree in 3 different GA cohorts, no significant differences. C) TSB is significantly higher with increasing BW, significant differences between all the cohorts. D) TSB is significantly different between the lowest GA cohort and the other 2 cohorts. E) UCBfree/TSB ratio is significantly lower in the highest BW cohort, compared to all lighter cohorts. F) UCBfree/TSB ratio is significantly higher in the lowest GA cohort, compared to the other 2 cohorts. G) Ka is significantly higher in the heaviest BW cohort, compared to all the lighter cohorts. H) Ka in 3 different GA cohorts, no significant differences. I) Albumin is significantly different between the BW cohorts, except for cohort 1250–1499 versus the lower 2 cohorts. J) Albumin increases significantly with increasing GA. K) B/A ratio increases significantly with increasing BW till 1500 g. L) B/A increases significantly with increasing GA.

antibiotics, the fast majority ($n = 41$) received a combination of amoxicillin and gentamycin. Ten children received a combination of amoxicillin, gentamycin and augmentin. No significant differences were detected between the different antibiotics groups for any of the bilirubin parameters (data not shown).

Caffeine was prescribed to 54 infants and 15 infants did not receive any caffeine. Caffeine improves neurodevelopmental outcome [24], is also a known bilirubin-albumin displacer [25], but this capacity has no known clinical implications on UCBfree or neurotoxicity. UCBfree levels were not increased by caffeine and no significant difference was detected in TSB, the UCBfree/TSB ratio, Ka, or the B/A ratio. Only albumin levels were significantly higher in infants who received caffeine; 33 ± 5 g/L in caffeine users vs. 30 ± 4 g/L in non-users (mean ± SD on day 4) ($p = 0.033$).

Besides postnatal medications, we assessed the use of antenatal steroids by the mother. The majority of the mothers in our cohort received at least one dose of antenatal steroids (61/72 = 85%), and only 6 mothers did not receive any. No significant differences were detected between antenatal steroid users and non-users, although mean UCBfree and UCBfree/TSB ratios seem slightly higher in non-users (Suppl. Fig. 1).

3.5. Effect of phototherapy on bilirubin parameters

The majority of our cohort received phototherapy during the first postnatal week (93%). Five infants did not receive phototherapy. Infants with phototherapy ($n = 67$) were compared with infants without phototherapy ($n = 5$). The mean maximal and minimal levels levels of all bilirubin parameters are calculated and compared (Suppl. Table 1), but no significant differences between the groups could be detected.

3.6. Auditory brainstem response

Auditory brainstem evoked responses were tested at discharge in the entire cohort and mild hearing loss was detected in 3 children. The mean bilirubin parameters of these 3 infants were compared with the entire cohort. All bilirubin parameters varied in the 3 infants with mild hearing loss, but did not display extreme values when compared to the entire cohorts. B/A ratios were relatively high in 2 out of 3 infants with abnormal hearing screening. One infant had a relatively high TSB, 1 had a relatively low Ka and 1 a relatively low albumin when compared with the entire cohort (Suppl. Table 2).

4. Discussion

This study characterizes the postnatal courses of UCBfree and UCBfree/TSB ratio, and describes their relationships with BW, GA, gender, postnatal age, serum albumin and other clinical characteristics.

We report considerable variability in UCBfree, and TSB levels and UCBfree/TSB, as well as B/A ratios during the first postnatal week. We also show that the variation in UCBfree is explained by postnatal age, not by BW or GA. UCBfree/TSB ratios are highest in infants with lowest BW and GA, who have lowest TSB levels. In contrast, B/A ratios decrease with lower birthweight and lower gestational age, also in the presence of low TSB levels. The latter confirms the well-known positive relationship between TSB and BW or GA, which, naturally, is also due to the lower TSB intervention thresholds for phototherapy in the smaller and younger preterm infants. UCBfree levels appear to be maximal one day after maximal TSB levels. At this fourth postnatal day, male preterm

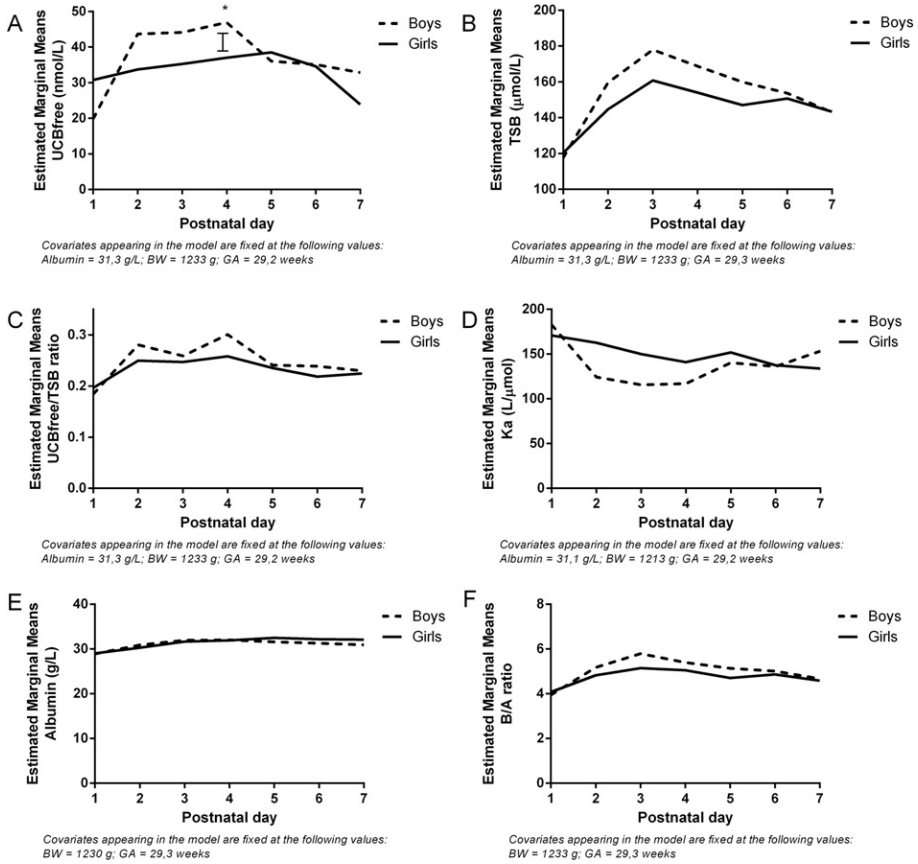

Fig. 3. Estimated Marginal Means (EMM) for bilirubin parameters in boys and girls. EMMs are generated by a Generalized Estimating Equation model, including albumin, BW and GA. EMMs are generated assuming fixed values for albumin, BW and GA, which are given below each figure. A) EMMs for UCBfree, with a significant difference between males and females on day 4. B) EMMs for TSB. C) EMMs for UCBfree/TSB ratio. D) EMM for Ka. E) EMMs for albumin, no significant difference between males and females. F) EMMs for B/A ratio, the minimal *p*-value for the difference between boys and girls is 0.068 (day 3).

infants have higher UCBfree levels when compared to female preterm infants.

TSB treatment thresholds for unconjugated hyperbilirubinemia in preterm infants are based on BW [19] or GA [1,2]. The choice for either of the two is not supported by firm evidence, although a strong association between the two exist. Cashore et al. showed a positive correlation between GA and BW on bilirubin-binding capacity [28,29], but to our knowledge this is the first study assessing relationship between UCBfree, UCBfree/TSB ratio, and BW or GA over several days with serial measurements in preterm infants. We did not find a significant effect of GA and BW on UCBfree levels.

TSB levels increase upon higher BW or GA. These relationships between TSB and either BW or GA can partially be attributed to applied treatment thresholds. According to the Dutch guidelines, the TSB thresholds at which phototherapy is started, are based on BW. BW will thereby directly affect TSB levels. The strong relationship between TSB and BW or GA might be predominantly responsible for the significant effect of GA and BW on the UCBfree/TSB ratio in the first week of life. Despite the fact that lower BW or GA children have lower TSB levels, their UCBfree/TSB ratio was higher, indicating that they might be at increased risk for developing BIND. Because the UCBfree/TSB ratio is a parameter that is closely associated with bilirubin neurotoxicity, this finding supports management of hyperbilirubinemia in preterm infants based on either BW or GA. Moreover, the rather high UCBfree levels despite low TSB levels indicate that current TSB treatment thresholds for (extremely) preterm infants may not be appropriate as assumed. In fact this is an argument in favor of UCBfree-based treatment thresholds, underlining the need for further research delineating the relation between UCBfree and neurotoxicity.

Of interest are the higher B/A ratios in more mature infants. Younger infants with a relatively high B/A ratios are considered to be at risk for bilirubin neurotoxicity [30]. Indeed, 2 out of 3 infants with abnormal neonatal hearing screening had relatively high B/A ratios when compared to the entire study population. Overall, the B/A ratio was lower in the youngest infants. Taken as a group, preterm infants treated according to current TSB treatment thresholds are not at risk of adverse outcomes. Three infants had an abnormal hearing screening (Suppl. Table 1). In this study, albumin levels were positively related to TSB, which could, at least in part, be explained by the finding that both TSB and albumin increase with increasing BW/GA and by the bilirubin binding of albumin; in plasma, bilirubin is for >99% bound to albumin [26]. Yet, albumin was not related to UCBfree levels, and despite the large UCBfree variation, hypoalbuminemia (serum albumin < 20 g/L) rarely occurred ($n = 6$ measurements). Low albumin levels may enhance bilirubin neurotoxicity, especially in the smallest and sick preterm infants, but our data might implicate an intact reserve binding capacity of albumin in this cohort of preterm infants with few clinical complications.

In contrast to national and international standardizations for TSB and albumin measurements [26], no 'gold standard' is available for UCBfree. Therefore, UCBfree values depend on the applied methodology and UCBfree values by different research groups vary considerably [12,27–31]. However, the establishment of a universal UCBfree measurement methodology and UCBfree calibrators is pivotal in the clinical application of UCBfree in hyperbilirubinemia management guidelines. One FDA-approved method (Arrows Co. LTD, Osaka, Japan) exists, but this is not readily available outside Japan. In addition, a prerequisite for this Arrows method is a 42-fold sample dilution before analysis, which has been shown to intrinsically alter the bilirubin-albumin binding. Arrows'

UCBfree measurements vary with different peroxidase concentrations [27]. These factors may, at least in part, underlie the differences in UCBfree concentration between Arrows and our study. Minimal sample dilution, and the use of more than one peroxidase concentration, as we performed in this study, is advocated to improve the accuracy of UCBfree measurement [27].

Ka was significantly related to both BW and GA during univariate analysis, but this was not apparent in the GEE model. This could be explained by the fact that when variables are simultaneously entered in a GEE model, they could correct each other. In case two variables are correlated, as is the case with BW and GA, they need to correct for each other's influence, to prevent over-estimation of the explanatory power of the model. When the explanatory power of the two variables is similar, both variables could potentially diminish each other's powers to a similar extent, resulting in 2 insignificant variables in the model.

Besides BW and GA, we did not find a relation between Ka and clinical risk factors. However, even within the same BW or GA cohort, we showed an extensive variation in Ka (Fig. 2), suggesting that the variation in UCBfree could be caused by an instable Ka. However, the Ka in our study is calculated from TSB, UCBfree and albumin, and is therefore correlated to these 3 variables. The calculation of Ka, as opposed to Ka measurement by direct titration or hematofluorometric assays, constitutes a limitation to our study [23,24]. The relatively small size and relative good health of our cohort is another limitation, resulting in insufficient power to detect significant correlations between bilirubin parameters and previously described hyperbilirubinemic and BIND risk factors [21,22]. Despite relatively good health, the cohort still showed extensive variation in bilirubin parameters, indicating that other factors, such as certain drugs or other mediators, might have played a role. Regarding the latter, levels of free fatty acids would have been worthwhile to study, but unfortunately, these were not at hand in our cohort. We assessed the effect of several drugs with known bilirubin interactions [23], and fortunately we did not find higher UCBfree levels in infants who were treated with these drugs. Only albumin levels were significantly higher in infants receiving caffeine. Although increased albumin levels are not reported as a side effect of caffeine, these higher albumin levels could, at least in theory, contribute to the neuroprotective effect of caffeine by decreasing UCBfree levels.

Concerning the effect of antenatal steroids on neonatal hyperbilirubinemia, no consensus exists in literature and both increased and decreased bilirubin levels have been reported [32–37]. In our cohort, the majority of the mothers received at least one dose of antenatal steroids, and only 6 mothers did not receive any. Therefore, no robust conclusions on this matter could be drawn from our data.

An alternate explanation for the observed variation in bilirubin parameters may reside in phototherapy treatment, which was started whenever TSB treatment thresholds were reached. Phototherapy is not only known to reduce TSB levels, but also UCBfree levels (up to 55% in specific animal models) [22,29,38]. Although inevitable, phototherapy might thereby conceal effects between bilirubin parameters and potential clinical risk factors. Unfortunately, the low number of infants who were not treated with phototherapy during the first postnatal week does not allow drawing any conclusion on his matter. Male gender could be a yet unidentified potential predictor of UCBfree in the first week of life. Gender differences in TSB levels have been reported previously in low BW infants [18], but have not been reported for UCBfree levels. Our data suggest an increased miscible pool of bilirubin (as reflected by higher TSB levels) and possibly a novel hyperbilirubinemia risk factor, i.e. higher UCBfree levels - at least at day 4 - in male infants. This finding is in line with the AAP-guideline of 2004, which mentions male gender as a minor risk factor. In addition, idiopathic kernicterus is more prevalent in males, whereas the prevalence of kernicterus with a known cause is equal between males and females [32]. It is tempting to attribute this finding to increased UCBfree levels in males. The cause of these increased levels are unknown, but could include gender differences in bilirubin conjugation maturation in analogy to experimental animal data [32].

We found no significant difference between males and females on day 4 regarding TSB, which could be explained by equal TSB-based treatment guidelines for both genders. Nor we found a significant difference in UCBfree/TSB ratio on day 4, which can be explained by the fact that by calculating a ratio, the absolute magnitude of both original variables vanishes. However, males could still be exposed to higher bilirubin levels at similar GA or BW. If both UCBfree and TSB are relatively equally increased in males, the ratio will be similar to the female UCBfree/TSB ratio. Further research is needed to show whether male gender could predispose to higher UCBfree levels.

Since UCBfree is considered to be mainly responsible for BIND, this study signifies the need for UCBfree treatment guidelines and better understanding of the clinical variables that affect UCBfree and thus bilirubin-bindingIn parallel, larger studies are needed to assess the influence of these variables and UCBfree on bilirubin neurotoxicity. This will also contribute to a better understanding of BIND at low TSB levels. Unraveling the factors that interfere with and affect UCBfree measurements is an important prerequisite for the eventual implementation of UCBfree reference values and treatment thresholds. Ideally, management of neonatal hyperbilirubinemia would be based on a comprehensive diagnostic test that includes all bilirubin parameters, including UCBfree, TSB and bilirubin-albumin binding [39].

5. Conclusions

UCBfree levels vary considerably in preterm infants, despite a low incidence of hyperbilirubinemic risk factors and similar TSB-based phototherapy treatment. UCBfree could not be predicted by GA or BW, but UCBfree/TSB ratios are highest in the smallest preterms, while they have the lowest TSB levels. Further research is needed to determine clinical variables of increased UCBfree levels, especially in male preterm infants, and to establish whether this leads to imminent bilirubin-neurotoxicity.

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