



## Molecular pathology of Crigler-Najjar type I and II and Gilbert's syndromes

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### ABSTRACT

*Background and Objective.* Crigler-Najjar syndromes type I and II and Gilbert's syndrome are familial unconjugated hyperbilirubinemias caused by genetic lesions involving a single complex locus encoding for bilirubin-UDP-glucuronosyltransferase which is involved in the detoxification of bilirubin by conjugation with glucuronic acid. Over the last few years a number of different mutations affecting this gene have been characterized. The aim of this work is to review the molecular pathology of Crigler-Najjar and Gilbert's syndromes, to discuss its impact on the clinical and genetic classification of these conditions, and on the diagnostic evaluation of clinical pictures associated with unconjugated hyperbilirubinemia.

*Evidence and Information Sources.* The authors of the present review are involved in the clinical management of patients with familial unconjugated hyperbilirubinemia as well as in the characterization of its molecular bases. Evidence from journal articles covered by the Science Citation Index<sup>®</sup> and Medline<sup>®</sup> has been reviewed and collated with personal data and experience.

*State of the Art and Perspectives.* It has been known for many years that mild to severe deficiency of bilirubin UDP-glucuronosyltransferase in the liver is the cause of two types of familial unconjugated hyperbilirubinemia, Crigler-Najjar syndromes I and II, and Gilbert's syndrome. Since the characterization of the gene encoding for bilirubin UDP-glucuronosyltransferase, a number of mutations affecting the expression of this gene have been identified. These mutations can be classified into three groups: mutations which result in a reduced production of a normal enzyme; mutations which give rise to the synthesis of a structurally abnormal and dysfunctional enzyme; mutations which completely abolish the expression of the affected allele. The combination of mutations affecting the coding region of the gene and of promoter mutations which reduce the expression of the gene accounts for the wide clinical spectrum of familial unconjugated hyperbilirubinemias, ranging from the clinically negligible Gilbert's syndrome to the severe and often fatal Crigler-Najjar type I syndrome. A better understanding of the genetics of these con-

ditions and the availability of molecular diagnosis will improve the diagnostic efficiency and afford better informed genetic counseling, not only for Crigler-Najjar and Gilbert's syndromes, but also for several acquired conditions characterized by unconjugated hyperbilirubinemia.

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Key words: hyperbilirubinemia, UDP-glucuronosyltransferase, Crigler-Najjar, Gilbert's syndrome, mutation analysis

**B**ilirubin is a toxic product of heme metabolism produced in large amounts from the normal turnover of hemoglobin and other hemoproteins. Serum bilirubin levels increase when its production from heme exceeds the metabolic capacity (4 mg/kg/die)<sup>1</sup> and excretion. Imbalance between production and clearance results either from excess release of bilirubin precursors into the bloodstream or impairment of the hepatic uptake, metabolism, or excretion.

Serum bilirubin concentrations in normal subjects range from 0.3 to 1.0 mg/dL. Approximately 80 percent of circulating bilirubin derives from senescent red blood cells destroyed by reticuloendothelial cells. The rest originates from other sources, including ineffective erythropoiesis and the metabolism of other heme-containing proteins, such as hepatic cytochromes and muscle myoglobin. More than 90 percent of serum bilirubin in normal individuals circulates as an albumin-bound complex in the unconjugated form. Unconjugated bilirubin enters the hepatocyte by diffusion or transport across the plasma membrane and is bound to ligandin, which prevents its efflux back into the plasma. In the endoplasmic reticulum of hepatocytes bilirubin detoxification takes place by glucuronidation by bilirubin UDP-glucuronosyltransferase (UGT) and conversion to excretable, water-soluble glucuronides. Soluble conjugated bilirubin is excreted by the hepatocyte with bile.

In several inherited disorders, the transfer of bilirubin from blood to bile is disrupted at a specific step. The study of these disorders has allowed better understanding of bilirubin metabolism in health and disease.

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### The UDP-glucuronosyltransferase gene: structure and regulation

Two human liver UGT cDNAs were isolated in 1991,<sup>2</sup> each encoding a bilirubin transferase isoenzyme capable of catalyzing the formation of physiological conjugates of bilirubin. Sequence analysis showed that they contained an identical 3' end, also identical to that of the human phenol transferase cDNA.<sup>3</sup> Subsequent studies demonstrated that the two bilirubin transferases as well as the phenol transferase, all having identical carboxyl termini, were all encoded by a single locus.<sup>4</sup> The transcriptional arrangement of the UGT locus is remarkable, consisting of an array of unique exon 1s encoding for 287-289 amino acids at the NH<sub>2</sub>-terminal of the enzyme, critical for substrate specificity of the isoform, in conjunction with four common exons which encode the identical carboxyl termini. The four common exons are concentrated in a 6 kb region, while the series of unique exon 1s is dispersed over a region of about 85 kb. Each exon 1 is differentially spliced to the four common exons to produce a unique mature mRNA. At least three of the transcripts are synthesized (phenol-UGT, bilirubin-UGT1, and bilirubin-UGT2) while one of the exons contains a single base mutation causing a frameshift and the synthesis of a truncated protein,<sup>4</sup> and two other exon 1s are not expressed.<sup>5</sup>

Each of the exon 1s has its own upstream promoter, thus providing independent regulation of each isoform. The promoter of exon 1\*1, encoding for the NH<sub>2</sub>-terminal of bilirubin-UGT1, the isoform responsible for 99% of bilirubin glucuronidating activity,<sup>6</sup> includes a peculiar TATAA element containing a TA repeat (A(TA)<sub>6</sub>TAA).<sup>7</sup> The TATAA element is the binding site for transcription factor IID, involved in the initiation of transcription.<sup>8</sup> A common elongated variant of the TATAA element in the promoter of exon 1\*1 containing an additional TA (A(TA)<sub>7</sub>TAA) has been shown to be associated with a reduced expression of bilirubin-UGT1.<sup>7</sup>

### Crigler-Najjar types I and II, and Gilbert's syndrome: clinical features

#### *Crigler-Najjar type I*

The clinical picture of a severe congenital familial non-hemolytic jaundice with kernicterus, later denominated Crigler-Najjar syndrome type I (CN-I), was described by Crigler and Najjar in 1952.<sup>9</sup> In that family and in several isolated patients and kindreds subsequently described, the clinical course of the disease was characterized by development of a progressive and severe neurologic syndrome resembling kernicterus, usually leading the affected subjects to death within the first two years of life. Rare cases with a more prolonged survival but with severe neurologic impairment have also been described.<sup>10,11</sup> Necropsy studies of CN-I patients show massive deposition of bilirubin in organs and tissues, deep jaundice of

the cerebral cortex and other structures of the central nervous system, and neuronal loss.<sup>9,12</sup>

In CN-I bilirubin production by erythroid cells and precursors is not increased and there is no evidence of liver dysfunction or abnormality of the biliary system. Total plasma bilirubin levels have been reported in a wide range from 15 to over 50 mg/dL. All bilirubin is unconjugated.<sup>13</sup>

Defective glucuronide conjugation of bilirubin was initially demonstrated by *in vivo* studies of the metabolism of non-bilirubin aglycones.<sup>14</sup> It was subsequently clarified that microsomes of CN-I patients lack the ability to conjugate bilirubin with UDP-glucuronic acid.<sup>15</sup> This defect is analogous to that observed in an animal model, the Gunn rat, which presents with a condition phenotypically similar to CN-I.<sup>16</sup>

CN-I is a rare disease, with only a few hundred cases described in detail in the literature. Analysis of pedigrees, in which consanguinity is frequently observed, is consistent with an autosomal recessive transmission, which has been basically confirmed by recent molecular genetics data.

The only effective treatment for CN-I patients is orthotopic liver transplantation. CN-I patients are not responsive to phenobarbital therapy that, in CN-II patients, accelerates hepatic bilirubin clearance by enzymatic induction.<sup>15</sup> Phototherapy has been effectively used to lower bilirubin levels in children,<sup>17</sup> but it is neither practical nor effective enough to be proposed as a lifelong measure. Exchange transfusion and exchange plasmapheresis have been used as emergency measures prior to liver transplantation.<sup>18</sup> The first successful liver transplant for CN-I was reported in 1982.<sup>19</sup> Liver transplantation should be considered early in CN-I, in order to prevent dramatic and irreversible neurologic damage.

#### *Crigler-Najjar type II*

The original group of patients with severe unconjugated hyperbilirubinemia leading to the definition of CN-II was described by Arias *et al.* in 1962.<sup>20</sup> As in CN-I icterus in the absence of increased bilirubin production or liver disease is the only physical finding. Bilirubin levels are lower than in CN-I (10-20 mg/dL), central nervous system damage is rare, and the majority of patients survive into adulthood without complications.<sup>13</sup> Patients with CN-II promptly respond to the administration of phenobarbital or other enzyme-inducing agents with a significant reduction of bilirubin levels.<sup>15</sup>

The bile of CN-I patients contains significant amounts of conjugated bilirubin, with a relative excess of bilirubin monoglucuronide.<sup>15, 21</sup> However, a severe deficiency of glucuronyl transferase activity, usually less than 10% of normal has been measured in the liver of affected patients.<sup>15, 20</sup> Since glucuronide synthesis is also reduced in the presence of non-bilirubin aglycones, the defect appears to involve multiple

isoforms of the enzyme, i.e. to depend on mutations affecting the common carboxyl termini of the protein encoded by exons 2-5.

The mode of inheritance of CN-II is not clear as in most cases of CN-I. Initial studies suggested an autosomal dominant trait with variable penetrance.<sup>15,20</sup> However several pedigrees do not conform to this mode of inheritance and it has also been hypothesized that CN-II could represent the homozygous state or a compound heterozygosity for the Gilbert's syndrome gene.<sup>13</sup>

#### Gilbert's syndrome

A new syndrome, designated "*cholémie simple familiale*" was described at the beginning of the century by Gilbert *et al.*<sup>22</sup> This syndrome is characterized by mild hyperbilirubinemia occurring in the absence of bilirubinuria or other evidence of hemolysis or liver disease. Mild icterus is the only abnormal physical finding in Gilbert's syndrome (GS) and even this is lacking in the majority of cases with mild hyperbilirubinemia. Unconjugated hyperbilirubinemia is the only abnormality of standard liver biochemistry.<sup>13</sup> Hepatic glucuronidation is reduced to approximately 30 percent of normal in subjects with GS.<sup>23,24</sup>

Serum bilirubin concentrations in GS are usually < 3 mg/dL, though higher as well as lower values are not uncommon. On the basis of serum bilirubin levels, 3-10% of the general population is estimated to have GS.<sup>13</sup> The fluctuation of bilirubin levels in subjects with GS, as well as in controls, however, makes the distinction between mild instances of GS and the normal condition sometimes blurred. The skewed distribution of serum bilirubin levels in the population does not allow definition of the normal range on a modal basis.<sup>25,26</sup> In the past this led to debate on the existence of GS, i.e. to questions on whether GS was a dis-

ease or rather a definition of subjects with the highest serum bilirubin levels in the population.<sup>27,28</sup> The characterization of the gene encoding bilirubin- and phenol-UDP-glucuronosyltransferase isoenzymes,<sup>4</sup> and the recent identification of a genetic variation of this gene strongly associated with increased levels of total and unconjugated serum bilirubin levels in several populations<sup>7,29-31</sup> provided a useful instrument for better understanding of the genetic mechanism and epidemiology of GS.

#### Molecular pathology of Crigler-Najjar and Gilbert's syndromes

At the molecular level CN-I is due to a number of different defects: nonsense (or frameshift) and missense mutations are represented in almost the same proportion both in homozygosity and in the genetic compound state.<sup>5, 32</sup> These mutations are described in the exon 1\*1 encoding for the substrate-specific region of bilirubin-UGT1, or are localized in one of the common exons. Obviously these latter mutations affect all the mRNAs produced. In the period 1992-98, 21 different mutations were described in 24 patients affected by CN-I, the majority abolishing the production of the protein (Figure 1). Some authors quantified the bilirubin-UGT activity; these data suggest the following model: the development of CN disease is entirely governed by the presence of mutations in the UGT1 gene; mutations in the constant or variable region, giving rise to inactive enzymes, result in CN-I. Looking at the distribution and molecular mechanism of CN-I mutations (Figure 1) it can be observed that the majority of them are located in the common region, and are missense or nonsense, whereas in the first exon the mutations are nonsense.

CN-I patients are heterogeneous regarding UGT

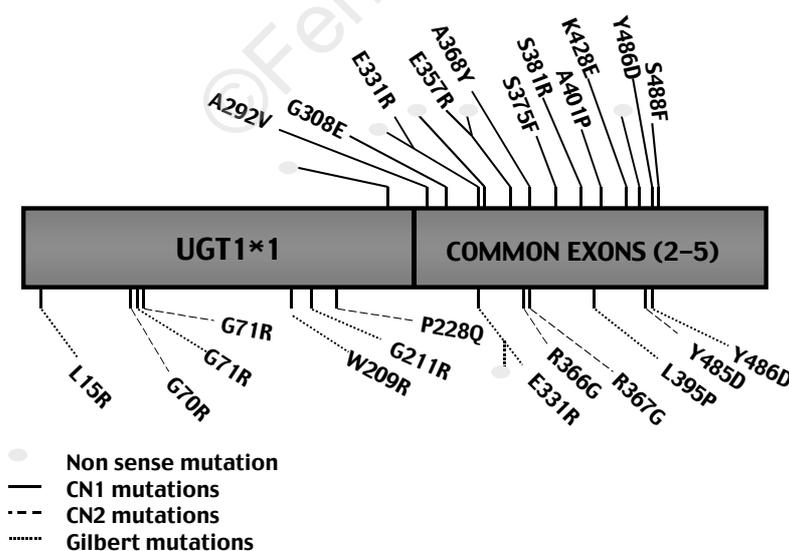


Figure 1. type and localization of mutations affecting the gene encoding UDP-glucuronosyltransferase characterized in patients with Crigler-Najjar and Gilbert's syndromes.

activity towards various substrates.<sup>33</sup> Whereas all CN-I patients lack UGT activity towards bilirubin, some also have deficiency of transferase activity towards phenolic substrates. The lack of bilirubin-UGT activity and the marked reduction of the activity toward 4-nitrophenol in these patients are readily explained by the presence of a stop codon (or similar lesion) in one of the common region exons. In other patients with CN-I, molecular lesions at other locations can be expected. In the Gunn rat, an animal model of CN, there is a severe reduction of phenolic substrate metabolism due to a deletion at nt1242. As hypothesized, there is a premature stop codon in the common region (C-terminus), which causes the loss of the terminal 150 amino acids. In the Gunn rat all the enzymatic activity codified by UGT1 produces truncated proteins without enzymatic activity.<sup>34</sup>

Rosatelli *et al.* reported that seven of eight mutant alleles from unrelated patients of Sardinian ancestry carry a deletion of the phenylalanine codon at position 170.<sup>35</sup> This may indicate that CN-I in Sardinia is prevalent because of a founder effect. A similar effect has been previously described in France, Portugal, Turkey and Tunisia; in particular the nt13 deletion of exon 2 was observed in three patients coming from different countries.<sup>32</sup> In general, the majority of patients with CN-I are double heterozygotes for two different mutations, whereas homozygosity for the same mutation suggests the presence of consanguinity.

The milder phenotype of CN-II patients is usually associated with homozygosity or compound heterozygosity for missense mutations.<sup>36</sup> More rarely it is due to compound heterozygosity for nonsense (or frameshift) and missense mutations<sup>37</sup> or to an interaction between missense mutations and a homozygous TA insertion in the TATAA promoter element, A(TA)<sub>7</sub>TAA, instead of the normal A(TA)<sub>6</sub>TAA.<sup>38, 39</sup> The position of the majority of mutations involved in CN-II is shown in Figure 1.

The inheritance mechanism of the CN syndromes seems to be genuinely recessive, although a possible dominant trait, a nonsense mutation at base position 991 (CAG→TAG),<sup>40</sup> was described. The results of a transfection assay strongly support the hypothesis that the severe manifestation of jaundice in this case might be caused by a dominant-negative mutation interfering with the assembly of four UGT monomers in a tetramer, which is normally the active form of the enzyme in the endoplasmic reticulum. However, since in no other cases has the presence of the heterozygous state for a nonsense mutation been associated with CN type II, it is also possible to hypothesize that in the above case an additive unidentified mutation, such as the (TA)<sub>7</sub> promoter variant could be present on the same allele or on the other.

In a recent paper we described the molecular characterization of a case of CN-II, which was due to the interaction of two different mutations (an AG deletion at codons 238/239/240, and a T→G transition

at codon 224, V224G), in the presence of homozygosity for the promoter polymorphism (TA)<sub>7</sub>.<sup>41</sup>

Looking at the distribution and type of mutations causing CN-II it can be observed that they are widespread in the exon 1\*1 and in the common exons. It is noteworthy that some of these mutations, in the heterozygous state, are also observed in GS. The mutation G71R appears to be very mild, since it was observed in the homozygous state in GS; when this mutation was observed in CN-II, the same chromosome carried an additional (cis) mutation (Y486D). Functional analysis by means of COS transfection showed that the enzymatic reduction caused by Y486D was greater.<sup>39</sup>

The association of GS with the presence of a TA insertion in the TATAA promoter element of the UGT1\*1 gene was initially described by Bosma *et al.* in 1995.<sup>7</sup> Homozygosity for the TA insertion reduces the expression of bilirubin-UGT1, and is associated with the typical mild form of GS.<sup>7</sup> The transfection of a construct containing the A(TA)<sub>7</sub>TAA promoter linked upstream to the firefly luciferase gene in a hepatic cell line led to reduced luciferase expression, 18 to 32% of that recorded in the presence of the normal TATA sequence motif A(TA)<sub>6</sub>TAA.<sup>7</sup> The frequency of homozygosity for the (TA)<sub>7</sub> promoter in normal populations is 10-16%. Fasting tests show enhanced bilirubin levels, but in normal conditions only one half of these subjects had a moderate hyperbilirubinemia.<sup>29</sup> Monaghan *et al.* confirmed the association studying a Scottish population, but they suggested that GS should be divided into two forms: mild and severe.<sup>29</sup> The mild type is associated with the TA insertion in the promoter on the UGT1\*1 gene and is genuinely recessive. The rarer, more severe and dominantly inherited form is due to heterozygosity for mutations in the coding region of the UGT1\*1 gene. These latter mutations have been extensively studied in the Japanese population. Koiwai *et al.* described four patients with mutations in exon 1\*1 and two others with mutations in exons 4 or 5.<sup>42</sup> Mutations found in exon 1\*1 might reduce the affinity of bilirubin for the catalytic site in the N-terminal half of the molecule, whereas mutations in exons 4 and 5 might reduce the affinity for UDP-glucuronic acid.

Mean bilirubin levels vary among different populations: black people have lower levels than whites, whereas bilirubin levels among Asians are higher than among whites. These findings prompted Beutler *et al.* to study the (TA)<sub>7</sub> mutation in these populations.<sup>31</sup> Surprisingly the prevalence of (TA)<sub>7</sub> polymorphism resulted to be inversely correlated with the mean bilirubin level of the populations. It is, therefore, likely that there are genetic differences between Africans and Europeans with respect to the metabolism of bilirubin other than the UGT1\*1 promoter polymorphism. In the same paper the presence of two abnormal promoters containing (TA)<sub>5</sub> and (TA)<sub>8</sub> was described among Africans. Interestingly the activity of

these promoters varies according to the (TA) repeat number, with (TA)<sub>5</sub> promoting the highest activity.

In this issue of the journal we describe a familial case of GS due to the presence of the (TA)<sub>8</sub> promoter.<sup>43</sup> The comparison of UGT1\*1 promoters containing the variable (TA) repeats using a luciferase reporter assay showed that the (TA)<sub>8</sub> promoter had a reduced activity. Analysis on a large population from the same area failed to find any other case of this variant. This variant is probably a *de novo* mutation, due to the intrinsic instability of repeated sequences, in a recent ascendant of the family we studied.

### The clinical and genetic classification of Crigler-Najjar and Gilbert's syndrome in the light of molecular pathology

The clinical classification of CN type I and type II syndromes is based on the severity of hyperbilirubinemia, the presence of kernicterus, and the inducibility of UGT activity by phenobarbital or other enzyme-inducing agents.<sup>13</sup> The recent characterization of a number of genetic lesions involving the UGT locus in CN patients does not substantially affect the clinical validity of these criteria, while providing a new frame for their pathophysiological interpretation at the molecular level. Analysis of the mutations causing CN syndromes allows two main conditions to be recognized: a) mutations inducing the synthesis of a dysfunctional enzyme and b) mutations which completely abolish enzymatic activity having bilirubin as substrate. Homozygosity or compound heterozygosity for mutations of the first type usually causes CN-I, while CN-II is more often associated with homozygosity or compound heterozygosity for mutations of the second type. The position of mutations within the gene, i.e. in exon 1 or in exons 2-5 does not seem to be critical in determining the severity of CN syndromes, though in the more severe CN-I non-sense or missense mutations in the common region prevail.

The variant promoter of exon 1 A(TA)<sub>7</sub>TAA does not appear to play a significant role in CN-I, since the expression of the gene is already abolished or severely reduced by mutations in the coding region. In CN-II, however, the interaction of the variant promoter with missense mutations in the coding region can significantly affect the clinical presentation of the disease.<sup>38,39</sup>

Understanding the molecular pathology of the UGT gene has introduced some complexity in the definition of GS and in its differentiation from the milder presentations of CN-II. The classical picture of GS is usually associated with homozygosity for the variant promoter of exon 1\*1, in the absence of other molecular lesions of the gene. This genotype, however, is not always sufficient to give rise to the mild hyperbilirubinemia of GS, and the interaction of other determinants affecting bilirubin production, hepato-

cellular uptake and storage has been advocated. The molecular genetic analysis of families of patients with CN syndromes revealed that a proportion of heterozygous carriers of UGT mutations show the typical clinical picture of GS. Therefore, the clinical picture of GS may be inherited as a recessive trait (homozygosity for A(TA)<sub>7</sub>TAA) or as a dominant trait (heterozygosity for mutations in the coding region of UGT1\*1). The latter condition is usually characterized by higher bilirubin levels.

In conclusion, while in most clinical contexts the classification of Crigler-Najjar and Gilbert's syndromes maintains its utility and allows diagnosis of three conditions with little overlapping, from the genetic point of view the milder type of GS and the most severe CN-I represent the two ends of a spectrum of conditions of increasing severity, affecting the same locus and deriving from the combination of several mutations.

### Gilbert's syndrome and neonatal jaundice

Every newborn baby has hyperbilirubinemia and about half of all neonates become clinically jaundiced during the first 5 days of life. Serum bilirubin is predominantly unconjugated. In the normal full term newborn, the serum bilirubin concentration increases rapidly from 1-2 to 5-6 mg/dL in approximately 72 hours and subsequently decreases to reach normal levels in approximately 7 to 10 days. Physiological jaundice of the newborn appears to result from a combination of increased bilirubin production and delayed maturation in the capability of the liver to dispose of bilirubin. Exaggeration of physiological jaundice can result in marked hyperbilirubinemia, with an attendant risk of kernicterus.

Several authors have analyzed the role of UGT1\*1 promoter polymorphism in the genesis of newborn jaundice. Bancroft *et al.*<sup>44</sup> demonstrated that newborns homozygous for the A(TA)<sub>7</sub>TAA polymorphism have a more rapid rise in their transcutaneous jaundice index over the first 2 days of life than other newborns. However, they speculated that the (TA)<sub>7</sub> polymorphism rarely, if ever, results in extreme jaundice in the absence of other historical, physical or biochemical jaundice risk factors.

In a similar study (our unpublished data) we analyzed 361 newborns subdivided into jaundiced or non-jaundiced, in the absence of other known risk factors for neonatal hyperbilirubinemia. Our conclusion was that the polymorphism associated with GS does not play a role in the pathogenesis of neonatal hyperbilirubinemia. 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 with the power to examine various combinations of jaundice risk factors.

## Interaction between Gilbert's syndrome and other conditions causing hyperbilirubinemia

Hyperbilirubinemia in the presence of normal liver function often occurs in disorders associated with increased bilirubin production. The serum bilirubin is unconjugated and rarely exceeds 3-4 mg/dL. Higher levels usually indicate hepatobiliary dysfunction in addition to bilirubin overproduction. The most common cause of increased bilirubin production is hemolysis such as that occurring in sickle-cell anemia, hereditary spherocytosis, and toxic or idiosyncratic drug reactions in susceptible individuals. These disorders are associated with premature destruction of erythrocytes: red cell morphology and life span are often abnormal. Ineffective erythropoiesis in thalassemia and in other hematologic disorders is often associated with hyperbilirubinemia.

The recent identification of a variant promoter in the UGT1A gene associated with Gilbert's syndrome allows the assessment of whether the presence of this polymorphism is a factor for the development of hyperbilirubinemia in subjects with inherited hematologic disorders. Hereditary spherocytosis (HS) is a common inherited hemolytic anemia with a prevalence of at least 1 case in 5,000 subjects. The clinical features of HS are anemia, jaundice and splenomegaly. HS frequently becomes clinically evident in the neonatal period and jaundice is the first and most frequent symptom, often requiring phototherapy and sometimes exchange-transfusion.

We examined 178 newborns affected with HS for the UGT1\*1 polymorphism associated with GS. In the examined HS population, 112 (63%) patients had received phototherapy during the first days of life. Jaundice requiring phototherapy was present in 97% of HS patients homozygous for the GS variant promoter. These results indicate that jaundice in newborns affected by HS is exacerbated by the interaction between hemolysis of spherocytes and the genetic variation in the bilirubin UGT1 gene promoter associated with Gilbert's syndrome. Very recently it was demonstrated that gallstone formation in HS patients is 4.5 times more frequent in homozygotes for (TA)<sub>7</sub> polymorphism.<sup>45</sup> Therefore the relative risk of developing gallstones in spherocytic patients (and probably in other hemolytic patients) seems to increase in an allele dose-dependent fashion, with the addition of one or two mutated UGT1\*1 alleles. Glucose-6-phosphate dehydrogenase deficiency (G6PD) is the most common red cell enzyme disorder in the Mediterranean area. We have investigated whether the variation of the A(TA)<sub>n</sub>TAA motif in the promoter of the UGT1\*1 gene may contribute to the pathogenesis of neonatal jaundice in G6PD deficient infants. The results of our study indicate that the presence of GS does not significantly influence the hyperbilirubinemia in these subjects, and that increased bilirubin production due to hemolysis is

not a frequent event in G6PD deficient newborns. Kaplan *et al.*<sup>46</sup> in a similar study performed on Sephardic Jewish neonates, found that the combination of G6PD deficiency and the presence of the variant UGT1\*1 promoter increased the incidence of hyperbilirubinemia. They concluded that this was the consequence of the combined effect of an increased bilirubin load, due to excessive hemolysis, and decreased bilirubin conjugation. These contrasting data might be at least partially explained by the extremely different genetic makeup of the populations investigated. We also analyzed the effect of the (TA)<sub>7</sub> polymorphism of the UGT1\*1 gene in G6PD deficient subjects during an acute hemolytic crisis (favic crisis), and found that G6PD deficient individuals with the expanded A(TA)<sub>7</sub>TAA motif had significantly higher bilirubin levels when compared to unit of decrease of hemoglobin during the favic crisis. Sampietro *et al.*<sup>47</sup> and Galanello *et al.*<sup>48</sup> examined the role played by GS in causing higher bilirubin levels in G6PD deficiency and  $\beta$ -thalassemia. The (TA)<sub>7</sub> genotype was rare in patients with normal or near-normal bilirubin levels, whereas it was the predominant genotype among patients with the higher bilirubin levels. Therefore, the expression of UGT1\*1 appears to be a major modifying factor in inherited hemolytic diseases accounting for a large proportion of bilirubin variability in these conditions. This is a clear example of the role of co-inherited modifying gene(s) in the determination of clinical heterogeneity in monogenic disorders.

## Clinical implications and perspectives after the definition of the molecular bases of Crigler-Najjar and Gilbert's syndromes

Although practically the clinical diagnosis of CN and GS has not been modified, in most instances, by the clarification of the molecular defects responsible for these syndromes, understanding the molecular genetics of these defects has not been clinically irrelevant. The first notable information for the clinician and the clinical geneticist is that CN and GS are closely related disorders, resulting from the combination of several mutations of variable severity at a single locus. Moreover, it has been shown that the promoter mutation usually associated with the mild form of GS may also be involved in CN syndromes,<sup>39</sup> contributing to the severity of hyperbilirubinemia by reducing the expression of a mutant allele in cis or of the normal allele in the presence of a structural mutation in trans. Therefore, the phenotype variability of these conditions is now, at least in part, readable in the light of genetic heterogeneity. Genetic counseling for these syndromes as well as molecular diagnosis, including antenatal diagnosis in families at risk for CN-I, will draw advantage from the new knowledge.

GS, at least from the genetic point of view, should now be considered as composed of two entities with

overlapping phenotypes. Homozygosity for A(TA)<sub>7</sub>TAA is the genotype associated with the classical mild form of GS,<sup>7</sup> while heterozygosity for mutations in the coding region of the gene may give rise to a slightly more severe hyperbilirubinemia and represents, in fact, the heterozygous state for CN syndromes.<sup>42</sup> In rare instances this information might be relevant for genetic counseling.

The characterization of a genetic defect which is present in the large majority of subjects with GS makes a genetic test for this disorder feasible, if not practical. Traditionally the diagnosis of GS has been a diagnosis of exclusion, which is usually less satisfactory for the clinician than positive confirmation. Moreover, not uncommonly patients with undiagnosed GS are at risk of invasive investigations prompted by the finding of jaundice. Genetic testing for GS has the potential to provide a positive diagnosis and to aid the management of the patient. The penetrance of the homozygous promoter mutation, however, appears to be well below 100%, and the prevalence of conditions other (and more harmful) than GS causing mild unconjugated hyperbilirubinemia is not negligible. The combination of these two factors reduces the potential effectiveness of direct genetic testing for GS as an alternative to the usual clinical investigations for liver and hemolytic disease.<sup>49</sup> Indeed, when indirectly diagnosing GS the primary goal is to exclude harmful causes of hyperbilirubinemia rather than to confirm the presence of a benign condition.

Understanding the molecular genetics of CN and GS has clinical implications that are not limited to the field of familial unconjugated hyperbilirubinemias. The promoter variant of the UGT1\*1 gene attains a very high frequency in some populations, with over 12-15% of individuals being homozygotes. In the presence of defects associated with an increased production of bilirubin, such as dyserythropoietic or hemolytic anemias, the reduced activity of bilirubin-UGT<sub>1</sub> induces higher bilirubin levels, apparently out of proportion to the severity of the known defect. The recognition of this rather common interaction may be a useful contribution to the differential diagnosis of unconjugated hyperbilirubinemias. Reduced activity of bilirubin-UGT1 may also have a role in modifying bilirubin levels in other clinical conditions such as the recurrent jaundice after porta-caval surgery,<sup>50</sup> liver transplantation<sup>51</sup> and hepatocellular toxicity caused by chemotherapy. In all these instances direct recognition of the role of GS may help in the diagnostic and therapeutic management of the patient, by ruling out more harmful causes of hyperbilirubinemia.

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