Effect of gene–gene and gene–environment interactions associated with antituberculosis drug-induced hepatotoxicity

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Objectives **This study evaluated the association between environmental factors and genetic variations in enzymes that metabolize antituberculosis (anti-TB) drugs [arylamine *N*-acetyltransferase 2, cytochrome P450 2E1 (CYP2E1), glutathione *S*-transferase theta 1 (GSTT1), and glutathione *S*-transferase mu 1] with antituberculosis drug-induced hepatotoxicity (ATDH). We also investigated the potential gene–gene and gene–environment interactions as well as their association with ATDH development in a population of hospitalized TB patients from Buenos Aires.**

Patients and methods **We investigated 364 TB patients who received anti-TB drugs. Physicians collected demographic and clinical data to identify environmental risk factors for ATDH development. Polymorphisms were detected using gene sequencing, PCR, and PCR-restriction fragment length polymorphisms. A binary logistic regression analysis was carried out to compare the results of TB patients with and without the development of hepatotoxicity. The multifactor dimensionality reduction method was used to examine genetic and environmental interactions in association with ATDH.**

Results **This study suggests that the slow acetylator profile [odds ratio (OR): 3.02; 95% confidence interval (CI): 1.82–5.00; $P < 0.001$], genotypes carrying the c2 variant (OR: 2.16; 95% CI: 1.33–3.51; $P = 0.002$) or the A4 variant of *CYP2E1* (OR: 2.13; 95% CI: 1.06–4.29; $P = 0.050$), and female sex (OR: 1.94; 95% CI: 1.20–3.14; $P = 0.006$) were independent predictor variables for ATDH. Patients carrying the slow acetylator profile and the c2 variant showed an increased risk (OR: 7.068; 95% CI: 3.34–14.95; $P < 0.001$). We also identified a synergic interaction (epistasis) between *GSTT1* and *CYP2E1* associated with an increased risk for ATDH. A meaningful gene–environment interaction was associated with an increased risk of ATDH [testing balance accuracy = 0.675 ($P = 0.001$) and cross-validation consistency = 10/10].**

Conclusion **ATDH is a severe and prevalent adverse drug reaction and leads to drug discontinuation in 11% of TB patients. Our study created a prediction model that properly classified the 67.5% of TB patients in their risk of developing ATDH. The considerable number of TB patients in our country supports the use of pharmacogenetic testing and a comprehensive clinical history to identify patients with a high risk of suffering hepatotoxicity.** ***Pharmacogenetics and Genomics*** **00:000–000**

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Introduction

Tuberculosis (TB) is the second leading cause of death from an infectious disease worldwide after HIV and it remains a major worldwide health problem, with an estimated 9.0 million new cases and 1.5 million deaths in 2013 [1,2]. Latent tuberculosis infection (LTBI) affects approximately one-third of the world's population. Approximately 10% of individuals with LTBI develop active TB disease in their lifetime, with most individuals developing it within 5 years after the initial infection [3]. LTBI is frequently treated with isoniazid (INH) monotherapy because of its widely known effectiveness against *Mycobacterium tuberculosis* [4]. Treatment of acute TB involves a multidrug therapy with first-line drugs [INH, rifampicin (RMP), ethambutol, and pyrazinamide (PZA)] for the first 2 months. Patients continue treatment with a sterilization phase of 4–6 months with INH and RMP [5].

Drug-induced liver injury (DILI) is becoming a significant public health issue because of its potential impact on patients, new drug development, and increased health costs [6]. Antituberculosis drug-induced hepatotoxicity (ATDH) is a severe, potentially fatal, and

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prevalent adverse drug reaction, and it leads to drug discontinuation in 11% of TB patients treated with a PZA, RMP, and INH regimen [7]. Several surveys found that INH was primarily responsible for liver injury [8–10]. A recently published meta-analysis suggested that INH monotherapy led to higher rates of hepatotoxicity than RMP monotherapy [11]. Sarma *et al.* [12] reported that combined INH and RMP treatment increased the risk of hepatotoxicity through the induction of amidase enzyme, which increased concentrations of a toxic metabolite of INH, hydrazine (Hz).

INH is metabolized in the liver and it is primarily excreted in the urine. INH is acetylated by *N*-acetyltransferase 2 (NAT2) in the liver to form acetyl isoniazid and hydrolyzed by amidase to acetyl hydrazine, which may be re-acetylated by NAT2 to diacetyl hydrazine (nontoxic). However, acetyl hydrazine may also be oxidized to hepatotoxic intermediates by cytochrome P450 2E1 (CYP2E1) [13]. An alternative pathway directly hydrolyzes INH to form Hz [13]. RMP is a potent inducer of CYP2E1 activity that also reduces NAT2 activity [14,15]. The formation of reactive metabolites may lead to oxidative stress, mitochondrial damage, and initiation of prodeath signaling pathways [16]. The detoxification of toxic INH metabolites involves conjugation reactions by glutathione *S*-transferases (GSTs), which is a family of phase II enzymes that play an important protective role as intracellular free radical scavengers [17,18].

Some studies of the pharmacogenetics of TB found an association between genetic variations in these enzymes and ATDH development [10,19–21]. The *NAT2* gene was studied widely, and several surveys found an association between the slow acetylator phenotype and ATDH development [10,19,20,22]. The single nucleotide polymorphism (SNP) *C-1053T* in the *CYP2E1* gene, also called *c1/c2* or the *RsaI* variant, is one of the most studied genetic variants that is also associated with ATDH susceptibility [21,23]. Deficiency in GST activity, caused by glutathione *S*-transferase mu 1 (*GSTM1*) and glutathione *S*-transferase theta 1 (*GSTT1*) null mutations, was associated with idiosyncratic DILI from a large variety of drugs [24] and ATDH development [18,25].

TB is a re-emerging infectious disease with a high level of hepatotoxicity (25.6%) in hospitalized TB patients in Argentina [19]. Therefore, the present survey investigated the environmental factors and pharmacogenetic variation involved in INH metabolism and evaluated their association with ATDH independently and/or through complex interactions between them in a sample of hospitalized TB patients in Buenos Aires.

Patients and methods

Patients

We enrolled a total of 364 patients who were treated for active TB between 2009 and 2015 at the Hospital of Infectious Disease 'Dr F. J. Muñiz' (Buenos Aires, Argentina). The ethics committee of the Hospital reviewed and approved the study protocol, which was performed according to the principles of the Declaration of Helsinki. All patients provided written informed consent to participate in the study. Pulmonologists carefully selected the TB patients, obtained written informed consent, and performed blood extractions and clinical data collection. Nineteen of the 364 patients had incomplete clinical data and were excluded. The inclusion and exclusion criteria have been described previously [19].

The following data were collected from patients: sex, age, nationality, BMI (BMI $< 16\ kg/m^2$ was considered severely underweight and indicative of malnourishment), smoking habits, alcohol abuse (>60 g/day in men and > 40 g/day in women), any associated diseases (diabetes, HIV infection, any immunosuppressive treatments, Chagas disease, and/or intestinal parasites), history of illegal drug abuse, and ATDH development. Hepatotoxicity was defined as alanine aminotransferase levels at least three times the upper limit of normal with hepatitis symptoms (nausea, vomiting, abdominal pain, and/or hyporexia) and/or jaundice reported or alanine aminotransferase level at least five times the upper limit of normal in the absence of symptoms [19].

All patients had normal liver tests before they received anti-TB drugs, which allowed us to differentiate healthy livers from acute or chronic alcoholic liver disease.

All patients were monitored clinically. Symptomatic patients were monitored using weekly biochemical assessments. Asymptomatic patients were monitored using biochemical assessments every 15 days.

DNA samples

Whole-blood samples from TB patients were collected in tubes containing ethylenediaminetetraacetic acid. Genomic DNA was extracted using an automated platform using the QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The extracted genomic DNA was analyzed using 2% agarose gel electrophoresis.

Genotyping

DNA samples from each patient were analyzed for polymorphisms in *CYP2E1*, *NAT2*, *GSTM1*, and *GSTT1* genes.

Seven of the most common *NAT2* polymorphisms [rs1801279 (G191A), rs1041983 (C282T), rs1801280 (T341C), rs1799929 (C481T), rs1799930 (G590A), rs1208 (A803G), and rs1799931 (G857A)] were analyzed. The entire open reading frame of the *NAT2* gene was amplified using PCR, and the product was purified and

sequenced on both strands to genotype polymorphisms. *NAT2* polymorphism genotyping has been described previously [26]. The NAT2PRED web server was used to infer the acetylator phenotype (rapid, intermediate, and slow acetylators) [27].

The variable-number tandem repeat (VNTR) polymorphism located in the 5′-untransleted region (−2173 and −1946) of the *CYP2E1* gene was screened using PCR-restriction fragment length polymorphisms (primers: F-5′-AAGCCTGAAGAACACCCTGC-3′ and R-5′-CTGTGGTCTGAGTCCTCTGG-3′). PCR amplification was performed as described previously, with omission of the final extension step [28]. Four different VNTR alleles were described: *A1* (five repeats), *A2* (six repeats; also named 1C), *A3* (six repeats), and *A4* (eight repeats; also named 1D) [29]. PCR products were digested with the restriction enzyme *NlaIV* as described by Catanzaro *et al.* [30] Genotyping for the *CYP2E1* SNP *C-1053T*, also named the *c1/c2* polymorphism and/or the *RsaI* variant (rs2031920), was performed using PCR-restriction fragment length polymorphisms. A 413-bp fragment was treated with an *RsaI* restriction enzyme. Loss of the *RsaI* restriction site indicates the presence of the *c2* allele. PCR and digestion conditions have been described previously [19].

The *GSTM1* null polymorphism was performed using multiplex PCR (primers: F-5′-GAACTCCCTGAAAAG CTAAAGC-3′ and R-5′-GACAGGGTTTCATCATGT TGG- 3′) under the following conditions: (a) an initial denaturation at 95°C for 5 min; (b) 37 thermal cycles of 1 min at 95°C to denature the DNA, 45 s at 55°C to anneal the primers, and 45 s at 72°C for DNA polymerization; and (c) a 10-min incubation at 72°C to ensure complete product extension. A 345-bp band (Factor II gene) and a 219-bp band (*GSTM1* gene) were identified in patients who were *GSTM1* wild type. The latter band was missing in individuals who were *GSTM1* null. The *GSTT1* null polymorphism was performed using multiplex PCR (primers: F-5′- TTCCTTACTGGTCCT CACATCTC-3′ and R-5′- TCACCGGATCATGGCC AGCA-3′) under the following conditions: (a) an initial denaturation at 95°C for 5 min; (b) 38 thermal cycles of 50 s at 95°C to denature the DNA, 40 s at 58°C to anneal the primers, and 45 s at 72°C for DNA polymerization; and (c) a 10-min incubation at 72°C to ensure complete product extension. *Factor II* gene was used as an internal control (primers: F-5′-TCTAGAAACCAGTTGCCTG GC-3′ and R-5′-ATAGCACTGGGAGCATTGAAGC-3′). A 345-bp band (*Factor II* gene) and a 459-bp band (*GSTT1* gene) were identified in wild-type *GSTT1*, and a single 345-bp band was observed in the *GSTT1* null genotype.

All PCR products and their corresponding digested fragments were analyzed using 2% agarose gel electrophoresis with SYBR Safe staining (Invitrogen, Paisley, UK).

Statistical analysis

The SPSS (version 23.0 for Windows; IBM SPSS Inc., Chicago, Illinois, USA) was used for statistical analyses. The data were not normally distributed, and results are presented as medians and percentiles (25 and 75) or percentages for categorical variables. Nonparametric tests (independent-samples median test) were used to compare the quantitative data and the χ^2-test was used to compare proportions.

To predict ATDH, we used the χ^2 outcomes and those independent variables with a significance *P*-value less than 0.1 were selected to a further binary logistic regression. A binary logistic regression (method: forward conditional) analysis was carried out to examine the association between ATDH and the independent variables selected. The odds ratio (OR) with a 95% confidence interval (CI) was estimated to assess the strength of this association. Inheritance models (recessive, dominant, additive, etc.) were established using a likelihood test ratio. We used the Hardy–Weinberg (HW) test to evaluate the heredity equilibrium.

Testing for genetic and environmental interactions in association with ATDH was performed using the multifactor dimensionality reduction (MDR) method [31], version beta 3.0.2. All two-way to five-way models were evaluated. Balanced accuracy in the context of 10-fold cross-validation was used to assess model quality. An overall best model was selected that had the maximum accuracy in the testing data. Cross-validation consistency (CVC) was recorded. Statistical significance was determined by comparing the average CVC from the observed data with the distribution of average consistencies under the null hypothesis of no associations derived empirically from 1000 permutations. The entropy decomposition provides distinguishable additive and nonadditive effects, and thus identifies the size and direction (synergy or redundancy) of epistasis among attributes. A positive information gain (positive entropy) indicates a synergistic or a nonadditive effect, whereas a negative information gain value (negative entropy) indicates redundancy or correlation. Interaction dendrograms were used to visualize interaction and hierarchical clustering was used to construct a dendrogram that places strongly interacting attributes close together at the leaves of the tree.

A *P*-value less than 0.05 was considered statistically significant.

Results

This study included 345 hospitalized TB patients and 96 patients developed ATDH (cases). The median age of the total study population was 29 (23–36) years and 128 (46.9%) patients were women. The median BMI was 20.8

(18.7–22.4) kg/m^2 and 5.7% were severely underweight. HIV (5.8%) and diabetes (3.2%) were the most frequently associated diseases. A total of 24.2% of the patients were smokers, 34.6% were alcohol abusers, and 14.1% were illicit drug users. Table 1 shows the demographic and clinical data of TB patients with and without ATDH development. Alcohol abuse was more frequent in controls ($P = 0.002$). An increased risk of ATDH development was linked to female sex (OR: 1.94; 95% CI: 1.20–3.14; $P = 0.006$).

Table 2 describes the genotypic frequencies of seven common *NAT2* SNPs. All polymorphisms were in HW equilibrium, except G191A, because of its uncommon occurrence in these populations. Therefore, G191A was not investigated further. We found that none of the six *NAT2* SNPs was associated with ATDH (data not shown)

Table 1 Demographic and clinical characteristics of patients with and without antituberculosis drug-induced hepatotoxicity

Variables	Without ATDH ($n = 249$)	With ATDH ($n = 96$)	*P*
Age (years)	28 (23.0–36.8)	30 (23.3–35.8)	0.279
Sex (female)	32.1 (80)	47.9 (46)	**0.006**
BMI (kg/height^2)	20.8 (18.8–22.2)	20.5 (18.4–23.4)	0.734
Severely underweight	6.1 (15)	4.5 (4)	0.790
Associated diseases	21.8 (54)	15.8 (15)	0.216
Smokers	26.1 (65)	18.8 (18)	0.152
Alcoholics	39.4 (98)	21.9 (21)	**0.002**
Drug abusers	16.1 (40)	8.3 (8)	0.063

Data are expressed as median with percentiles (25 and 75) or *n* (%).
ATDH, antituberculosis drug-induced hepatotoxicity.
Significant associations are indicated by bold text.

Table 2 Distribution of genotypic frequencies of *N*-acetyltransferase 2 variants in cases with antituberculosis drug-induced hepatotoxicity and controls

Genotypes	Without ATDH ($n = 247n$)	With ATDH ($n = 96$)	*P*
C282T			0.175
CC	40.1 (99)	38.5 (37)	
CT	47 (116)	40.6 (39)	
TT	12.9 (32)	20.8 (20)	
T341C			0.971
TT	51.4 (127)	50 (48)	
TC	39.3 (97)	40.6 (39)	
CC	9.3 (23)	9.4 (9)	
C481T			0.880
CC	51.8 (128)	50 (48)	
CT	38.9 (96)	41.7 (40)	
TT	9.3 (23)	8.3 (8)	
G590A			0.392
GG	73.7 (182)	67.7 (65)	
GA	25.5 (63)	30.2 (29)	
AA	0.8 (2)	2.1 (2)	
A803G			0.858
AA	49.8 (123)	46.9 (45)	
AG	38.5 (95)	41.7 (40)	
GG	11.7 (29)	11.5 (11)	
G857A			0.152
GG	62.8 (155)	55.2 (53)	
GA	30.8 (76)	32.3 (31)	
AA	6.4 (16)	12.5 (12)	

ATDH, antituberculosis drug-induced hepatotoxicity; NAT2, *N*-acetyltransferase 2.

and for the genotype distribution, we did not find a significant deviation from HW equilibrium.

The slow acetylator profile was higher in patients who developed hepatotoxicity ($P < 0.001$) (Table 3). We tested the inheritance model on the acetylator profile using logistic regression. The best model fits when the slow acetylator profile is used as a recessive variable, and rapid and intermediate acetylator as only one variable (rapid acetylator). A significant association was observed between a slow acetylator profile and ATDH development (OR: 2.93; 95% CI: 1.78–4.82; $P < 0.001$) (Table 3).

The *c1/c1* genotype of the *CYP2E1* gene was the most frequent in our population and both groups studied. Variants that contained the *c2* allele (*c1/c2* and *c2/c2* genotypes) were more prevalent in patients who developed hepatotoxicity ($P = 0.004$). We tested the inheritance model on the *CYP2E1 C-1056T* genotype using logistic regression and the model fit using the *c2* allele as a dominant variable (*c1/c2* and *c2/c2* vs. *c1/c1*). Our results indicated that the c2 allele was associated with ATDH development (OR: 2.19; 95% CI: 1.32–3.48; $P = 0.002$). The VNTR polymorphism of the *CYP2E1* gene was genotyped, and only four variants were found: *A1/A2*, *A2/A2* (*or 1C/1C*), *A2/A4* (*or 1C/1D*), and *A4/A4* (*or 1D/1D*). Genotypes containing the *A3* allele were not detected in our population. Only one patient was a carrier of the *A1* allele and this patient was not considered in further analyses. The homozygous wild-type genotype (*A2/A2*) was the most frequent in all the groups studied (>83%). The *A4* variant was poorly represented in our studied population, but we found that patients who were carriers of the *A4* allele showed a trend for an increased risk of ATDH (OR: 1.99; 95% CI: 1.00–3.96; $P = 0.050$) (Table 3).

GSTM1 and *GSTT1* genotyping showed an increased frequency of the *GSTM1* null genotype in our population (46.7%), but the *GSTT1* null genotype frequency was lower and present in 19.1% of TB patients. Analyses of the combined variant of GSTT1 and GSTM1 indicated that only 7% of patients showed alterations of both GST (null/null). These frequency distributions were maintained between the groups and no significant differences were observed between the groups in individual or combined *GST* genotypes (Table 3).

OR estimations related to genetic variables were adjusted for sex, which was the only environmental variable associated with an increased risk for ATDH development to avoid environmental factors as potential confounders. Slow acetylator profile and the *CYP2E1* polymorphisms *c2* and *A4* were independent predictors for ATDH.

Gene–gene and gene–environment interactions

The combined distributions of the acetylator profiles of NAT2 with *CYP2E1 c1/c2* genotypes were analyzed. An increased risk was observed in patients who were slow

Table 3 Genetic frequencies of cytochrome P450 2E1, glutathione *S*-transferases, and *N*-acetyltransferase 2 acetylator profile in cases with antituberculosis drug-induced hepatotoxicity and controls

	Total sample ($n=345$)	Without ATDH ($n=249$)	With ATDH ($n=96$)	*P*	OR (95% CI) (*P*)	OR adjusted by sex (95% CI) (*P*)
NAT2 phenotype						
RA	12.8 (44)	12.5 (31)	13.5 (13)	**< 0.001**	SA vs. others: 2.931 (1.783–4.819) ($P<0.001$)	SA vs. others: 3.017 (1.822–4.996) ($P<0.001$)
IA	38.8 (133)	46.2 (114)	19.8 (19)			
SA	48.4(166)	41.3 (102)	66.7 (64)			
CYP2E1						
C-1053T						
c1/c1	63.5 (219)	68.7 (171)	50 (48)	**0.004**	c2 carriers vs. c1/c1: 2.192 (1.355–3.548) ($P=0.001$)	c2 carriers vs. c1/c1: 2.156 (1.325–3.507) ($P=0.002$)
c1/c2	31.6 (109)	26.5 (66)	44.8 (43)			
c2/c2	4.9 (17)	4.8 (12)	5.2 (5)			
VNTR						
A2/A2	88.7 (305)	90.8 (226)	83.2 (79)	**0.047**	A4 carriers vs. others: 1.990 (1.001–3.958) ($P=0.050$)	A4 carriers vs. others: 2.131 (1.059–4.290) ($P=0.050$)
A2/A4	11 (38)	8.8 (22)	16.8 (16)			
A4/A4	0.3 (1)	0.4 (1)	0			
GSTT1						
Wild	80.9 (279)	80.7 (201)	81.3 (78)	0.911	–	–
Null	19.1 (66)	19.3 (48)	18.8 (18)			
GSTM1						
Wild	53.3 (184)	54.2 (135)	51 (49)	0.596	–	–
Null	46.7 (161)	45.8 (114)	49 (47)			
GSTT1 and GSTM1						
Wild/wild	41.2 (142)	42.2 (105)	38.5 (37)	0.741	–	–
Wild/null	51.9 (179)	50.6 (126)	55.2 (53)			
Null/null	7 (24)	7.2 (18)	6.3 (6)			

ATDH, antituberculosis drug-induced hepatotoxicity; CI, confidence interval; CYP2E1, cytochrome P450 2E1; GSTM1, glutathione *S*-transferase mu 1; GST, glutathione S-transferase; IA, intermediate acetylator; NAT2, *N*-acetyltransferase 2; OR, odds ratio; RA, rapid acetylator; SA, slow acetylator; VNTR, variable number tandem repeat. Significant associations are indicated by bold text.

acetylators and carried the *c2* allele of *CYP2E1* (OR: 6.60; 95% CI: 3.14–13.86; $P<0.001$) in ATDH prediction (Table 4). After adjusting for sex, this increased risk remained. Therefore, we used the MDR method to improve our understanding of the gene–gene and gene–environment interactions [32].

Table 5 presents the MDR analysis for each factor considered and summarizes the average CVC, average testing balance accuracy (TBA) obtained, and the empirical *P*-value derived from permutation testing. The best model of gene–gene analysis that maximized TBA and CVC was the two-factor model that included the genotypic variant of *NAT2* and the genetics variants of *CYP2E1* (marked as SNP C-1053T and VNTR). This model showed a maximum TBA of 0.653 ($P=0.001$), which indicates the ability to properly classify 65.3% of the individuals included in the analysis, and a CVC of 10 out of 10 (10/10), which indicates that this particular marker combination was chosen by MDR across the 10 runs. Gene–environmental interaction analyses that only included the identified environmental risk factor (sex) showed that the best model that maximized both parameters was the three-factor model [TBA = 0.675 ($P=0.001$) and CVC = 10/10] that included sex in the gene–gene model.

The interaction dendrogram (Fig. 1a) showed a strong synergistic effect between the markers analyzed in *GSTT1* and in *CYP2E1* genes associated with the risk for ATDH. Sex and the NAT2 acetylator profile showed the highest degree of redundancy in their interactions. We also found that the sex/NAT2 cluster and the *GSTT1*/*CYP2E1* cluster showed a redundant interaction, but to a lower degree. No interaction was found between *GSTM1* and other variables. The interaction entropy graph indicated that the NAT2 acetylator profile eliminated 4.88% of entropy and the main large independent effect in ATDH development, followed by *CYP2E1* (2.15%) and sex (1.54%). The synergistic interaction between *GSTT1* and *CYP2E1* genes removed a considerable percentage of the entropy (1.44%) (Fig. 1b).

Discussion

To our knowledge, this report is the first study to evaluate environmental factors and polymorphisms in enzymes that metabolize anti-TB drugs together and investigate potential gene–gene and gene–environment interactions and their association with ATDH development in a population of hospitalized TB patients from Buenos Aires. Our primary finding was a prediction model that properly identified 67.5% of the TB patients at risk of developing ATDH. We also showed for the first time a synergistic effect between *CYP2E1* and *GSTT1*, which suggests a potential gene–gene interaction (epistasis) associated with increased risk for ATDH.

Several environmental factors, including sex, age, BMI, nutrition status, diseases in course, comedication, and environmental agents, influence drug effects. We analyzed whether some of these influential variables were

Table 4 Combined distribution of genotypic frequencies of cytochrome P450 2E1 variants and *N*-acetyltransferase 2 acetylator profile in cases with antituberculosis drug-induced hepatotoxicity and controls

NAT2 acetylator profile	*CYP2E1* C-1053T genotype	Without ATDH [*n* (%)]	With ATDH [*n* (%)]	OR (95% CI) (*P*)	OR adjusted by sex (95% CI) (*P*)
RA	c1/c1	66.7 (96)	48.4 (15)	1 (ref)	1 (ref)
RA	c2 carriers	33.3 (48)	51.6 (16)	2.065 (0.949–4.493) ($P = 0.067$)	2.218 (1.003–4.903) ($P = 0.049$)
SA	c1/c1	71.8 (74)	50.8 (33)	2.521 (1.282–4.957) ($P = 0.007$)	3.045 (1.526–6.074) ($P = 0.002$)
SA	c2 carriers	28.2 (29)	49.2 (32)	6.597 (3.141–13.858) ($P < 0.001$)	7.068 (3.341–14.951) ($P < 0.001$)

ATDH, antituberculosis drug-induced hepatotoxicity; CI, confidence interval; CYP2E1, cytochrome P450 2E1; IA, intermediate acetylator; OR, odds ratio; RA, rapid acetylator; Ref, reference; SA, slow acetylator; VNTR, variable number tandem repeat.

associated with ATDH development in our TB population and we found that female sex was an important risk factor for ATDH. This result is consistent with previously reported findings that women are particularly predisposed to ATDH [19,33]. Other clinical variables studied that could affect our results (i.e. drug abuse, alcohol abuse, etc.) were not observed to be predictive for ATDH. Surprisingly, alcohol abuse was higher in the control group than in the cases; therefore, this variable was not taken into account for further analysis.

TB treatment involves a multidrug scheme, but some reports indicate that alterations in the INH metabolic pathway are primarily responsible for the occurrence of ATDH. This hypothesis likely stemmed from the ability of RMP to act as a potent inducer of CYP2E1 activity and as a reducer of NAT2 activity, which increase the accumulation of Hz and toxic metabolites [14,15]. PZA increases the hepatotoxicity of INH [34], and nontoxic concentrations of INH and hydrazine increase in-vitro PZA hepatotoxicity [35]. Several surveys reported that genetic alterations in the enzymes involved in the metabolic pathways of INH were associated with hepatotoxicity [36–39]. We investigated the pharmacogenetics of INH, and our results support this hypothesis.

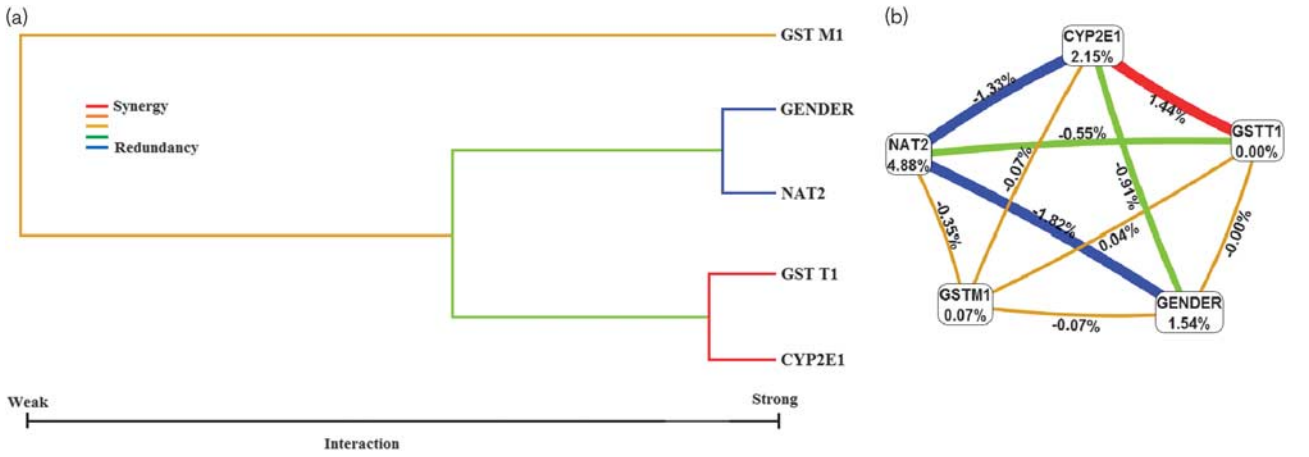
The *CYP2E1 c1/c2* polymorphism and ATDH susceptibility were studied widely in several populations [19,21, 23,40–42]. Several surveys found an association between the *c1/c1* (*RsaI* +) variant and the development of ATDH [13,21], but other studies found no associations [19,41,42]. Notably, a significant association was observed between *c2* allele carriers and the development of ATDH, which suggests that this allele is an independent risk factor for hepatotoxicity in our population (Table 3). Our results are contradictory to some studies, but consistent with Watanabe *et al.* [43], who suggested that the *c2* variant shows higher transcriptional activity, protein levels, and enzyme activity than the wild-type variant (*c1*). Sharma *et al.* [44] reported similar findings in a North Indian population and proposed a re-examination of the previous report adjusting for confounder factors. However, few studies have examined whether VNTR polymorphisms of the *CYP2E1* gene were associated with ATDH susceptibility [10,45,46]. We found that *A4* carriers show an increased risk of ATDH development (Table 3). This result is consistent with previous studies that proposed that the transcriptional activity of the *A4* allele (containing eight repetitive units, also named **1D*) was higher than the *A2* allele (containing six repetitive units, also named **1C*) [47]. A higher transcriptional activity of CYP2E1 would promote the hepatotoxic pathway. To the best of our knowledge, this study is the first report to identify an association between the *A4* allele and ATDH susceptibility. It is possible that other groups did not find an association because of the low number of ATDH patients recruited to achieve statistical power [Santos *et al.* [46]

Table 5 Multifactor dimensionality reduction: models detected and their performance

Number of markers considered	Best candidate model	CVC[a]	TBA[b]	*P**
Gene–gene interaction				
1	NAT2	10/10	0.614	0.008
2	**NAT2, CYP2E1 (C-1053T + VNTR)**	**10/10**	**0.653**	**0.001**
3	NAT2, CYP2E1 (C-1053T + VNTR), GSTT1	9/10	0.606	0.014
4	NAT2, CYP2E1 (C-1053T + VNTR), GSTM1, GSTT1	10/10	0.603	0.026
Gene–environment interaction (sex)				
1	NAT2	10/10	0.614	0.020
2	NAT2, CYP2E1 (C-1053T + VNTR)	10/10	0.653	0.001
3	**NAT2, CYP2E1 (C-1053T + VNTR),** sex	**10/10**	**0.675**	**0.001**
4	NAT2, CYP2E1 (C-1053T + VNTR), sex, GSTT1	9/10	0.625	0.008
5	NAT2, CYP2E1 (C-1053T + VNTR), sex, GSTT1, GSTM1	10/10	0.613	0.020

The bold fonts indicate the overall best MDR model.
CVC, cross-validation consistency; CYP2E1, cytochrome P450 2E1; GSTM1, glutathione *S*-transferase mu 1; GSTT1, glutathione *S*-transferase theta 1; MDR, multifactor dimensionality reduction; TBA, testing balanced accuracy; VNTR, variable-number tandem repeat.
[a]CVC: measure of how many times out of 10 divisions of the data, MDR found the same best model.
[b]TBA: measure of the predictive performance of the model, the one with the highest TBA represents the best overall model.
Number of markers considered.
**P*-value based on 1000 permutations: shows the statistical significance of the model.

Fig. 1



(a) Interaction dendrogram for the markers tested in the multifactor dimensionality reduction (MDR) analysis to evaluate associations with antituberculosis drug-induced hepatotoxicity (ATDH). The lines represent a continuum from synergy to redundancy of gene–gene and gene–sex interactions with a variable strength. The color of the line indicates the type of interaction between factors. The red line suggests a high degree of synergy. The gold line represents independence or additivity. The blue line suggests a high degree of redundancy or correlation, and the green line indicates a lower degree of redundancy. (b) Entropy-based interaction circle graph for ATDH with MDR. Entropy values in the cells of particular factors indicate the main independent effects and the entropy values on the lines connecting two factors represent the entropy of the interaction. The NAT2 phenotype removes 4.88% of entropy, which, in theory, means that this factor eliminates 4.88% of 'uncertainty' in the prediction of ATDH. VNTR and c1/c2 polymorphism in *CYP2E1* removes 2.15% of 'uncertainty'. The environmental factor sex removes 1.54% of entropy. Interaction between *GSTT1* and *CYP2E1* removes 1.44% entropy and this range of information is not explained by any of these factors treated separately (synergistic interaction). ATDH, antituberculosis drug-induced hepatotoxicity; CYP2E1, cytochrome P450 2E1; GSTT1, glutathione *S*-transferase theta 1; MDR, multifactor dimensionality reduction; NAT2, *N*-acetyltransferase 2; VNTR, variable-number tandem repeat.

($n = 18$) and Cho *et al.* [10] ($n = 18$)]. Kudryashov *et al.* [45] found an association between the *A2/A2* genotype with an increase in serum alanine aminotransferase activity within the normal range of values, but no hepatotoxicity criteria were defined.

GSTM1 and *GSTT1* null mutations are associated with idiosyncratic DILI caused by a large variety of drugs [24] and ATDH development [18,25]. Fukino *et al.* [36] reported that the Hz concentration was higher in *GSTM1*-null than wild-type patients. No individual or combined association between *GSTT1* null and *GSTM1* null with hepatotoxicity was found in our population (Table 3). The lack of individual influence of GSTs in ATDH development may be because these enzymes act downstream in the INH metabolic pathway and its action only became important in the presence of a slow acetylator profile or in *c2* carriers. Another hypothesis is that other detoxifying enzymes are involved in INH toxic metabolites that scavenge the bypassing GSTT1 and GSTM1 pathway (Table 3).

Analysis of the acetylator profile showed that ATDH primarily developed in patients who were slow acetylators (66.7%). The slow acetylator profile was an independent variable that was associated with an increased risk of developing ATDH (Table 3), which is consistent with our previously reported data [19]. This study examined the interaction of the acetylator profile with other risk factors in the prediction of ATDH. The combination of the *c1/c2* with acetylator status analyses showed that a defective NAT2 enzyme (slow acetylator) and wild-type *CYP2E1 (c1/c1)* increased the risk of ATDH. Our hypothesis is that the alternative metabolic pathway prevails and INH is hydrolyzed directly to form Hz. However, the risk of developing ATDH is augmented when NAT2 is acetylated normally, but the *c2* variant of *CYP2E1* is present. This hypothesis may be explained by the fact that the *c2* variant shows higher transcriptional activity, protein levels, and enzyme activity than the wild-type variant. Therefore, CYP2E1 may oxidize more acetyl hydrazine to hepatotoxic intermediates. Additive effects are observed when these alterations occur simultaneously, with a higher risk of hepatotoxicity than patients who show only a slow acetylator profile or are *c2* carriers (Table 4).

We analyzed gene–gene and gene–sex interactions to gain a better understanding of ATDH mechanisms. MDR analysis indicated that the NAT2 acetylator profile was the highest individual risk factor for ATDH. The best model for ATDH prediction from gene–gene interaction was the interaction between *NAT2* and *CYP2E1*. This model supported the findings in Table 4. However, the model fit better when a gene–sex interaction was analyzed. Therefore, inclusion of the sex variable in the genotypic classification of CYP2E1 and acetylator profile increased to the 67.5% the ability to properly classify individuals in their risk to develop ATDH.

The interaction entropy graph provides more detailed information on the interaction of variables in the prediction of ATDH. Contrary to our expectation and the results of the logistic regression in Table 4, these results suggest that the interaction between all markers considered in the best model provides redundant information. This result means that each marker removes more entropy on its own that the interactions between them. However, a synergistic interaction was found between *GSTT1* and *CYP2E1* genes, which indicates possible epistasis between variants in these two genes in ATDH cases, even when *GSTT1* did not show an individual association with the adverse effect. The presence of a synergistic effect in the absence of main effects would fit the classical definition of epistasis (Fig. 1b). One explanation for this finding is that increased activity of CYP2E1 in TB patients with a *c1/c2* or *c2/c2* genotype leads to an augmented concentration of hepatotoxins that cannot be scavenged by the GSTT1 pathway (in null genotypes), which increases the risk of ATDH.

This study elucidated the interaction of different genetic polymorphisms in enzymes involved in INH metabolism with each other and female sex increased the risk of ATDH. However, one limitation of our investigation was the low number of cases, which should be higher to achieve greater statistical power. The population of TB patients analyzed received a four-drug treatment, and we cannot obviate drugs interactions and their influence in the hepatotoxicity explained above. Therefore, further pharmacogenetics studies of the metabolic pathway of PZA and RMP should be carried out to gain a better understanding of ATDH mechanisms. Our study achieved a very acceptable prediction model that may aid early detection in patients with an increased risk of ATDH.

ATDH is a severe, potentially fatal, and prevalent adverse drug reaction, and it leads to drug discontinuation in 11% of TB patients. Our study designed a prediction model that adequately identified 67.5% of patients with TB in their risk of developing ATDH. The considerable number of TB patients in our country supports the fact that the use of early pharmacogenetic testing and a comprehensive clinical history may be useful in identifying patients with a high risk of suffering hepatotoxicity. This type of research further supports the use of personalized medicine in the treatment of TB and might be further carried out on the new anti-TB acrylamide-derived drugs [48] because the genotoxicity of this vinyl monomer is known [49].

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Conflicts of interest

There are no conflicts of interest

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