Review Article

The contribution of genetic factors to hyperbilirubinemia and kernicterus risk in neonates: a targeted update

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Abstract: The genesis of neonatal hyperbilirubinemia is characterized by etiologic heterogeneity, environmental modulation, and the interaction of multiple gene loci. In addition to inherited hemolytic conditions, common icterogenic gene variants may act as modifiers of hyperbilirubinemia and kernicterus risk. The current review targets the effect of biologic sex, uridine diphosphate glucuronosyltransferase isoenzyme *UGT1A1* gene variants of Gilbert syndrome, including their role in breastmilk jaundice, and the co-expression of icterogenic alleles have on potentiating hyperbilirubinemia risk in neonates. Notably, evidence accrued during the past two decades, from around the globe, confirm that breastmilk jaundice is a prevalent Gilbert syndrome phenotype in neonates. Moreover, novel data from humanized murine models suggest an important repressive effect of breastmilk oligosaccharides on intestinal (as opposed to hepatic) *UGT1A1* expression in driving breast milk jaundice risk. More specifically, human milk oligosaccharides block intestinal Toll-like receptor activation and downstream IκB kinase phosphorylation. This in turn represses newborn intestinal UGT1A1 activity. Formula feeding, by contrast, activates IκB and induces intestinal (but not hepatic) UGT1A1 activity thereby lowering the total serum bilirubin (TSB). Whether this phenomenon is operative in human neonates is unclear. Although *UGT1A1* is expressed in adult intestine, there are no comparable developmental data on intestinal UGT1A1 expression in the human fetus or neonate, a knowledge gap that is ripe for clinical investigation.

Keywords: Next generation sequencing; uridine diphosphate glucuronosyltransferase; Gilbert syndrome; male; breastmilk jaundice

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Introduction

The genesis of significant neonatal hyperbilirubinemia [total serum bilirubin (TSB) ≥17 mg/dL (291 µmol/L)] (1) is characterized by etiologic heterogeneity, environmental modulation, and the interaction of multiple gene loci (2-6). Comprehensive reviews of specific genetic contributors to neonatal jaundice have been published and suggest that in addition to inherited hemolytic conditions such as hereditary spherocytosis, common icterogenic gene variants with individually small effects may act as modifiers of hyperbilirubinemia and kernicterus risk (2-6). Damaging mutations (e.g., those of Crigler-Najjar type I) also contribute to the overall genetic architecture of neonatal hyperbilirubinemia but, fortunately, are rare. The current review targets the effect biologic sex, uridine diphosphate glucuronosyltransferase isoenzyme *UGT1A1* (OMIM *191740) gene variants of Gilbert syndrome (OMIM #143500), and co-expression of icterogenic alleles have on potentiating hyperbilirubinemia risk in neonates.

Sexual dimorphism in neonatal hyperbilirubinemia and kernicterus risk

One of the most frequently reported, yet often overlooked, genetic based contributors to hyperbilirubinemia and

kernicterus risk is the biologic sex of the neonate. Male neonates have higher TSB concentrations (7-9), higher rates of non-physiologic hyperbilirubinemia [TSB >12 mg/dL; >205 μmol/L (10)] and are at greater risk for hospital readmission for neonatal jaundice (9,11,12) than female neonates. Similarly, a male sex bias typifies extreme (TSB ≥25 mg/dL) and hazardous (TSB ≥30 mg/dL) hyperbilirubinemia cohorts (13) as well as kernicterus registries (1,14-20). The greater male susceptibility to bilirubin-induced brain damage, on an order of more than 2:1 in some reports (1,14,20), is a consistent finding across many countries, including the United States (1,14), the United Kingdom and Ireland (15), Canada (16), Egypt (17), Denmark (19), Sweden (18), and China (20). Earlier studies reported a male preponderance in preterm kernicterus (21), in neonatal mortality attributed to kernicterus in erythroblastosis fetalis (22), and correspondingly in autopsy case series of kernicterus (23).

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked condition consistently overrepresented as a cause of kernicterus across the globe (24), in all probability contributes to the male preponderance in bilirubin-induced brain damage. Similarly, Gilbert syndrome, a hyperbilirubinemia potentiating genetic condition, is more prevalent in males (25-28). It is doubtful, however, that these clinical entities alone account for the greater numbers of affected males among kernicterus cases.

Although recognition of this male sex bias has no relevance to the care of neonates (female neonates are at risk for kernicterus and are evaluated and managed the same as their male counterparts), it is surprising the male sex kernicterus bias has received limited examination and not been exploited to enhance our understanding of bilirubin-induced brain injury.

The Gunn rat model of neonatal hyperbilirubinemia mirrors humans in sharing a male sex bias for kernicterus and thereby lends itself as a tool for investigating the nature of sexual dimorphism in kernicterus (29,30). Existing data on male-female differences in jaundiced (j/j) hyperbilirubinemic Gunn rat pups, including those collected during sulfadimethoxine induced acute bilirubin encephalopathy, are limited and shown in *Table 1*. They demonstrate that advanced stages of neuromotor dysfunction and kernicterus in hyperbilirubinemic male j/j Gunn rat pups are associated with a two-fold greater cerebellar and brainstem bilirubin content than in their jaundiced female j/j littermate pairs (30). Given similar baseline TSB, serum albumin, and calculated free bilirubin levels (30), one would presume the sulfadimethoxine induced CNS bilirubin exposure itself should be similar between male and female j/j Gunn rat pups. The notable greater cerebellar bilirubin content in j/j male pups may therefore reflect sex specific differences in CNS bilirubin uptake and clearance (30). CNS bilirubin content is modulated in part by the efflux transporter P-glycoprotein (33-35) an ATP binding cassette (ABC) transmembrane protein encoded by the *ABCB1* gene and expressed in microvessel endothelial cells of the blood-brain barrier in human neonates (36). There are, however, no data on sex specific CNS P-glycoprotein expression patterns or the impact of non-synonymous *ABCB1* gene variants (37,38) on P-glycoprotein function in the neonatal period.

Table 1 also highlights a more robust microglial response in j/j male pups during sulfadimethoxine induced acute bilirubin encephalopathy. Although neuroinflammation routinely accompanies bilirubin induced brain damage (39), it is unclear if the greater number of microglia, including amoeboid activated microglia, in encephalopathic male j/j Gunn rat pup cerebellum simply mirrors or contributes to the bilirubin-induced injury.

One potential experimental approach to explore male-female j/j Gunn rat pup differences in susceptibility to bilirubin-induced brain injury is hormonal manipulation. Plasma estradiol in the female Gunn rat pups is protein bound and does not exert an effect in the CNS. By contrast, males produce testosterone which is able to cross the BBB and enter the CNS where it either exerts a direct androgenic effect or is converted to estradiol by tissue aromatase in a region-specific fashion (40). Notably, aromatase levels are undetectable in both male and female rat pup cerebellum (41,42) including the Gunn rat strain (unpublished observations). Other possible experimental manipulations could include castrating Gunn rat male pups or treating females with testosterone. Regardless, the male sex bias in bilirubin-induced brain injury merits further study in the continued effort to more fully understand the cascade of events leading to kernicterus.

Gilbert syndrome and the neonate

Gilbert syndrome is a common congenital inborn error of hepatic bilirubin conjugation wherein UGT1A1 isoenzyme activity is reduced by ~70% or more (43,44). Many pediatricians have long speculated a role for Gilbert syndrome in potentiating neonatal hyperbilirubinemia (45-48). Following the seminal genetic characterizations of Gilbert syndrome (43,49-51) more than twenty-five

Table 1 Male-female differences in hyperbilirubinemic j/j Gunn rat pups in sulfadimethoxine-induced acute bilirubin encephalopathy

Variables	Male	Female	Reference
% BIND score* ≥2	~80%	~50%	Unpublished observations
Total serum bilirubin (mg/dL)**	7.1±1.2	7.5±1.1	(30)
Serum albumin (g/dL)**	3.2±0.6	3.1±0.5	(30)
Calculated unbound bilirubin (μmol/L)**	0.149±0.028	0.153±0.021	(30)
Cerebellar bilirubin content (ug/g tissue)	17.9±8.8[#]	9.2±6.8	(30)
Brainstem bilirubin content (ug/g tissue)	10.8±8.1	6.8±2.9	(30)
Microglia (per hpf)[+]	39.8±27.8	18.9±7.4	Unpublished observations
Activated microglia (per hpf)[+]	17.0±11.2	10.5±8.2	Unpublished observations
Activated microglia in granular layer (per hpf)[+]	14.0±5.8	6.7±6.1	Unpublished observations
Kernicterus[†]	57.6%	40.5%	(29)

Except where otherwise indicated, data were measured 24 hours following sulfadimethoxine triggered acute bilirubin encephalopathy. *, bilirubin-induced neurologic dysfunction (BIND) score quantifies gait abnormalities and dystonia in hyperbilirubinemic j/j Gunn rats. Score ranges from 0 to 5 based on the following signs: 0= normal; 1= mildly abnormal with slight hindlimb ataxia; 2= mild hindlimb ataxia, dystonia and gait abnormality with impaired righting reflex; 3= abnormal as in 2, but with more severe movement disorder and prolonged righting reflex; 4= severe failure of locomotion, general lack of spontaneous movement with occasional bursts of hyperactivity and no righting reflex; 5= moribund including seizures and/or agonal respirations (31,32). **, prior to sulfadimethoxine dosing. [#], $P<0.02$ compared with female cerebellum (Cannon 2006). [+], cerebellar microglia CD11b/c immunofluorescence (OX-42, Serotec, Raleigh, NC) counts were characterized as activated microglia if they displayed a larger, more rounded amoeboid soma and thicker less ramified processes. Counts were expressed as number of microglia per high powered field (400×) based on a minimum of 3 non-overlapping fields (mean ± SD). [†], in absence of sulfadimethoxine induced bilirubin encephalopathy.

years ago, support for this conjecture has grown as has our understanding of the roles genetic heterogeneity and *UGT1A1* variant allele co-expression play in this condition.

Molecular genetics of Gilbert syndrome

A schematic diagram of the *UGT1A1* gene is shown in *Figure 1*. Originally defined by an extra thymine-adenine (TA) dinucleotide repeat within the A(TA)$_n$TAA element of the *UGT1A1* TATAA box promoter (*UGT1A1*28*) in European populations (43,49) and missense mutations in *UGT1A1* exons (*UGT1A1*6*, *UGT1A1*7*, *UGT1A1*27*, *UGT1A1*29*) in Japan (50,51), at least 14 additional *UGT1A1* variant alleles have been identified in association with a Gilbert syndrome phenotype (*Table 2*). Generally held to be an autosomal recessive condition (63), Gilbert syndrome may be inherited in an autosomal dominant manner when *UGT1A1* exon variants are operative; *UGT1A1*6* is a prime example (50,51,64-66). Inheritance is subject to variable penetrance and expressivity (43) depending on the nature of the *UGT1A1* variant, the co-expression of modifying alleles, and the presence of environmental factors.

Adding to this complexity, Ehmer *et al.* report that Gilbert syndrome often represents an expanded genetic haplotype encompassing co-expression of *UGT1A3*, *UGT1A6*, and *UGT1A7* variants in addition to those of *UGT1A1* (*Figure 1*) (53). Greater than three quarters of individuals homozygous for *UGT1A1*28* from the Ehmer *et al.* white northern European cohort were concurrently homozygous for *UGT1A3-66 T>C*, *UGT1A6*2a* and *UGT1A7*3* (53). Moreover, higher TSB levels were observed in those carrying the expanded four gene *UGT1A* haplotype than in those homozygous for *UGT1A1*28* alone (53). It is unclear, however, whether this or an analogous expanded haplotype is expressed in other populations. Similarly, it is uncertain how the expanded four gene UGT1A haplotype enhances hyperbilirubinemia given that only UGT1A1 effectively conjugates bilirubin (67). Nothing is known about the perinatal, neonatal or postnatal expression of this expanded *UGT1A* haplotype or how it might impact neonatal hyperbilirubinemia risk. Regardless, this review will only examine *UGT1A1* variant polymorphisms of Gilbert syndrome.

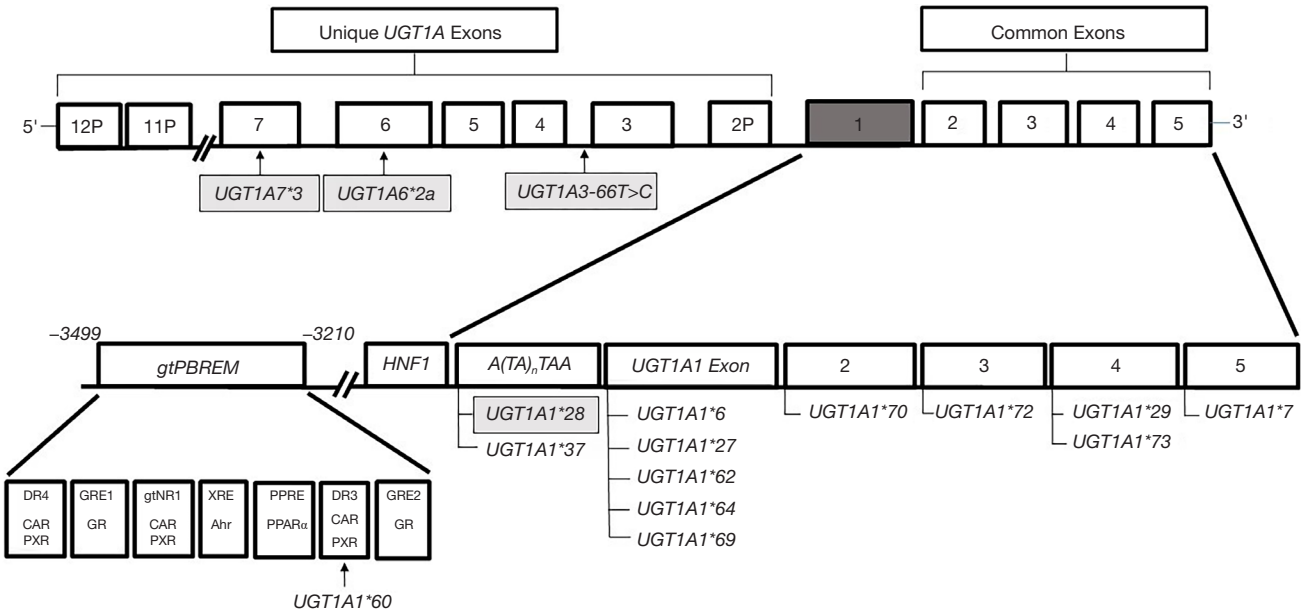

Figure 1 Diagram of the human *UGT1A* gene located on chromosome 2q37. The uppermost panel represents the entire *UGT1A* gene complex which includes *UGT1A1* (dark gray), additional exons 3–7 that encode functional proteins (exon 8–10 and 13 not shown), three pseudogenes (2P,11P,12P), and the common exons 2–5, shared across all *UGT1A* transcripts. The lower panel shows the unique *UGT1A1* exon coupled with common exons 2–5, the upstream glucuronosyltransferase phenobarbital responsive enhancer module (*gtPBREM*) encompassing seven nuclear receptor elements (DR4, GRE1, gtNR1, XRE, PPRE, DR3, GRE2), their transcription factors CAR, PXR, GR (glucocorticoid receptor), Ahr (aryl hydrocarbon receptor), and PPARα, as well as the TATA box promoter sequence [adapted from (3,52)]. The hepatocyte nuclear factor 1 (*HNF1*) region is located between *gtPBREM* and the TATA box and contains the HNF1α binding site (52). Specific *UGT1A1* hypomorphic Gilbert syndrome allele variants and their location are labelled using given numbers preceded by an asterisk. The *UGT1A3-66T>C*, *UGT1A6*2a*, *UGT1A7*3*, *UGT1A1*28* haplotype reported in over three quarters of Gilbert syndrome subjects of northern European descent is highlighted in light gray (53). *UGT1A7*3* combines *UGT1A7* coding sequence variants p.N129K/p.R131K and p.W208R (53); whereas the *UGT1A6*2a* genotype combines *UGT1A6* coding sequence variants p.Ser7Ala, p.Thr181Ala, and p.Arg184Ser (53).

Gilbert syndrome and neonatal hyperbilirubinemia risk

Bancroft *et al.* in 1998 were the first to explore the relationship between a Gilbert syndrome genotype, specifically *UGT1A1*28*, and neonatal hyperbilirubinemia (68). Their findings, from a largely white-non-Hispanic cohort, demonstrated an increased rate of rise in transcutaneous bilirubin levels during the first two days of life in *UGT1A1*28* homozygous neonates (68). Despite the enhanced rate of rise, peak transcutaneous bilirubin levels in neonates with Gilbert syndrome did not differ from wild type controls (68). Bancroft *et al.* concluded that the "determination of the relative role of this genetic variable in the assessment of overall neonatal jaundice risk will require completion of a prospective study with multivariate analysis to examine various combinations of jaundice risk factors" (68).

Numerous such studies published over the ensuing decades support a potentiating role for Gilbert syndrome in neonatal hyperbilirubinemia risk, depending on the specific genotype, study population, the presence of breastmilk feeding, and/or hemolytic disease. The most prevalent polymorphic gene variants involved are *UGT1A1*28*, *UGT1A1*6*, and *UGT1A1*60*.

*UGT1A1*28*

The Gilbert syndrome promoter sequence variant *UGT1A1*28* is common to individuals of European and African ancestry (57,69,70). Several studies (2,3,64,71-76), but not all (10,65,77,78), suggest that *UGT1A1*28* alone poses limited to no enhanced neonatal hyperbilirubinemia

Table 2 Individual *UGT1A1* gene variants reported in Gilbert syndrome

Allele	Nucleotide change	Amino acid change	Variant location	Reference
*UGT1A1*1*	$A(TA)_6TAA$	Wild type	Promoter	
*UGT1A1*6*	211(G>A)	G71R	Exon 1	(54)
*UGT1A1*28*	$A(TA)_6TAA$ to $A(TA)_7TAA$	n/a	Promoter	(43)
*UGT1A1*60*	–3279(T>G)	n/a	Promoter	(55,56)
*UGT1A1*7*	1456(T>G)	Y486D	Exon 5	(54)
*UGT1A1*27*	686(C>A)	P229Q	Exon 1	(51)
*UGT1A1*37*	$A(TA)_6TAA$ to $A(TA)_8TAA$	n/a	Promoter	(57)
*UGT1A1*62*	247(T>C)	F83L	Exon 1	(58)
*UGT1A1*64*	488-491 dupACCT	Frameshift	Exon 1	(59)
*UGT1A1*65*	–1126(C>T)	n/a	Promoter	(59)
*UGT1A1*66*	997-82(T>C)	n/a	Intron 2	(59)
*UGT1A1*67*	–85 to –83 ins CAT	n/a	Promoter	(60)
*UGT1A1*68*	–63(G>C)	n/a	Promoter	(60)
*UGT1A1*69*	476(T>C)	I159T	Exon 1	(60)
*UGT1A1*70*	962(C>G)	A321G	Exon 2	(60)
*UGT1A1*72*	1075(G>A)	D359N	Exon 3	(60)
*UGT1A1*73*	1091(C>T)	P364L	Exon 4	(60)
*UGT1A1*81*	–64(G>C)	n/a	Promoter	(61)

Alleles shaded in gray are polymorphic. Adapted updated and modified from reference (62). Reproduced with permission of Taylor & Francis Inc.

risk. Published meta-analyses confirm the same (64) or at least less icterogenic potential than *UGT1A1*6* (64). The *UGT1A1*28* variant, however, when co-expressed with *UGT1A1* coding sequence variants or icterogenic conditions such as breastfeeding and hemolytic disease, appears to augment hyperbilirubinemia risk.

*UGT1A1*6*

In marked contrast to *UGT1A1*28*, it is increasingly apparent that the *UGT1A1* exon 1 coding sequence variant *UGT1A1*6*, common to Asian populations, in itself exerts an icterogenic effect. *UGT1A1*6* acts as an independent risk factor for neonatal hyperbilirubinemia (64-66,79-82) and contributes to, and in fact may primarily underlie, the widely recognized increased neonatal hyperbilirubinemia risk among Asian populations (83-85). This association is robust as confirmed by at least three large meta-analyses, irrespective of genetic modeling approach (homozygous, heterozygous, dominant or recessive) (64-66), a fundamental effect that may relate to the greater reduction in UGT1A1 enzymatic activity (~14% of wild type levels) associated with this variant (50). Not surprisingly, co-expression of *UGT1A1*6* with other coding sequence variants, promoter variants, hemolytic conditions, and breastfeeding further increases hyperbilirubinemia risk.

*UGT1A1*60*

The *UGT1A1*60* variant has been of great interest in efforts to understand the genetic nature of Gilbert syndrome. This promoter variant is located in the glucuronosyltransferase phenobarbital responsive enhancer module (gtPBREM) cluster (*Figure 1*), a regulatory element containing multiple binding sites for nuclear receptor motifs including the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), the xenobiotic responsive element (XRE), peroxisome proliferator-activated receptor alpha (PPARα),

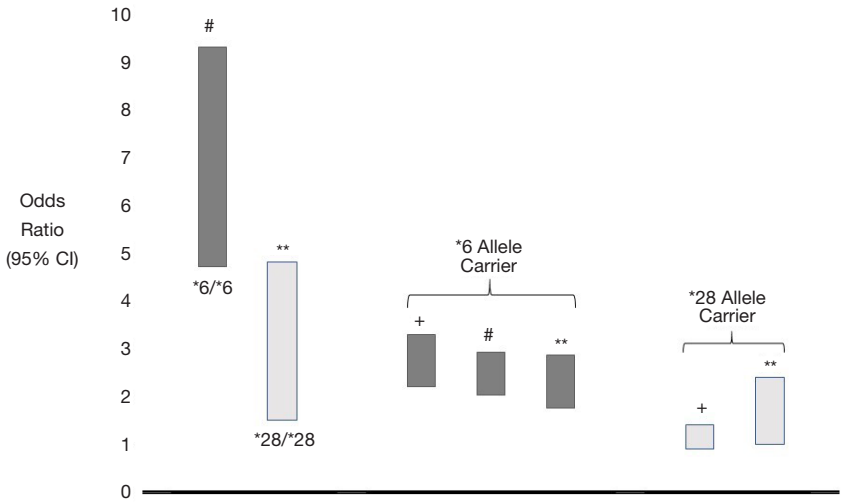

Figure 2 Plot of 95% confidence interval odds ratio for neonatal hyperbilirubinemia risk as a function of *UGT1A1*6* and *UGT1A1*28* Gilbert syndrome polymorphism expression. Homozygous *UGT1A1*6* expression: (**6/*6*), homozygous *UGT1A1*28* expression: (**28/*28*), *UGT1A1*6* allele carrier: **6/*6 + *6/*1 vs. *1/*1*, *UGT1A1*28* allele carrier: **28/*28 + *28/*1 vs. *1/*1*. Data from references (64) (+), (65) (**), and (66) (#).

and glucocorticoid responsive elements (GRE) (52). *UGT1A1*60*, in contrast to most other variants, is common across studied populations regardless of biogeographic heritage with allele frequencies generally between 0.25 and 0.50 (3). Studies report a high degree of linkage disequilibrium between *UGT1A1*60* and *UGT1A1*28*, an association asserted by some (69) [but not all (86,87)] as essential to the genesis of Gilbert syndrome. Linkage of *UGT1A1*60* with *UGT1A1*6* has also been reported (28). However, investigations suggest that homozygous expression of *UGT1A1*60* alone can be associated with reduced *UGT1A1* transcriptional activity (55,56) and itself account for Gilbert syndrome in some populations (56). A recent meta-analysis shows that *UGT1A1*60* is associated with a significant increased risk for neonatal hyperbilirubinemia, albeit the study did not rule out linkage with other variants as the mechanism (88). Given the cluster of regulatory elements contained in gtPBREM and their potential roles in regulating the developmental expression of *UGT1A1*, it is important to further clarify the nature of variants localized to that region of the *UGT1A1* promoter (52,89,90).

Spectrum of neonatal hyperbilirubinemia risk in Gilbert syndrome

Taken together the *UGT1A1*28* and *UGT1A1*6* studies detailed above suggest there is a spectrum of neonatal hyperbilirubinemia risk across Gilbert syndrome genotypes (64-66), depending on the variant(s) involved and expression mode. Neonates homozygous for *UGT1A1*6* demonstrate the highest reported odds ratios for neonatal hyperbilirubinemia risk among Gilbert syndrome variants (65,66), one that is notably higher than those homozygous for *UGT1A1*28* (*Figure 2*). *UGT1A1*6* allele carriers also evidence a significantly increased neonatal hyperbilirubinemia risk compared to wild type (64-66) whereas allele carriers for *UGT1A1*28* show borderline to non-significant associations with neonatal hyperbilirubinemia risk (64,65) (*Figure 2*). Monaghan *et al.* suggested a similar divergence in hyperbilirubinemia risk in their 1996 study of adults when they asserted there were both "mild and more severe forms of Gilbert's syndrome" (49). The 'mild' form was related to expression of *UGT1A1* promoter variants whereas 'more severe forms' were due to *UGT1A1* coding sequence variants (49). In fact, promoter variants alone may not always be sufficient to develop a Gilbert syndrome phenotype (43), whereas biallelic expression of coding sequence variants associated with Gilbert syndrome may lead to TSB levels intermediate between Gilbert syndrome and Crigler-Najjar syndrome type II (91). Indeed, the genetic characterization of the non-hemolytic hyperbilirubinemias Gilbert syndrome, Crigler-Najjar syndrome type II and Crigler-Najjar syndrome type I are not as invariant and sharply demarcated as once thought,

but similarly reflect a spectrum of hyperbilirubinemia severity and risk.

UGT1A1 gene variants and hemolysis

Hemolysis is the dominant cause of extreme hyperbilirubinemia, acute bilirubin encephalopathy, and kernicterus (92,93). Expression of *UGT1A1* gene variants of Gilbert syndrome with hemolytic conditions may augment the risk of significant hyperbilirubinemia (2-5,24,94,95). Kaplan *et al.* was the first to highlight the importance of this phenomenon in G6PD deficiency, itself a leading cause of kernicterus worldwide (94). In their seminal study, there was a dose dependent genetic interaction of *UGT1A1*28* alleles in hemizygous G6PD Mediterranean deficient males that enhanced neonatal hyperbilirubinemia (TSB >15 mg/dL) risk (94). A similar association has been reported between *UGT1A1*6* and G6PD deficiency in China (95). Others have documented an association between UGT1A1*28 and hyperbilirubinemia risk in symptomatic ABO hemolytic disease of the newborn, hereditary spherocytosis, G6PD deficiency, and beta-thalassemia (96-100). Correspondingly, a recent report from China demonstrates co-expression of *UGT1A1*6* and *ANK1* mutations of hereditary spherocytosis (101). The Gilbert syndrome variants icterogenic augmenting effect in hemolytic conditions does not appear to be related to any change in heme catabolism (96). Collectively, these studies illustrate the importance of coupling genetically determined hemolytic conditions with gene polymorphisms that reduce hepatic bilirubin clearance in increasing the risk of developing severe neonatal hyperbilirubinemia.

Breastmilk jaundice: a prevalent Gilbert syndrome phenotype

Neonatal hyperbilirubinemia is more common and TSB levels are significantly higher in breastfed than in formula-fed neonates (102,103). Hyperbilirubinemia in association with suboptimal breastmilk intake during the first week of life is termed "breast-feeding jaundice"; whereas prolonged jaundice in thriving breastfed neonates extending into the second to third week of life, occasionally longer, is termed "breastmilk jaundice" or "breastmilk jaundice syndrome" (102,103). Despite decades of investigation, the operative mechanism(s) underlying breastmilk jaundice syndrome remain a source of debate; the condition is likely multifactorial in nature.

Clinical evidence accrued during the past two decades, from around the globe, confirm that breastmilk jaundice is a prevalent Gilbert syndrome phenotype in neonates (10,104-106). Monaghan *et al.* were the first to highlight the association between *UGT1A1* gene variants and prolonged unconjugated hyperbilirubinemia in breastfed term neonates (104). Their study of breastfed Scottish neonates showed that those homozygous for the Gilbert syndrome *UGT1A1*28* promoter variant had a more than four-fold increased rate (27%) of prolonged jaundice (TSB >150 µmol/L at 14 day) than breast fed infants who were homozygous for the wild type *UGT1A1*1* allele (6%) (104). Zaja *et al.* demonstrated a similar four-fold impact of homozygous *UGT1A1*28* expression on the risk of breastmilk jaundice of greater than 21 days duration in their large Croatian cohort (10). Forty percent were homozygous for *UGT1A1*28* Gilbert genotype (10).

Maruo *et al.* demonstrated an analogous relationship between breastmilk jaundice and the *UGT1A1*6* Gilbert variant in Japan (105). They reported that 16 of 17 breastfed Japanese infants with prolonged unconjugated hyperbilirubinemia [TSB >10 mg/dL (171 µmol/L) at 3–4 weeks of age] carried at least one *UGT1A1*6* variant allele. Nine of the 16 were either homozygous for *UGT1A1*6* (n=8) or compound heterozygous for *UGT1A1*6* and *UGT1A1*28* (n=1), both classic Gilbert syndrome genotypes. The homozygous subset evidenced a median TSB of 18.8 mg/dL (range, 10.3–31.8 mg/dL) at 3–4 weeks of age. In a subsequent expanded set of 170 infants with breastmilk jaundice syndrome, Maruo *et al.* observed that 88 (51.8%) were homozygous for *UGT1A1*6*, as opposed to none in breastfed controls without breast milk jaundice (106). They also reported 23 neonates who were compound heterozygous for Gilbert variant alleles, bringing to a total of 122 (72%) the number of breastmilk jaundice infants who carried a Gilbert genotype. If one further considers heterozygosity for *UGT1A1*6* a Gilbert genotype, a widely held premise, then another 26 breastmilk jaundice neonates would be added to the total, resulting in 148 of the 170-breastmilk jaundice cohort (87%) carrying a Gilbert syndrome genotype (106).

Several other studies have demonstrated a strong association between the risk (80,81), degree (79,80,107), and duration (107) of hyperbilirubinemia and a Gilbert syndrome genotype in breastfed neonates from China, Taiwan, and Greece. Other findings from these studies include a synergistic effect of *UGT1A1*6* and breastfeeding

on the risk of meriting phototherapy (81) and developing a TSB of ≥20 mg/dL (342 µmol/L) (79).

How does breastmilk combine with Gilbert syndrome to produce prolonged indirect hyperbilirubinemia? Ramos *et al*. hypothesized that both a breastmilk inhibitor of bilirubin conjugation and an impaired hepatic bilirubin conjugating system are required for the clinical expression of breastmilk jaundice syndrome (108). This conjecture is consistent with the fact that not all breastfed neonates develop breast milk jaundice syndrome and that formula-fed infants with Gilbert syndrome do not evidence prolonged indirect hyperbilirubinemia. Several different substances in breast milk have been suggested to inhibit hepatic bilirubin conjugation including pregnane-3α,20β-diol, nonesterified fatty acids, and oligosaccharides. Studies by Tukey and colleagues using a translational hyperbilirubinemic humanized *UGT1* mouse model suggest an important repressive effect of breastmilk oligosaccharides on intestinal (as opposed to hepatic) *UGT1A1* expression in driving breast milk jaundice risk (90,109,110). More specifically, human milk oligosaccharides block intestinal Toll-like receptor activation and downstream IκB kinase phosphorylation (90,109,110). This in turn represses newborn intestinal UGT1A1 activity (90). Formula feeding, by contrast, activates IκB and induces intestinal (but not hepatic) UGT1A1 activity thereby lowering the TSB (90). Whether this phenomenon is operative in human neonates is unclear. UGT1A1 is expressed in adult small intestine (duodenum, jejunum and ileum) (111,112), stomach, and colon (112) where it is localized to the epithelial cell layer of the mucosa, most prominently at the apical portion of the crypt enterocytes (111). There are, however, no comparable developmental data on intestinal UGT1A1 expression in the human fetus or neonate, a knowledge gap that is ripe for clinical investigation.

It is also possible that breastmilk and Gilbert syndrome exert their effects via the enterohepatic circulation of bilirubin to produce breastmilk jaundice. Breastmilk increases intestinal bilirubin absorption (113,114) independent of any augmentation of intestinal beta-glucuronidase activity. An important feature of Gilbert syndrome is a predominance of bilirubin monoglucuronides over bilirubin diglucuronides (115,116). This condition should increase enterohepatic bilirubin circulation as hydrolysis of monoglucuronides back to unconjugated bilirubin occurs at rates 4–6 time that of the diglucuronide (117). Combined, these breastmilk and Gilbert syndrome enterohepatic circulation enhancing effects would increase the hepatic bilirubin load while at the same time limit the liver's capacity to conjugate that load, producing an increased prolonged unconjugated hyperbilirubinemia risk.

Biogeographic distribution of UGT1A1 Gilbert syndrome variants

As detailed above, the most prevalent genotype underlying Gilbert syndrome in European populations is the TATA box promoter variant *UGT1A1*28* (3,43,69,118). Similarly, *UGT1A1*28* underlies Gilbert syndrome in Sub-Saharan Africa and individuals of African biogeographic heritage where the less frequent *UGT1A1*37* promoter variant allele is also observed (57,69,119). Coding sequence missense variants associated with Gilbert syndrome (e.g., *UGT1A1*6*) are distinctly uncommon in Northern European or African populations (3,69).

In marked contrast, *UGT1A1*6* is the most common variant underlying Gilbert syndrome across many Asian populations, whereas *UGT1A1*28* is less common with the exception being populations of the Indian subcontinent (India, Bangladesh, Sri Lanka) (77,119). Not surprisingly, subgroup analyses by East Asian ethnicity document that *UGT1A1*6* allele carriers have a significantly increased risk of neonatal hyperbilirubinemia in Northeast Asia, Southeast Asia, China, Japan, and Malaysia (3,64,82).

The nature of these biogeographic differences in Gilbert syndrome genotypes remains a focus of investigation; but distinct genotypes leading to a Gilbert syndrome phenotype is consistent with convergent evolution (120). Correspondingly, investigators postulate that *UGT1A1* variant alleles represent balanced polymorphisms in human evolution maintained by natural selection (57,69). Whether this is the case is uncertain (119), as is whether bilirubin is the source of selective pressure (69,120,121). There is growing evidence, however, that a mildly elevated TSB, an endogenous antioxidant, is associated with relative protection against an array of diseases (120,122) and may thereby provide an evolutionary advantage (3,57,69,120,121).

Co-expression of icterogenic gene polymorphisms

Co-expression of gene polymorphisms that potentiate bilirubin production, limit hepatic bilirubin uptake, reduce hepatic bilirubin conjugation and clearance is common (2,3,123) and contributes to neonatal hyperbilirubinemia

Table 3 Reported compound heterozygous *UGT1A1* genotypes in Gilbert syndrome

Co-expressed UGT1A1 variant alleles	Allele location	Ref
*UGT1A1*6/UGT1A1*7*	Exon 1/exon 5	(27)
*UGT1A1*6/UGT1A1*27*	Exon 1/exon1	(91)
*UGT1A1*6/UGT1A1*28*	Exon 1/promoter	(27)
*UGT1A1*6/UGT1A1*60*	Exon 1/enhancer	(27)
*UGT1A1*6/UGT1A1*73*	Exon 1/exon 4	(125)
*UGT1A1*7/UGT1A1*73*	Exon 1/exon 4	(125)
*UGT1A1*27/UGT1A1*60*	Exon 1/enhancer	(91)
*UGT1A1*28/UGT1A1*7*	Promoter/exon 5	(27)
*UGT1A1*28/UGT1A1*27*	Promoter/exon 1	(125)
*UGT1A1*28/UGT1A1*29*	Promoter/exon 4	(91)
*UGT1A1*28/UGT1A1*60*	Promoter/enhancer	(27)
*UGT1A1*28/UGT1A1*73*	Promoter/exon 4	(125)
*UGT1A1*60/UGT1A1*81*	Enhancer/promoter	(28)
*UGT1A1*6/UGT1A1*27/UGT1A1*28*	Exon 1/exon 1/promoter	(125)
*UGT1A1*6/UGT1A1*28/UGT1A1*60*	Exon 1/promoter/enhancer	(27)
*UGT1A1*6/UGT1A1*28/UGT1A1*73*	Exon 1/promoter/exon 4	(125)
*UGT1A1*6/UGT1A1*60/UGT1A1*73*	Exon 1/enhancer/exon 4	(27)
*UGT1A1*28/UGT1A1*27/UGT1A1*60*	Promoter/exon1/enhancer	(27)
*UGT1A1*28/UGT1A1*60/UGT1A1*73*	Promoter/enhancer/exon 4	(27)
*UGT1A1*6/UGT1A1*27/UGT1A1*28/UGT1A1*60*	Exon 1/exon 1/promoter/enhancer	(27)
*UGT1A1*28/UGT1A1*27/UGT1A1*60/UGT1A1*73*	Promoter/exon1/enhancer/exon 4	(27)

Co-expressed *UGT1A1* allele genotypes compiled from references (27,28,91,125).

risk (2,3,79,124). Such co-expression includes two interesting and clinically relevant phenomena: compound and synergistic heterozygosity. Compound heterozygosity refers to the inheritance of alternate alleles from each parent located at different loci within the same gene. A prime example is compound heterozygosity for *UGT1A1* variants of Gilbert syndrome, a phenomenon more frequent than previously thought (*Table 3*) (27). Sun *et al.* recently reported as many as three and four *UGT1A1* mutation sites in individuals with Gilbert syndrome (*Table 3*) (27).

In contrast, synergistic heterozygosity refers to heterozygosities across different genes that combine to produce a range of subtle to more severe phenotypes (126). Although initially applied to inborn errors of energy metabolism, synergistic heterozygosity is a concept with broad clinical applicability including the numerous genes involved in bilirubin production, metabolism, and clearance (3). Partial defects in one or more of these pathways may contribute to hyperbilirubinemia risk (3). Zangen and co-workers described the occurrence of fatal kernicterus in a female neonate heterozygous for the *G6PD* Mediterranean mutation and the *UGT1A1*28* Gilbert syndrome variant as a paradigm for this phenomenon in neonatal hyperbilirubinemia (127).

Next generation sequencing in the diagnostic evaluation of extreme or hazardous neonatal hyperbilirubinemia and kernicterus

In almost all reported case series of extreme or hazardous hyperbilirubinemia and kernicterus, the etiology of the marked hyperbilirubinemia is often unidentified

(1,14,18,19). This unfortunate situation reflects the limited investigative repertoire available to providers in the clinical arena. Once maternal antibody mediated hemolysis and G6PD deficiency are ruled out, the etiology often remains unclear or is conjectured based on the red cell smear and red cell indices. A firm diagnosis is not made. In the US Pilot Kernicterus Registry, idiopathic cases comprised 43% of the total demonstrating a median TSB of 36.0 mg/dL (615 µmol/L) and range of 20.7–52.0 mg/dL (354–889 µmol/L) (14). Few neonates have the capacity to generate TSB levels in a hazardous range; many, if not most have an underlying hemolytic condition (92,93). Christensen and colleagues have utilized a next-generation sequencing gene panel targeted on heritable causes of hemolytic anemia and disorders of hepatic bilirubin uptake and conjugation to clarify the nature of extreme hyperbilirubinemia (92,128) and kernicterus (93). They have recently established a Neonatal Acute Bilirubin Encephalopathy Registry (NABER) that will correlate clinical data with the results of sequencing 28 genes involved in bilirubin production and metabolism to clarify the nature of hyperbilirubinemia in acute bilirubin encephalopathy (129). Molecular diagnosis holds an important key in improving our understanding of the pathogenesis of hazardous hyperbilirubinemia and kernicterus and in identifying means of preventing their occurrence (129).

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