Hemolysis and Bilirubin Conjugation in Association With UDP-Glucuronosyltransferase 1A1 Promoter Polymorphism

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Hemolysis may contribute to hyperbilirubinemia in Gilbert's syndrome. The authors examined blood carboxyhemoglobin corrected for inspired CO (COHbc) to index heme catabolism and serum conjugated bilirubin fractions to reflect bilirubin conjugation. Both parameters were related to UDP-glucuronosyltransferase 1A1 (UGT) promoter polymorphism, associated with Gilbert's syndrome, in term male newborns. COHbc was expressed as percentage of total hemoglobin, and total conjugated bilirubin (TCB) value as a percentage of serum total bilirubin (STB), (TCB/STB[%]). A production/conjugation index, COHbc/(TCB/STB[%]), represented bilirubin production divided by conjugation. UGT promoter genotype was designated according to the number of promoter TA insertions in each allele: 6/6, homozygous normal; 6/7, heterozygous; 7/7, homozygous variant. STB and COHbc values were higher in the 7/7 subgroup than the other counterparts ($P < .01$). The COHbc/(TCB/STB[%]) was higher in the 7/7 than either the 6/6 or 6/7 subsets (1.93 [1.31-2.88] vs. 0.85 [0.51-1.72] and 0.84 [0.53-1.87], respectively; $P < .01$). In conclusion, 7/7 UGT promoter polymorphism was associated with increased blood COHbc values (unexpected finding) as well as diminished serum total conjugated bilirubin ratios (expected finding). The increased hemolysis may contribute to the pathogenesis of increased STB values seen in Gilbert's syndrome, and exacerbate neonatal hyperbilirubinemia associated with the promoter polymorphism. (HEPATOLOGY 2002;35:905-911.)

Gilbert's syndrome, a commonly occurring inherited condition usually diagnosed first during adolescence, is benign in adults. It is characterized by mild, indirect bilirubinemia in the presence of normal serum liver enzyme values. In neonates, the condition has been associated with the development of hyperbilirubinemia. In contrast to the Crigler-Najjar syndromes, in which diminished bilirubin conjugation is the result of an abnormally structured UDP glucuronosyltransferase 1A1 (UGT) enzyme, emanating from a mutation in the coding area of the UGT gene, in Gilbert's syndrome diminished enzyme activity results from decreased expression of normally structured UGT.[1,2,3]

The most frequent genetic finding in white patients with Gilbert's syndrome is a polymorphism of the gene encoding UGT, in which an additional TA base pair is inserted the TATAA box of the gene promoter. Affected individuals are homozygous for the variant promoter and have 7 TA repeats—$(TA)_7TAA$ (7/7)—instead of the more usual 6 repeats—$(TA)_6TAA$ (6/6). Heterozygotes have one allele of each of the normal and variant promoter (6/7).[4,5] An inverse relationship exists between the number of TA repeats and the promoter activity, so that individuals with the 7/7 sequence have diminished promoter activity compared with 6/7 or 6/6 counterparts, which leads to the decreased enzyme

Abbreviations: UGT, UDP glucuronosyltransferase 1A1; COHb, carboxyhemoglobin; COHbc, COHb corrected for ambient CO; G-6-PD, glucose-6-phosphate dehydrogenase; STB, serum total bilirubin; TCB, total conjugated bilirubin; RBC, red blood cell; TCB/STB(%) serum total conjugated bilirubin expressed as percentage of serum total bilirubin; COHbc/(TCB/STB[%]), bilirubin production/conjugation index.

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expression.[4,6] However, not all individuals homozygous for the variant UGT promoter display the clinical features of Gilbert's syndrome, and additional icterogenic factors, such as decreased hepatic uptake of unconjugated bilirubin, may be necessary for full clinical expression.[7-9]

Although Gilbert's syndrome is not associated with overt evidence of hemolysis,[1,2,3] a shortened red cell life span has been found in some affected individuals.[10-17] To clarify the role of UGT 1A1 promoter polymorphism in moderating bilirubin production and conjugation, we measured blood carboxyhemoglobin (COHb) corrected for inspired carbon monoxide (COHbc), an index of heme catabolism and ultimately bilirubin production,[18-20] as well as serum unconjugated and conjugated bilirubin fractions, to reflect bilirubin conjugation,[21] in a cohort of neonates categorized for the three UGT promoter genotypes. We hypothesized that homozygosity for the variant 7/7 UGT gene promoter would be associated with diminished bilirubin conjugation relative to bilirubin production when compared with those homozyous for the normal 6/6 promoter, and that these neonates would not respond as expected to an increased bilirubin load by increasing bilirubin conjugation.

Patients and Methods

The study protocol was approved by the Institutional Review Board of the Shaare Zedek Medical Center. As there was no randomization of patients, clinical or therapeutic trial, or additional risk to babies, and the small amounts of blood required for the study purposes were sampled simultaneously with routine metabolic screening, this Board gave blanket approval to perform the study. The patient cohort consisted of healthy, consecutively born male neonates delivered at greater than 37 weeks' gestational age to Sephardic Jewish mothers at the Shaare Zedek Medical Center. Data from this cohort previously have been included in a study of bilirubin production and conjugation in the pathogenesis of neonatal bilirubinemia.[22] Because glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is not uncommon in Israel and is known to increase the risk of neonatal hyperbilirubinemia in neonates homozygous for the variant UGT promoter,[23] all babies were tested for G-6-PD Mediterranean, the mutation known to occur in this population subgroup,[23,24] and those with G-6-PD deficiency were excluded from the current analysis. Males only were studied so as to avoid encountering the G-6-PD heterozygous state that may be present in females, which cannot be diagnosed by regular biochemical tests and is more difficult to perform even on molecular testing. Also excluded were infants with other conditions that may have affected bilirubin metabolism, including direct Coombs positive hemolytic disease, sepsis, maternal diabetes, cephalhematoma or extensive bruising, and Down syndrome. All babies received routine clinical management during their nursery stay.

Blood was sampled for the study on the third day of life, concomitant with routine, predischarge metabolic screening, to avoid a special blood-taking procedure. Whole blood for COHb determination (150 μL) was collected into custom-made capillary tubes containing heparin and saponin, as previously described[25,26] and stored at −18°C. Before shipping, the samples were allowed to thaw and were transported on ice to Stanford University. Simultaneously with the blood collection, a sample of room air from the nursery in which the baby was being cared for was collected and stored in a special stainless steel container until determination of its CO concentration (Bistable Gas Sampler, Chemical Projects Limited, Toronto, ON, Canada).

For analysis of the bilirubin fractions, 0.5 mL serum was separated under dim light, stored at −70°C in the dark, and shipped on dry ice to the University of Padua, Italy. Blood for DNA extraction was collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes.

Laboratory Methods. COHb was determined at Stanford University by a gas chromatographic method, and its concentrations expressed as a percentage of total hemoglobin, which was quantitated by the cyanmethemoglobin method, as previously described.[25,26] With this method, the within-day and between-day coefficients of variation for reference blood samples are 3% and 8%, respectively.[27] The CO concentation of the room air specimens was determined at the Shaare Zedek Medical Center using a sensitive electrochemical CO analyzer supplied by Stanford University.[28] Room air CO concentrations, which ranged from 0.15 to 1.33 ppm (mean 0.46 ppm) were used to correct measured COHb for the effect of inspired CO (COHbc) by a previously derived formula [COHbc = measured COHb − 0.17 (μL CO/L room air)].[29]

Quantitation of the unconjugated, monoconjugated, and diconjugated bilirubin fractions was performed by the alkaline methanolysis technique followed by reverse-phase high-performance liquid chromatography, according to the method of Muraca and Blanckaert.[30] Actual unconjugated, monoconjugated, and diconjugated bilirubin were separated and their concentrations measured. The internal standard used was made up of crystalline xanthobilirubinic acid methyl ester in methanol: 36.2×10^3 L/cm^{-1}. For this method, the within-day coefficient

of variation is 5% to 8% and that for between day is 6% to 13%.

DNA was prepared from peripheral leukocytes by a salt extraction method.[31] The presence or absence of nt 563, the nucleotide mutated in G-6-PD Mediterranean, was determined at the Scripps Research Institute, La Jolla, CA (Ernest Beutler, MD) by polymerase chain reaction followed by allele-specific oligonucleotide hybridization, as published elsewhere.[23] UGT promoter genotype was determined at Shaare Zedek Medical Center by PCR mediated site directed mutagenesis as previously described.[23]

Data Analysis. The sum of measured values for serum unconjugated, monoconjugated, and diconjugated bilirubin values comprised the total bilirubin (STB), while that of the monoconjugated and diconjugated fractions only compromised the total conjugated bilirubin (TCB) value. To facilitate comparison of TCB between the various UGT promoter genotypes with differing STB values, TCB was expressed as a percentage of STB ([TCB/STB(%)]; see Discussion). A lower percentage value will signify diminished bilirubin conjugation, whereas a higher percentage will reflect augmented conjugation. The percentage value for each infant was individually calculated, and then the median and interquartile range of these calculations was computed. To assess the combined role of bilirubin load and conjugation in the pathogenesis of jaundice, an index calculated by dividing COHbc by TCB/STB(%) (COHbc/[TCB/STB(%)]), representing bilirubin production divided by bilirubin conjugation, was used. Lower bilirubin production rates along with efficient bilirubin conjugation will result in a lower index, whereas increased hemolysis relative to conjugation will result in a higher index.

UGT genotypes were assigned according to the gene promoter TATAA box sequence: individuals bearing the sequence $[TA]_6TAA$ in the promoter of both alleles were classified as normal homozygotes (6/6), those with the sequence $[TA]_7TAA$ in both alleles as variant homozygotes (Gilbert's syndrome) (7/7), and those with one of each allele as heterozygotes (6/7).[4,5]

Continuous variables across the 3 UGT promoter genotype groups were compared by ANOVA as an initial statistical test. In the event of significance ($P < .05$), the ANOVA was followed by the Student-Newman-Keuls test or Dunn's test, as appropriate, to determine which variables contributed to the significance. Further statistical analysis was performed using Student's t test, which was replaced by the Mann-Whitney rank sum test for data which did not have a normal distribution. Parametric data are presented as mean ± SD, and nonparametric data as median (interquartile range).

Table 1. Demographic Data of the 131 Babies Enrolled in the Study

Category	Result
Birth weight	3,336 ± 470 g
Gestational age	39.6 ± 1.2 wks
Vaginal delivery (n)	119 (91%)
Exclusively breastfed (n)	100 (73%)

Results

A total of 131 male babies, whose demographic data appear in Table 1, were appropriate for inclusion in the study. Mean (± SD) age at the time of sampling was 52 ± 8 hours. Allele frequency for the variant promoter UGT was 0.32. Sixty-three neonates were normal homozygotes for the UGT promoter (6/6), 50 were heterozygotes (6/7), and 18 were homozygotes for the variant UGT promoter (7/7).

STB values were significantly higher in those homozygous for the variant promoter (154 ± 37 μmol/L) than both homozygote normals (103 ± 50 μmol/L) and heterozygotes (114 ± 41 μmol/L) ($P < .001$). COHbc values were higher in the 7/7 subgroup (0.88 ± 0.19) than in 6/6 or 6/7 counterparts (0.73 ± 0.19% and 0.74 ± 0.19%, respectively; $P < .01$). Measured TCB values were not significantly different between the 3 UGT promoter genotype subgroups (0.69 [0.42-1.31] μmol/L, 0.84 [0.54-1.20] μmol/L, and 0.81 [0.41-1.21] μmol/L for the homozygous normal, heterozygous, and homozygous variant UGT promoter genotypes, respectively). However, TCB/STB(%) ratios were significantly lower in those homozygous (7/7) for the variant promoter than in homozygous (6/6) normals (0.48 [0.24-0.78] vs. 0.81 [0.47-1.27]; $P = .035$), with a trend to also being lower than 6/7 heterozygotes (0.74 [0.48-1.32], $P = .06$). The COHbc/(TCB/STB[%]) was significantly higher in those with the 7/7 variant promoter (1.93 [1.31-2.88]) than both subgroups with the 6/6 or 6/7 promoters (0.85 [0.51-1.72] and 0.84 [0.53-1.87], respectively; $P < .01$). These results are all displayed graphically in Fig. 1. Within the subgroup of infants homozygous for the variant UGT promoter, COHbc values did not correlate with STB values ($r = 0.06$, $P = .8$), nor was there any difference in STB values between the subgroups with COHbc values below mean or greater than mean (156 ± 42 μmol/L versus 155 ± 32 μmol/L); implying uniform distribution of hemolysis throughout the subgroup.

Discussion

In this study, both increased blood COHbc values as well as diminished serum conjugated bilirubin fractions

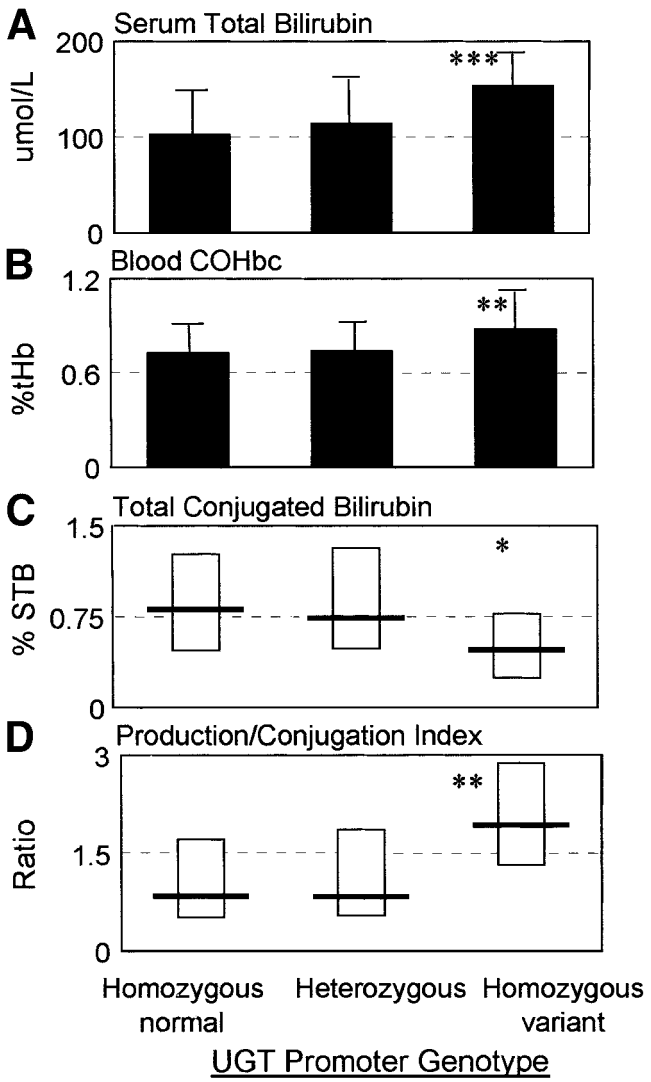

Fig. 1. Graphic presentation of (A) STB values, (B) COHbc, (C) TCB/STB(%), and (D) COHbc/(TCB/STB[%]). **Left panel** for all 4 graphs, normal homozygous UGT promoter genotype (6/6); **middle panel**, heterozygous UGT promoter genotype (6/7); **right panel**, homozygous variant UGT promoter genotype (7/7). Data are presented in graphs A and B as mean ± SD (**vertical bar graphs**), and in C and D as median (interquartile range), as appropriate. In the latter, **horizontal bars** represent median value, and the lower and upper poles of the **vertical bars** represent twenty-fifth and seventy-fifth percentiles, respectively. $*P < .005$, $**P < .01$, $***P < .001$.

were found in association with homozygosity for the variant UGT gene promoter. The low-serum conjugated bilirubin ratios noted in those neonates homozygous for the variant UGT promoter fit in the Gilbert's syndrome sequence of reduced promoter activity[4,6]; diminished enzyme expression and, therefore, activity in hepatic tissue[32]; decreased conjugating capacity[21,33]; and finally, elevated STB values.[4,5] What was surprising was the significantly higher mean COHbc value in the 7/7 subgroup, making it apparent that the underlying abnormalities associated with 7/7 UGT promoter polymorphism include both increased hemolysis and diminished bilirubin conjugation.

Although both reduced red blood cell (RBC) lifespan[10-17] and diminished serum conjugated bilirubin fractions[21,33] have been described previously in some patients with Gilbert's syndrome, these subjects were identified because of clinically apparent Gilbert's syndrome (*i.e.,* mildly visible jaundice or incidentally detected elevated serum bilirubin levels). In contradistinction to these studies, our neonatal cohort was identified according to UGT promoter polymorphism. Not only did we identify individuals many years younger than the age at which the signs of Gilbert's syndrome become manifest, but also only a fraction of our patient cohort can be expected to develop elevated STB levels when they reach adulthood. Consequently, in our series, there could have been no bias in regard to jaundice in case selection.

Within the 7/7 subgroup, lack of correlation between STB and COHbc values and similar STB values in those with COHbc values less than mean or greater than mean imply that there was not a subset of infants who were dependent on greater degrees of hemolysis for the development of higher levels of STB. Rather, distribution of patients with the increased hemolysis was consistent throughout the 7/7 subgroup. This contrasts with the role of defective hepatic bilirubin uptake in the pathogenesis of increased STB values, which are encountered primarily in the subgroup of individuals both homozygotic for the 7/7 promoter and with the lowest hepatic bilirubin uptake rates.[7-9]

The mechanism for the increased hemolysis associated with 7/7 UGT promoter polymorphism is currently unknown and will require further study. Although direct bilirubin toxicity to red blood cells *in vitro* has been described, such toxicity occurred at concentrations of bilirubin far in excess of those encountered in the present study population.[34,35] Further evidence that plasma bilirubin itself does not cause hemolysis *in vivo* is that hemolysis is not seen in patients with Crigler-Najjar syndrome.[36] Thus, in the absence of either icterus as an identifying factor or presence of very high STB levels, it is now evident that both increased hemolysis as well as diminished bilirubin conjugation are inherited as genetically determined defects associated with the variant UGT gene promoter.

Determination of blood COHbc values and serum unconjugated and conjugated bilirubin profiles have been developed as minimally invasive methods of assessment of bilirubin production and conjugation, respectively, and are particularly useful in neonatal investigations. Red blood cell indices, and haptoglobin or hemopexin determinations, are unreliable indicators of hemolysis in new-

borns because of overlap between normal and hemolytic states.[37,38] The principle of COHbc determinations in the assessment of heme catabolism is that equimolar quantities of CO and biliverdin (subsequently bilirubin) are released from heme by the action of the enzyme heme oxygenase. Measurement of COHbc reflects the endogenous CO production, offering accurate assessment of bilirubin production. In the human body, the predominant (85%) endogenous source of CO is from the degradation of heme by heme oxygenase, with only a small proportion originating from non-heme sources; therefore, measurement of blood COHbc reflects primarily the rate of bilirubin formation from hemoglobin.[18-20]

Physiologically, a small fraction of bilirubin conjugates effluxes from the hepatocyte to the serum,[21,39-41] and the serum conjugated bilirubin profile parallels the intrahepatocytic profile. Because the liver is the only organ that can esterify bilirubin to any significant extent and conjugated bilirubin compounds are not absorbed from the bowel, in the absence of hepatocellular disease or cholestasis, the profile is believed to reflect the intrahepatocytic bilirubin profile. This method has been used in the minimally invasive assessment of bilirubin conjugation in neonates,[42-44] children,[45,46] and adults[21,33] alike.

There are data showing that bilirubin may induce its own conjugation and, as a result, TCB concentrations should be expected to fluctuate in concert with STB values.[21,47-50] Measured conjugated bilirubin fractions can be used for comparisons between individuals or groups with similar total bilirubin concentrations,[21,43] but comparisons may be misleading when comparing individuals or groups with varying STB values. This effect may become even more pronounced in individuals with diminished bilirubin conjugating capacity, such as in Gilbert's syndrome. In contrast, serum total conjugated bilirubin values expressed as a percentage of serum total bilirubin, used for analysis in our study, reflect the actual ability of the conjugating system to respond to a bilirubin load. The differences between these 2 methods of analyzing serum conjugated bilirubin values can be readily appreciated when comparing our measured TCB values versus calculated values for serum total conjugated bilirubin as a percentage of STB. The former were similar between the 3 UGT promoter subgroups, despite the increased bilirubin load in the patients homozygous for the variant 7/7 UGT promoter; however, when TCB values were evaluated as a percentage of the STB, it immediately becomes apparent that, in the latter UGT promoter subgroup, the TCB/STB(%) ratios are in fact significantly lower than in the heterozgyous or normal homozygous counterparts, signifying the inability of these patients to respond to the increased load by increasing bilirubin conjugation.

In the absence of additional icterogenic factors, it is likely that the otherwise normal neonate with the 7/7 UGT promoter variant can cope with this increased rate of heme catabolism and diminished bilirubin conjugating capacity without developing significant hyperbilirubinemia.[51] In the presence of further increased hemolysis associated with G-6-PD deficiency[52,53] or hereditary spherocytosis,[54] affected neonates may be unable to respond by increasing bilirubin conjugation in parallel to the additional bilirubin load as expected and imbalance between bilirubin production and conjugation, with resultant hyperbilirubinemia, will result. The imbalance is reflected in the COHbc/(TCB/STB[%]), which was significantly higher in the 7/7 promoter subgroup than in both 6/6 and 6/7 counterparts.

Although the majority of infants were exclusively breast fed, the authors were not concerned that pooling of nursing and bottle-fed infants would lead to any discrepancy in the interpretation of the results. Rubaltelli et al.[55] have shown that conjugated bilirubin profiles on the third and fifth day of life are similar between breast-fed and formula-fed infants, whereas Stevenson et al.[56] have shown similar end tidal CO concentrations between these groups. Although the distribution of the variant UGT promoter genotype is similar between the sexes,[57,58] there is a male preponderance in the Gilbert's syndrome phenotype,[1-3] and our results should not be extrapolated to females. Further studies should be performed to confirm that increased hemolysis associated with the variant UGT promoter occurs in both adults as well as in females.

Unfortunately, the tests used in this study are not readily available for implementation in routine clinical practice; however, the data generated have expanded our understanding of both neonatal hyperbilirubinemia and the function of the UGT 1A1 gene. The pathogenesis of neonatal hyperbilirubinemia is multifactorial and is dependent on factors that both increase bilirubin production and diminish its elimination.[59] Homozygosity for UGT promoter polymorphism may alter the baseline equilibrium by increasing bilirubin production and relative to conjugation. The effect of additional, superimposed pathologic disturbances in bilirubin production or elimination will be exacerbated by the lack of baseline equilibrium, thereby predisposing to hyperbilirubinemia.

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