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ORIGINAL ARTICLE

Clinical and genetic risk factors for moderate hyperbilirubinemia in Brazilian newborn infants

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Objective: To identify clinical and genetic risk factors for moderate hyperbilirubinemia during the first week of life.

Study Design: Using univariate and multivariate multiple regression analyses, the RR for clinical factors, the African variant of glucose-6-phosphate dehydrogenase (G6PD) deficiency (G202A/A376G), and $(TA)_n$ UGT1A1 polymorphisms were established in a cohort of 608 Brazilian newborn infants. Hyperbilirubinemia was monitored until 134.5 ± 49.8 h of life (IQR, 111.0 to 156.7). The dependent variable was total bilirubinemia (TB) $\geqslant 12.9$ mg per 100 ml estimated by transcutaneous or plasma bilirubin measurements.

Result: The African variant of G6PD deficiency and $(TA)_7/(TA)_7$ and $(TA)_7/(TA)_8$ polymorphisms present in 6.1 and 12.0% of newborns, respectively, were not risk factors for moderate hyperbilirubinemia. Coexpression of G6DP deficiency and UGT1A1 polymorphisms occurred in 0.49% of the subjects. Independent clinical predictors for TB $\geqslant 12.9$ mg per 100 ml were gestational age <38 weeks and reference curve percentiles $>$P40th.

Conclusion: In this study, G6PD deficiency and UGT1A1 gene promoter polymorphisms were not risk factors for moderate hyperbilirubinemia. Genetic factors may vary considerably in importance among different populations.

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Introduction

Neonatal hyperbilirubinemia is a common condition that has a multifactorial etiology. When total serum bilirubin rises to very high levels and for a prolonged period of time, it may cause acute bilirubinic encephalopathy and kernicterus.[1,2]

Glucose-6-phosphate dehydrogenase (G6PD) enzyme deficiency affects 300 to 400 million people worldwide and has been considered as a risk factor for kernicterus.[3] A strong association between G6PD deficiency and hyperbilirubinemia has been reported in populations of specific ethnic groups and cultures, because of the high prevalence rates of the Mediterranean variant.[4,5] In contrast, there are conflicting results on the incidence and risk of hyperbilirubinemia associated with the African variant in the USA,[6,7] although 20 to 30% of the reported cases of kernicterus are related to G6DP deficiency.[1]

The World Health Organization recommends universal neonatal screening for G6PD deficiency when its frequency in the male gender surpasses 3 to 5%.[8] However, there is a lack of consensus on whether this measure is effective at preventing significant hyperbilirubinemia and reducing kernicterus.[9]

Approximately 50% of G6DP-deficient individuals coexpress the uridine diphosphoglucuronosyl transferase (UGT1A1) promoter polymorphisms in at least one gene.[10,11] Homozygous individuals have about 30% of the normal UGT1A1 activity.[12] Thus, homozygosity and heterozygosity for this polymorphism are considered determinants for increased bilirubinemia levels alone[5] or combined with G6PD deficiency.[11]

Until recently, few authors[7,13] have shown a higher hyperbilirubinemia risk and phototherapy use in black males carrying the African variant of G6PD deficiency. In Brazil, where the African variant predominates,[14] the clinical and genetic factors for hyperbilirubinemia have not been investigated.

This study was conducted to determine the risk of moderate hyperbilirubinemia in the first week of life, according to the presence of G6PD deficiency, UGT1A1 polymorphisms, and some clinical factors. The purpose of the study was to identify the relative importance of genetic alterations in the occurrence of hyperbilirubinemia in newborns assisted in a hospital with a kernicterus prevention program.[15]

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Methods

A prospective cohort study was conducted in a public university hospital from June 2008 to October 2008 for total bilirubinemia (TB) monitoring in newborns during the first week of life. Any infant born in the institution was included in the study if healthy, birth weight $\geqslant$2000 g, and gestational age $\geqslant$35weeks. Moreover, at least one outpatient visit after hospital discharge was required in the first week of life for TB evaluation.

Exclusion criteria were neonatal intensive care unit admission, hemolytic disease because of Rh incompatibility, and Down's syndrome. Subjects with ABO hemolytic disease, along with those who did not return for a perinatal visit (TB monitoring) or those who had their first perinatal visit after 192 h of life, were discontinued.

Blood group, indirect Coombs' test, and elution test[16] were performed in the umbilical cord blood of all subjects whose mothers had type O blood.

Subjects were breastfed without restriction and maintained in rooming-in care from birth until hospital discharge. Neonatologists used the following criteria to supplement breastfeeding with formula: weight loss above 8% and clinical signs of low ingestion. If breastfeeding was contraindicated, milk formula was administered.

Evaluation of TB during admission

Regardless of earlier measurements, transcutaneous TB (TcB) was assessed in all subjects on the morning of hospital discharge, using Bilicheck (Georgia Respironics, Kennesaw, GA, USA) as in routine practice.[15] When TcB $\geqslant$ 14.0 mg per 100 ml, plasma total bilirubinemia (PTB) was measured by the bilirubinometer Unistat-Leica (Leica, Buffalo, NY, USA) during neonatal screening. Mean values of two PTB measurements were used.

TB values at discharge (TcB or PTB) were plotted on a reference curve[17] and the corresponding risk percentiles for hyperbilirubinemia were established.

Follow-up of TB after discharge

The frequency of return visits was individualized, according to risk percentile.[17] When TB at hospital discharge surpassed the P95th, the return visit was scheduled for the following day or discharge was postponed. Between the P95th and the P75th, the infant was reevaluated within 48 h. When TB <75th percentile, the return visit occurred between 72 and 192 h after discharge. All newborns with gestational age between 35 and 38 weeks, including those with TB <P75th, were reevaluated 48 h after discharge. When the newborn was discharged after 72 h of life and TB <P40th, the TB measurement at discharge was used as the outcome value.

After the first return visit, subsequent consultations were scheduled for 24 to 72 h intervals, according to the risk percentile and hours of life. Similar to monitoring during hospital admission, PTB was measured when TcB $\geqslant$ 14.0 mg per 100 ml.

When TB reached <12.9 mg per 100 ml, follow-up ended. In prolonged hospitalization, standard TB monitoring was maintained. A reference curve percentile[17] was not established when discharge took place after 168 h of life and phototherapy was used during hospital stay after delivery. Follow-up of infants treated with phototherapy was similar to untreated subjects, measuring TcB only 48 h after phototherapy was discontinued.

Phototherapy was indicated when PTB $\geqslant$ 20.0 mg per 100 ml for newborns at term and PTB $\geqslant$ 18.0 mg per 100 ml for preterm newborns, regardless of chronological age. For jaundice identified before 24 h of life, phototherapy was indicated after two consecutive TB measurements above 8.0 mg per 100 ml and a more rapid increase compared with the reference curve.[17] The double phototherapy devices used had 14 special blue lights (Philips TL52), mean spectral radiance above 45 $\mu W\,cm^{-2}\,nm^{-1}$, and were periodically calibrated.[18]

The highest TB value obtained during the follow-up period was the dependent variable categorized into two levels: $\geqslant$12.9 and $\geqslant$15.0 mg per 100 ml.

Screening, quantification of enzyme activity, and molecular analysis for G6PD deficiency and UGT1A1 genotyping

Umbilical cord blood was tested by the Laboratory of Molecular Biology of the institution. All samples were tested for methemoglobin reduction.[19] G6PD enzyme activity was quantified by the Beutler method in positive samples. Normal values used were 10.0 to 14.1 UI g Hb^{-1}.[20] Regardless of these preliminary results, the African variant (G202A/A376G mutation) was investigated in all subjects. The Mediterranean variant was assessed when the African variant was not identified.

DNA extraction was performed by the modified phenol/chloroform technique, and samples were quantified in NanoDrop ND-1000 spectrophotometers (NanoDrop Technologies, USA). Genomic DNA was amplified by polymerase chain reaction technique[21] to identify exons 4 and 6 of the G6PD gene. Two pairs of primers were used for each mutation. For the digestion of exons 4 and 6, NlaIII restriction endonuclease and MboII enzyme were used, respectively. Severe enzyme deficiency was considered when G6PD enzyme activity levels <4.0 UI g Hb^{-1} were confirmed by molecular analysis.

To detect $(TA)_n$ UGT1A1 polymorphisms, the UGT1A1 gene promoter region was amplified by polymerase chain reaction using primers, in which the last base of the sense primer was labeled with a fluorescent dye (FAM). The MegaBACE 1000 automatic sequencer (Amersham Pharmacia Biotech, Cleveland, OH, USA) and the Fragment Profiler software, v1.2 were used.

Clinical variables

In addition to genetic factors, the independent clinical variables studied were maternal age, parity, and maternal diabetes defined by the glucose tolerance test, birth weight, gestational age,[22]

and gender. Race was established according to maternal physical characteristics and was stratified into white, black, or Asian. Other variables studied were cephalohematoma or large ecchymoses (grouped in a single variable), percentage of weight loss at hospital discharge, and type of breastfeeding (exclusive or supplemented). ABO incompatibility was defined when a mother was type O blood and the infant was type A or B blood with positive elution test, independent of the Coombs' test result. Reference curve percentiles[17] at hospital discharge were also evaluated as predictors.

Sample size

The minimum sample size for 563 subjects was established from estimated rates of 5.9% G6PD deficiency and 14.7% $(TA)_7/(TA)_7$ in Brazilian adults,[23] based on an incidence of 5% TB $\geqslant$ 12.9 mg per 100 ml.[24] A sampling error of 1.8% and a significance level of 5% were adopted.

Statistical analysis

For ratio comparison, the χ^2 and the Fisher's exact tests were used. Numerical variables were compared by the Mann–Whitney test, Student's *t*-test, and the Kruskall–Wallis test. Univariate and stepwise Cox multiple regression analyses were used. In various models, the variables were inserted regardless of the significance level of bivariate analysis. Models with and without the percentile reference curve were tested. On regression analysis, gestational age was stratified into two categories: $\geqslant$38 and <38 weeks, as hyperbilirubinemia risk did not differ for gestational ages above 38 weeks. G6PD deficiency was tested in the following categories: heterozygotes and hemizygotes/homozygotes, and carriers or non-carriers of severe enzyme deficiency. The SAS System for Windows version 9.1.3 was used (Statistical Analysis System, Service Pack 3, SAS Institute, 2002 to 2003, Cary, NC, USA). Significance level was set at 5%. Study protocol was approved by the local Research Ethics Committee and a written informed consent was obtained from all parents.

Results

Of the 784 potentially eligible infants born during the study period, 666 (84.9%) were included. (Figure 1) Excluded from the study were 118 subjects because of various causes (Figure 1). At discharge, the TB in 49 subjects who did not complete the follow-up was 7.3 ± 3.0 mg per 100 ml (IQR, 7.7 to 11.9). Only one newborn had TB $\geqslant$ 12.9 mg per 100 ml and two newborns were above the P75th. Three subjects were G6PD deficient (6.1%), six had $(TA)_7/(TA)_7$ (12.2%), and 17 (34.7%) had $(TA)_6/(TA)_7$ genotype.

Of the 608 subjects studied, 119 (19.6%) reached TB $\geqslant$ 12.9 mg per 100 ml, 70 (10.3%) developed TB $\geqslant$ 15.0 mg per 100 ml, and 13 (2.1%) had TB $\geqslant$ 20.0 mg per 100 ml. The main characteristics studied are shown in Table 1, according to outcome

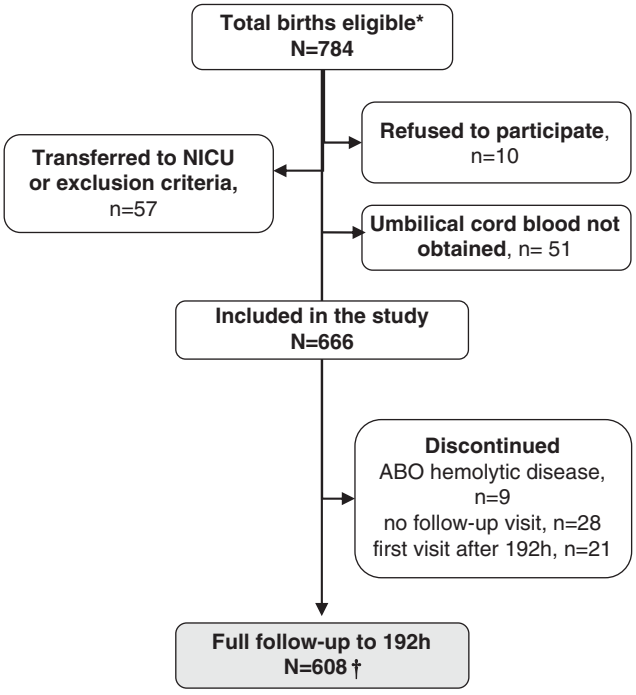

Figure 1 Cohort study design. *Eligible = weighing $\geqslant$2000 g and gestational age $\geqslant$35 weeks; †19.6% of subjects developed TB $\geqslant$ 12.9 mg per 100 ml, 10.3% developed TB >15.0 mg per 100 ml, and 2.1% with TB $\geqslant$ 20.0 mg per 100 ml. NICU, neonatal intensive care unit; TB, total bilirubin.

(peak TB $\geqslant$ 12.9 mg per 100 ml). Exclusive breastfeeding was the predominant type of feeding and 57 (9.4%) infants received formula supplementation. Six subjects were fed with formula only.

Of the total subjects studied, 76.2% had one outpatient return visit, 12.6% had two or more return visits, and 11.2% were watched for jaundice during their stay in the well-baby nursery. Follow-up finished at a mean of 134.5 ± 49.8 h of life (IQR, 111.0 to 156.7), and eight subjects (1.3%) had >12.9 mg per 100 ml at the end of follow-up. The mean TB at the end of follow-up was 6.8 ± 3.7 (IQR, 3.7 to 10.0).

On bivariate analysis, TB levels $\geqslant$ 12.9 mg per 100 ml were associated with the presence of maternal diabetes, lower gestational age, white race, breastfeeding supplemented, greater weight loss at discharge, and higher frequency of infants above the P75th. A longer hospital stay and earlier first consultation were associated with TB levels $\geqslant$ 12.9 mg per 100 ml (Table 1).

The G202A/A376G mutation was detected in 37 (6.1%) subjects. Of these, 19 (3.1% of the total sample) had a very low enzyme activity (18 male hemizygotes and one female heterozygote) (Table 2). Mediterranean variants were not identified. The Brewer test identified 97.3% of cases with G202A/A376G mutation (only one boy had a test Brewer false-negative result).

The rate of G6PD-deficient black males was 8.8% (10/114): two of these had TB >12.9 mg per 100 ml and one >20.0 mg per 100 ml. Unadjusted risk estimate was not significant (RR = 1.60; CI 95% = 0.36 to 7.09).

Table 1 Subject characteristics according to maximum bilirubin values in the first week of life ($N = 608$)

	TB < 12.9 (n = *489*)	*TB ⩾ 12.9* (n = *119*)	P-*value*
Maternal age (years)	25.2 ± 6.5	25.6 ± 7.2	
Median	24	24	0.935[a]
IQR	20–30	20–30	
Primiparity (*n*)	230	60	0.574[b]
Diabetic mother (*n*)	14	11	*0.004*[c]
Birth weight (g)	3250.0 ± 459.6	3243.5 ± 491.5	0.892[d]
Median	3250.0	3280.0	
IQR	2942.5–3565.0	2855.0–3560.0	
Gestational age (weeks)	39.1 ± 1.4	38.4 ± 1.8	
Median	39	39	*<0.001*[a]
IQR	38–40	37–40	
Gender; male:female (*n*)	251:238	57:62	0.502[b]
Race; white:black (*n*)[e]	260:203	80:35	*0.008*[b]
Cephalohematoma+bruising (*n*)	14	8	0.055[c]
ABO incompatibility (*n*)	47	15	0.333[b]
Breastfeeding; exclusive:supplemented (*n*)[f]	448:35	97:22	*<0.001*[b]
Weight loss at discharge (%)	(−5.9) ± (−2.8)	(−7.0) ± (−2.4)	*<0.001*[a]
Median	−6.3	−7.2	
IQR	(−4.3)–(−7.7)	(−5.5)–(−8.6)	
G6PD deficiency (*n*)[g]	17	2	0.555[c]
G6PD heterozygotes (*n*)	13	5	0.368[c]
Homozygotes UGT1A1 (*n*)[h]	62	11	0.301[b]
Heterozygotes UGT1A1 (*n*)[i]	215	60	0.204[b]
Discharge (h)	64.6 ± 19.1	71.1 ± 24.9	
Median	61	64	*0.017*[a]
IQR	52.0–69.0	53.0–82,5	
TB at discharge (mg per 100 ml)	7.5 ± 2.9	12.5 ± 2.4	[j]
Median	7.9	12.8	
IQR	5.7–9.6	10.8–14.0	
Percentile >75th at discharge (*n*)[k]	28	54	*<0.001*[b]
First return visit (h)	126.1 ± 27.1	121.4 ± 32.3	
Median	124	117	*0.028*[a]
IQR	105.5–144.0	98.0–139.7	
Maximum TB in the first week (mg per 100 ml)	7.9 ± 3.0	16.3 ± 2.8	
Median	8.2	15.9	[j]
IQR	5.8–10.2	13.7–18.8	

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; IQR, interquartile range; TB, total bilirubin.
Values presented in mean ± s.d. and absolute values.
[a]Mann–Whitney test.
[b]χ^2 test.
[c]Fisher's exact test.
[d]Student's *t*-test.
[e]Missing information on 28 subjects and only two Asians.
[f]Exclusive formula ($n = 6$).
[g]Low enzyme activity and positive molecular analysis.
[h]Homozygotes or heterozygotes with Gilbert phenotype: $(TA)_7/(TA)_7$, $(TA)_7/(TA)_8$.
[i]$(TA)_5/(TA)_7$, $(TA)_6/(TA)_7$, $(TA)_6/(TA)_8$.
[j]Comparisons were not made.
[k]Without information of percentile in 19 subjects by phototherapy use and discharge after 168 h.

Table 2 Frequency of hyperbilirubinemia (TB $\geqslant$ 12.9 m l^{-1}) according to G6PD genotypes and UGT1A1 polymorphisms ($N = 608$)

	n	*TB $\geqslant$ 12.9 mg per 100 ml (%)*	P-*value*
G6PD deficiency (n) (%)			0.415[a]
With low activity[b]	19	2 (10.5)	
With intermediary or normal activity	18	5 (27.7)	
Without mutation	571	112 (19.6)	
Genotypes UGT1A1 (n) (%)[c]			0.363[a]
Homozygotes	73	11 (15.0)	
Heterozygotes	275	60 (21.8.)	
No polymorphisms	260	48 (18.5)	
G6PD and UGT1A1 genotype coexpression (n) (%)			0.133[d]
G6PD homozygotes and UGT1A1 homozygotes or heterozygotes	8	1 (12.5)	
G6PD heterozygotes and UGT1A1 homozygotes or heterozygotes	12	5 (41.7)	
No coexpression	588	113 (19.2)	

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase; TB, total bilirubin.
[a]χ^2 test.
[b]Includes only one heterozygous girl with a very low activity (1.6 UI gHb^{-1}).
[c]Homozygote or heterozygote with Gilbert phenotype: $(TA)_7/(TA)_7$, $(TA)_7/(TA)_8$; heterozygote: $(TA)_5/(TA)_7$, $(TA)_6/(TA)_7$, $(TA)_6/(TA)_8$; no polymorphisms: $(TA)_5/(TA)_5$, $(TA)_5/(TA)_6$, $(TA)_6/(TA)_6$.
[d]Fisher's exact test.

Compatible genotypes with phenotypic expression of the Gilbert syndrome, that is $(TA)_7/(TA)_7$ ($n = 65$) or $(TA)_7/(TA)_8$ ($n = 8$), occurred in 73 (12.0%) subjects (Table 2).

The mean peak TB values did not differ, according to the presence of G6PD deficiency (hemizygotes, 10.7 ± 4.3 mg per 100 ml; heterozygotes, 10.2 ± 4.0 mg per 100 ml; and no mutation, 9.5 ± 4.6 mg per 100 ml) ($P = 0.235$), or according to UGT1A1 genotypes (homozygotes, 9.3 ± 3.6 mg per 100 ml; heterozygotes, 9.6 ± 4.5 mg per 100 ml; and without polymorphism, 9.4 ± 4.8 mg per 100 ml) ($P = 0.843$). Three subjects (0.49%) coexpressed the $(TA)_7/(TA)_7$ genotype with G6PD mutation and a very low enzymatic activity. None developed TB $\geqslant$ 12.9 mg per 100 ml.

Phototherapy was used in 34 subjects (5.6%): 15 were treated after birth while still in the hospital and 19 during follow-up. One was treated in two stages. The mean PTB of these 34 subjects was 20.0 ± 1.5 mg per 100 ml (range, 17.7 to 24.3) at the start of phototherapy. In this group, five newborns had $(TA)_6/(TA)_7$ genotype, one had $(TA)_7/(TA)_7$ genotype, and two boys were G6PD deficient. No exchange transfusion was performed.

Univariate regression analysis did not detect any significant interaction between weight loss and UGT1A1 genotypes for TB $\geqslant$ 12.9 mg per 100 ml ($P = 0.680$). On regression analysis, no interaction occurred between heterozygosity of G202A/A376G mutation and heterozygosity of UGT1A1 polymorphisms for TB $\geqslant$ 12.9 and $\geqslant$ 15.0 mg per 100 ml ($P = 0.995$ and 0.993).

Univariate regression analysis identified maternal diabetes, gestational age <38 weeks, white race, breastfeeding supplemented, weight loss >7%, and reference curve percentiles as risk factors for TB $\geqslant$ 12.9 mg per 100 ml (Table 3). On multivariate regression analysis, the independent predictors for TB levels $\geqslant$ 12.9 mg per 100 ml were gestational age <38 weeks ($P = 0.010$) and reference curve percentiles above P40th ($P < 0.0001$). Suppressing the reference curve percentiles of the model, gestational age <38 weeks remained a risk factor for TB $\geqslant$ 12.9 mg per 100 ml ($P < 0.0001$) and other variables appeared on the model: weight loss ($P = 0.002$) and white race ($P = 0.038$) (Table 3).

Multivariate regression for TB levels $\geqslant$ 15.0 mg per 100 ml, in addition to percentiles >P40th, identified the following independently associated variables, RR (CI 95%): white race 2.1 (1.1 to 4.2), $P = 0.025$ and gestational age <38 weeks 1.8 (1.0 to 3.4), $P = 0.033$. Risk estimates, taking as a reference the P40th were P40th to P75th 6.5 (2.4 to 17.9), $P = 0.0002$; P75th to P95th 16.0 (5.8 to 44.3), $P < 0.0001$; and >P95th 42.1 (14.9 to 118.5), $P < 0.0001$.

Suppression of the reference curve percentiles of this model showed four independently associated predictive variables, RR (CI 95%): gestational age <38 weeks 1.9 (1.1 to 3.3), $P = 0.016$; white race 2.5 (1.4 to 4.5), $P = 0.002$; ecchymoses/cephalohematoma 2.6 (1.1 to 6.0), $P = 0.024$; and breastfeeding supplementation 2.3 (1.3 to 4.1), $P = 0.006$.

Discussion

In the sample studied, G6PD deficiency (G202A/A376G mutation) and gene promoter polymorphisms encoding UGT1A1 were not risk factors for moderate hyperbilirubinemia. The reference curve

Table 3 Univariate and multiple analysis regression for TB > 12.9 mg per 100 ml in the first week of life ($N = 608$)

Variables	*Univariate analysis RR (CI 95%)*	P-*value*	*Multivariate analysis*	*Multivariate analysis (excluding percentile at discharge)*
Maternal age, per year	1.00 (0.97–1.03)	0.660	[a]	[a]
Primiparity	1.10 (0.77–1.58)	0.575	[a]	[a]
Diabetic mother	*2.4 (1.2–4.4)*	*0.006*	[a]	[a]
Birth weight, per 500 g	1.00 (1.0–1.0)	0.903	[a]	[a]
Gestational age				
<38	*2.1 (1.4–3.0)*	*<0.0001*	*1.7 (1.1–2.7)*	*2.0 (1.3–2.9)*
⩾38	Ref			
Gender			[a]	[a]
Female	1.1 (0.7–1.6)	0.547		
Male	Ref			
Race			[a]	
White	*1.6 (1.0–2.3)*	*0.020*		*1.5 (1.0–2.3)*
Black	Ref			
Bruising	*1.9 (0.9–3.9)*	*0.074*	[a]	[a]
ABO incompatibility	1.3 (0.7–2.1)	0.374	[a]	[a]
Breastfeeding			[a]	[a]
Supplemented	*2.1 (1.3–4.3)*	*<0.001*		
Exclusive	Ref			
Weight at discharge >7%[b]	*1.8 (1.3–2.6)*	*0.0007*	[a]	*1.1 (1.0–1.2)*
G6PD deficiency				
Hemizygote[c]	0.5 (0.1–2.1)	0.373	[a]	[a]
Heterozygote	1.3 (0.5–3.2)	0.520	[a]	[a]
No mutation	Ref			
UGT1A1 genotypes[d]		0.445	[a]	[a]
Homozygote	0.8 (0.4–1.6)			
Heterozygote	1.2 (0.85–1.8)			
$(TA)_6$ $(TA)_6$	Ref			
Percentile at discharge		*<0.0001*		
>P 95	*33.2 (15.6–71.0)*		*30.5 (13.9–66.6)*	
P75–95	*19.4 (9.6–39.3)*		*17.0 (8.3–34.8)*	
P40–75	*8.6 (4.3–17.2)*		*7.6 (3.8–15.4)*	
<P40	Ref		Ref	

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase; TB, total bilirubin.
[a]Did not remain in the model.
[b]Included in multiple analysis as a continuous variable.
[c]Includes only one heterozygous female with a very low enzyme activity.
[d]Homozygotes or heterozygotes with Gilbert phenotype: $(TA)_7/(TA)_7$, $(TA)_7/(TA)_8$, heterozygotes: $(TA)_5/(TA)_7$, $(TA)_6/(TA)_7$, $(TA)_6/(TA)_8$.

percentiles at discharge (>P40th) and gestational age (<38 weeks) were predictors independently associated with TB ⩾ 12.9 mg per 100 ml. For TB values ⩾ 15.0 mg per 100 ml, the white race added to those variables was also an independent risk factor. Curve percentiles suppressed from the multiple regression models allowed the expression of the following variables: weight loss, ecchymoses/cephalohematomas, and supplementation to breastfeeding.

We chose to establish risk factors for two hyperbilirubinemia levels ($\geqslant$12.9 and $\geqslant$15.0 mg per 100 ml) as TcB may be inaccurate. However, the predictive variables identified and their RR for both TB levels were similar.

The incidence of hyperbilirubinemia, G202A/A376G mutation, and $(TA)_7/(TA)_7$ and $(TA)_7/(TA)_8$ genotypes were in agreement with other reports.[8,10,23,25] In addition, these frequencies are in agreement with the sample size calculated, resulting in a study with adequate power. Furthermore, TB monitoring in the first week of life was strict until values <12.9 mg per 100 ml (IQR, 3.7 to 10.0 mg per 100 ml), to ensure that all cases of hyperbilirubinemia were identified.

To the best of our knowledge, this is the first study to investigate the function of UGT1A1 genotypes and the A^- variant of G6PD deficiency, diagnosed by molecular analysis and enzyme quantification in the occurrence of hyperbilirubinemia in a large cohort of Brazilian children. The prevalence of G6DP-deficient individuals obtained by molecular analysis was similar to a study of a North-American population.[6] In both studies, G6PD deficiency did not predict moderate TB levels.

In certain ethnic groups[4] or newborns with severe hyperbilirubinemia,[5] G6PD deficiency was associated with higher TB levels and kernicterus.[5] A high prevalence rate of the Mediterranean variant,[5] different study designs,[4] cultural factors, and type of neonatal healthcare provided probably contributed toward these results. In contrast, evaluating a general population, without overrepresentation of risk groups, the main factor related to increased bilirubin level was breastfeeding.[26] G6PD deficiency accounted for only 10% of indications of phototherapy.[26]

G6PD deficiency was detected in 7.7% of infants reaching a TB>20.0 mg per 100 ml and 5.9% (2/34) of those using phototherapy. The two newborns treated with phototherapy had the most significant predictors (35 weeks of gestational age and TB above the P95th at discharge). Thus, even with unknown genetic factors, the risk of hyperbilirubinemia could be successfully identified in both subjects.

In this study, considering the group at highest risk (black males), G6PD deficiency was not predictive of hyperbilirubinemia. The TB$\geqslant$12.9 mg per 100 ml ratio in this group was similar to that of the general population, 20.0 $\times$ 19.6%, respectively. However, the sample size of this subgroup may have been inadequate for analysis.

As it is difficult to quantify G6PD enzyme activity in female heterozygotes,[27] we evaluated whether heterozygosity for the G202A/A376G mutation could predict hyperbilirubinemia, as suggested in other studies.[28] However, although RR was higher than in male hemizygotes, the confidence interval was not significant.

About 57.2% of all newborns studied and 54.0% of G6PD-deficient subjects in this study had one or more additional base pairs, $(TA)_7$ or $(TA)_8$, in at least one gene encoding UGT1A1, similar to an earlier description.[10] The incidence of $(TA)_7/(TA)_7$ and $(TA)_7/(TA)_8$ genotypes was 12% in newborn infants. During the neonatal period, homozygotes and heterozygotes may have a more rapidly rising or higher TB levels, because of reduced glucuronization activity, especially in association with hemolysis.[11] Despite the high frequency of these polymorphisms, the occurrence of hyperbilirubinemia and mean TB values neither vary among different genotypes, nor did they predict hyperbilirubinemia.

Coexpression of UGT1A1 polymorphisms with G6PD deficiency was rare (0.49%) in this study, almost 10 times lower than the frequency reported earlier.[29] This low frequency made assessment of the risk of hyperbilirubinemia more difficult in this cohort study. Other recently recognized genetic factors, such as the OATP1B1[30] transporter polymorphisms, require further investigation in this population.

Although hyperbilirubinemia has different definitions, the clinical risk factors identified in this study have been consistently reported.[3,6,24,31,32] The reference curve percentiles had the highest RR, producing with gestational age the best predicting model of hyperbilirubinemia, as described earlier.[6,32] The use of risk curves as the only predicting instrument may determine false-negative results.[33]

In this study, weight loss and breastfeeding supplemented were variables that increased the risk of hyperbilirubinemia, in agreement with the well-known association between inadequate breastfeeding and increased TB.[6,24] In contrast, weight loss did not show any interaction with UGT1A1 genotypes in the occurrence of hyperbilirubinemia as suggested earlier.[34]

Some limitations may be cited in this study. Only the A^- variant was identified in our sample. However, 2% of Brazilian blood donors are carriers of the Mediterranean variant.[14] This finding is probably due to a population of predominantly African origin assisted at a public university hospital. We should not rule out the additive effects of homozygosity of the UGT1A1 genotypes and G6PD deficiency in hyperbilirubinemia, resulting from the rare coexpression of both conditions. Certain clinical and epidemiological variables related to hyperbilirubinemia[3] have not been studied or were very rare such as the Asian race and early-onset jaundice. Phototherapy in earlier children has not been evaluated, because of the heterogeneous indications for this treatment in our setting.

Considering the risk factors found in this study, the best approach to severe hyperbilirubinemia prevention seems to be universal bilirubin screening through transcutaneous bilirubinemia before discharge. Both the reference curve percentiles[17,32] and clinical factors[32] should be highlighted, providing follow-up for children at higher risk.

In conclusion, despite its importance in suspected cases, the implementation of a newborn screening program for G6PD deficiency may not be an effective measure to prevent hyperbilirubinemia in our population. The reason is the low prevalence and risk rate of this disorder, in addition to its rare

association with homozygosity for the variant of the UGT1A1 gene promoter.

Conflict of interest

The authors declare no conflict of interest.

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