FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM MEDICINA

2013/2014

Luís Filipe Barbosa Amado Belo

Body fat percentage is a major determinant of total bilirubin levels independently of *UGT1A1*28* polymorphism in obese children and adolescents

março, 2014

FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

Luís Filipe Barbosa Amado Belo

Body fat percentage is a major determinant of total bilirubin levels independently of *UGT1A1*28* polymorphism in obese children and adolescents

Mestrado Integrado em Medicina

Área: Pediatria

Trabalho efetuado sob a Orientação de:
Professora Doutora Carla Rêgo

Trabalho organizado de acordo com as normas da revista:
"PLOS ONE"

março, 2014

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR PROJETO DE OPÇÃO DISSERTAÇÃO / MONOGRAFIA

Projeto de Opção do 6º ano - DECLARAÇÃO DE INTEGRIDADE

Eu, Luís Filipe Barbosa Amado Belo, abaixo assinado, nº mecanográfico 199100955, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro ter atuado com absoluta integridade na elaboração deste projeto de opção.

Neste sentido, confirmo que **NÃO** incorri em plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria de um determinado trabalho intelectual, ou partes dele). Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores, foram referenciadas, ou redigidas com novas palavras, tendo colocado, neste caso, a citação da fonte bibliográfica.

Este trabalho, do qual sou o primeiro autor, foi publicado em 2013 na Dissertação de Doutoramento de Henrique da Silva Ferrão Nascimento, aluno da Faculdade de Farmácia da Universidade do Porto.

Faculdade de Medicina da Universidade do Porto, 10/03/2014

Assinatura conforme cartão de identificação:

Luís Filipe Barbosa Amado Belo

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR PROJETO DE OPÇÃO DISSERTAÇÃO / MONOGRAFIA

Projecto de Opção do 6º ano – DECLARAÇÃO DE REPRODUÇÃO

NOME

Luís Filipe Barbosa Amado Belo

CARTÃO DE CIDADÃO OU PASSAPORTE (se estrangeiro)	E-MAIL	TELEFONE OU TELEMÓVEL
10034258	luisbelo@ff.up.pt	914 883 033

NÚMERO DE ESTUDANTE	DATA DE CONCLUSÃO
199100955	2014

DESIGNAÇÃO DA ÁREA DO PROJECTO

Pediatria

TÍTULO DA DISSERTAÇÃO

Body fat percentage is a major determinant of total bilirubin levels independently of *UGT1A1*28* polymorphism in obese children and adolescents

ORIENTADOR

Professora Doutora Carla Maria Barreto da Silva de Sousa Rêgo

COORIENTADOR (se aplicável)

É autorizada a reprodução integral desta Dissertação para efeitos de investigação e de divulgação pedagógica, em programas e projectos coordenados pela FMUP.

Faculdade de Medicina da Universidade do Porto, 10/03/2014

Assinatura conforme cartão de identificação: Luís Filipe Barbosa Amado Belo

À Professora Carla Rêgo,

pelo extraordinário apoio dado durante a minha formação em Medicina

A todos os que colaboraram neste trabalho

À memória do meu Pai,

João Belo

À minha mãe, Conceição e à minha irmã, Sara

Aos meus filhos,

Hugo e Sofia

À minha fonte de encanto,

Márcia

Body fat percentage is a major determinant of total bilirubin levels independently of *UGT1A1*28* polymorphism in obese children and adolescents

Luís Belo[1,2*], Henrique Nascimento[1,2], Michaela Kohlova[3], Elsa Bronze-Rocha[1,2], João Fernandes[1,3], Elísio Costa[1,2], Cristina Catarino[1,2], Luísa Aires[4,5], Helena Ferreira Mansilha[6], Petronila Rocha-Pereira[7], Alexandre Quintanilha[2,8], Carla Rêgo[9], Alice Santos-Silva[1,2]

[1]Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, 4050-313 Porto, Portugal;

[2]Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, 4150-180 Porto, Portugal;

[3]IBILI, Faculdade de Medicina, Universidade de Coimbra, 3000-548 Coimbra, Portugal.

[4]CIAFEL, Faculdade de Desporto, Universidade do Porto, 4200-450 Porto, Portugal;

[5]Instituto Superior da Maia (ISMAI), Av. Carlos Oliveira Campos, 4475-690 S. Pedro Avioso. Portugal.

[6]Departamento da Infância e Adolescência/Serviço de Pediatria do Centro Hospitalar do Porto; 4099-001 Porto, Portugal;

[7]Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6200-506 Covilhã, Portugal:

[8]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, 4099-003 Porto, Portugal;

[9]Centro da Criança e do Adolescente. Hospital Cuf Porto. Faculdade de Medicina, Universidade do Porto; 4200-319 Porto, Portugal.

*Corresponding author. E-mail: luisbelo@ff.up.pt

Abstract

Background: Bilirubin has potential antioxidant and anti-inflammatory properties. The *UGT1A1*28* polymorphism (TA duplication in the TATA box region of the *UGT1A1* gene promoter) is a major determinant of bilirubin levels and recent evidence suggest that raised adiposity may also be a contributing factor.

Objective: To study the interaction between *UGT1A1*28* polymorphism and both haematological and anthropometric variables on total bilirubin levels in young individuals.

Methods: We studied 350 obese (mean age of 11.6 years; 52% females) and 79 controls (mean age of 10.5 years; 59% females). Anthropometric data, *UGT1A1*28* polymorphism, haemogram and plasma levels of total bilirubin, C-reactive protein (CRP), glucose and insulin were evaluated. In a subgroup of 74 obese and 40 controls body composition was analyzed by dual-energy X-ray absorptiometry.

Results: The *UGT1A1* genotype frequencies in all studied individuals were 49.9%, 42.7% and 7.5% for 6/6, 6/7 and 7/7 genotypes, respectively. Patients with 7/7 genotype presented the highest total bilirubin levels, followed by 6/7 and 6/6 genotypes. Compared to controls, obese patients presented higher erythrocyte count, haematocrit values and haemoglobin, insulin and CRP levels, but no differences in bilirubin or in *UGT1A1* genotype distribution. Body fat percentage was negatively correlated with bilirubin in obese patients but not in controls. This negative association was observed either in 6/7 or 6/6 genotype obese patients. The *UGT1A1*28* polymorphism and body fat percentage were the main factors affecting bilirubin levels within obese patients, by linear regression analysis (standardised Beta: 0.348, -0.291; $p = 0.002$ and $p = 0.009$, respectively).

Conclusions: In obese children and adolescents, body fat composition and *UGT1A1*28* polymorphism are independent determinants of total bilirubin levels. Raised inflammation and oxidative stress in obese patients may trigger the consumption of bilirubin.

Introduction

Bilirubin is the ultimate product of the haem group catabolism and serves as a diagnostic marker of liver and blood disorders [1]. Bilirubin is a water-insoluble compound that circulates bounded to albumin and requires glucuronidation by a microsomal enzyme, the uridine diphosphate glucuronosyltransferase (UGT) 1A1, to be excreted. The *UGT1A1* gene locus has been mapped to chromosome 2q37 [2] and one of the most common genetic variants that affects the glucuronidation of bilirubin is a TA duplication polymorphism in the TATA box region of the gene promoter. Homozygous individuals carrying the A(TA)7TAA allele have higher levels of unconjugated bilirubin (UCB), caused by a reduction of 30% in the *UGT1A1* transcription [3]. The estimated frequency of this allele is 0.35 in Caucasians, leading to a homozygous genotype in about 10% of the population, but the frequency is highly variable in different ethnicities [4, 5]. Homozygosis for the TA duplication was considered as the main cause of Gilbert syndrome in Caucasian population [3, 4], and justify some of the inter-individual variations in bilirubin levels [6].

Under certain conditions bilirubin can be toxic [7]. High plasma concentrations are associated with deleterious effects in new-borns, increasing the risk of neurological dysfunction [7, 8], as a result of its toxic effect on neuronal tissue. However, recent investigation has recognized that UCB exerts anti-oxidant and anti-inflammatory activities, and that mild hyperbilirubinaemia might have positive health effects. UCB inhibits lipid peroxidation [9] and suppresses inflammation in activated neonatal neutrophils [10], and population studies documented that individuals with higher circulating UCB have a reduced incidence of cardiovascular problems [11-13] and of carcinoma in general [14]. Furthermore, subjects with Gilbert syndrome seem to present low levels of oxidative stress associated with hyperbilirubinaemia [15].

Obesity, a low-grade inflammatory disease [16], is increasing all over the world and is a significant risk factor for cardiovascular disease (CVD). This is of particular concern in our country, considering the very high prevalence of overweight/obesity (31.5%) in Portuguese children when compared to other European countries [17]. In obesity, cardiovascular morbidity and mortality are associated with classic risk factors, namely dyslipidaemia, hypertension and impaired glucose metabolism. These risk factors, known as predictive of CVD, are characteristic of the metabolic syndrome (MS) [18]. Moreover, serum bilirubin levels are inversely associated with the MS and systemic inflammation in adults [19-21], as well as in children and adolescents [22]. In particular, abdominal obesity *per se* seems to be associated with low serum bilirubin levels [21-23]. Furthermore, a recent study hypothesized that circulating bilirubin levels might be already altered in overweight asymptomatic middle-aged individuals before full development of the MS [24].

The aim of our work was to evaluate how total bilirubin (TB) levels are influenced by *UGT1A1*28* polymorphism, haematological, biochemical and anthropometric variables in Portuguese obese children and adolescents.

Material and methods

Study population

Obese children and adolescents, aged 4-18 years, were identified from medical records, at the outpatient clinics of pediatric obesity in two hospitals in Porto - Portugal. A group of children from 5 primary and 2 middle and high public schools from Oporto suburban setting, were also recruited to this study, providing a control group and enlarging the obese group.

Smokers, subjects with diabetes mellitus, endocrine disorders, hereditary diseases, inflammatory or infectious diseases or under any therapy that could interfere with our results were excluded from the study.

Ethics approval and patient consent

The study protocol was approved by the Committee on Ethics of Oporto Hospital Centre, the Committee on Ethics of Hospital São João, the Review Committee of the Scientific Board of the Faculty of Sport of the University of Porto as well as by the Foundation of Science and Technology.

A total of 350 obese children and adolescents and 79 controls participated in the study after informed and written consent of their parents.

Anthropometric characterization and clinical evaluation

All participants were subjected to clinical examination. Height and weight were measured. Obesity was defined as body mass index (BMI) z-score greater than +1.65 for age and gender, according to 2000 Centre for Disease Control and Prevention (CDC) growth charts. Body composition was evaluated by dual-energy X-ray absorptiometry (DEXA) in a subgroup of participants (74 obese and 40 controls).

Blood samples

Blood was collected by venipuncture in EDTA containing tubes, after overnight fasting (10-12h) and processed within 2h of collection. Aliquots of buffy-coat and plasma were made, and immediately stored at -80^{o}C until assayed.

Haematological data

Red blood cell (RBC) count, haematocrit (Ht), haemoglobin (Hb) concentration and haematimetric indices [mean cell volume (MCV), mean cell Hb (MCH) and mean cell Hb concentration (MCHC)] were measured by using an automatic blood cell counter (ABX Micros 60-OT).

DNA analysis

Genomic DNA was extracted from buffy-coat by proteinase K/salt precipitation method [25, 26]. Genotyping TA duplication in the TATA box of the *UGT1A1* promoter was performed by polymerase chain reaction (PCR) (forward: 5´-TAACTTGGTGTATCGATTGGTTTTTG-3´; reverse: 5´-ACAGCCATGGCGCCTTTGCT-3´). PCR was followed by electrophoresis in 15% polyacrylamide gel in a Tris/Borate/EDTA buffer. The gel was stained with silver nitrate, photographed and samples were classified.

Plasma analysis

The plasma levels of C-reactive protein (CRP) were determined by immunoturbidimetry [CRP (latex) High-Sensitivity, Roche Diagnostics] and quantification of TB was performed by a colorimetric test (diazotized sulfanilic acid reaction, Roche Diagnostics).

The determination of circulating levels of glucose and insulin was performed by using routine automated technology (ABX Diagnostics). Homeostasis model assessment of insulin resistance ($HOMA_{IR}$) was calculated [27].

Statistical analysis

The distributions of continuous variables were analysed using Kolmogorov-Smirnov tests. Normally distributed variables are presented as mean ± SD and those non-normally

distributed are presented as median (interquartile range). Comparisons between two groups were performed using Student's unpaired *t*-test or Mann-Whitney *U* test. Adjustment for confounding factors was performed using ANCOVA. The association between categorical variables was analysed using chi-squared (χ^2) test and Fisher's exact test.

The strength of the association between the variables was estimated by Pearson correlation coefficient, after log transformation of the variables (whenever necessary). To evaluate the contribution of the different variables to TB levels, multiple regression analysis was performed, using stepwise selection, with an entry criteria of $p < 0.05$.

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS), version 20.0 (IBM, Armonk, NY, USA). Statistical significance was accepted at p less than 0.05.

Results

The anthropometric data, *UGT1A1* genotypes, haematological and biochemical parameters of the obese children and adolescents (n=350) and controls (n=79), according to gender, are presented in Table 1.

Comparing males and females within the control group, body fat and trunk fat percentages were significantly lower for boys, whereas RBC count, Hb levels and MCHC values were significantly higher. Within obese patients, RBC count, Hb levels and Ht values were significantly higher for boys, whereas insulin levels and $HOMA_{IR}$ values were lower. No statistical significant differences were found in the distribution of subjects with respect to *UGT1A1* genotypes or in TB levels between boys and girls, within both groups.

Compared to controls (independently of gender), obese patients presented significantly higher height, weight, BMI, BMI z-score, body fat and trunk fat percentages, erythrocyte

count, Ht and $HOMA_{IR}$ values and Hb, insulin and CRP levels, but no significant differences in TB levels or in *UGT1A1* genotype distribution.

The *UGT1A1* genotype frequencies in all studied individuals were 49.9%, 42.7% and 7.5% for 6/6, 6/7 and 7/7 genotypes, respectively. *UGT1A1*28* polymorphism was associated with different TB levels (Fig. 1A); patients with 7/7 genotype presented the highest TB levels, followed by 6/7 and 6/6 genotypes ($p<0.01$ between all groups). No significant differences were observed between obese and control individuals, for the different *UGT1A1* genotypes (Fig. 1B).

Within the control group ($n=79$), TB levels correlated positively and significantly with age ($r=0.304$, $p=0.007$), height ($r=0.360$, $p=0.001$), weight ($r=0.390$, $p<0.001$), BMI ($r=0.370$, $p=0.001$), Ht ($r=0.247$, $p=0.028$), MCV ($r=0.292$, $p=0.009$), and correlated negatively and significantly with MCHC ($r=-0.258$, $p=0.022$). Within the obese group ($n=350$), TB levels correlated positively and significantly with age ($r=0.284$, $p<0.001$), height ($r=0.285$, $p<0.001$), weight ($r=0.219$, $p<0.001$), BMI ($r=0.123$, $p=0.021$), Hb ($r=0.305$, $p<0.001$), Ht ($r=0.352$, $p<0.001$), MCV ($r=0.394$, $p<0.001$), MCH ($r=0.301$, $p<0.001$) and correlated negatively and significantly with BMI z-score ($r=-0.131$, $p=0.014$), MCHC ($r=-0.149$, $p=0.006$) and CRP ($r=-0.178$, $p=0.001$).

The characteristics of obese patients that evaluated their body composition by DEXA ($n=74$) are presented in Table 2. These obese patients were divided in two groups according on having a body fat lower or higher/equal than 42.5% (cut-off that corresponds to the median value for the obese group). The two groups of obese patients were matched for gender and *UGT1A1* genotype distribution, but not for age. Patients presenting higher body fat had lower bilirubin and higher CRP levels (Table 2). These differences were similar to both sexes (Fig. 2) and remained statistically significant after adjustment for age. No significant differences in $HOMA_{IR}$ values were found between the two groups.

Associations between body and trunk fat were only accessed in participants that evaluated their body composition by DEXA (74 obese and 40 controls). Body fat and trunk fat percentages were negatively and significantly related with TB levels in obese patients (r =-0.287, p=0.013 and r=-0.245, p=0.038) but not within controls (r=0.012, p=0.941 and r=0.014, p=0.002).

By linear regression analysis, the *UGT1A1*28* polymorphism and body weight were the only factors associated to bilirubin levels within controls (Ln TB= 1.143 + 0.462 *UGT1A1*28* polymorphism + 0.014 weight; standardised Beta: 0.598 and 0.490; p<0.001 and p=0.001, respectively). Within obese patients, the *UGT1A1* polymorphism and body fat percentage were the main determinant factors of bilirubin levels (Ln TB= 2.761 + 0.251 *UGT1A1*28* polymorphism -0.020 body fat; standardised Beta: 0.348, -0.291; p=0.002 and p=0.009, respectively). For a better visualization of the results (graphically), obese participants were divided on the basis of their *UGT1A1* genotype and on having a body fat lower or higher/equal than 42.5% (cut-off that corresponds to the median value for the obese group; Fig. 3).

Discussion

As far as we know, this is the first report assessing the concomitant influence of *UGT1A1*28* polymorphism and adiposity markers on bilirubin levels in obese children and adolescents. We demonstrated that body fat percentage is a major determinant of TB levels independently of *UGT1A1*28* polymorphism in obese children and adolescents.

It is known that *UGT1A1* polymorphisms are associated with bilirubin levels and our data is in agreement with previous reports in young patients and adults [15, 28-30]. Patients and controls with 7/7 genotype presented the highest TB levels, followed by 6/7 and 6/6 genotypes (Fig. 1A and 1B).

The frequency of 7/7 homozygotes (7.5% in the whole population) was lower than that observed in other works, namely in healthy Greek [29] and Slovenian [30] populations, with frequencies of 14.8% and 13.6%, respectively. However, the distribution of subjects with respect to *UGT1A1* genotypes was similar to that found in previous studies involving Portuguese children with Hereditary spherocytosis, with a 7/7 frequency of 8.8% [28], as well as Portuguese healthy subjects, with frequencies observed in two studies of 6.3 and 9.9% [28, 31]. Thus, it seems reasonable to assume that the frequency of 7/7 homozygotes in the Portuguese population may be lower than that observed in other Caucasian populations.

Other potential variables could influence TB levels. Within both controls and obese patients TB levels were positively and significantly correlated with age, height, weight, BMI, and Ht. However, BMI z-score, body fat and trunk fat percentages were negatively and significantly related with TB levels in obese patients, but not within controls. In multiple regression analysis, the *UGT1A1*28* polymorphism and body weight were the only factors associated to bilirubin levels within controls, whereas the *UGT1A1*28* polymorphism and body fat percentage were the main determinant factors of bilirubin levels within obese patients.

In the present study, the evaluation of body composition by DEXA was performed in a subgroup of participants. Despite the lower number of participants in this sub-analysis, the negative relation between bilirubin and body fat percentage was highly statistically significant and independent of the effect of *UGT1A1*28* polymorphism. Furthermore, this negative relation is in agreement with a previous study involving 41 lean and obese adult men and women [23].

Bilirubin derives mainly from the haem present in Hb, released during breakdown of senescent erythrocytes [1]. Thus, in healthy conditions, it would be assumed that increases in Hb levels are generally associated with increases in TB. This explains our positive association

between the age of the participants and TB, as in young individuals there is a physiological increase in Hb levels with age. It is well known that Hb and Ht increase substantially during childhood, whereas RBC count remains almost constant [32]. Differences according to gender become prominent in the second decade of life; with menstruation, these three variables tend to be lower in females. The inclusion in our study of subjects with a range of age between 4 and 18 years old justifies the higher values of RBC and Hb observed in males within both controls and obese patients (Table 1). The differences were particularly evident in obese patients, not only because of the large number of subjects included in this group but also due to the enhanced puberty development in obesity. Actually, the increasing prevalence of obesity in children worldwide is a major cause of precocious pubertal maturation, verified during the past decades [33].

Total and direct bilirubin levels were reported to be higher in normal weight adult males than in females [24, 34, 35], but similar within overweight patients [24]. In our study, we observed no statistical significant differences between boys and girls regarding TB levels, either in controls or in obese patients. Within these two groups, males and females were matched for age, weight, BMI and distribution of *UGT1A1* genotypes (Table 1) and, therefore, the analysis of TB was not affected by these factors.

In the present manuscript, obese patients and controls were matched for age and distribution of *UGT1A1* genotypes, allowing the comparison of groups. Independently of gender, obese patients presented higher RBC count, Hb levels and Ht values compared with controls (Table 1). The higher weight and BMI in obese patients trigger the stimulation of erythropoiesis in order to supply adequate oxygenation to increased body tissues. However, TB levels were similar between groups. This may be explained by the fact that obesity is associated with increased inflammation [16, 36, 37] and oxidative stress [38, 39], and that bilirubin, presenting antioxidant and anti-inflammatory properties [9, 10], may be somewhat

consumed. In fact, oxidative stress increases with increasing BMI and age [34]. In line with this, we found that bilirubin levels are negatively correlated with body and trunk fat percentages and CRP levels within obese patients. Moreover, when obese patients were divided in two groups according to the median value of body fat presented by this group (42.5%), patients presenting higher body fat presented lower bilirubin and higher CRP levels (Table 2). The negative relation that we found between bilirubin and CRP levels is in line with the bilirubin's anti-inflammatory activity, as previously reported [40-43].

In obese patients, insulin resistance may also underlie the association between lower bilirubin levels and higher body fat percentages. Indeed, it seems that the activity of heme oxygenase-1, the rate-limiting enzyme of bilirubin production, is impaired in insulin resistant states [44, 45]. Also, the up-regulation of heme oxygenase-1 in adipocyte by insulin was recently demonstrated [46]. In this work obese patients presented with higher $HOMA_{IR}$ values compared to controls (Table 1). Obese patients with body fat percentages higher than 42.5% also presented with higher $HOMA_{IR}$ values, although without statistical significance. However, no significant correlation was obtained between $HOMA_{IR}$ and bilirubin. Thus, association between insulin resistance and bilirubin might not be so clear in paediatric populations.

A previous work from our group demonstrated that BMI z-score is significantly and independently related to the lipid profile in obese children and adolescent [47]. However, in the present study BMI z-score was poorly related with TB levels in obese patients and it was not an independent predictor of bilirubin plasma concentration. This suggests that body fat percentage is a better indirect marker of oxidative stress, rather than BMI z-score. Actually BMI z-score is calculated using the BMI of patients, adjusted to age and gender, but it may not necessarily express the degree of obesity.

Individuals with a higher physical fitness index (which serves as an aerobic assessment) seem to present with higher bilirubin levels [24] and a study performed in overweight and obese adult patients demonstrated an increase in bilirubin levels due to short-term weight loss [35]. It seems that high doses of exercise training are necessary to significantly increase bilirubin levels in overweight and obese women [48]. The fact that bilirubin levels increase as a function of weight loss may be of particular importance in obese individuals with *UGT1A1* genotypes associated to lower bilirubin levels, as we here demonstrated effects on TB by body fat composition in addition to the *UGT1A1*28* polymorphism. It is important to keep in mind that atherosclerosis is a multifactorial disease that initiates early in life, involving the interplay of genetic and environmental factors. The lifestyle improvement is conditioned by environmental factors (such as nutritional behaviour and practice of physical activity) and may be particularly worthy in obese individuals with a less favourable genetic background.

Despite the new data reported here, this work presented some limitations. Obesity was defined according to CDC although a novel criteria is now recommended for the Portuguese population, causing us to have probably underestimated the degree of obesity. Nevertheless, at the beginning of this study the criteria recommend by the Portuguese Ministry of Health was that of CDC. Also, the evaluation of body composition by DEXA was performed only in a subgroup of participants due to logistical constraints and equipment availability. Furthermore, we did not evaluate the association between bilirubin and the MS as a large proportion of our obese patients were under the age of 10, not allowing their classification according to the International Diabetes Federation (IDF) definition.

In conclusion, body fat percentage is a major determinant of TB levels independently of *UGT1A1*28* polymorphism in obese children and adolescents. This may have a particular relevance, as obese individuals, particularly those with 6/6 *UGT1A1* genotype and higher body fat mass, may benefit from a closer clinical follow-up, considering their increased risk

for other comorbidities. Moreover, lifestyle modifications at low ages, when good habits can be created, should be highly encouraged in such obese children and adolescents.

Acknowledgements

The authors wish to thank the technician team from Porto Hospital Centre and Hospital São João for their expert assistance on blood collection. The authors would also like to thank all the participants that were involved in this project and their parents.

References

1. Fevery J (2008) Bilirubin in clinical practice: a review. Liver Int 28: 592-605.
2. Gong QH, Cho JW, Huang T, Potter C, Gholami N, et al. (2001) Thirteen UDPglucuronosyltransferase genes are encoded at the human UGT1 gene complex locus. Pharmacogenetics 11: 357-368.
3. Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, et al. (1995) The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. N Engl J Med 333: 1171-1175.
4. Beutler E, Gelbart T, Demina A (1998) Racial variability in the UDP-glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism? Proc Natl Acad Sci U S A 95: 8170-8174.
5. Kaniwa N, Kurose K, Jinno H, Tanaka-Kagawa T, Saito Y, et al. (2005) Racial variability in haplotype frequencies of UGT1A1 and glucuronidation activity of a novel single nucleotide polymorphism 686C> T (P229L) found in an African-American. Drug Metab Dispos 33: 458-465.
6. Clementi M, Di Gianantonio E, Fabris L, Forabosco P, Strazzabosco M, et al. (2007) Inheritance of hyperbilirubinemia: evidence for a major autosomal recessive gene. Dig Liver Dis 39: 351-355.
7. Tiribelli C, Ostrow JD (2005) The molecular basis of bilirubin encephalopathy and toxicity: report of an EASL Single Topic Conference, Trieste, Italy, 1-2 October, 2004. J Hepatol 43: 156-166.
8. Gourley GR (1997) Bilirubin metabolism and kernicterus. Adv Pediatr 44: 173-229.
9. Wu TW, Fung KP, Wu J, Yang CC, Weisel RD (1996) Antioxidation of human low density lipoprotein by unconjugated and conjugated bilirubins. Biochem Pharmacol 51: 859-862.

10. Weinberger B, Archer FE, Kathiravan S, Hirsch DS, Kleinfeld AM, et al. (2013) Effects of bilirubin on neutrophil responses in newborn infants. Neonatology 103: 105-111.
11. Lin JP, O'Donnell CJ, Schwaiger JP, Cupples LA, Lingenhel A, et al. (2006) Association between the UGT1A1*28 allele, bilirubin levels, and coronary heart disease in the Framingham Heart Study. Circulation 114: 1476-1481.
12. Novotny L, Vitek L (2003) Inverse relationship between serum bilirubin and atherosclerosis in men: a meta-analysis of published studies. Exp Biol Med (Maywood) 228: 568-571.
13. Horsfall LJ, Nazareth I, Petersen I (2012) Cardiovascular events as a function of serum bilirubin levels in a large, statin-treated cohort. Circulation 126: 2556-2564.
14. Temme EH, Zhang J, Schouten EG, Kesteloot H (2001) Serum bilirubin and 10-year mortality risk in a Belgian population. Cancer Causes Control 12: 887-894.
15. Maruhashi T, Soga J, Fujimura N, Idei N, Mikami S, et al. (2012) Hyperbilirubinemia, augmentation of endothelial function, and decrease in oxidative stress in Gilbert syndrome. Circulation 126: 598-603.
16. Nascimento H, Costa E, Rocha-Pereira P, Rego C, Mansilha HF, et al. (2012) Cardiovascular risk factors in portuguese obese children and adolescents: impact of small reductions in body mass index imposed by lifestyle modifications. Open Biochem J 6: 43-50.
17. Padez C, Fernandes T, Mourao I, Moreira P, Rosado V (2004) Prevalence of overweight and obesity in 7-9-year-old Portuguese children: trends in body mass index from 1970-2002. Am J Hum Biol 16: 670-678.
18. Alexander CM, Landsman PB, Grundy SM (2008) The influence of age and body mass index on the metabolic syndrome and its components. Diabetes Obes Metab 10: 246-250.

19. Hwang HJ, Kim SH (2010) Inverse relationship between fasting direct bilirubin and metabolic syndrome in Korean adults. Clin Chim Acta 411: 1496-1501.

20. Jo J, Yun JE, Lee H, Kimm H, Jee SH (2011) Total, direct, and indirect serum bilirubin concentrations and metabolic syndrome among the Korean population. Endocrine 39: 182-189.

21. Wu Y, Li M, Xu M, Bi Y, Li X, et al. (2011) Low serum total bilirubin concentrations are associated with increased prevalence of metabolic syndrome in Chinese. J Diabetes 3: 217-224.

22. Lin LY, Kuo HK, Hwang JJ, Lai LP, Chiang FT, et al. (2009) Serum bilirubin is inversely associated with insulin resistance and metabolic syndrome among children and adolescents. Atherosclerosis 203: 563-568.

23. Devries MC, Samjoo IA, Hamadeh MJ, Tarnopolsky MA (2008) Effect of endurance exercise on hepatic lipid content, enzymes, and adiposity in men and women. Obesity (Silver Spring) 16: 2281-2288.

24. Jenko-Praznikar Z, Petelin A, Jurdana M, Ziberna L (2013) Serum bilirubin levels are lower in overweight asymptomatic middle-aged adults: an early indicator of metabolic syndrome? Metabolism 62: 976-985.

25. Olerup O, Zetterquist H (1992) HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39: 225-235.

26. Gaffney D, Campbell RA (1994) A PCR based method to determine the Kalow allele of the cholinesterase gene: the E1k allele frequency and its significance in the normal population. J Med Genet 31: 248-250.

27. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, et al. (1985) Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28: 412-419.
28. Rocha S, Costa E, Ferreira F, Cleto E, Barbot J, et al. (2010) Hereditary spherocytosis and the (TA)nTAA polymorphism of UGT1A1 gene promoter region--a comparison of the bilirubin plasmatic levels in the different clinical forms. Blood Cells Mol Dis 44: 117-119.
29. Karatzas A, Giannatou E, Tzortzis V, Gravas S, Aravantinos E, et al. (2010) Genetic polymorphisms in the UDP-glucuronosyltransferase 1A1 (UGT1A1) gene and prostate cancer risk in Caucasian men. Cancer Epidemiol 34: 345-349.
30. Ostanek B, Furlan D, Mavec T, Lukac-Bajalo J (2007) UGT1A1(TA)n promoter polymorphism--a new case of a (TA)8 allele in Caucasians. Blood Cells Mol Dis 38: 78-82.
31. Rodrigues C, Vieira E, Santos R, de Carvalho J, Santos-Silva A, et al. (2012) Impact of UGT1A1 gene variants on total bilirubin levels in Gilbert syndrome patients and in healthy subjects. Blood Cells Mol Dis 48: 166-172.
32. Yip R, Johnson C, Dallman PR (1984) Age-related changes in laboratory values used in the diagnosis of anemia and iron deficiency. Am J Clin Nutr 39: 427-436.
33. Chauhan A, Grissom M. (2013) Disorders of childhood growth and development: precocious puberty. FP Essent 410:25-31.
34. Wonisch W, Falk A, Sundl I, Winklhofer-Roob BM, Lindschinger M (2012) Oxidative stress increases continuously with BMI and age with unfavourable profiles in males. Aging Male 15: 159-165.
35. Andersson C, Weeke P, Fosbol EL, Brendorp B, Kober L, et al. (2009) Acute effect of weight loss on levels of total bilirubin in obese, cardiovascular high-risk patients: an

444 analysis from the lead-in period of the Sibutramine Cardiovascular Outcome trial.
445 Metabolism 58: 1109-1115.
446 36. Taube A, Schlich R, Sell H, Eckardt K, Eckel J (2012) Inflammation and metabolic
447 dysfunction: links to cardiovascular diseases. Am J Physiol Heart Circ Physiol 302:
448 H2148-2165.
449 37. Greco EA, Francomano D, Fornari R, Marocco C, Lubrano C, et al. (2013) Negative
450 association between trunk fat, insulin resistance and skeleton in obese women. World J
451 Diabetes 4: 31-39.
452 38. Bondia-Pons I, Ryan L, Martinez JA (2012) Oxidative stress and inflammation
453 interactions in human obesity. J Physiol Biochem 68: 701-711.
454 39. D'Archivio M, Annuzzi G, Vari R, Filesi C, Giacco R, et al. (2012) Predominant role of
455 obesity/insulin resistance in oxidative stress development. Eur J Clin Invest 42: 70-78.
456 40. Hwang HJ, Lee SW, Kim SH (2011) Relationship between bilirubin and C-reactive
457 protein. Clin Chem Lab Med 49: 1823-1828.
458 41. Ohnaka K, Kono S, Inoguchi T, Yin G, Morita M, et al. (2010) Inverse associations of
459 serum bilirubin with high sensitivity C-reactive protein, glycated hemoglobin, and
460 prevalence of type 2 diabetes in middle-aged and elderly Japanese men and women.
461 Diabetes Res Clin Pract 88: 103-110.
462 42. Yoshino S, Hamasaki S, Ishida S, Kataoka T, Yoshikawa A, et al. (2011) Relationship
463 between bilirubin concentration, coronary endothelial function, and inflammatory stress
464 in overweight patients. J Atheroscler Thromb 18: 403-412.
465 43. Yu K, Kim C, Sung E, Shin H, Lee H (2011) Association of Serum Total Bilirubin with
466 Serum High Sensitivity C-reactive Protein in Middle-aged Men. Korean J Fam Med 32:
467 327-333.

468 44. Abraham NG, Tsenovoy PL, McClung J, Drummond GS (2008) Heme oxygenase: a
469 target gene for anti-diabetic and obesity. Curr Pharm Des 14: 412-421.
470 45. Bruce CR, Carey AL, Hawley JA, Febbraio MA (2003) Intramuscular heat shock protein
471 72 and heme oxygenase-1 mRNA are reduced in patients with type 2 diabetes: evidence
472 that insulin resistance is associated with a disturbed antioxidant defense mechanism.
473 Diabetes 52: 2338-2345.
474 46. Chang CL, Au LC, Huang SW, Fai Kwok C, Ho LT, et al. (2011) Insulin up-regulates
475 heme oxygenase-1 expression in 3T3-L1 adipocytes via PI3-kinase- and PKC-dependent
476 pathways and heme oxygenase-1-associated microRNA downregulation. Endocrinology
477 152: 384-393.
478 47. Nascimento H, Silva L, Lourenco P, Weinfurterova R, Castro E, et al. (2009) Lipid
479 profile in Portuguese obese children and adolescents: interaction of apolipoprotein E
480 polymorphism with adiponectin levels. Arch Pediatr Adolesc Med 163: 1030-1036.
481 48. Swift DL, Johannsen NM, Earnest CP, Blair SN, Church TS (2012) Effect of different
482 doses of aerobic exercise training on total bilirubin levels. Med Sci Sports Exerc 44: 569-
483 574.
484

Figure 1. **Total bilirubin levels in all participants according to the number of TA repeats in the promoter region of *UGT1A1* gene (A) and also according to group (B), control and obese.** The boxes represent the interquartile range (IQR), with the upper and lower edges of the boxes representing the 75th and 25th percentiles, respectively. The central horizontal lines within the boxes represent median levels for each group. The vertical whiskers above and below the boxes represent the range of outlying data points up to 1.5 times the IQR.

Figure 2. **Total bilirubin (A) and C-reactive protein (B) levels in obese participants according to gender and to body fat percentage (*n*=74), using the cut-off value of 42.5 % (cut-off that corresponds to the median value for the obese group).** The boxes represent the interquartile range (IQR), with the upper and lower edges of the boxes representing the 75th and 25th percentiles, respectively. The central horizontal lines within the boxes represent median levels for each group. The vertical whiskers above and below the boxes represent the range of outlying data points up to 1.5 times the IQR.

Figure 3. **Effect of body fat percentage on total bilirubin levels according to *UGT1A1*28* polymorphism on obese patients.** For a better visualization of the results we used for body fat percentage a cut-off of 42.5% (cut-off that corresponds to the median value for the obese group). Results are presented as mean ± standard error of mean. The influence of body fat percentage, adjusted for *UGT1A1* polymorphism, on total bilirubin levels, was highly significant ($p = 0.009$), by multiple regression analysis.

Table 1. Anthropometric data, *UGT1A1*28* polymorphism, haematological and biochemical parameters of the participants in the study

	Controls (*n* =79)			Obese patients (*n* =350)				
	Females	Males	*p*	Females	Males	*p*	$p^{\dagger}$	$p^{\dagger\dagger}$
Number of participants	47	32		182	168			
Age (years)	10.5 ± 4.0	10.7 ± 3.6	0.830	11.6 ± 2.9	11.7 ± 2.9	0.559	0.083	0.113
Height (cm)	139.7 ± 17.9	143.8 ± 17.6	0.317	151.3 ± 13.2	155.4 ± 15.4	0.008	<0.001	<0.001
Weight (kg)	37.0 ± 14.6	39.7 ± 15.8	0.440	72.1 ± 22.5	76.2 ± 27.4	0.128	<0.001	<0.001
BMI (kg/m²)	18.1 ± 2.9	18.3 ± 2.9	0.691	30.7 ± 5.8	30.5 ± 6.4	0.762	<0.001	<0.001
BMI z-score	0.17 ± 0.65	0.24 ± 0.77	0.636	2.22 ± 0.34	2.30 ± 0.40	0.046	<0.001	<0.001
Body fat (%)	30.8[a] ± 4.1	25.4[b] ± 5.2	0.001	43.5[c] ± 4.1	39.8[d] ± 6.6	0.156	<0.001	<0.001
Trunk fat (%)	25.6[a] ± 4.8	21.9[b] ± 6.0	0.045	41.1[c] ± 8.9	37.8[d] ± 7.9	0.107	<0.001	<0.001
***UGT1A1* genotype**								
6/6, *n* (%)	21 (44.7%)	12 (37.5%)	0.298	92 (50.6%)	89 (53.0%)	0.433	0.455	0.085
6/7, *n* (%)	21 (44.7%)	19 (59.4%)		79 (43.4%)	64 (38.1%)			
7/7, *n* (%)	5 (10.6%)	1 (3.1%)		11 (6.0%)	15 (8.9%)			
RBC ($x10^{12}$/L)	4.62 ± 0.29	4.77 ± 0.29	0.031	4.78 ± 0.32	5.03 ± 0.39	<0.001	0.003	<0.001
Hb (g/dL)	13.1 ± 0.9	13.6 ± 1.2	0.029	13.6 ± 0.8	14.2 ± 1.2	<0.001	0.001	0.017
Ht (L/L)	0.39 ± 0.03	0.40 ± 0.04	0.263	0.40 ± 0.02	0.42 ± 0.03	<0.001	0.033	0.003
MCV (fL)	84.9 ± 4.6	84.0 ± 6.1	0.486	84.2 ± 5.1	83.8 ± 4.7	0.454	0.432	0.846
MCH (pg)	28.4 ± 1.7	28.6 ± 2.0	0.684	28.5 ± 1.7	28.2 ± 1.6	0.185	0.909	0.239
MCHC (g/dL)	33.4 ± 1.2	34.0 ± 1.1	0.025	33.8 ± 1.0	33.7 ± 1.1	0.271	0.027	0.081
Total bilirubin (µmol/l)	8.89 (5.47-13.34)	7.52 (5.30-11.54)	0.463	8.89 (6.16-11.63)	9.23 (6.84-12.65)	0.232	0.919	0.079
Acute phase protein								
CRP (mg/L)	0.26 (0.20-0.73)	0.36 (0.26-0.83)	0.121	1.83 (0.85-3.73)	1.64 (0.85-3.54)	0.527	<0.001	<0.001
Glucose metabolism								
Glucose (mg/dl)	85.3 ± 9.3	87.0 ± 6.5	0.365	84.0 ± 8.9	85.8 ± 12.6	0.121	0.384	0.419
Insulin (µU/ml)	6.8 (5.0-9.9)	5.3 (4.1-8.5)	0.051	16.6 (11.7-23.2)	12.8 (9.1-20.0)	0.001	<0.001	<0.001
$HOMA_{IR}$	1.41 (1.06-2.05)	1.14 (0.82-1.83)	0.130	3.39 (2.21-4.87)	2.75 (1.88-4.06)	0.006	<0.001	<0.001

Values are given as mean ± SD or median (interquartile range), unless otherwise indicated.

BMI, body mass index; RBC, red blood cells; Hb, haemoglobin; Ht, haematocrit; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; CRP, C-reactive protein; $HOMA_{IR}$, homeostasis model assessment insulin resistance.

a, *n*=25; b, *n*=15; c, *n*=34; d, *n*=40.

† Controls *versus* obese patients (females)

†† Controls *versus* obese patients (males)

Table 2. Anthropometric data, *UGT1A1*28* polymorphism, haematological and biochemical parameters of obese patients according to body fat percentage (*n*=74) lower or higher/equal than 42.5% (median value for the obese group)

	Obese patients (*n* =74)		
	Body fat ≤ 42.5%	Body fat > 42.5%	*p*
Number of participants	37	37	
Female, *n* (%)	13 (35.1%)	21 (56.8%)	0.102
Age (years)	11.0 ± 3.0	9.5 ± 2.5	0.022
Height (cm)	149.2 ± 14.3	144.0 ± 14.2	0.126
Weight (kg)	59.8 ± 18.9	61.4 ± 23.9	0.749
BMI (kg/m²)	26.0 ± 4.0	28.4 ± 5.5	0.041
BMI z-score	1.98 ± 0.24	2.31 ± 0.26	<0.001
Body fat (%)	36.8 ± 4.3	46.1 ± 2.6	<0.001
Trunk fat (%)	34.5 ± 5.5	45.3 ± 3.4	<0.001
***UGT1A1* genotype**			
6/6, *n* (%)	21 (56.8%)	19 (51.4%)	0.359
6/7, *n* (%)	14 (37.8%)	18 (48.6%)	
7/7, *n* (%)	2 (5.4%)	0 (0%)	
RBC ($x10^{12}$/L)	4.83 ± 0.38	4.91 ± 0.34	0.389
Hb (g/dL)	13.9 ± 1.0	13.6 ± 0.8	0.268
Ht (L/L)	0.41 ± 0.03	0.41 ± 0.02	0.798
MCV (fL)	85.4 ± 4.9	83.8 ± 4.6	0.156
MCH (pg)	28.7 ± 1.7	27.8 ± 1.5	0.017
MCHC (g/dL)	33.6 ± 0.8	33.2 ± 0.9	0.020
Total bilirubin (µmol/l)	11.29 (8.72-14.36)	8.89 (7.69-11.63)	0.013
Acute phase protein			
CRP (mg/L)	1.31 (0.84-2.30)	2.00 (1.43-3.54)	0.017
Glucose metabolism			
Glucose (mg/dl)	83.5 ± 7.6	81.0 ± 8.6	0.191
Insulin (µU/ml)	11.6 (8.9-14.6)	15.3 (7.5-22.9)	0.272
$HOMA_{IR}$	2.25 (1.91-3.01)	3.15 (1.57-4.56)	0.361

Values are given as mean ± SD or median (interquartile range), unless otherwise indicated.

BMI, body mass index; RBC, red blood cells; Hb, haemoglobin; Ht, haematocrit; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; CRP, C-reactive protein; $HOMA_{IR}$, homeostasis model assessment insulin resistance.

Fig. 1

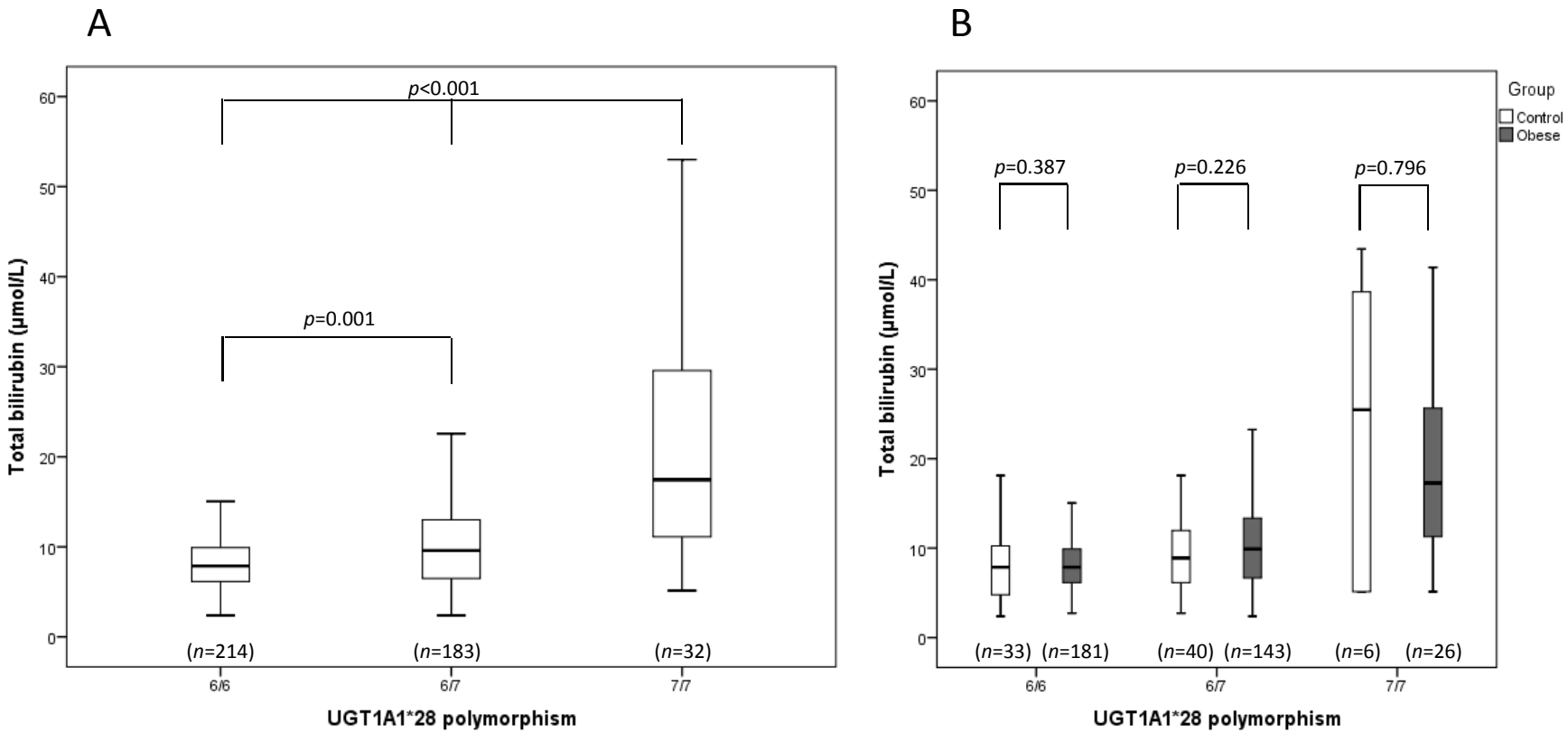

A
p<0.001
p=0.001
Total bilirubin (µmol/L)
60
50
40
30
20
10
0
(n=214)
(n=183)
(n=32)
6/6
6/7
7/7
UGT1A1*28 polymorphism
B
Group
Control
Obese
p=0.387
p=0.226
p=0.796
Total bilirubin (µmol/L)
(n=33)
(n=181)
(n=40)
(n=143)
(n=6)
(n=26)
6/6
6/7
7/7
UGT1A1*28 polymorphism

Fig. 2

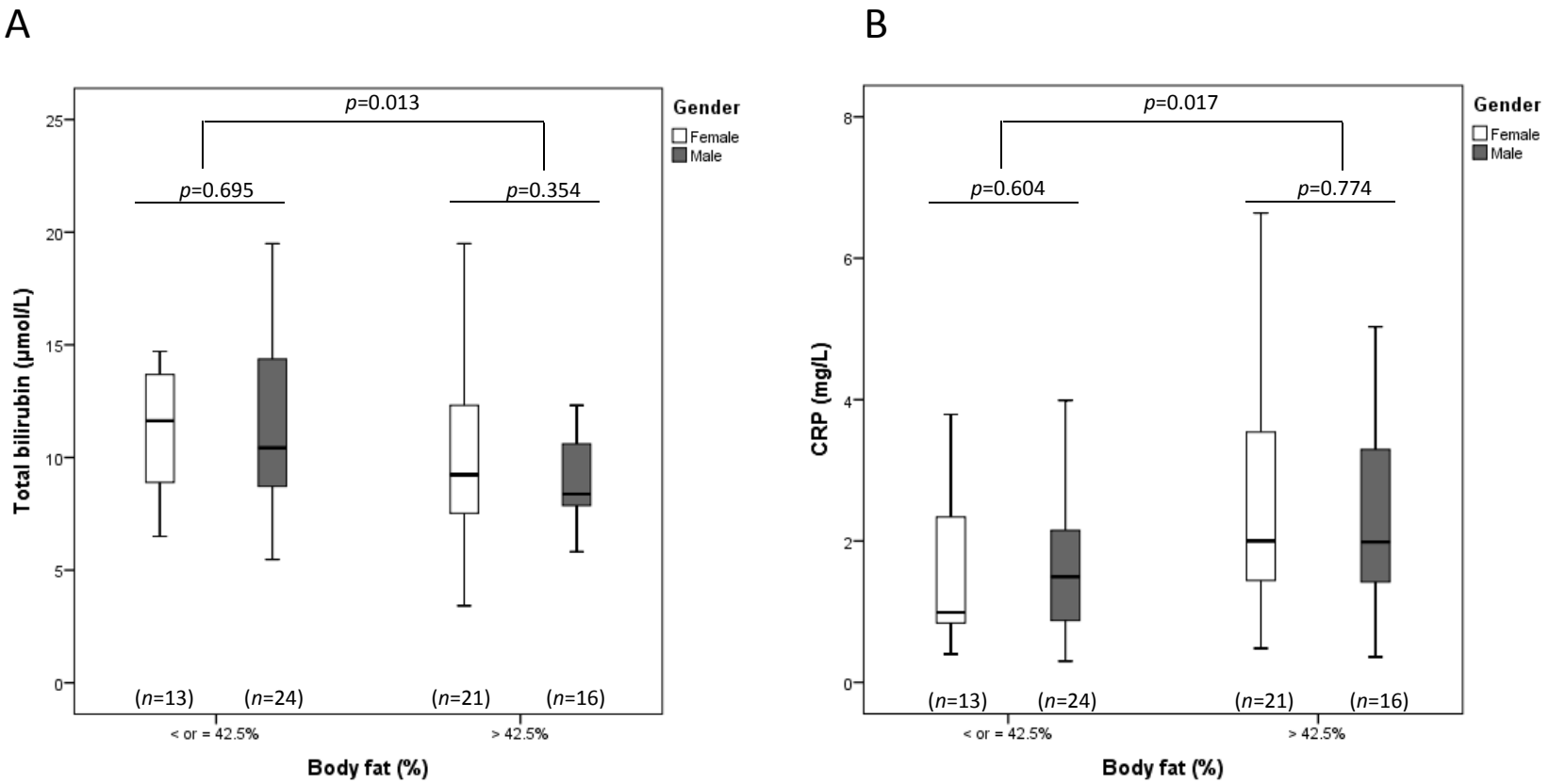
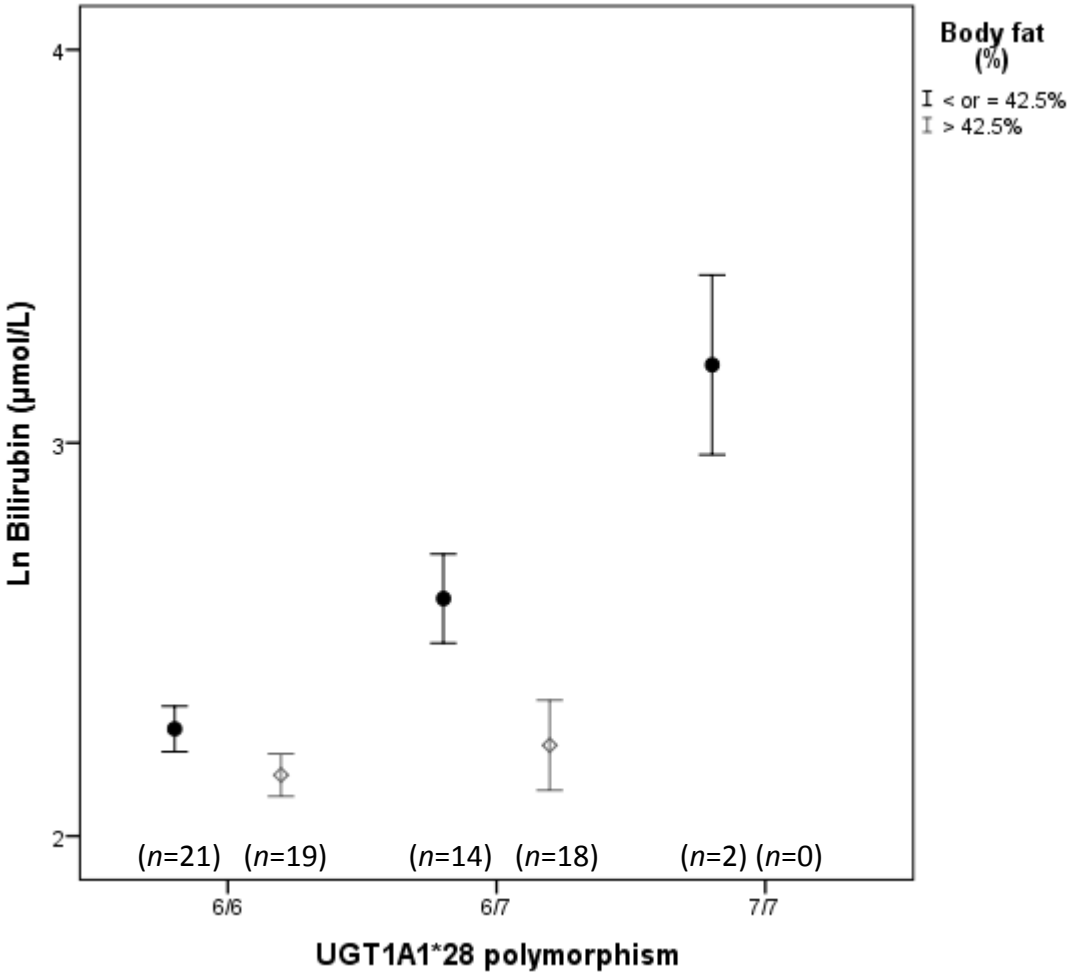

A
p=0.013
p=0.695
p=0.354
Gender
Female
Male
Total bilirubin (μmol/L)
25
20
15
10
5
0
(n=13)
(n=24)
(n=21)
(n=16)
< or = 42.5%
> 42.5%
Body fat (%)
B
p=0.017
p=0.604
p=0.774
Gender
Female
Male
CRP (mg/L)
8
6
4
2
0
(n=13)
(n=24)
(n=21)
(n=16)
< or = 42.5%
> 42.5%
Body fat (%)

Fig. 3


Body fat (%)
< or = 42.5%
> 42.5%
Ln Bilirubin (µmol/L)
4
3
2
(n=21)
(n=19)
(n=14)
(n=18)
(n=2)
(n=0)
6/6
6/7
7/7
UGT1A1*28 polymorphism

APPENDIX I

DATA ENTERED ONLY DURING THE ONLINE SUBMISSION PROCESS

Short Title: Major determinants of bilirubin in young obese patients

Financial Disclosure: This work was funded by FEDER funds through the Operational Competitiveness Programme – COMPETE and by National Funds through FCT – Fundação para a Ciência e a Tecnologia under the project FCOMP-01-0124-FEDER-028613 (PTDC/DTP-DES/0393/2012). A PhD grant was attributed to H. Nascimento by FCT (SFRH/BD/48060/2008). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest statement: The authors have no conflict of interest to disclosure.

APPENDIX II

PLOS ONE

MANUSCRIPT GUIDELINES

1. Format Requirements

PLOS ONE does **not** consider presubmission inquiries. All submissions should be prepared with the following files:

- Cover letter
- Manuscript, including tables and figure legends
- Figures (guidelines for preparing figures can be found at the Figure and Table Guidelines)

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service." Submissions are **not** copyedited before publication.
Submissions that do not meet the *PLOS ONE* Publication Criterion for language standards may be rejected.

Cover Letter

You should supply an approximately one page cover letter that:

- Concisely summarizes why your paper is a valuable addition to the scientific literature
- Briefly relates your study to previously published work
- Specifies the type of article you are submitting (for example, research article, systematic review, meta-analysis, clinical trial)
- Describes any prior interactions with PLOS regarding the submitted manuscript
- Suggests appropriate *PLOS ONE* Academic Editors to handle your manuscript.
- Lists any recommended or opposed reviewers

Your cover letter should **not** include requests to reduce or waive publication fees. Should your manuscript be accepted, you will have the opportunity to include your requests at that time. See *PLOS ONE* Editorial Policy for more information regarding publication fees.

Manuscript Organization

PLOS ONE considers manuscripts of any length. There are no explicit restrictions for the number of words, figures, or the length of the supporting information, although we encourage a concise and accessible writing style. We will **not** consider monographs.
All manuscripts should be double-spaced and include line numbers and page numbers.
Manuscripts should begin with the ordered sections:

- Title
- Authors
- Affiliations
- Abstract
- Introduction

and end with the sections of:

- Acknowledgments
- References
- Figure Legends
- Tables

Figures should not be included in the main manuscript file. Each figure must be prepared and submitted as an individual file.

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.
There are no explicit requirements for section organization between these beginning and ending sections. Articles may be organized in different ways and with different section titles, according to the authors' preference. In most cases, internal sections include:

- Materials and Methods
- Results
- Discussion
- Conclusions (optional)

PLOS ONE has no specific requirements for the order of these sections, and in some cases it may be appropriate to combine sections. Guidelines for individual sections can be found below.
Abbreviations should be kept to a minimum and defined upon first use in the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.
Standardized nomenclature should be used as appropriate, including appropriate usage of species names and SI units.

Manuscript File Type Requirements

Authors may submit their manuscript files in Word (as .doc or .docx), LaTeX (as .pdf), or RTF format. Only RTF and .doc files can be used during the production process. Word files must not be protected.
LaTeX Submissions. If you would like to submit your manuscript using LaTeX, you must author your article using the *PLOS ONE* LaTeX template and BibTeX style sheet. Articles prepared in LaTeX may be submitted in PDF format for use during the review process. After acceptance, however, .tex files and formatting information will be required as a zipped file. Please consult our LaTeX guidelines for a list of what will be required.
Submissions with equations. If your manuscript is or will be in .docx format and contains equations, you must follow the instructions below to make sure that your equations are editable when the file enters production.
If you have not yet composed your article, you can ensure that the equations in your .docx file remain editable in .doc by enabling "Compatibility Mode" before you begin. To do this, open a new document and save as Word 97-2003 (*.doc). Several features of Word 2007/10 will now be inactive, including the built-in equation editing tool. You can insert equations in one of the two ways listed below.
If you have already composed your article as .docx and used its built-in equation editing tool, your equations will become images when the file is saved down to .doc. To resolve this problem, re-key your equations in one of the two following ways.
1. Use MathType to create the equation (recommended)
2. Go to Insert > Object > Microsoft Equation 3.0 and create the equation
If, when saving your final document, you see a message saying "Equations will be converted to images," your equations are no longer editable and PLoS will not be able to accept your file.

2. Guidelines for Standard Sections

Title

Manuscripts must be submitted with both a full title and a short title, which will appear at the top of the PDF upon publication if accepted. Only the full title should be included in the manuscript file; the short title will be entered during the online submission process.
The full title must be 250 characters or fewer. It should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).
Examples:

- Impact of Cigarette Smoke Exposure on Innate Immunity: A *Caenorhabditis elegans* Model
- Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial

The short title must be 50 characters or fewer and should state the topic of the paper.

Authors and Affiliations

All author names should be listed in the following order:

- First names (or initials, if used),
- Middle names (or initials, if used), and
- Last names (surname, family name)

Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.
This information cannot be changed after initial submission, so please ensure that it is correct.
To qualify for authorship, a researcher should contribute to **all** of the following:

1. Conception and design of the work, acquisition of data, or analysis and interpretation of data
2. Drafting the article or revising it critically for important intellectual content
3. Final approval of the version to be published

All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author must have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments.
When a large group or center has conducted the work, the author list should include the individuals whose contributions meet the criteria defined above, as well as the group name.
One author should be designated as the corresponding author, and his or her email address or other contact information should be included on the manuscript cover page. This information will be published with the article if accepted. See the *PLOS ONE* Editorial Policy regarding authorship criteria for more information.

Abstract

The abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should **not** include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

This section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and

protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.
We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information.
Methods sections of papers on research using **human or animal subjects and/or tissue or field sampling** must include required ethics statements. See the Reporting Guidelines for human research, clinical trials, animal research, and observational and field studies for more information.
Methods sections of papers with **data that should be deposited in a publicly available database** should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication.
Methods sections of papers using **cell lines** must state the origin of the cell lines used. See the Reporting Guidelines for cell line research for more information.
Methods sections of papers adding **new taxon names** to the literature must follow the Reporting Guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Results, Discussion, and Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.
Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn. Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.
PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Publication Criteria for more information.

Acknowledgments

People who contributed to the work but do not fit the *PLOS ONE* authorship criteria should be listed in the acknowledgments, along with their contributions. You must ensure that anyone named in the acknowledgments agrees to being so named.
Funding sources should **not** be included in the acknowledgments, or anywhere in the manuscript file. You will provide this information during the manuscript submission process.

References

Only published or accepted manuscripts should be included in the reference list. Manuscripts that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only as “unpublished data.”
References must be listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, citations should be indicated by the reference number in brackets. Journal name abbreviations should be those found in the NCBI databases. A number of reference software companies supply PLOS style files (e.g., Reference Manager, EndNote).
References should be formatted as follows:

- Published papers. Hou WR, Hou YL, Wu GF, Song Y, Su XL, et al. (2011) cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res 10: 1576-1588.

  Note: Use of a DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers.

- Accepted, unpublished papers. Same as above, but “In press” appears instead of the page numbers.

- Electronic journal articles. Huynen MMTE, Martens P, Hilderlink HBM (2005) The health impacts of globalisation: a conceptual framework. Global Health 1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14. Accessed 25 January 2012.

- Books. Bates B (1992) Bargaining for life: A social history of tuberculosis. Philadelphia: University of Pennsylvania Press. 435 p.

- Book chapters. Hansen B (1991) New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health. pp. 21-28.

Tables

Tables should be included at the end of the manuscript. All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above. Tables occupying more than one printed page should be avoided, if possible. Larger tables can be published as Supporting Information. Please ensure that table formatting conforms to our Guidelines for table preparation.

Figure Legends

Figures should **not** be included in the manuscript file, but figure legends should be.
Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations.
Further information about figure legends can be found in the Figure Guidelines.

Striking Images

Authors are encouraged to upload a "striking image" that may be used to represent their paper online in places like the journal homepage or in search results. The striking image must be derived from a figure or supporting information file from the paper, ie. a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows. If no striking image is uploaded, a figure from the paper will be designated as the striking image.

Please keep in mind that PLOS's Creative Commons Attribution License applies to striking images. As such, do not submit any figures or photos that have been previously copyrighted unless you have express written permission from the copyright holder to publish under the CCAL license. Note that all published materials in PLOS ONE are freely available online, and any third party is permitted to read, download, copy, distribute, and use these materials in any way, even commercially, with proper attribution.
Care should be taken with the following image types in particular:

1. PLOS ONE is unable to publish any images generated by Google software (Google Maps, Street View, and Earth)
2. Maps in general are usually copyrighted, especially satellite maps
3. Photographs
4. Commercial or government images, slogans, or logos
5. Images from Facebook or Twitter

Authors must also take special care when submitting manuscripts that contain potentially identifying images of people. Identifying information should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF).

3. Specific Reporting Guidelines

Human Subject Research

Methods sections of papers on research using human subject or samples must include ethics statements that specify:

- The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subject research, see the Publication Criteria and Editorial Policies.