FMUC FACULDADE DE MEDICINA
UNIVERSIDADE DE COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

MARIA JOÃO SÃO MIGUEL PERESTRELO CAVACO

Susceptibility to antituberculosis drugs-induced hepatotoxicity: a multivariate model

ARTIGO CIENTÍFICO

ÁREA CIENTÍFICA DE GENÉTICA

Trabalho realizado sob a orientação de:

PROFESSORA DOUTORA HENRIQUETA ALEXANDRA MENDES BREDA LOBO COIMBRA SILVA

DOUTORA MARIA CELESTE DA SILVA ALCOBIA

NOVEMBRO/2018

Susceptibility to antituberculosis drugs-induced hepatotoxicity: a multivariate model

Maria Cavaco[1], Celeste Alcobia[2], Bárbara Oliveiros[3], Luís Mesquita[4], Fátima Castro[4], Miguel Vilar[5], Henriqueta C. Silva[4]

[1] Faculdade de Medicina, Universidade de Coimbra
[2] Centro Hospitalar e Universitário de Coimbra
[3] Laboratório de Bioestatística e Informática Médica da Faculdade de Medicina, Universidade de Coimbra
[4] Instituto de Genética Médica da Faculdade de Medicina, Universidade de Coimbra
[5] Centro de Diagnóstico Pneumológico de Vendas Novas

Table of Contents

Abstract

Tuberculosis remains a pressing public health problem at a global scale, due to its incidence, morbidity and mortality. Drug-induced liver injury (DILI) is one of the most feared side effects associated with antituberculosis treatment, INH being frequently implicated. DILI is a multifactorial phenotype, influenced by the interaction of both genetic and non-genetic factors.

The present study aimed to identify clinical and genetic variables associated with susceptibility to antituberculosis drugs-induced hepatotoxicity in patients with pulmonary tuberculosis. Clinical variables analyzed included age, gender, weight, smoking habits and presence of chronic diseases. Variants of four candidate genes, *NAT2* (INH-metabolism), *ABCB11* (bile-salts transport), *IL6* (immunoinflammation and liver regeneration) and *NBAS* (liver failure) were assessed. The population sample included a total of 217 Caucasian patients with pulmonary tuberculosis, of whom 96 developed hepatotoxicity. Three grades of hepatotoxicity were considered: grade 1 (mild, 45 patients), grade 2 (DILI, 46 patients) and grade 3 (need for liver transplant or death, 5 patients). A logistic multivariate regression analysis was used to identify risk variables. Hardy-Weinberg equilibrium was tested by a chi-squared test.

Age ≥ 60 years old (OR: 2.93; 95% CI: 1.58 – 5.43; p = 0.001) was found to be associated with an increased risk of hepatotoxic events in the global group with hepatotoxicity, as well as in subgroups comprising only females, males, grade 1 or grade 2 hepatitis. Female gender (OR: 2.16; 95% CI: 1.16 – 4.03; p = 0.015) and slow acetylator genotype (OR: 1.82; 95% CI: 1.02 – 3.27; p = 0.044) were also identified as predictors of hepatotoxicity. The model explains 15% of susceptibility to liver injury. For grade 1 hepatitis, the correlation between slow acetylator genotype and hepatotoxicity was also observed in the subgroup of females < 60 year old, but not in males' subgroup. For males with grade 1 hepatitis, the presence of the CC genotype of rs2287622 variant in *ABCB11* gene was associated with increased risk only for men ≥ 60 years old (OR: 7.39; 95% CI: 1.33 – 41.04; p = 0.022). For grade 2 hepatitis, only older age and female gender were identified as risk factors.

In conclusion, antituberculosis drugs-induced hepatotoxicity is a complex phenotype, difficult to predict, for which age, female gender and variants of *NAT2* and *ABCB11* genes, have a modest contribution. Until the mechanisms involved are revealed, a close clinical and analytical monitoring and eventually, an INH dosage adapted to the acetylator status, are the only disposable preventive measures.

Keywords: Isoniazid (INH), DILI, Hepatotoxicity, N-acetyltransferase 2 (NAT2), Interleukin 6 (IL6), ABCB11, NBAS

Resumo

A tuberculose continua a ser um importante problema de saúde pública à escala global, devido à sua incidência, morbidade e mortalidade. A hepatite induzida por drogas (DILI) é um dos efeitos secundários mais temidos associados aos fármacos antituberculosos, estando a isoniazida (INH) frequentemente envolvida. A DILI é um fenótipo multifatorial, influenciado pela interação de fatores genéticos e não genéticos, sendo difícil identificar um perfil de suscetibilidade.

O presente estudo teve como objetivo identificar variáveis clínicas e genéticas associadas à suscetibilidade à hepatotoxicidade induzida por fármacos antituberculosos em pacientes com tuberculose pulmonar. As variáveis clínicas analisadas incluíram idade, sexo, peso, tabagismo e presença de doenças crónicas. Caracterizaram-se variantes de quatro genes candidatos, *NAT2* (metabolismo da INH), *ABCB11* (transporte de sais biliares), *IL6* (imunoinflamação e regeneração hepática) e *NBAS* (insuficiência hepática). A amostra populacional incluiu 217 pacientes com tuberculose pulmonar, 96 dos quais desenvolveram hepatotoxicidade. Três graus de hepatotoxicidade foram considerados: grau 1 (leve, 45 pacientes), grau 2 (tóxica ou DILI, 46 pacientes) e grau 3 (necessidade de transplante hepático ou morte, 5 pacientes). Foi realizada uma regressão logística multivariada para identificar as variáveis de risco. O equilíbrio de Hardy-Weinberg foi testado pelo teste de qui-quadrado.

Idade ≥ 60 revelou-se um fator de risco quer quando se consideraram todos os doentes com hepatotoxicidade (OR: 2.93; 95% CI: 1.58 – 5.43; p = 0.001), quer para os subgrupos incluindo apenas mulheres, homens, grau 1 ou 2 de hepatite. O sexo feminino (OR: 2.16; 95% CI: 1.16 – 4.03; p = 0.015) e o genótipo de acetilador lento (OR: 1,82; 95% CI: 1,02 – 3,27; p = 0,044) também foram identificados como preditores de hepatotoxicidade. O modelo explica 15% de suscetibilidade a lesão hepática. Para a hepatite grau 1, a correlação entre genótipo de acetilador lento e hepatotoxicidade também foi observada em mulheres com menos de 60 anos de idade, mas não no grupo dos homens. Para os homens com hepatite de grau 1, a presença do genótipo CC da variante rs2287622 de gene *ABCB11* associou-se a risco aumentado apenas em homens ≥ 60 anos (OR: 7.39; 95% CI: 1.33 – 41.04; p = 0.022). Para hepatite de grau 2, apenas a idade ≥ 60 anos e sexo feminino foram identificados como fatores de risco.

Conclui-se que a hepatotoxicidade induzida por fármacos antituberculosos é um fenótipo complexo, difícil de prever, para o qual a idade, sexo feminino e variáveis dos genes *NAT2* e e *ABCB11* têm uma contribuição modesta. Até que os mecanismos envolvidos sejam

desvendados, a monitorização clínica e analítica e, eventualmente, uma dosagem de INH adaptada ao genótipo de acetilação, são os únicos recursos disponíveis.

Palavras-chave: Isoniazida (INH), DILI, Hepatotoxicidade, N-acetyltransferase 2 (NAT2), Interleucina 6 (IL6), ABCB11, NBAS

Introduction

Tuberculosis (TB), an infectious disease caused by Mycobacterium tuberculosis bacillus, is one of the top 10 causes of death worldwide, still affecting developed and developing countries. In 2017, it caused an estimated 1.3 million deaths among HIV-negative people plus 300 000 deaths among HIV-positive people. About 1.7 billion people are estimated to have a latent tuberculosis infection, and are thus at risk of developing active tuberculosis disease during their lifetime.[1]

The treatment recommended for the newly diagnosed disease consists of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZ) and ethambutol for 2 months followed by 4 months of INH and RIF.[2] As a prophylaxis it is consensual to administer INH for 6 to 9 months or INH with RIF for 3 months.[3]

Due to its high efficacy, INH remains a gold-standard drug for treatment and prophylaxis of tuberculosis despite being the most frequent cause of drug-induced liver injury (DILI) associated with antituberculosis treatment.[4] RIF and PZ have also been associated with this side effect and can potentiate INH-DILI.[5,6] This adverse effect, occurring in 1-36% of treated patients, has a very broad spectrum of presentation, ranging from asymptomatic elevations of liver aminotransferases in 20% of patients to acute liver failure in 1-5% of patients.[7–9]

Being considered an idiosyncratic event, INH-DILI is a complex phenotype involving multiple low penetrance variables, including variants in genes regulating the metabolism of the drug and the organism's immunoinflammatory response, and non-genetic variants, namely individual biological and clinical characteristics and environmental factors.[4,10] Identifying susceptibility profiles is an essential step towards the development of multifactorial predictive models that may improve clinical decisions and diminish therapeutic risk. Age, female gender, black race, alcoholism, pre-existing liver disease and concomitant medications such as RIF and PZ are some of the known risk factors for INH-DILI.[11] In fact, causality assessment may be impossible to establish.

The first genetic variants to be studied were single nucleotide polymorphisms (SNP) from the N-acetyltransferase 2 (*NAT2*) gene, which encodes the polymorphic NAT2 enzyme, responsible for the acetylation of INH. Based on *NAT2* genotype, individuals can be classified into slow (SA), intermediate (IA) or rapid acetylators (RA).[12] According to most studies, SA have increased susceptibility to the development of INH-induced hepatotoxicity.[13,14] However, not all authors confirmed this correlation.[12]

Genetic variants in genes encoding proteins involved in bile salt transport may also be implicated, as is the case of the loss-of-function, missense (V444A) SNP rs2287622, in the *ABCB11* gene. This gene encodes the bile salt export pump (BSEP), an ATP-binding

cassette transporter (ATP-binding cassette, subfamily B, member11) located at the canalicular membrane of the hepatocyte. This SNP has been related with increased risk of intrahepatic cholestasis in different clinical situations.[15–21]

IL6 is a cytokine with pleiotropic activity, rapidly produced in response to emergent events like infection and tissue injury, liver and immunoinflammatory cells being two important targets.[22] In the liver, IL6 is a major inducer of the acute phase response after infection, improving hepatic regeneration and repair, but studies show that in more chronic exposure, it can actually sensitizes the liver to injury and cell-death.[23] These two opposing liver responses have been associated with the *IL6* SNP rs1800797, an adenine to guanine transition in the gene regulatory region that may interfere with IL6 expression.[24,25]

In 2015, a study was published associating variants in neuroblastoma amplified sequence (*NBAS*) gene with the occurrence of fever-triggered recurrent liver failure (RALF) in children.[26] Since then, there have been new reports from different parts of the world,[27–31] making *NBAS* a candidate gene for DILI.

The aim of the present study was to identify clinical and genetic variables associated with susceptibility to antituberculosis drugs-induced hepatotoxicity in patients with pulmonary disease. Clinical variables analyzed included age, gender, concomitant chronic diseases, weight and smoking habits. Variants of four candidate genes, *NAT2*, *ABCB11*, *IL6* and *NBAS* were also assessed.

Materials and methods

1. Study Design and characterization of patient sample

Patients (217) were followed mainly in the Coimbra Hospital and University Centre (CHUC) and in the Centre of Pneumologic Diagnostic of Coimbra, with 20 patients being recruited from the Centre of Pneumologic Diagnostic of Vendas Novas. This case control study has two set of patients. The first group was retrospectively selected from clinical records of tuberculosis patients treated in CHUC and at the Centre of Pneumologic Diagnostic from 2004 to 2009. Since 2009, the study was prospective and observational. For all patients that agree to participate, a specific clinical record was created. Blood samples for genotyping were collected from all patients with the diagnosis of hepatotoxicity (HT) and from a similar number of controls (the first or two next patients according to the numbers of clinical records). In the prospective study, whenever a control developed HT during treatment (becoming a case), another control was included.

All blood samples sent to the genetic laboratory were referred by code number. All participants were informed about the study and signed an informed consent. The study was approved by the Ethics Committee of the CHUC. This study is part of a doctoral project of Dr. Celeste Alcobia.

The eligibility criteria were pulmonary tuberculosis patients without contraindication to a multi drug therapeutic scheme including standard dose of INH, expectation of good therapeutic compliance or TOD (therapy observed directly), unrelated Caucasian subjects, age between 17 and 80 years old, weight > 40kg, a complete medical record (for retrospective set of patients), normal baseline laboratory testing including: blood cells and platelet count, serum creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin and γgamma-glutamyltransferase (γ-GT). Human hepatitis virus (A, B and C) and HIV serology should be negative. Pregnant women, intellectually disable patients and patients with a history of alcohol abuse, hepatic alcoholic disease or hepatitis (any cause) were excluded.

A standard therapeutic antituberculosis treatment including isoniazid was applied. Patients had a first follow up appointment after 15 days of therapy and monthly thereafter. In each appointment, a clinical record was fulfilled and blood levels of liver enzymes were evaluated.

Three grades of hepatotoxicity, from 1 to 3, were considered. Criteria for DILI (grade 2 or toxic hepatitis) associated with antituberculosis drugs were the following: 1) ALT value $\geq$ 5 x the upper limit of normal (ULN) or ALT value $\geq$ 3 x ULN and TBRB $\geq$ 2 x ULN or ALP value $\geq$

2 x ULN and concomitant increase in γ-GT in the absence of any bone disease;[31] (2) absence of serological evidence of infection with hepatitis A, B or C and 3) normalization in abnormal liver chemistry results after withdrawal of drugs. Grade 3 included patients submitted to liver transplant or deceased. We also consider as having mild hepatotoxicity (grade 1) patients with serum ALT or AST levels > 2 x baseline levels and > 1.5 x the ULN. Smoking habits were categorized as non-smoker, former smoker (> 1 year), smoker consuming < 20 pack-year or consuming ≥ 20 pack-years. Chronic diseases were defined as any disease lasting 3 months or more imposing chronic pharmacologic therapeutic.

3. Data collection

Extraction of DNA was performed in 1.5 ml peripheral blood samples collected beforehand into tubes containing EDTA (Ethylenediamine Tetraacetic Acid) using the "NZY Blood gDNA Isolation" kit. DNA quantification and purity were checked in a Nano Drop spectrophotometer (NanoDrop ND- 1000) with all samples showing values within normal range (between 1.8 – 2.0 and 1.8 – 2.2 for 260/280 nm and 260/230 nm wavelengths reads, respectively). DNA samples were stored at -20ºC until further use.

For the *NAT2* gene, 10 single nucleotide polymorphisms (SNPs), localized in the coding region, were evaluated: 111 T>C (rs72554615) , 191 G>A (rs1801279), 282 C>T (rs1041983) , 341 T>C (rs1801280), 434 A>C (rs72554616), 481 C>T (rs1799929) , 590 G>A (rs1799930), 803 A>G (rs1208), 845 A>C (rs56054745) and 857 G>A (rs1799931). Characterization of these SNPs allows identification and classification of patients as rapid (RA), intermediate (IA) or slow (SA) acetylators. To characterize these SNPs two gene segments were amplified and submitted to Sanger sequencing.

The SNPs rs1800796 from *IL6* gene and rs2287622 from *ABCB11* gene were also identified by Sanger sequencing.

For the SNP rs2052438 of the *NBAS* gene, first, 10 samples were sequenced to identify different genotypes; these samples were subsequently used as controls in a real time PCR protocol with TaqMan probes applied to the remaining samples (TaqMan SNP genotyping assay rs2052438; PN4351375).

All first PCR were carry out in a 25 µl of reaction volume containing NZYTech Buffer 1x, 200 µM of dNTPs, 1.5 mM of MgCl, 0.2 µM primers (tab.X), 0.03 U/µl of NZYTech Taq polymerase and 150 ng of DNA. PCRs of NAT2 included 5% DMSO. The amplification reaction was performed in a thermocycler "MyCycler" (Bio-Rad). Primers and annealing temperatures are described in Table 1. The same primers were used for Sanger sequencing.

Real time PCRs were carry out in a 20 µl of reaction volume containing SensiFast Probe No-ROX kit (Bioline)1x, 0.5 µl of probes and primers mix (TaqMan SNP genotyping assay rs2052438; PN4351375) and 100 ng of DNA. Cycling conditions included 40s at 72ºC as described in SensiFast Probe No-ROX kit protocol. A CFX96 Real Time System (Bio-Rad) was used.

Quality of the first PCR (specificity and absence of contamination) was confirmed by electrophoresis on 2% agarose gel stained with Safe-Green (abm) after visualization in UV transilluminator. Loading buffer type IV and 1µl HiperLadder II™ molecular weight marker (Invitrogen, CA, USA) were used.

For Sanger sequencing PCR samples were submitted to column purification (Genomed Jet quick PCR product purification, spin kit/250). Sequencing reactions were carry using 2 µl Big Dye terminator buffer (BDv.1.1 Applied biosystems, CA, USA). Cycling conditions included 1 min denaturation at 96ºC, followed by 25 cycles of 10 sec at 96ºC, 5 sec at 50ºC and 4 min at 60ºC, and a final infinite extension at 4ºC. A thermocycler ""MyCycler"" (Bio-Rad) was used. Samples were submitted to a second purification in Sephadex column (Ilustra Sephadex G-50). About 4 µl of purified product in 12 µl of Hi-DiTM formamide were than applied in an AbiPrism 3130 Genetic Analyzer. Software 3130 Data Collection v3.0 and Sequencing Analysis Software v5.2 were used for data collection and analysis, respectively.

Table 1 – Primers used in amplification and sequencing reactions.

Gene	Primer	Annealing temperature
NAT2	F: 5'ACACGAGGAAATCAAATGCTAAAG 3' R: 5'CTGCCACATCTGGGAGGAG 3'	59ºC
	F: 5' GCTGGGTCTGGAAGCTCCTC 3' R: 5' TTGGGTGATACATACACAAGGG 3'	59ºC
IL6	F: 5' GGAGACGCCTTGAAGTAACTGC 3' R: 5' AGTTTCCTCTGACTCCATCGCAG 3'	56.5ºC
ABCB11	F: 5' ACA CCG AGT ATC AAC ACA AAG C 3' R: 5' CCA GGA CAG TCT CAA TGT ATG C 3'	56ºC
NBAS	F: 5' GTTCTTTCAACTCTGTATTTCTGCC 3' R: 5' TGAAAGGTAGCAGAGGAAGGATG 3'	58ºC

4. Data analysis

The software Excel 2016 (6) (Microsoft Corporation) was used to process the obtained data and the software SPSS Statistics - Version 25 (SPSS, Inc., IBM Company) for its later statistical analysis. Hardy-Weinberg equilibrium was tested for all genes applying a chi-squared test. A *P* value of less than 0.05 was considered significant.

Univariate analyses were performed separately for each risk factor using the Fisher's exact test. Subsequently, a refinement of the evaluation was made by recoding as binary the variables mentioned before with respect to the presence of the characteristic that presented greater risk for the development of hepatotoxicity. Multicollinearity was determined between independent variables using Fisher's exact test. The identification of predictors of hepatotoxicity, as well as predictors of mild hepatitis and toxic hepatitis, either globally or by subgroups of age and gender, was performed through logistic regression. The adequacy of the logistic regression models was evaluated by the Hosmer-Lemeshow test.

Results

Of the 217 patients included in the study, 44.2% (96) developed some degree of antituberculosis drugs–induced HT, equally distributed between grade 1 and grade 2 plus 5 patients being classified as grade 3 (Table 2). All had ages between 17 and 85 years old, the mean age being 50 years old and weighting between 33 and 103 kg. Males correspond to 68.2% (148 subjects) of the population sample (Table 3).

Table 2 – Population characterization based on the grade of hepatotoxicity.

Presence of hepatotoxicity	**n (%)**
Without hepatotoxicity	121 (55.8%)
With hepatotoxicity	96 (42.2%)
Mild hepatotoxicity (grade 1)	45 (20.7%)
Toxic Hepatitis (grade 2)	46 (21.2%)
Liver transplantation or death (grade 3)	5 (2.3%)
Total	217 (100%)

n – number of patients

Table 3 describes the clinical characteristics assessed.

Table 3 – Characterization of non-genetic risk factors.

Characteristics	**HT n (%)**	**no HT n (%)**	**Global n (%)**	***p***	**OR (95% IC)**
Age					
< 60 years	53 (36.55%)	92 (63.45%)	145 (66.8%)		0.39 (0.22 - 0.69)
≥60 years	**43 (59.72%)**	**29 (40.28%)**	**72 (33.2%)**	**0.001**	**2.57 (1.44 - 4.6)**
Mean (sd)	54 (17.85)	46 (16.52)	50 (17.45)		
Gender					
Female	**38 (55.07%)**	**31 (44.93%)**	**69 (31.8%)**	**0.04**	**1.9 (1.07 - 3.39)**
Male	58 (39.19%)	90 (60.81%)	148 (68.2%)		0.53 (0.29 - 0.94)
Chronic Diseases					
No	31 (37.35%)	52 (62.65%)	83 (38.2%)	0.123	0.63 (0.36 - 1.11)
Yes	65 (48.51%)	69 (51.49%)	134 (61.8%)		1.58 (0.9 - 2.76)
Smoking habits					
No	47 (45.63%)	56 (54.37%)	103 (47.5%)	0.424	1.29 (0.74 - 2.26)
Former	17 (48.57%)	18 (51.43%)	35 (16.1%)		1.34 (0.65 - 2.79)
< 20 py	10 (37.04%)	17 (62.96%)	27 (12.4%)		0.77 (0.33 - 1.77)
≥ 20 py	12 (32.43%)	25 (67.57%)	37 (17.1%)		0.59 (0.28 - 1.25)
Weight (Kg)					
Mean (sd)	62.26 (11.48)	62.30 (11.94)	62.28 (11.66)		

HT – hepatotoxicity; n – number of patients; *p* – *p* value; OR -odds ratio; CI – confidence interval; sd – standard deviation; py – pack-year.

The frequencies of the *NAT2* genotypes found in this study are described in Table 4. Some genotypes within the same acetylator phenotype were ambiguous, and in these cases, if according to the literature there was a more frequent allele, the more frequent genotype was assumed.[32] The most frequent genotypes in the population were **4/*5B* and **4/*6A*, corresponding to the intermediate acetylators, and **5B/*6A* and **5B/*5B*, corresponding to slow acetylators. A total of 113 patients (52.1%) were genotyped as slow acetylators, 91 (42%) as intermediate acetylators and the other 13 patients (6%) were rapid acetylators (Table 4). The genotypic and phenotypic frequencies found in this study were similar to those previously described for the Portuguese population.[32,33]

Table 4 – Frequencies of *NAT2* genotypes and phenotypes.

Genotypes	**HT** **n (%)**	**no HT** **n (%)**	**Global** **n (%)**
Rapid Acetylator	**7 (53.85%)**	**6 (46.15%)**	**13 (6%)**
**4/*4*	6 (6.3%)	5 (4.1%)	11 (5%)
**4/*12[a]*	1 (1%)	0 (0%)	1 (0.5%)
**12A/*12[a]*	0 (0%)	1 (0.8%)	1 (0.5%)
Intermediate Acetylator	**30 (33.33%)**	**61 (66.67%)**	**91 (42%)**
**4/*5A*	1 (1%)	2 (1.7%)	3 (1.4%)
**4/*5B (*5A/*12A)*	14 (14.6%)	32 (26.4%)	46 (21.2%)
**4/*5D*	0 (0%)	1 (0.8%)	1 (0.5%)
**4/*6A (*6B/*13)*	10 (10.4%)	19 (15.7%)	29 (13.4%)
**4/*6C (*6A/*12A or *6B/*12B)*	2 (2.1%)	2 (1.7%)	4 (1.8%)
**5A/*14G or *5B/*14B (or *13/*14C)*	1 (0.8%)	0 (0%)	1 (0.5%)
**5A/*12B or *5B/*13*	2 (2.1%)	2 (1.7%)	4 (1.8%)
**6A/*13*	0 (0%)	2 (1.7%)	2 (0.9%)
**13/*14C*	0 (0%)	1 (0.8%)	1 (0.5%)
Slow Acetylator	**59 (52.21%)**	**54 (47.79%)**	**113 (52.1%)**
**5A/*5B*	0 (0%)	2 (1.7%)	2 (0.9%)
**5B/*6C (*5A/*6A)*	2 (2.1%)	2 (1.7%)	4 (1.8%)
**5B/6A (*5A/*6C)*	22 (22.9%)	15 (12.4%)	37 (17.1%)
**5A/*14E or *5B/*14A*	1 (1%)	0 (0.%)	1 (0.5%)
**5B/*5B*	17 (17.7%)	17 (14%)	34 (15.7%)
**5B/*5C*	0 (0%)	2 (1.7%)	2 (0.9%)
**5B/*7B*	1 (1%)	0 (0%)	1 (0.5%)
**5C/*6A*	2 (2.1%)	0 (0%)	2 (0.9%)
**6A/*6A*	11 (11.5%)	11 (9.1%)	22 (10.1%)
**6A/*7B*	2 (2.1%)	4 (3.3%)	6 (2.8%)
**6A/*14B*	1 (1%)	1 (0.8%)	2 (0.9%)

HT – hepatotoxicity; n – number of patients. For ambiguous genotypes, the less probable genotypes are in brackets, when the probability is similar “or” was used.

For statistical analysis, *NAT2* genotypes were grouped according to acetylator phenotype (Table 5). The frequencies of genotypes and alleles of the SNPS analyzed for *ABCB11*, *IL6*

and *NBAS* genes, in the global sample and in the two groups, with and without hepatotoxicity, are also described in table 5. Hardy-Weinberg equilibrium was confirmed for genotypes of the 4 genes analyzed (p > 0.05).

Table 5 – Frequencies of *NAT2* acetylator status and of genotypes and alleles from *ABCB11*, *IL6 597* and *NBAS* SNPs.

Genotypes/ Alleles	**HT n (%)**	**no HT n (%)**	**Global n (%)**	***p***	**OR (95% IC)**
Acetylation status					
SA	**59 (52.21%)**	**54 (47.79%)**	**113 (52.1%)**		**1.98 (1.15 - 3.41)**
IA	30 (33.33%)	61 (66.67%)	91 (42%)	**0.023**	0.46 (0.26 - 0.81)
RA	7 (53.85%)	6 (46.15%)	13 (6%)		1.51 (0.49 - 4.64)
IA+RA	**37 (35.58%)**	**67 (64.42%)**	**103 (48%)**	**0.014**	**0.51 (0.29 - 0.87)**
ABCB11					
CC	38 (53.52%)	33 (46.48%)	29 (13.4%)		1.75 (0.99 - 3.1)
TT	9 (31.03%)	20 (68.97%)	71 (32.7%)	0.095	0.52 (0.23 - 1.21)
TC	49 (41.88%)	68 (58.12%)	117 (53.9)		0.81 (0.47 - 1.39)
TT+TC	**58 (39.73%)**	**88 (60.27%)**	**178 (86.6%)**	**0.06**	**0.57 (0.32 - 1.01)**
C			0.597		
IL6					
GG	36 (37.5%)	60 (62.5%)	22 (10.1%)		0.61 (0.35 - 1.05)
AA	12 (54.55%)	10 (45.45%)	96 (44.2%)	0.176	1.59 (0.65 - 3.85)
AG	48 (48.48%)	51 (51.52%)	99 (45.6%)		1.37 (0.8 - 2.35)
AA+AG	**60 (49.59%)**	**61 (50.41%)**	**195 (89.9%)**	**0.098**	**1.64 (0.95 - 2.83)**
G			0.670		
NBAS					
TT	29 (47.54%)	32 (52.46%)	61 (28.1%)		1.2 (0.66 - 2.18)
CC	25 (45.45%)	30 (54.55%)	55 (25.3%)	0.747	1.07 (0.58 - 1.98)
TC	42 (41.58%)	59 (58.42%)	101 (46.5%)		0.82 (0.48 - 1.4)
T			0.514		

HT – hepatotoxicity; n – number of patients; *p* – *p* value; OR -odds ratio; CI – confidence interval.

A logistic multivariate analysis was performed including variables with a $p < 0.1$ in univariate analysis: age $\geq$ 60 years old ($p = 0.01$), female gender ($p = 0.04$), acetylator status ($p = 0.014$), *IL6* SNP ($p = 0.098$) and *ABCB11* SNP ($p = 0.06$) (Tables 3 and 5). Results (Table 6) show that age $\geq$ 60 years old ($p = 0.001$), female gender ($p = 0.015$) and SA status ($p = 0.044$) are associated with susceptibility to INH-induced HT. The possibility of HT is about the double for female patients (OR: 2.16; 95% CI: 1.16 – 4.03) and for subjects aged $\geq$ 60 years old the risk is about 3 times the risk of younger patients (OR: 2.93; 95% CI: 1.58 –

5.43). For slow acetylators, HT is 1.8 times more frequent than for rapid or intermediate acetylators. The presented model explains 15% of the susceptibility to HT.

The presence of CC genotype for *ABCB11* SNP or of AA genotype for the *IL6* SNP, although not reaching statistically significant values (p = 0.083 and p = 0.147, respectively) are associated with a tendency to increase the chance of HT (Table 6).

An analysis by age and gender was also performed but no specific variables for these subgroups were identified (Supplement Table 1).

Table 6 – Results of logistic multivariate analysis.

Variables	OR	95% CI	*P*
Age ≥ 60 years	**2.93**	**1.58 - 5.43**	**0.001**
Female	**2.16**	**1.16 - 4.03**	**0.015**
Slow Acetylator	**1.82**	**1.02 - 3.27**	**0.044**
ABCB11- CC	1.73	0.93 - 3.23	0.083
IL6 597 – AA	2.10	0.78 - 5.13	0.147

OR – odds ratio; CI – confidence interval; p – p value.

A logistic multivariate analysis was also performed for patients with grade 1 and grade 2 liver injury (Tables 7 and 9). For mild hepatitis (grade 2), age and SA status were the only risk factors identified (p = 0.027 and p = 0.043, respectively), with the presence of the CC genotype for the SNP of *ABCB11* gene showing an almost statistically significant effect (p = 0.055) (Table 7).

Table 7 - Results of logistic multivariate analysis for mild hepatitis.

Variables	OR	95% CI	*p*
Age ≥ 60 years	**2.47**	**1.11 -5.53**	**0.027**
Female	1.68	0.73 - 3.84	0.219
Slow Acetylator	**2.17**	**1.02 - 4.59**	**0.043**
ABCB11- CC	2.11	0.97 - 4.50	0.055
IL6 – AA	2.46	0.82 - 7.272	0.107

OR – odds ratio; CI – confidence interval; p – p value.

We than analyzed if there were different risk profiles for subgroups of patients with mild hepatitis according to age and gender (Table 8). The subgroup of women with age $\geq$ 60 years old was not assessed because of the reduced number of patients in this category. For women age < 60, SA status is a significant risk factor (OR: 6.97; 95% CI: 1.33 – 36.45; p = 0.022). For men, if age < 60, only SA status show a tendency to increase susceptibility to HT, whereas if age $\geq$ 60, genotype CC of *ABCB11*, is the best predictor (OR: 7.39; 95% CI: 1.33 – 41.04; p = 0.022) (Table 8).

Table 8 – Multivariate analysis of risk factors for mild hepatitis per age and gender.

Age	Gender	Risk factor	OR	95% CI	*p*
< 60 years	Female	**Slow Acetylator**	**6.97**	**1.33 - 36.54**	**0.022**
		ABCB11- CC	0.37	0.06 - 2.19	0.275
		IL6 – AA	1.59	0.14 - 18.42	0.709
	Male	**Slow Acetylator**	**3.35**	**0.93 - 12.00**	**0.063**
		ABCB11- CC	2.45	0.74 - 8.14	0.143
		IL6 – AA	2.48	0.51 - 12.07	0.260
≥ 60 years	Male	Slow Acetylator	0.58	0.12 - 2.88	0.581
		***ABCB11*- CC**	**7.39**	**1.33 - 41.04**	**0.022**
		IL6 – AA	0.61	0.04 - 8.84	0.722

OR – odds ratio; CI – confidence interval; *p* – *p* value.

In the subgroup with toxic hepatitis (grade 2), only age and gender remain significant (*p* = 0.000 and *p* = 0.005, respectively) (Table 9). Analysis by age and gender was also performed but no specific risk factor was identified (Supplement Table 2).

Given the reduced number of patients in the grade 3 it was not possible to obtain an adequate analysis for this category.

Table 9 – Logistic multivariate analysis for toxic hepatitis.

Variables	OR	95% CI	*p*
Age ≥ 60 years	**4.218**	**1.926 - 9.236**	**0.000**
Female	**3.084**	**1.410 - 6.743**	**0.005**
Slow Acetylator	1.893	0.869 - 4.125	0.108
ABCB11- CC	1.557	0.678 - 3.576	0.297
IL6 – AA	2.243	0.643 - 7.822	0.205

OR – odds ratio; CI – confidence interval; *p* – *p* value.

Discussion

In this study, we searched for genetic and non-genetic variants associated with susceptibility antituberculosis drugs-induced hepatotoxicity in a Caucasian population. Regarding non-genetic risk factors, results show that older age (≥ 60 years old) was associated with an increased risk of hepatotoxicity, not only when considering all affected patients but also when separating mild and toxic hepatitis. Other studies confirm that older age is a risk factor for INH-DILI.[34,35] However, the reasons for this association are unclear.[36] Aging is known to cause a decrease in renal function, blood flow, hepatic mass, and in cytochrome-mediated hepatic metabolism, all of which might affect drug pharmacokinetics'.[9] Despite these changes, liver function is essentially preserved in healthy older humans without additional diseases. It is also important to consider that other cellular stress factors may influence the development of INH-HT in susceptible patients. Polypharmacy, which is more common among the elderly, can lead to drug interactions, and might be another important factor.[36] Yet, in our population, chronic diseases imposing medication were not associated with increased risk.

Concerning gender, the risk of HT is about the double for female patients. This correlation has been previously described[37] although some studies failed to corroborate this finding.[38] It is important to notice that this association is based on the observation of a preponderance of women in many published retrospective and prospective DILI cohorts and may be partly explained by a greater follow-up of the female subjects during anti-TB therapy.[37]

The association between *NAT2* genotype and DILI susceptibility has been extensively explored**.** *NAT2* gene is highly polymorphic with SNPs at positions 191, 341, 434, 590 and 857 creating *missense* variants responsible for reduced enzyme stability, altered affinity for substrate or protein degradation by proteasome.[12] In this study, an association between SA status and hepatotoxicity was found, with HT being 1.8 times more frequent in SA. This value rises to 3.6 for females with < 60 years of age. A recent meta-analysis also describes a higher incidence of INH-induced liver injury in patients with genotypes conferring a slow acetylator phenotype (OR: 3.08; 95% CI: 2.29 – 4.15).[13] The more accepted explanation relies on the fact that NAT2 enzyme is responsible for about 88% of INH clearance, and plasma concentrations of INH and of INH-derived toxic metabolites such as acetyl-hydrazine are higher in SA than in AR or AI.[39–41]

Some recent trials compared standard INH dosing with pharmacogenetic-based dosing, demonstrating the significant therapeutic potential of the *NAT2* genotype-guided dosing of INH. According to these authors, the new dosing regimen, with lower doses for SA, significantly reduced the incidence of INH-DILI and early treatment failure. This strategy

allowed a 31% reduction in absolute risk of unfavorable events.[42] It was also suggested that non-SA could safely undergo treatment with INH with limited monitoring for DILI. Conversely, patients with SA status would likely benefit from closer surveillance.[11] This new regimes are also very important considering the fact that different populations have very different frequencies of acetylator status.

However, some studies have yielded contradictory results,[12] and in our study, unexpectedly, when analyzing only patients with grade 2 HT, genotype-defined acetylator status loss statistical significance. Differences in population genetic background, in environmental exposures and in study design may account for some discrepancies between studies. For instance, most studies identifying SA as a risk factor only perform univariate analysis. Another issue is that genotypes associated with slow acetylator phenotype may actually show differences in enzyme activity, which may interfere with the ability to detect significant associations. Also, for rare alleles no clear genotype/phenotype association has been established.

According to the literature, the most frequent Caucasian-specific SNP in the *ABCB11* gene is the rs2287622.[19] BSEP, which is encoded by *ABCB11*, transports many different molecules through the cellular membrane, namely bile acids, and a reduction of its expression may lead to excessive accumulation of toxic metabolites within cells. Loss-of-function variants in *ABCB11* gene are of considerable interest in relation to DILI, especially for cholestatic forms of the disease where bile acids accumulate within hepatocytes, resulting in local toxicity.[20] We could not highlight a statistically significant difference in the frequency of SNP rs2287622 between patients with or without HT. However, patients with CC genotype show a slightly increased risk of HT. This correlation is stronger in the group of patients with mild hepatitis, especially in older male individuals. No correlation was found when only females were considered. A recent study establish that rs2287622 was significantly associated with cholestatic/mixed liver injury.[19] Moreover, an *in vitro* study proved that the association of INH with RFP significantly down-regulated the expression of BSEP in liver extracts of mice.[21] A correlation between this polymorphism and intrahepatic cholestasis of pregnancy was confirmed by several studies.[15,16] It is believed, that during pregnancy, BSEP expression could be further reduced by the action of reproductive hormones, leading to cholestasis.[15,16] Intrahepatic cholestasis associated with consumption of oral contraceptives[17] and increased risk of DILI,[15,18,19] especially for cholestatic forms, was also described.[21,43] In our population, the lack of association with female gender could be due to the small number of women under study or to the fact that this SNP may only be associated with increased risk in women exposed to an abnormal hormonal profile.

In this study, AA genotype of *IL6* SNP, rs1800797, presented a tendency to increase the susceptibility to HT, although without statistically significance. This is in agreement with previous studies in animal models that show the influence of IL6 in the mechanisms of cell proliferation and apoptosis, especially with an increased expression of IL6 in the hepatic response to aggression.[23,44] This polymorphism is located in *IL6* gene regulatory region and has been associated with other pathologies, such as increased risk of colorectal cancer and more severe forms of distal interphalangeal osteoarthritis, suggesting it may influence the interleukin expression.[45,46]

The present study is, to our knowledge, the first to assess *NBAS* SNP rs2052438, localized in the gene regulatory region, as a risk factor for antituberculosis drugs-induced HT. The SNP is described as an A>G or C>T (c.-523C>T) depending on the DNA strand (reverse or forward) used as reference. *NBAS* is a highly conserved gene encoding a protein that seems to be involved in Golgi-to-endoplasmic reticulum retrograde transport of vesicles and to play a role as regulator of nonsense-mediated mRNA decay pathway[47]. Yet, the specific mechanisms involved in *NBAS* role in liver disease are not fully understood.[48] Variants in the *NBAS* gene have been associated with fever-triggered recurrent liver failure in children[26] and with a rare multisystemic phenotype including short stature, optic nerve atrophy, skeletal dysplasia and Pelger-Huet anomaly (SOPH syndrome, MIM614800), but without liver failure.[49] According to our data, the SNP evaluated is not associated with susceptibility to antituberculosis drugs-induced HT.

In this study, when mild and more severe liver injury were separately evaluated, different risk profiles emerged. Specifically for grade 2, only age $\geq$ 60 years old and female gender could be assigned as risk factors. Acetylator status was no longer determinant. It is possible, that particularly for progression of liver injury, other factors than INH toxic metabolites play a major role. The fact that considering all patients with HT, and exploring 4 genes and 5 clinical variables only 15% of the risk could be predicted supports the idiosyncratic nature of antituberculosis drugs-induced HT and its multifactorial and polygenic behavior.

Recent evidences also suggest that an immune mediated response may explain some INH-induced DILI events.[4] Immune responses may be associated with mild injury, allowing recovery by immune tolerance, or with severe hepatic lesion that make it difficult for patients to recover even if the drug is stopped, often leading to liver transplantation or death.[4] In fact, in both grade 1 and grade 3, it is more difficult to established causality, as in the first situation drugs are usually not interrupted, and in the second case, reintroduction of drugs is not possible. Unfortunately, *in vitro* studies to assess immune response to INH are usually not performed. If the injury is immune mediated, particularly if it is mediated by lymphocytes, treatment with agents such as anti-thymocyte globulin could be effective.[4]

This study has several limitations. First, the patients were submitted to treatment with 4 drugs simultaneously (isoniazid, rifampicin, pyrazinamide and etambutol), and although isoniazid is thought to be the most frequently involved in DILI**,** we cannot rule out the influence of antituberculosis drugs, namely of pyrazinamide in grade 1 and grade 3 patients. It is known that the incidence of DILI appears to be higher when INH is combined with rifampicin[5] and the risk increases further with the addition of pyrazinamide.[6] Ideally, in order to assess INH-DILI susceptibility factors, studies including genotyping and plasms INH toxic metabolites monitoring should be performed on individuals treated with isoniazid alone, as in latent TB.[50] Second, even though the present study is, to our knowledge, one with a higher number of cases analyzed, sample size should be extended, particularly with patients with grade 2 HT. Future studies combining multiple cohorts will be needed to create an appropriately powered sample size. Also, some variants previously associated with INH-DILI were not included. However, most variants were from single studies or were not significant in meta-analyses, like *GSTM1* and *GSTT1* null genotypes and *CYP2E1* rs6413432. Likewise, there was lack of information on other potential non-genetic risk factors, in particular dietary habits and body mass index. Lastly, the possible role of epigenetic factors, which may by themselves contribute to risk for DILI, or may modify effects of DNA sequence variants was not evaluate.[51,52]

Conclusion

Genetic variables, such as the *NAT2* SNPs determining slow acetylator phenotype and CC genotype for *ABCB11* SNP rs2287622, and non-genetic factors, specifically female gender and older age, were identified as risk factors for antituberculosis drugs-induced liver injury. However, the overall effect of the studied variables is modest, which suggests a complex interaction of many, still unknown genetic, epigenetic and environmental factors.

Knowledge of pharmacogenetic markers paves the way for more personalized forms of treatment and may even allow the development of integrated risk model to assess individual risk of DILI. This would permit a more appropriate selection of drugs and adjustment of their dosage, leading to reduction of possible adverse reactions, better adherence to therapy and lower dropout rate from the therapeutic plan. Personalized treatment is also beneficial in preventing and combating the resistant forms of tuberculosis. Unfortunately, translation of pharmacogenetics to the clinical practice is limited to few drugs. Antituberculosis drugs-induced DILI will continue to be a challenge to clinicians and researchers.

Acknowledgements

To Prof. Dr. Henriqueta C. Silva for awakening my interest in Genetics, for accepting to coordinate this project and for always being an example of dedication, availability, sincerity, rigor and hard work.

To Dr Celeste Alcobia, for co-coordinating this project, especially for collecting the data and clarifying the many doubts as they appeared.

To Dr. Bárbara Oliveiros, for collaborating in the statistical analyses and for helping me to understand better the world of Biostatistics.

To Dr. Luís Mesquita, for the knowledge transmitted, for the patience and constant stimulus to pursue scientific investigation.

To Dr. Fátima Castro and Dr. Miguel Villar, for their contribution to this work, making it a more complete and interesting project.

To my parents, for their unconditional support, encouragement and patience.

To all my friends and family, for their companionship, strength and support.

References

1. WHO. *Global Tuberculosis Report 2018*. 2018.

2. World Health Organization. Treatment of tuberculosis. *World Heal Organ Doc*. 2010.

3. Kim HW, Kim JS. Treatment of latent tuberculosis infection and its clinical efficacy. *Tuberc Respir Dis (Seoul)*. 2018;81(1):6-12.

4. Metushi I, Uetrecht J, Phillips E. Mechanism of isoniazid-induced hepatotoxicity: Then and now. *Br J Clin Pharmacol*. 2016;81(6):1030-1036.

5. Steele MA, Burk RF, DesPrez RM. Toxic Hepatitis with Isoniazid and Rifampin. *Chest*. 1991;99(2):465-471.

6. Durand F, Bernuau J, Pessayre D, et al. Deleterious influence of pyrazinamide on the outcome of patients with fulminant or subfulminant liver failure during antituberculous treatment including isoniazid. *Hepatology*. 1995;21(4):929-932.

7. Huang YS, Chern H Der, Su WJ, et al. Cytochrome P450 2E1 genotype and the susceptibility to antituberculosis drug-induced hepatitis. *Hepatology*. 2003;37(4):924-930.

8. Saukkonen JJ, Cohn DL, Jasmer RM, et al. An official ATS statement: Hepatotoxicity of antituberculosis therapy. *Am J Respir Crit Care Med*. 2006;174(8):935-952.

9. Ortega-Alonso A, Stephens C, Lucena MI, Andrade RJ. Case characterization, clinical features and risk factors in drug-induced liver injury. *Int J Mol Sci*. 2016;17(5):1-22.

10. Woodhead JL, Watkins PB, Howell BA, Siler SQ, Shoda LKM. The role of quantitative systems pharmacology modeling in the prediction and explanation of idiosyncratic drug-induced liver injury. *Drug Metab Pharmacokinet*. 2017;32(1):40-45.

11. Chan SL, Chua APG, Aminkeng F, et al. Association and clinical utility of NAT2 in the prediction of isoniazid-induced liver injury in Singaporean patients. *PLoS One*. 2017;12(10):1-16.

12. McDonagh EM, Boukouvala S, Aklillu E, et al. *Very Important Pharmacogene Information for N-Acetyltransferase 2*. Vol 24.; 2014 8:409-425.

13. Shi J, Xie M, Wang J, Xu Y, Liu X. Susceptibility of N-acetyltransferase 2 slow acetylators to antituberculosis drug-induced liver injury: a meta-analysis. *Pharmacogenomics*. 2015;16(18):2083-2097.

14. Sun F, Chen Y, Xiang Y, Zhan S. Drug-metabolising enzyme polymorphisms and predisposition to anti-tuberculosis drug-induced liver injury: A meta-analysis. *Int J Tuberc Lung Dis*. 2008;12(9):994-1002.

15. Dixon PH, Van Mil SWC, Chambers J, et al. Contribution of variant alleles of ABCB11 to susceptibility to intrahepatic cholestasis of pregnancy. *Gut*. 2009;58(4):537-544.

16. Piątek K, Kurzawińska G, Magiełda J, et al. The role of ABC transporters' gene polymorphism in the etiology of intrahepatic cholestasis of pregnancy. *Ginekol Pol*. 2018;89(7):393-397.

17. Meier Y, Zodan T, Lang C, et al. Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 1331T>C polymorphism in the bile salt export pump. *World J Gastroenterol*. 2008;14(1):38-45.

18. Lang C, Meier Y, Stieger B, et al. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet Genomics*. 2007;17(1):47-60.

19. Ulzurrun E, Stephens C, Crespo E, et al. Role of chemical structures and the 1331T>C bile salt export pump polymorphism in idiosyncratic drug-induced liver injury. *Liver Int*. 2013;33(9):1378-1385.

20. Daly AK. Are Polymorphisms in Genes Relevant to Drug Disposition Predictors of Susceptibility to Drug-Induced Liver Injury? *Pharm Res*. 2017;34(8):1564-1569.

21. Guo YX, Xu XF, Zhang QZ, et al. The inhibition of hepatic bile acids transporters Ntcp and Bsep is involved in the pathogenesis of isoniazid/rifampicin-induced hepatotoxicity. *Toxicol Mech Methods*. 2015;25(5):382-387.

22. Kishimoto T. IL-6: From its discovery to clinical applications. *Int Immunol*. 2010;22(5):347-352.

23. Jin X, Zimmers TA, Perez EA, Pierce RH, Zhang Z, Koniaris LG. Paradoxical effects of short- and long-term interleukin-6 exposure on liver injury and repair. *Hepatology*. 2006;43(3):474-484.

24. Chen D, Fan J, Guo F, Qin S, Wang Z, Peng Z. Novel Single Nucleotide Polymorphisms in Interleukin 6 Affect Tacrolimus Metabolism in Liver Transplant Patients. *PLoS One*. 2013;8(8):8-12.

25. Wang Z, Wu S, Liao J, et al. Interleukin-6 and rs1800796 locus single nucleotide polymorphisms in response to hypoxia/reoxygenation in hepatocytes. *Int J Mol Med*. 2016;38(1):192-200.

26. Haack TB, Staufner C, Köpke MG, et al. Biallelic Mutations in NBAS Cause Recurrent Acute Liver Failure with Onset in Infancy. *Am J Hum Genet*. 2015;97(1):163-169.

27. Hasosah MY, Iskandarani AI, Shawli AI, Alsahafi AF, Sukkar GA, Qurashi MA. Neuroblastoma amplified sequence gene mutation: A rare cause of recurrent liver failure in children. *Saudi J Gastroenterol*. 2017;23(3):206-208.

28. Li J-Q, Qiu Y-L, Gong J-Y, et al. Novel NBAS mutations and fever-related recurrent acute liver failure in Chinese children: a retrospective study. *BMC Gastroenterol*. 2017;17(1):77.

29. Wang J, Pu Z, Lu Z. Targeted next-generation sequencing reveals two novel mutations of NBAS in a patient with infantile liver failure syndrome-2. *Mol Med Rep*. 2018;17(2):2245-2250.

30. Calvo PL, Tandoi F, Haak TB, et al. NBAS mutations cause acute liver failure: When acetaminophen is not a culprit. *Ital J Pediatr*. 2017;43(1):4-9.

31. Aithal GP, Watkins PB, Andrade RJ, et al. Case Definition and Phenotype Standardization in Drug-Induced Liver Injury. *Clin Pharmacol Ther*. 2011;89(6):806-815.

32. Lemos MC, Regateiro FJ. N-acetyltransferase genotypes in the Portuguese population. *Pharmacogenetics*. 1998;8(6):561-564.

33. Lemos MC, Cabrita FJ, Silva HA, Vivan M, Placido F, Regateiro FJ. Genetic polymorphism of CYP2D6, GSTM1 and NAT2 and susceptibility to haematological neoplasias. *Carcinogenesis*. 1999;20(7):1225-1229.

34. Fountain FF, Tolley E, Chrisman CR, Self TH. Isoniazid Hepatotoxicity Associated With Treatment of Latent Tuberculosis Infection. *Chest*. 2005;128(1):116-123.

35. Onji M, Fujioka S, Takeuchi Y, et al. Clinical characteristics of drug-induced liver injury in the elderly. *Hepatol Res*. 2009;39(6):546-552.

36. Chalasani N, Bjornsson E. Risk Factors for Idiosyncratic Drug-Induced Liver Injury. *Gastroenterology*. 2011;138(7):2246-2259.

37. Lauren N. Bell, Ph.D.1 and Naga Chalasani MD. Epidemiology of Idiosyncratic Drug-Induced Liver Injury. *Genet Program Evolvable Mach*. 2008;9(2):107-108.

38. Ghabril M, Chalasani N, Björnsson E. Drug-induced liver injury: a clinical update. *Curr Opin Gastroenterol*. 2010;26(3):222-226.

39. Kinzig-schippers M, Tomalik-scharte D, Jetter A, et al. Should We Use N-Acetyltransferase Type 2 Genotyping To Personalize Isoniazid Doses?. *Society*. 2005;49(5):1733-1738.

40. Donald PR, Parkin DP, Seifart HI, et al. The influence of dose and N-acetyltransferase-2 (NAT2) genotype and phenotype on the pharmacokinetics and pharmacodynamics of isoniazid. *Eur J Clin Pharmacol*. 2007;63(7):633-639.

41. Kubota R, Ohno M, Hasunuma T, Iijima H, Azuma J. Dose-escalation study of isoniazid in healthy volunteers with the rapid acetylator genotype of arylamine N-acetyltransferase 2. *Eur J Clin Pharmacol*. 2007;63(10):927-933.

42. Azuma J, Ohno M, Kubota R, et al. NAT2 genotype guided regimen reduces isoniazid-induced liver injury and early treatment failure in the 6-month four-drug standard treatment of tuberculosis: A randomized controlled trial for pharmacogenetics-based therapy. *Eur J Clin Pharmacol*. 2013;69(5):1091-1101.

43. Chen R, Wang J, Tang S, et al. Role of polymorphic bile salt export pump (BSEP, ABCB11) transporters in anti-tuberculosis drug-induced liver injury in a Chinese cohort. *Sci Rep*. 2016;6(January):1-7.

44. Streetz K, Luedde T, Manns M, Trautwein C. Interleukin 6 and liver regeneration. *Gut*. 2000;47(2):309-312.

45. Curtin K, Wolff RK, Herrick JS, Abo R, Slattery ML. Exploring multilocus associations of inflammation genes and colorectal cancer risk using hapConstructor. *BMC Med Genet*. 2010;11(1):170.

46. Kämäräinen OP, Solovieva S, Vehmas T, et al. Common interleukin-6 promoter variants associate with the more severe forms of distal interphalangeal osteoarthritis. *Arthritis Res Ther*. 2008;10(1):1-9.

47. Longman D, Hug N, Keith M, et al. DHX34 and NBAS form part of an autoregulatory NMD circuit that regulates endogenous RNA targets in human cells, zebrafish and Caenorhabditis elegans. *Nucleic Acids Res*. 2013;41(17):8319-8331.

48. Staufner C, Haack TB, Köpke MG, et al. Recurrent acute liver failure due to NBAS deficiency: phenotypic spectrum, disease mechanisms, and therapeutic concepts. *J Inherit Metab Dis*. 2016;39(1):3-16.

49. Maksimova N, Hara K, Nikolaeva I, et al. Neuroblastoma amplified sequence gene is associated with a novel short stature syndrome characterised by optic nerve atrophy and Pelger-Huët anomaly. *J Med Genet*. 2010;47(8):538-548.

50. Ng CS, Hasnat A, Al Maruf A, et al. N-acetyltransferase 2 (NAT2) genotype as a risk factor for development of drug-induced liver injury relating to antituberculosis drug treatment in a mixed-ethnicity patient group. *Eur J Clin Pharmacol*. 2014;70(9):1079-1086.

51. Urban TJ, Goldstein DB, Watkins PB. Genetic basis of susceptibility to drug-induced liver injury: What have we learned and where do we go from here? *Pharmacogenomics*. 2012;13(7): 735-738.

52. Murata K, Hamada M, Sugimoto K, Nakano T. A novel mechanism for drug-induced liver failure: Inhibition of histone acetylation by hydralazine derivatives. *J Hepatol*. 2007;46(2):322-329.

Supplement Tables

Supplement Table 1 – Multivariate Analysis of Risk Factors for DILI per age and gender.

	Female <60Y			**Female ≥60Y**			**Male <60Y**			**Male ≥60Y**		
Variables	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
Slow Acetylator	**3.59**	**1.04 - 12.41**	**0.043**	-	-	-	2.15	0.88-5.23	0.093	0.84	0.24 - 2.95	0.787
ABCB11 - CC	1.12	0.31 - 4.10	0.862	-	-	-	1.47	0.57-3.78	0.419	4.12	0.96 - 17.62	0.057
IL6 - AA	2.41	0.30 - 19.22	0.406	-	-	-	1.88	0.52-6.89	0.339	0.99	0.12 - 8.24	0.993

OR – odds ratio; CI – confidence interval; *p* – *p* value.

Supplement Table 2 – Multivariate Analysis of Risk Factors for Toxic Hepatitis per age and gender.

Age	**Gender**	**Variables**	**OR**	**95% CI**	***p***
< 60 anos	F	Slow Acetylator	2.522	0.433 - 14.693	0.304
		ABCB11- CC	2.426	0.376 - 15.665	0.352
		IL6 – AA	4.700	0.255 - 86.472	0.298
	M	Slow Acetylator	2.841	0.776 - 10.398	0.115
		ABCB11- CC	1.155	0.306 - 4.365	0.831
		IL6 – AA	2.108	0.353 - 12.583	0.413
≥ 60 years	M	Slow Acetylator	0.58	1.228 - 0.240	6.271
		ABCB11- CC	7.39	1.932 - 0.287	12.989
		IL6 – AA	0.61	1.474 - 0.113	19.180

OR – odds ratio; CI – confidence interval; *p* – *p* value.