

Bilirubin Dependent on *UGT1A1* Polymorphisms, Hemoglobin, Fasting Time and Body Mass Index

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Abstract: In humans, bilirubin levels are influenced by different factors. This study aims to evaluate the influence of several nongenetic factors (hematologic data, smoking status, alcohol intake, fasting time, physical activity, oral contraceptive therapy and caloric intake) and the genetic contribution of *UGT1A1* polymorphisms for the bilirubin levels, in a cohort of young women. Hematologic data, bilirubin and screening of TA duplication in the TATA box region of the *UGT1A1* gene were performed in 146 young white women. Body mass index (BMI) and body fat were determined, and a questionnaire about fasting time, smoking habits, oral contraceptive therapy, caloric intake and physical activity was performed. Participants were divided into 3 groups according to the tertiles of bilirubin levels. Subjects from the second and third tertile had significant increases in hemoglobin (Hb) concentration, hematocrit, mean cell Hb and mean cell Hb concentration compared with those in the first tertile. Red blood cell count was significantly increased in subjects in the third tertile. A significant increased frequency was found for the c.-41_-40dupTA allele in homozygosity for both second and third tertiles. Multiple linear regression analysis showed that the c.-41_-40dupTA allele, Hb, BMI and fasting hours were independent variables associated with bilirubin serum levels. Hb concentration, fasting time and BMI were identified as nongenetic causes, together with the genetic *UGT1A1* polymorphisms, as the main factors associated with variations in bilirubin levels in a healthy female population.

Key Indexing Terms: Bilirubin levels; *UGT1A1*; Genetic and nongenetic factors; Hemoglobin; Gilbert's syndrome. [Am J Med Sci 2011; XXX(X):1.]

Bilirubin is the main metabolic end product of heme breakdown, and it is a key marker of liver and hematologic disorders. Bilirubin itself is a water-insoluble compound that requires glucuronidation by a microsomal enzyme, the uridine diphosphate glucuronosyltransferase (*UGT1A1*), to be excreted.¹ *UGT1A1* has other endogenous and exogenous substrates, apart from bilirubin, and is implicated in several processes, such as estradiol metabolism and detoxification of

potential carcinogens and mutagens.^{2,3} Changes in *UGT1A1* activity are therefore able to modify water-soluble bilirubin glucuronidation, drug metabolism.

The *UGT1A1* gene locus has many variants, and genotyping data describe >100 single-nucleotide polymorphisms within the *UGT1A1* gene promoter and coding sequences.^{2,3} Wild-type activity is associated with 6 TA repeats in TATA box region of the gene, and the *UGT1A1* gene expression decreases with increasing number of TA repeats. Moderate to severe unconjugated hyperbilirubinemia results from a severe reduction or from the absence of *UGT1A1* activity, occurring in the Crigler-Najjar syndrome type I and type II, respectively, whereas a mild hyperbilirubinemia occurs in the Gilbert's syndrome (GS).⁴ Homozygosity for the c.-41_-40dupTA allele is the most common cause for the Gilbert's syndrome.⁵ Statistics based on incidence and genotype frequencies provided evidence that other inherited and/or acquired factors affect the bilirubin metabolism; however, the *UGT1A1* gene is known as the major factor.⁶

Bilirubin levels present slight daily changes, increasing during fasting and in adolescence. A slight increase in bilirubin concentration has been reported also during physical exercise, stress or menstruation.⁷ The activity of *UGT1A1* can also be enhanced with alcohol intake and by some drugs,⁸⁻¹² or it can be down-regulated during infection and inflammation.¹²⁻¹⁴

Bilirubin in high concentrations is a dangerous metabolite for human health, namely, in newborns that are susceptible to toxicity from unconjugated bilirubin, leading to Kernicterus if it is not immediately treated.¹⁵ Recent evidences suggest that mild bilirubin levels are strong physiologic antioxidants that may give protection against atherosclerosis, coronary heart disease and inflammation, all known as oxidative stress conditions.^{16,17}

In addition to the referred genetic background that regulates serum bilirubin levels, there are other genetic and nongenetic factors that may contribute for bilirubin concentration. Because hemoglobin (Hb) degradation is the main source of bilirubin, it is possible that individual differences in bilirubin concentrations could be due, mainly, to differences in Hb concentration. Indeed, it was, recently, described that an increase in red cell mass, probably, plays a crucial role in hyperbilirubinemia levels, because they would increase to unexpected higher values in case of GS,¹⁸ eventually triggering the clinical manifestation of the disease or its worsening.

The aim of this study was to evaluate the influence of Hb concentration and of several nongenetic factors, such as smoking status, alcohol intake, physical activity, oral contraceptive therapy, fasting time and caloric intake, and the genetic contribution of *UGT1A1* polymorphisms for the bilirubin levels, by performing a cohort study in young Portuguese women.

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MATERIALS AND METHODS

Subjects

We studied a group of 146 randomly selected young white female students (20.7 ± 2.6 years old) from the north of Portugal. All participants gave their informed consent to participate in this study, and those with liver and/or hematologic disorders, chronic or acute infection, under medication, other than contraceptives and with a history of malignancy were excluded from the study.

All participants were asked to respond to a questionnaire, including questions about fasting time, smoking habits and oral contraceptive therapy. To assess physical activity, we applied the Short Form of the International Physical Activity Questionnaire.¹⁹ Body weight and height were determined by standard anthropometric techniques. Body mass index (BMI) was calculated according to World Health Organization recommendations. Body fat measurement was performed by a bioelectrical impedance system (Model BC532; TANITA, Tokyo).

Samples and Measurements

Blood samples were collected (ethylenediaminetetraacetic acid as anticoagulant) to obtain whole blood, plasma and buffy coat for DNA extraction. Plasma samples were aliquoted and stored at -70°C , until assayed.

Red blood cell count, total and differential white blood cell count, Hb concentration, hematocrit (Ht) and hematologic indices [mean cell volume, mean cell Hb (MCH), mean cell Hb concentration (MCHC) and red cell distribution width] were measured by using an automatic blood cell counter (ABX Micros 60-OT; Horiva-ABX, France). Plasmitic total bilirubin concentration was determined using a colorimetric method (diazotized sulfanilic acid reaction; Roche Diagnostics Co., IN).

Genotype Determination

All participants were screened for the presence of the TA duplication in the TATA box region of the *UGT1A1* gene. This TATA box region was analyzed by polymerase chain reaction (PCR) amplification, using the primers previously described by Bancroft et al,²⁰ with the introduction of a fluorochrome label in the reverse primer, for subsequent analysis by automated capillary electrophoresis. In brief, genomic DNA was extracted from blood samples by using the standard salting out method. The TATA box region of *UGT1A1* gene was analyzed by PCR amplification. For the reaction mixture, we used the PCR Master Mix (Promega, Madison, WI). To 25 μL of this mixture (with dNTPs, DNA Taq polymerase and MgCl_2) was added 1 μL of each primer (10 pmol/ μL), 1 μL of genomic DNA (100 ng/ μL) and water, for a final volume of 50 μL . The protocol of cycling was performed on a DNA thermocycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The first step of denaturation at 95°C for 10 minutes was followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 45 seconds and extension at 72°C for 1 minute; and a final extension at 72°C for 10 minutes was performed. After PCR, 1 μL of the products was separated by automated capillary electrophoresis (ABI Prism 3130xl Genetic Analyser; Applied Biosystems) and analyzed by GeneScan software v3.7 along with the molecular weight marker GeneScan ROX-500 Size Standard (Applied Biosystems).

Statistical Analysis

For statistical analysis, we used the Statistical Package for Social Sciences (version 17.0; SPSS, Chicago, IL). Kolmogorov-Smirnov statistics were applied to evaluate sample

normality distribution. Continuous variables without normal distribution were log transformed. The participants were divided by tertiles of total bilirubin, and multiple comparisons between the obtained groups were performed by 1-way analysis of variance, supplemented with Tukey's HSD *post hoc* test. For categorical variables, we used χ^2 test or Fisher's exact test to compare groups. Pearson correlation coefficient was applied to evaluate relationships between sets of data. Multiple-regression analysis (stepwise method) was used to find independent variables associated with total bilirubin levels. The genotype of the TATA box polymorphism was coded using 2 dummy variables, one to compare the homozygous for the wild-type allele (reference category) with heterozygous for the c.-41_-40dupTA, and the other to compare the wild-type allele with homozygous for c.-41_-40dupTA. A *P* value of <0.05 was considered statistically significant.

RESULTS

To analyze the contribution of Hb and other factors to the bilirubin blood levels, we divided our population of young female participants into 3 groups, according to the tertiles of total bilirubin concentration, the first group $\leq 6 \mu\text{mol/L}$, the second group between 6 and $9 \mu\text{mol/L}$ and the third group $\geq 9 \mu\text{mol/L}$.

No differences were observed between the 3 groups when comparing the age of the participants (Table 1). The hematologic studies showed that subjects from the second and third tertile had significantly higher Hb concentration, Ht, MCH and MCHC and lower platelet counts compared with those in the first tertile (Table 1). The subjects from the third tertile maintained the same significant changes, and a significant increase in red blood cell count, compared with the first tertile values. No changes were observed between the second and the third tertile despite the increasing bilirubin concentration. No significant differences were found between groups for total and differential white blood cell counts (Table 1).

The frequency for the c.-41_-40dupTA allele in homozygosity increased from the first to the third tertile because a significant increase was observed between the first and the second tertile, and the frequency for the latter was significantly lower than that observed for the third tertile (Table 1). Allele frequencies of 0.31 for c.-41_-40dupTA allele and of 0.69 for normal allele were obtained in all population, which are in Hardy-Weinberg equilibrium (χ^2 test; $P = 0.029$). Moreover, when compared, total bilirubin concentration, according to *UGT1A1* genotype, a significant and progressive increase was observed, from the [=] + [=] to the homozygous c.-41_-40dupTA allele (Figure 1).

No significant differences were observed between the 3 tertile groups of bilirubin concentration, when considering physical activity, smoking habits, oral contraceptive therapy, body fat, alcohol ingestion and fasting time. However, a significant decrease in BMI was observed in the third tertile compared with the first tertile ($P = 0.009$; Table 2).

Significant positive correlations were found between total bilirubin concentration and Hb ($r = 0.336$; $P < 0.0001$), Ht ($r = 0.244$; $P = 0.003$), MCH ($r = 0.276$; $P = 0.001$) and MCHC ($r = 0.326$; $P < 0.0001$).

Multiple linear regression analyses showed that the TATA box gene polymorphism, Hb, BMI and fasting time account for approximately 44% of the variation in serum total bilirubin levels (adjusted $R^2 = 0.439$; $P < 0.0001$). The model identified the c.-41_-40dupTA allele as the main contributor for total bilirubin variation ($\beta = 0.481$, $P < 0.0001$ when in

AQ: 5

T1, AQ:6

AQ: 3

F1

T2

AQ: 4

TABLE 1. Hematologic data, bilirubin levels and *UGT1A1* genotype distribution by tertiles of total bilirubin in all population

	Participants (n = 146)	Tertiles of total bilirubin levels (μmol/L)			P ^a
		T1 (≤6.0, n = 49)	T2 (6.0–9.2, n = 49)	T3 (≥9.2, n = 48)	
Age (yr)	20.5 (2.3)	20.9 (2.8)	20.3 (2.1)	20.4 (2.0)	0.332
Blood count					
RBC (×10 ¹² /L)	4.6 (0.3)	4.5 (0.2)	4.6 (0.3)	4.7 (0.3) ^b	0.149
Hemoglobin (g/dL)	13.6 (1.1)	13.0 (1.1)	13.8 (1.0) ^b	13.9 (1.0) ^b	<0.0001
Hematocrit (%)	40.7 (2.4)	39.8 (2.5)	41.1 (2.2) ^b	41.3 (2.4) ^b	0.005
MCV (fL)	88.4 (3.9)	87.6 (4.1)	88.8 (4.1)	88.8 (3.5)	0.231
MCH (pg)	29.4 (1.7)	28.7 (1.8)	29.8 (1.7) ^b	29.9 (1.4) ^b	0.001
MCHC (g/L)	33.3 (1.1)	32.8 (1.0)	33.5 (1.1) ^b	33.7 (1.1) ^b	<0.0001
RDW-CV (%)	12.9 (0.9)	13.1 (0.9)	12.8 (0.8)	12.7 (0.9)	0.341
WBC (×10 ⁹ /L)	6.4 (1.3)	6.5 (1.3)	6.6 (1.3)	6.3 (1.6)	0.390
Neutrophil (×10 ⁹ /L)	3.4 (1.4)	3.5 (1.4)	3.4 (1.5)	3.2 (1.4)	0.493
Eosinophil (×10 ⁹ /L)	0.13 (0.5)	0.12 (0.5)	0.15 (0.5)	0.12 (0.5)	0.377
Basophil (×10 ⁹ /L)	0.03 (0.06)	0.02 (0.06)	0.03 (0.05)	0.02 (0.06)	0.075
Monocyte (×10 ⁹ /L)	0.4 (0.2)	0.4 (0.1)	0.5 (0.3)	0.4 (0.1)	0.085
Lymphocyte (×10 ⁹ /L)	2.4 (0.7)	2.3 (0.6)	2.5 (0.7)	2.3 (0.8)	0.606
Platelets (×10 ⁹ /L)	248 (56.9)	267.9 (62.7)	242.9 (54.4) ^b	234.4 (48.4) ^b	0.019
TA duplication in <i>UGT1A1</i> gene					
[=] + [=] [%, (n)]	48.8 (71)	67.3 (33)	53.1 (26) ^b	25.0 (12) ^{b,c}	
[=] + [c.-41_-40dupTA] [%, (n)]	40.8 (59)	33.3 (16)	43.1 (21) ^b	45.8 (22) ^b	<0.0001
c.-41_-40dupTA [%, (n)]	10.9 (16)	0.0 (0)	3.9 (2) ^b	29.2 (14) ^{b,c}	

Data are presented as mean (standard deviation) for continuous variables and as percentage (number) for categorical variables.

^a P value for 1-way analysis of variance or χ^2 tests.

^b P < 0.05 vs. T1.

^c P < 0.05 vs. T2.

RBC, red blood cells; WBC, white blood cells; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW-CV, coefficient of variation of the red cell width.

homozygosity; $\beta = 0.381$, $P < 0.0001$ when in heterozygosity), followed by Hb ($\beta = 0.297$, $P < 0.0001$), fasting hours ($\beta = 0.292$, $P < 0.0001$) and BMI ($\beta = -0.235$, $P = 0.004$).

DISCUSSION

Several studies aimed to clarify the influence of genetic and nongenetic factors in bilirubin levels.^{2,3,21–27} Most of them

focused only on genetic or nongenetic factors and included individuals of both sex and with a wide range of ages. In this work, we analyzed a cohort of young women with narrow age range to eliminate the potential interference of age and sex.

Our results showed that TA duplication in the promoter region of *UGT1A1* gene, Hb concentration, fasting time and BMI are the main determinants of bilirubin levels.

According to bilirubin tertiles, our population showed an increase in bilirubin concentration that follows the genetic polymorphism present at the promoter region of the *UGT1A1* gene. In fact, we found a higher prevalence of c.-41_-40dupTA allele in subjects with the highest bilirubin concentrations. Moreover, when the population was stratified by *UGT1A1* genotype, we found significant differences for bilirubin levels between the 3 different genotypes (Figure 1), showing a strong association between higher bilirubin levels and the presence of TA duplication in the promoter region of *UGT1A1* gene. This finding is in accordance with published data that defined the c.-41_-40dupTA allele as the most important cause of hyperbilirubinemia in white population.^{6,27}

Because most of plasma bilirubin results from the catabolism of Hb in the macrophages from the reticuloendothelial system, it is expected that bilirubin concentration will increase as Hb concentration increases. Our results showed that Hb, MCH and MCHC were increased in women with bilirubin levels >6 μmol/L (second and third tertile). Moreover, we found positive and significant correlations between bilirubin levels and Hb, Ht, MCH and MCHC. These results strongly suggest that red cell mass and Hb concentration are associated

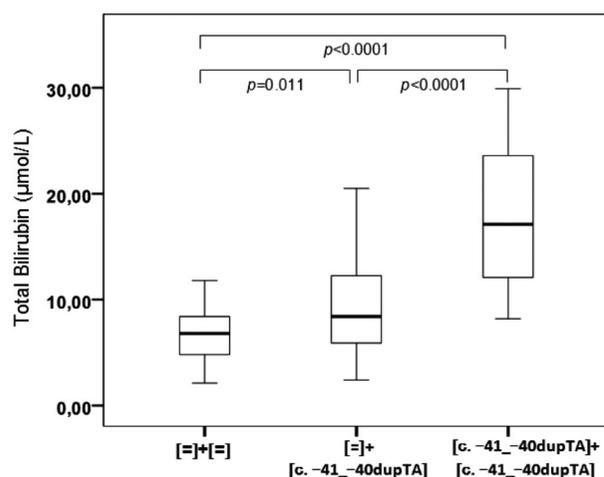


FIGURE 1. Total bilirubin plasma levels according to *UGT1A1* genotype.

TABLE 2. Data from physical activity, smoking habits, oral contraceptive therapy, body fat, alcohol ingestion, fasting time, caloric intake distribution and body mass index by tertiles of total bilirubin in all population

	Participants (n = 135) ^a	Tertiles of total bilirubin levels ($\mu\text{mol/L}$)			P ^a
		T1 (≤ 6.0 , n = 45)	T2 (6.0–9.2, n = 44)	T3 (≥ 9.2 , n = 46)	
Physical activity					
Low [%, (n)]	14.8 (20)	20.0 (9)	10.9 (4)	14.3 (7)	
Moderate [%, (n)]	55.6 (75)	48.9 (21)	56.5 (26)	61.9 (28)	0.470
High [%, (n)]	29.6 (40)	33.3 (15)	32.6 (14)	23.8 (11)	
Smokers [%, (n)]	11.9 (16)	14.9 (7)	13.0 (6)	7.1 (3)	0.504
With OCT [%, (n)]	42.2 (56)	48.9 (23)	35.6 (16)	35.4 (17)	0.420
BMI (kg/m^2)	22.0 (4.3)	22.6 (4.4)	22.8 (5.4)	20.8 (2.2) ^b	0.099
Body fat (%)	25.7 (6.9)	27.4 (7.7)	26.1 (7.2)	24.3 (5.7)	0.211
Alcohol intake (mg/d)	4.0 (6.6)	5.3 (9.2)	3.7 (5.0)	2.9 (4.2)	0.248
Fasting time (hr)	11.1 (2.0)	11.0 (2.1)	11.0 (2.0)	11.4 (1.6)	0.492

Data are presented as mean (standard deviation) for continuous variables, and as percentage (number) for categorical variables.

^a P value for 1-way analysis of variance or χ^2 tests.

^b P < 0.05 vs. T2.

OCT, oral contraceptive therapy; BMI, body mass index.

with interindividual variations in bilirubin levels. This finding was previously reported in a study with patients with GS¹⁸; our study reports for the first time, in a young female population, an increase in Hb concentration that follows bilirubin levels (bilirubin $\geq 6 \mu\text{mol/L}$). We also found lower platelet counts in subjects with bilirubin levels higher than $6 \mu\text{mol/L}$. Low Hb concentration has been associated with an increased risk for cardiovascular disease events and mortality in patients with end-stage renal disease,²⁸ and to heart failure²⁹ in the general population.³¹ We doubt whether lower levels of bilirubin that probably occur in these cases represent an additional risk, because bilirubin may have an important role in the prevention of cardiovascular diseases,^{31,32} as a result of its antioxidant effect.³¹

We also found that fasting time is an independent variable associated with total bilirubin concentration. This association has been previously reported in subjects who were not stratified according to *UGT1A1* genotype.^{24,25} The increase of bilirubin levels with fasting time seems to result from a decrease of the intestinal motility and elimination of bile pigments, causing bilirubin accumulation in the intestine—leading to an increased reflux of bilirubin into the plasma.³³ In fasting subjects with a normal capacity of bilirubin conjugation, the accumulation of bilirubin in the intestine can be higher compared with those with a low capacity of bilirubin conjugation because of *UGT1A1* polymorphisms, explaining the higher bilirubin levels found in women with normal number of TA repeats and with *UGT1A1* polymorphisms, with the same fasting time.

Multivariate regression analysis also showed BMI as an independent variable associated with total bilirubin concentration. No significant differences were established for body fat between the 3 tertiles of total bilirubin levels; however, a trend for lower values was observed from the lowest to the highest bilirubin tertile. An inverse correlation between body fat and BMI with bilirubin concentration has been reported,^{34,35} but this association is still poorly clarified.

Regarding the influence of lifestyle variables, data from literature are still controversial.^{21,22,26} We also analyzed the effect of other nongenetic factors in bilirubin levels, such as physical activity, tobacco smoking, alcohol intake and oral

contraceptive therapy. We did not find any association between these factors and the changes in bilirubin concentration. This can be due to the characteristics of our sample, which, for instance, included only young women who did not have strong habits of smoking or did exceed the recommended limit of alcohol ingestion (data not shown).

In conclusion, we studied the effect of several factors that could contribute for total bilirubin concentration. We found that Hb concentration, fasting time and BMI are independent variables significantly associated with total bilirubin concentration in a female population. In the same way, total bilirubin concentration was strongly affected by the polymorphism presented in the promoter region of the *UGT1A1* gene. Further studies must be performed to determine the effect of these factors in different ages, in both men and women, and determine whether *UGT1A1* variations, other than the TA duplication in the promoter region, could affect the total bilirubin levels.

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AQ: 7

AUTHOR QUERIES

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- 1—Kindly confirm whether the short title is OK as given.
 - 2—Per journal style, unstructured abstracts should be ≤ 150 words. Kindly modify the abstract to match the word limit.
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 - 4—Please confirm whether location for the manufacturer “SPSS” is OK as given.
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