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Differing Pathogenesis of Perinatal Bilirubinemia in Glucose-6-Phosphate Dehydrogenase-Deficient *Versus* -Normal Neonates

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ABSTRACT

The objective was to compare the contribution to perinatal bilirubinemia of hemolysis and UDP-glucuronosyltransferase (UGT) gene promoter polymorphism, seen in Gilbert's syndrome, between glucose-6-phosphate dehydrogenase (G-6-PD)-deficient and -normal neonates. Serum total bilirubin (STB) values from 52 G-6-PD-deficient and 166 G-6-PD-normal term, male neonates, sampled within 3 h of delivery (first sample) and on d 3 (second sample), were analyzed in relation to blood carboxyhemoglobin corrected for inspired CO (COHbc), an accurate index of hemolysis, and UGT promoter genotype. COHbc values (% total Hb) were greater in G-6-PD-deficient neonates than controls: first sample 1.00 ± 0.25% *versus* 0.84 ± 0.24%, $p < 0.0001$; second sample 0.83 ± 0.20% *versus* 0.76 ± 0.19%, $p = 0.002$. First sample COHbc and STB values did not correlate in either the G-6-PD-deficient or control groups, whereas second sample COHbc values correlated significantly with corresponding STB values in the control population only ($r = 0.28$, $p = 0.0007$). At second sampling, there was a higher allele frequency of the variant UGT promoter among those with STB values $\geq 75^{th}$ percentile than those $< 75^{th}$ among the G-6-PD-deficient neonates (0.60 *versus* 0.33, respectively, $p = 0.025$), but not controls (0.31 *versus* 0.40, respectively, $p = 0.24$). Among those infants with at least one variant UGT promoter allele, STB values were higher in the G-6-PD-deficient neonates than controls at second sampling only (181 ± 56 μM *versus* 149 ± 46 μM, respectively, $p = 0.03$). Both within and between the G-6-PD-deficient and control groups, our data demonstrate changing and differing contributions of hemolysis and UGT promoter polymorphism to bilirubinemia during the first 3 d of life. **(*Pediatr Res* 50: 532–537, 2001)**

Abbreviations:
COHb, carboxyhemoglobin
COHbc, carboxyhemoglobin corrected for inspired carbon monoxide
G-6-PD, glucose-6-phosphate dehydrogenase
RBC, red blood cell
STB, serum total bilirubin
tHb, total Hb
UGT, UDP-glucuronosyltransferase 1A1

G-6-PD deficiency, a commonly occurring enzyme defect, may be associated with severe neonatal hyperbilirubinemia with potential to cause bilirubin encephalopathy or even death (1–3). Although some COHb studies, reflecting heme degradation and therefore bilirubin production, have demonstrated markedly increased hemolysis in certain situations (4, 5), this is not consistently found. For example, in G-6-PD-deficient Sephardic Jewish neonates, moderately increased hemolysis occurred not only in those infants who become significantly hyperbilirubinemic, but in those who remained only mildly jaundiced as well (6). Rather, in babies from the same population subgroup, Kaplan *et al.* (7, 8) have shown that crucial to the pathogenesis of hyperbilirubinemia in G-6-PD-deficient newborns was a deficiency in bilirubin conjugation, over and above the physiologic immaturity of the bilirubin conjugation system encountered in normal, term newborns. This diminished bilirubin conjugating ability was the functional manifestation of an interaction between G-6-PD deficiency and $(TA)_7TAA$ promoter polymorphism for the gene that encodes the bilirubin conjugating enzyme, UGT, seen in Gilbert's syndrome (9–11).

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In addition to increased risk for hyperbilirubinemia, G-6-PD-deficient neonates have also been shown to have significantly higher STB levels than controls both immediately after delivery, reflecting the *in utero* situation, and also on d 3 of life (12). In both groups, the very early STB values correlated with both d 3 STB levels and with those who subsequently developed hyperbilirubinemia, implying that neonatal icterus has its origins in the *in utero* or immediate postnatal period. Based on the aforementioned studies, we hypothesized that the increased STB values in G-6-PD-deficient neonates, at both these points in time, would be the result of a combination of increased bilirubin production and presence of the variant UGT promoter. As a result, COHb values could be expected to be increased in the G-6-PD-deficient neonates, compared with controls, both immediately after delivery, as well as on d 3 of life. Increased STB values in the G-6-PD-deficient neonates could be anticipated to be limited to those who also carried one or two variant promoter UGT genes, and this latter subgroup could be predicted to have higher STB values than G-6-PD-normal neonates with the identical UGT promoter genotypes.

In this study, we sought to extend previous observations to an earlier phase in the perinatal period and to expand knowledge regarding the factors leading to the hyperbilirubinemia. As immediate postnatal bilirubinemia correlates with subsequent hyperbilirubinemia (12), we asked whether the effects of increased hemolysis and UGT promoter polymorphism were also in force, and to what extent, *in utero* and on d 3 of life.

METHODS

Study protocol. In this study, we analyzed data from the same cohort of babies previously reported to have higher STB values immediately after delivery (12). Consecutively born, healthy males, ≥37 wk gestation, born at the Shaare Zedek Medical Center to Sephardic Jewish mothers at high risk for G-6-PD deficiency, were enrolled in cohort fashion. Babies with any other condition likely to exacerbate hyperbilirubinemia, such as cephalhematoma, direct Coombs' positive isoimmunization, maternal diabetes, sepsis, or Down's syndrome were excluded from the study. Within the first 3 h after delivery, to reflect the *in utero* status (first sample) and again on d 3 of life (second sample), blood was drawn for COHb determination (simultaneous with the previously reported STB values). At one of these samplings, blood was collected for DNA extraction. At the time of the COHb sampling, room air was sampled for CO analysis. Smoking, which can affect early COHb values, is extremely unusual among the women delivering at this hospital. Certainly by d 3 of life any effect of smoking on neonatal COHb values should no longer be apparent (4, 13). Routine medical care has been previously described (14).

The study was approved by the Institutional Review Board of the Shaare Zedek Medical Center and was further sanctioned by the Israel Government Ministry of Health authorities. As there was no randomization of patients, clinical or therapeutic trial or any other deviation from routine clinical management and no additional risk to the babies, these authorities gave blanket approval to perform this study.

Laboratory methods. DNA was extracted from peripheral blood leukocytes using a high-salt extraction procedure (15) and used to determine the UGT gene promoter G-6-PD genotypes.

G-6-PD classification was performed at The Scripps Research Institute, La Jolla, CA (Ernest Beutler, MD). PCR followed by allele-specific oligonucleotide hybridization was used to determine the presence or absence of nt 563, the nucleotide mutated in G-6-PD Mediterranean (16) and which is seen in the Sephardic-Jewish population studied (9, 17). Details of the procedure have been published elsewhere (9).

UGT promoter genotype was determined using PCR-mediated site-directed mutagenesis with the primers Bili F2: 5' - GCT CCA CCT TCT TTA TCT CTGAA-3' and Bili F: 5'-CGC CCT CTC CTA CTT ATA TAC CTA T - 3', which insert a BslI restriction site in the wild-type allele. PCR was carried out at an annealing temperature of 54°C for 35 cycles in a buffer containing 1.5 mM $MgCl_2$. Amplification was followed by digestion with BslI (New England Biolabs, Beverly, MA, U.S.A.) and electrophoresis on 3% agarose, to separate the wild-type and polymorphic alleles.

Blood for COHb analysis was collected into custom-made tubes containing saponin and heparin, stored at –18°C, and allowed to thaw for transfer on ice to Stanford University. COHb was determined by gas chromatography, and tHb by a cyanmethemglobin method, both as previously described (18, 19). The within-day and between-day coefficients of variation for reference blood samples using this method are 3% and 8%, respectively (20). COHb values are expressed as a percentage of tHb. The CO content of the room air specimens was determined using a sensitive electrochemical CO analyzer supplied by Stanford University (21). Ambient CO concentrations were used to correct measured COHb for the effect of inspired CO (COHbc) by a previously derived formula: COHbc = measured COHb − 0.17(concentration of CO in room air in μL/L) (22). STB values were determined by reflectance spectrophotometry using an Ektachem analyzer (Vitros 700c/750XRC Chemistry System, Johnson & Johnson Clinical Diagnostics, Rochester, NY, U.S.A.).

Data analysis. G-6-PD-deficient neonates (study group) were compared with those who were G-6-PD normal (control group). Based on their UGT promoter genotype, infants were further subdivided into two subgroups, comprising either those homozygous for the normal UGT promoter, or those with at least one variant promoter gene, who were pooled into the variant promoter subgroup. We felt that this was an acceptable strategy as previously both variant UGT promoter genotypes were instrumental in significantly increasing the incidence of hyperbilirubinemia when in combination with G-6-PD deficiency (9). First and second sample STB values were compared between the UGT promoter subgroups in the G-6-PD-deficient and control populations, respectively. First and second sample COHbc values were compared between the study and control groups. To compare changes within groups, the percentage change between the first and second COHbc measurements was determined by first calculating the change for each individual infant, and then comparing the percentage changes between groups. To further define the pathogenesis of the

hemolysis, in addition to measured COHbc and tHb values, the mathematical expression, the fraction of COHbc divided by tHb concentration (COHbc/tHb) was calculated. This ratio was thought to supplement the assessment of the relative contribution of RBC breakdown to a given COHbc concentration by indexing CO production to tHb. Assuming similar CO elimination rates and therefore COHbc, individuals with high COHbc/tHb ratios will imply lower tHb, indicative of a comparatively high RBC breakdown rate, whereas the converse would imply a lower rate of RBC destruction (23, 24). Values with a normal distribution were displayed as mean ± SD, whereas median (interquartile range) was used for those that did not have a normal distribution. For continuous variables, *t* test was used to compare data with a normal distribution, and was replaced by the Mann-Whitney rank sum test in the data that did not have a normal distribution. Linear regression analysis was used to examine for possible associations of study variables. Categorical variables were compared using χ^2 analysis. For standardization with previous studies in this population group (6–9, 12, 14) hyperbilirubinemia was defined as an STB ≥256 μM during the first week of life. The alpha level of significance was defined as $p < 0.05$.

RESULTS

Demographic Data

As reported in the original description of this cohort, 52 G-6-PD deficient and 166 G-6-PD normal infants were enrolled. Mean (± SD) birth weights were 3189 ± 462 g and 3316 ± 457 g, and mean gestational age 39.0 ± 1.5 wk and 39.5 ± 1.0 wk, for the G-6-PD deficient and control infants, respectively (NS). Eighty-one percent of the G-6-PD-deficient babies, and 93% of the controls, were delivered vaginally ($p = 0.02$), whereas 50% and 75%, respectively, were exclusively breast-fed ($p = 0.001$). STB values at the time of the first sampling (1.7 ± 0.8 h and 1.9 ± 0.7 h for the G-6-PD-deficient and control groups, respectively) were 50 ± 12 μM and 44 ± 10 μM ($p = 0.003$), respectively. For the third day sampling (age 53 ± 10 h and 52 ± 8 h, respectively) respective STB values were 174 ± 53 μM and 152 ± 51 μM ($p = 0.007$). Sixteen (30.8%) of the G-6-PD-deficient neonates, compared with 10 (6%) of the controls developed hyperbilirubinemia (relative risk 5.11, 95% confidence interval 2.47–10.56).

COHbc Studies

First sample COHbc levels of four infants (one G-6-PD deficient, three controls) were excessively high, ranging from 3.37 to 4.24%, exceeding the range of mean + 10 SD. As this phenomenon did not apply to corresponding second sample COHbc values for the same infants, and as neither first nor second sample STB values in any of these infants were greater than mean + 1 SD, and none of these babies developed hyperbilirubinemia, they were regarded as outliers with regard to the first COHbc values, possibly the result of maternal smoking, and were excluded from the first COHbc analyses.

COHbc values were significantly greater in the G-6-PD-deficient neonates than in controls at both sampling points (first sample 1.00 ± 0.25% *versus* 0.84 ± 0.24%, $p < 0.0001$, and second sample 0.83 ± 0.20% *versus* 0.76 ± 0.19%, $p = 0.002$, respectively) (Fig. 1). Within groups, whereas values for both G-6-PD-deficient and control neonates decreased significantly between the first and second sampling ($p = 0.0002$ and $p = 0.0008$, respectively), the median percentage change was double in the G-6-PD-deficient neonates than in the controls (−16.74% [−0.59 to −31.4%] *versus* −8.59% [−4.16 to −22.7%], $p = 0.028$ [median, interquartile range]). First sample COHbc values did not correlate with first sample STB values in either the G-6-PD-deficient ($r = 0.175$, $p = 0.23$) or control ($r = 0.06$, $p = 0.5$) groups. Second sample COHbc values correlated significantly with second sample STB values in the control population only ($r = 0.28$, $p = 0.0007$), but not in the G-6-PD-deficient population ($r = 0.04$, $p = 0.77$).

First sample tHb values were significantly lower in the G-6-PD-deficient neonates than in controls (186 ± 36 g/L *versus* 199 ± 23 g/L, $p = 0.002$, respectively) but, by the second sampling, significant differences for tHb values between the groups was no longer evident (185 ± 24 g/L *versus* 190 ± 24 g/L, $p = 0.2$). Values (median [interquartile range]) for COHbc/tHb ratio at the first sampling were 519 (432–850) and 399 (350–478) ($p < 0.001$) for the G-6-PD-deficient and control infants, respectively, and at the second sampling were 451 (346–527) and 388 (319–459), respectively, $p = 0.004$.

UGT Promoter Polymorphism Studies

Distribution. The allele frequencies for the variant promoter UGT genotype were similar for the G-6-PD-deficient and control cohorts (0.40 and 0.34, respectively, $p = 0.3$), whereas 14.0% and 13.7% individual babies, respectively, were homozygous for the variant UGT promoter gene (Gilbert's syndrome). Forty percent of all variant UGT promoter alleles in the G-6-PD-deficient group were found among those neonates who developed hyperbilirubinemia, compared with 9% of the variant UGT promoter genes in the controls ($p < 0.0001$).

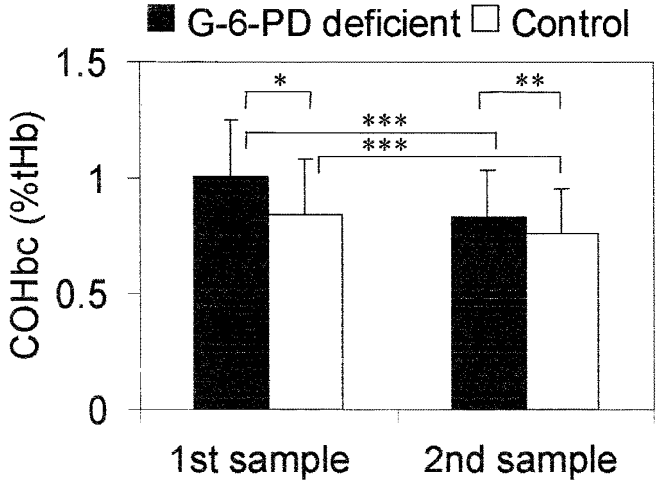

Figure 1. Carboxyhemoglobin values corrected for inspired CO in G-6-PD-deficient and normal neonates. Values were significantly higher in G-6-PD-deficient neonates than controls at both samplings. Within groups, first COHbc values were significantly higher than second sample values for both G-6-PD-deficient and control neonates. *$p < 0.0001$; **$p = 0.02$; ***$p < 0.001$.

To determine the role of the variant UGT promoter gene in the pathogenesis of the bilirubinemia, the allele frequency of the variant gene was determined for those with lower STB values (<75th percentile) and compared with those with STB values ≥75th percentile, for the first and second samples for the G-6-PD-deficient and control infants, respectively (Table). In the first samples, there was a similar distribution of the variant promoter gene among those with higher and lower STB values. However, by d 3, in the G-6-PD-deficient group, the allele frequency was significantly greater among those with STB values ≥75th percentile, whereas a similar imbalance was not seen in the control group.

Relation to STB values. First and second sample STB values, analyzed in relation to UGT promoter genotypes, are displayed graphically in Figures 2 and 3, respectively. Contrary to expectations, presence of one or two variant UGT promoter genes did not significantly increase first sample STB values in the G-6-PD-deficient neonates compared with controls (50 ± 12 μM *versus* 48 ± 10 μM, respectively, $p = 0.45$) (Fig. 2, *right series*). However, among those who were homozygous for the normal UGT promoter, first sample STB values were significantly higher in the G-6-PD-deficient neonates than in the controls (50 ± 12 μM *versus* 41 ± 10 μM, respectively, $p = 0.003$) (Fig. 2, *left series*). Within groups, G-6-PD-deficient neonates had similar first sample STB values both for those homozygous for the normal UGT promoter as well as those with the variant UGT promoter genotype. These findings contrasted with the control group, in whom STB values were significantly increased in those with one or two variant UGT promoters, compared with those homozygous for the normal promoter ($p < 0.001$).

By the third postnatal day, as expected, STB values of the G-6-PD-deficient neonates who carried the variant UGT promoter gene were significantly higher than control neonates of the identical UGT promoter genotype (181 ± 56 μM *versus* 159 ± 46 mg/dL, respectively, $p = 0.03$) (Fig. 3, *right series*). However, the previously noted difference between STB values between G-6-PD-deficient and control neonates of those homozygous for the normal UGT promoter gene was now less marked and no longer reached statistical significance (164 ± 41 μM *versus* 142 ± 55 μM, respectively, $p = 0.12$) (Fig. 3, *left series*). Within groups, STB values were not significantly different between G-6-PD-deficient neonates with normal or variant UGT promoter genotypes ($p = 0.3$), but were significantly so among the control infants ($p = 0.03$).

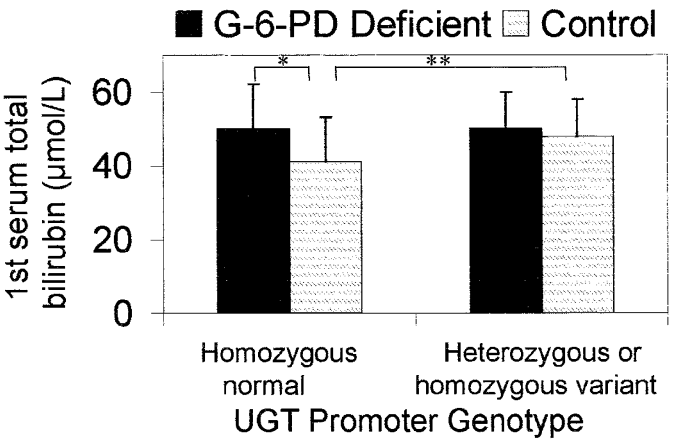

Figure 2. First sample STB values in relation to UGT promoter genotype. Among those who were homozygous for the normal promoter, STB values were higher in G-6-PD-deficient neonates than controls. This effect was not seen in those with at least one variant UGT promoter allele. Within groups, those with the variant UGT promoter had significantly higher STB values than those homozygous for the normal UGT promoter in the control group, whereas such an effect was not seen within the G-6-PD-deficient group. *$p = 0.003$; **$p < 0.001$.

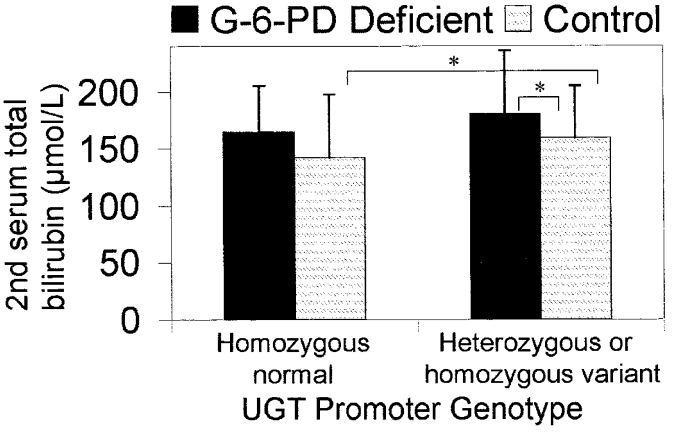

Figure 3. Second sample STB values in relation to UGT promoter genotype. Among those with the variant UGT promoter, STB values were now significantly greater in the G-6-PD-deficient group than controls. This difference no longer reached statistical significance among those homozygous for the normal UGT promoter. Within groups, STB values of the control infants with the variant UGT promoter were significantly higher than those homozygous for the normal UGT promoter. *$p = 0.03$.

Table 1. *Allele frequency of the variant UGT promoter gene among those with lower STB values (≤75th percentile) and those with STB values ≥75th percentile*

	G-6-PD deficient		Control	
Percentile	<75th	>75th	<75th	>75th
1st sample				
STB (μmol/L)	<58	≥58	<50	≥50
Allele frequency	0.39	0.42	0.31	0.41
Significance	$p = 0.96$		$p = 0.13$	
2nd sample				
STB (μmol/L)	<212	≥212	<183	≥183
Allele frequency	0.33	0.60*	0.31	0.40
Significance	*$p = 0.025$		$p = 0.24$	

DISCUSSION

Previous studies in this population group (9) have related to the development of hyperbilirubinemia, defined arbitrarily as a STB value ≥256 μM in the first week of life. In the current study, we assessed the dynamics of the development of jaundice, before the development of hyperbilirubinemia, at two junctures in time. The study period reflects the transition between intra- and extrauterine life, a time when, physiologically, dynamic changes in STB values can be expected. Furthermore, as only a fraction of the babies develop hyperbilirubinemia, use of STB values allowed for

both hyperbilirubinemic neonates as well as those who did not develop high STB values to be included in the analysis.

Although the rate of hemolysis was greater in the G-6-PD-deficient neonates than controls, lack of correlation between COHbc and simultaneously drawn STB values indicated that excessively increased hemolysis could not be implied as the cardinal factor in the pathogenesis of bilirubinemia in the G-6-PD-deficient neonates at either of the sampling points. These findings contrast with those of Widness *et al.* (20), who studied fetuses with increased hemolysis, albeit of a different etiology: alloimmunization. In that study, COHb and STB values, sampled by cordocentesis, were highly correlated, and implied that increased hemolysis was the major factor in the pathogenesis of jaundice in those patients. Thus, whereas increased hemolysis is seen in both alloimmunization and G-6-PD deficiency, the relative roles of the hemolytic processes in the pathogenesis of *in utero* bilirubinemia varies from etiology to etiology, from a strong association in the former condition, to a weaker correlation in the latter. These findings imply that additional factors such as deficient bilirubin conjugation must be dominating bilirubin metabolism in the G-6-PD-deficient group. By the third day in the control infants, but not in the G-6-PD-deficient group, a positive correlation between COHbc values and STB readings had developed, implying a more prominent role for hemolysis in the pathogenesis of bilirubinemia in that group compared with the G-6-PD-deficient neonates.

The dynamics of COHbc changes during this transition period are no less intriguing. The transition from fetal to neonatal life is one from a relatively oxygen-deprived environment to that of an oxygen rich one (25). It would be presumed that G-6-PD-deficient neonates, with their inherently diminished antioxidative defenses, would increase the degree of hemolysis in parallel to the increasing oxygen content of their environment. In fact, the converse occurred: the degree of hemolysis actually decreased from first to second sample at a rate that was greater than that of the control infants. Despite this greater rate of decrease, third day COHbc values were still higher in the G-6-PD-deficient neonates than in the controls.

Contrary to expectations, the predicted interaction between G-6-PD deficiency and UGT promoter polymorphism was not apparent at the first sampling. An effect was noted at the second sampling, albeit less dramatic than was anticipated from the previously documented interactions (8, 9). There seems, therefore, to be a time-related progression of the manifestation of this gene interaction. We believe that this progression is the result of gradual bilirubin buildup over the first few days of life. The STB value at any point in time reflects the combined forces of bilirubin production, on the one hand, and bilirubin conjugation and elimination, on the other (26). Imbalance between these forces probably occurred because of increased heme breakdown in the face of diminished UGT enzyme expression in those infants with a variant UGT gene promoter. This imbalance resulted in STB values increasing to a greater extent in G-6-PD-deficient neonates than controls through the first days of postnatal life, with subsequent hyperbilirubinemia developing in those in whom the imbalance was greatest.

The most dramatic change from intrauterine to extrauterine life is that from total dependence on the placenta, to complete independence. *In utero*, the conjugation process is poorly developed (27), the result of decreased UGT activity in the fetal liver (28, 29). However, it is very unusual for a baby to be born clinically jaundiced as unconjugated bilirubin is readily transferred across the placenta to the mother in whose liver it is conjugated and excreted (30). It seems likely that this process must be moderating bilirubin metabolism *in utero* in G-6-PD-deficient neonates, minimizing any effect of a variant UGT promoter. With the loss of the placenta at birth, the neonatal liver now has to contend with a rapidly increasing bilirubin load, and any abnormality in conjugation, such as the effect of UGT promoter polymorphism, may now become apparent.

In this study, we have demonstrated that the pathogenesis of bilirubinemia, with regard to the role of hemolysis and UGT promoter polymorphism, differs between G-6-PD-deficient and -normal newborns during the immediate perinatal period. Within groups, dynamic changes in the contribution of these factors were also documented during this time period. Many additional factors may also contribute to increasing bilirubin production relative to conjugation. These may be moderated by presence of the placenta in the bilirubin metabolism pathway, interaction between G-6-PD deficiency and the variant UGT promoter gene, and varying degrees of inherent antioxidant deficiency with resultant hemolysis. Researchers of neonatal jaundice, in geographic areas in which the frequency of G-6-PD deficiency is considerable, should beware of pooling data. They should be aware that this condition may result in differing rates of bilirubin production and conjugation, which may be confounding factors in bilirubin-related data analysis.

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