

Genetic Risk Factors in Drug-Induced Liver Injury Due to Isoniazid-Containing Antituberculosis Drug Regimens

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Drug-induced liver injury (DILI) is a complication of treatment with antituberculosis (TB) drugs, especially in isoniazid (INH)-containing regimens. To investigate genetic risk factors, we performed a genomewide association study (GWAS) involving anti-TB DILI cases (55 Indian and 70 European) and controls (1,199 Indian and 10,397 European). Most cases were treated with a standard anti-TB drug regimen; all received INH. We imputed single nucleotide polymorphism and HLA genotypes and performed trans-ethnic meta-analysis on GWAS and candidate gene genotypes. GWAS found one significant association (rs117491755) in Europeans only. For HLA, *HLA-B*52:01* was significant (meta-analysis odds ratio (OR) 2.67, 95% confidence interval (CI) 1.63–4.37, $P = 9.4 \times 10^{-5}$). For N-acetyltransferase 2 (NAT2), *NAT2*5* frequency was lower in cases (OR 0.69, 95% CI 0.57–0.83, $P = 0.01$). *NAT2*6* and *NAT2*7* were more common, with homozygotes for *NAT2*6* and/or *NAT2*7* enriched among cases (OR 1.89, 95% CI 0.84–4.22, $P = 0.004$). We conclude HLA genotype makes a small contribution to TB drug-related DILI and that the NAT2 contribution is complex, but consistent with previous reports when differences in the metabolic effect of *NAT2*5* compared with those of *NAT2*6* and *NAT2*7* are considered.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Antituberculosis (TB) drugs, including isoniazid (INH), are a common cause of drug-induced liver injury (DILI). Previous reports suggest N-acetyltransferase 2 (*NAT2*) genotype and some HLA alleles are risk factors but not all studies agree on this.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ We aimed to identify novel genetic risk factors for DILI due to anti-TB drugs, including INH, in European and Indian cases and consolidate understanding on relevance of HLA and *NAT2* genotypes to risk DILI.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ The study provides further support for *NAT2*6* and *NAT2*7* variants of *NAT2* as risk factors for development of anti-TB drug-related DILI and for *NAT2*5* being protective. There may also be increased risk in those carrying the *HLA-B*52:01* allele.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ The study provides further support for *NAT2* and HLA contributions to risk of DILI from TB drugs, adding to knowledge that may lead to genetic tests capable of identifying those patients at risk.

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Received July 20, 2020; accepted October 15, 2020. doi:10.1002/cpt.2100

Up to 20% of patients receiving isoniazid (INH) either as mono or combination therapy for tuberculosis (TB) may develop transient asymptomatic elevation of liver enzymes but this elevation usually resolves without drug discontinuation.^{1,2} In a recent large prospective cohort study based in China, 5.4% of patients on anti-TB combination therapy developed drug-induced liver injury (DILI)³ as defined by the International DILI Expert Working Group.⁴ In 16% of these cases, DILI was accompanied by other symptoms of hepatotoxicity, including jaundice, and 5.3% developed acute liver failure.³ Recurrence of DILI upon retreatment (called positive rechallenge) has been reported to occur in ~9–25% with at least one of the anti-TB drugs.^{1,2,5,6} The incidence of DILI when INH is combined with rifampicin appears higher than for INH alone, with addition of pyrazinamide increasing the risk further.⁷

A number of drug-specific and host-related factors influence the susceptibility of a patient to DILI with anti-TB drugs.⁸ Potential mechanisms related to INH DILI have been the most widely investigated. Acetylhydrazine is generally considered to be a key INH metabolite contributing to INH-induced DILI and is produced by N-acetyltransferase 2 (NAT2). It can undergo further metabolism by cytochrome P450 to a toxic metabolite or by NAT2 to the less toxic diacetylhydrazine. It has been suggested that fast acetylators (those with NAT2 activity within the normal range) will form diacetylhydrazine efficiently and therefore levels of both acetylhydrazine and toxic P450 metabolites will be low.⁹ It has also been proposed that slow acetylators who lack NAT2 activity may form higher concentrations of the toxic metabolite hydrazine by cleavage of the amide bond on INH to form isonicotinic acid.¹⁰ There are also data to suggest formation of a reactive metabolite directly from INH oxidation, which may contribute to liver toxicity through the formation of protein adducts¹¹ inducing an inappropriate immune response.¹²

Because of the important contribution by acetylation to INH metabolism and the existence of common loss of function polymorphisms in *NAT2*, the gene encoding the acetylation enzyme, there have been a large number of studies examining these variants as DILI risk factors. At least four different meta-analyses, including large numbers of cases, have concluded that slow acetylators have an increased risk of TB drug DILI with an overall odds ratio (OR) varying from 1.59 to 6.42, although the risk varied depending on the precise genotypic definition of slow acetylation and the population studied.^{13–16} Although many of these studies involved a limited number of patients experiencing only mild liver injury, the *NAT2* association has also been observed in a study involving moderate to severe DILI cases only.¹⁷ A clinical trial based in Japan with differential dosing with INH on the basis of *NAT2* genotype found a lower incidence of DILI when slow acetylators were given a lower dosing regimen.¹⁸ A genomewide association study (GWAS) involving patients from Thailand recently described a genomewide significant signal for *NAT2*, suggesting a stronger risk for DILI development in those positive for slow acetylator alleles in line with many of the earlier candidate gene studies.¹⁹ However, two GWAS, which included small numbers of European cases with anti-TB DILI, did not find any genomewide significant association with the *NAT2* slow acetylator genotype.^{20,21}

In addition to *NAT2* variants, other candidate genetic risk factors for anti-TB DILI have also been investigated, with reports suggesting that genotypes for genes relevant to anti-TB drug disposition and oxidative stress, such as *CYP2E1*,²² *SOD2*,²³ *GST* isoforms,²³ carboxylesterase (*CES*) isoforms,²⁴ and *PXR* (*NR1I2*)²⁵ may modulate risk. HLA genotype is a strong risk factor for a number of forms of DILI²¹ and it has been suggested that HLA class II genotype is relevant to risk of DILI due to anti-TB drugs in an Indian population.²⁶ However, further studies using either candidate gene or genomewide approaches have failed to confirm a role for HLA genotype in susceptibility to anti-TB drug-related DILI in Europeans.^{20,21,27} A recent study of DILI due to anti-TB drugs in an Ethiopian population who were also HIV-positive and receiving anti-HIV treatment found an interesting association with the class I HLA B*57 alleles,²⁸ but this form of DILI showed distinct phenotypic differences from that normally associated with anti-TB drugs. A non-HLA immunogenetic risk factor for DILI generally in the gene *PTPN22* has also been identified recently but its relevance to anti-TB drug-related DILI has not been investigated in detail to date.²⁹

The aims of the current study were to perform a GWAS together with additional candidate gene studies on a newly recruited group of Indian patients with moderate to severe anti-TB drug DILI and on an enlarged European cohort, which includes some cases studied previously.^{17,20,21}

METHODS

Study design

This study combined in a trans-ethnic meta-analysis framework results from GWAS conducted separately in subjects with European and Indian ancestry. The study was conducted according to the Declaration of Helsinki (Hong Kong Amendment) and Good Clinical Practice (European guidelines). All participants provided written informed consent and each study was approved by the appropriate local (Department of Gastroenterology, St. John's Medical College Hospital, Bangalore, India, and Christian Medical College, Vellore, India), national or institutional ethical review boards, as reported previously.^{21,29}

Indian cohort

Patients ($n = 55$) who developed DILI after exposure to INH in combination with rifampicin, pyrazinamide, and ethambutol (all 4 drugs for the first 2 months and INH and rifampicin for a further 4 months), 105 patients treated with these drugs without DILI development and 104 healthy South Indian adults of mixed ancestries were enrolled from August 2009 to February 2014 at St. John's Medical College, Bengaluru and Christian Medical College, Vellore, South India. Roussel Uclaf Causality Assessment Method (RUCAM) was used for case adjudication, as described previously.⁴ To further increase study power, we added to the control set a total of 990 ethnically matched samples comprising 356 from 1,000 G project and 634 controls of Indian descent from Charles Bronfman Institute for Personalized Medicine BioMe BioBank (phs000925.v1.p1) identified by principal component analysis (see **Supplementary Materials**).

European cohort

We analyzed 70 European ancestry DILI cases exposed at least to INH alone or in combination with one or more of rifampicin, pyrazinamide, and ethambutol, collected by the DILIGEN, iDILIC, and DILIN consortia. Of these cases, 43 were treated with INH alone as standard TB prophylaxis and 27 had been treated with any combination of INH and one or more additional anti-TB drugs with 11

of those exposed to all 4 drugs. These cases all form part of a large cohort of DILI cases previously analyzed by GWAS,²⁹ with some also included in earlier studies, as summarized in **Table S1**. A subgroup of 12 UK cases (DILIGEN study) had been included in an earlier study involving direct *NAT2* genotyping only¹⁷ but subsequently also underwent genomewide genotyping.²⁰ European ancestry controls ($n = 10,397$) were used.²⁹

Clinical characterization of DILI

Criteria used for case definition, categorization of DILI pattern as well as grading of severity of DILI were harmonized across all cohorts using previous guidelines.⁴ Causality assessment was done by RUCAM score for the DILIGEN and iDILIC cohorts and by both structured expert opinion and RUCAM score for the DILIN cohort, as previously reported.^{20,21}

DNA preparation and genotyping

For the Indian cases and controls, DNA was isolated from whole blood samples using the standard phenol-chloroform method. DNA samples were genotyped in one batch using the Illumina Human Core Exome-24 BeadChip by the Department of Molecular Genetics, Madras Diabetes Research Foundation, India. DNA isolation and genotyping of European cases from the DILIGEN, iDILIC, and DILIN studies was as described previously.^{20,21}

Population structure and imputation

Quality control checks on the initial genotype data were performed as described previously.²⁹ To assess the extent of population structure of the study cohorts, and derive eigenvectors to account for confounding, we applied principal component analysis on each cohort separately, using the smartPCA program from the EIGENSTRAT package (version 3.0)³⁰ on the overlapping single nucleotide polymorphisms (SNPs; minor allele frequency > 0.01) across the range of genotyping arrays used for typing cases and controls. We used 1000 Genome Project samples as the reference panel to select cases and controls of white and South Indian ancestries. SNP imputation was performed in batches dividing the samples according to ethnicity and genotyping platforms. For each batch imputation was carried out using Michigan Imputation Server, as described previously.²⁹

GWAS analysis and meta-analysis

We tested for association of each SNP with DILI, separately in Indian and European GWAS, in a logistic regression framework, under an additive genetic model, with adjustment for the principal components from smartPCA to account for population structure using PLINK version 1.07.³¹ No other additional covariates were included in the model because we did not have clinical information for controls. Association summary statistics from the two cohorts were combined using effective sample size weighted *Z*-score fixed-effects meta-analysis, implemented in METAL.³² Allelic ORs across the two cohorts were obtained through inverse-variance weighting of effect sizes, with heterogeneity assessed with Cochran's *Q* statistic,³³ implemented in METAL. We reported only those SNPs that attained, in addition to genomewide significance, nominal evidence of association ($P < 0.05$) with the same direction of effect on DILI in both GWAS phases (internal validation). Genomewide significance with clinical outcomes was defined using a common threshold of $P < 5 \times 10^{-8}$. Because the phenotypes studied are rare, the number of cases analyzed was limited. All detailed analyses and Manhattan plots were produced with R (version 3.0.2, The R Project for Statistical Computing, <http://www.r-project.org>). Regional plots were drawn by LocusZoom, as described previously.²¹

HLA analysis

For each cohort, HLA alleles were inferred using HIBAG³⁴ using the reference predictor panels specific for the genotyping chip and ancestry

provided in the software webpage. To impute Indian samples, we used the provided Asian reference data.³⁴ In total, we imputed 217 HLA alleles in the overall European cohort and 192 in the Indian cohort. We set the major histocompatibility complex (MHC) regionwide significance *P* value threshold for the HLA allele association to 2.5×10^{-4} to correct for multiple testing (Bonferroni correction for 200 predicted HLA alleles). Association tests for each HLA allele and meta-analysis were carried out as reported above. Haplotype analysis was performed by Plink version 1.07, including the most significantly associated HLA alleles.

NAT2 genotypes

We predicted *NAT2* alleles using genotypes for rs1801280, rs1799930, and rs1799931 from the GWAS (*NAT**5, *6, and *7 alleles, respectively) by haplo.stats (<https://cran.r-project.org/web/packages/haplo.stats/index.html>) and extracted the best haplotype predictions based on posterior probability. We tested for association between DILI and allele by logistic regression with adjustment for the principal components in Plink. *NAT2* genotypes were recorded and samples were divided into different acetylator status groups as originally proposed³⁵ and refined more recently³⁶: (a) rapid (*4/*4); (b) intermediate (*4/*5, *4/*6, and *4/*7); (c) slow (*5/*5, *5/*6, and *5/*7); and (d) ultraslow (*6/*6, *6/*7, and *7/*7). We then tested for association between DILI and acetylator status groups and genotypes in a multivariate regression model, including principal components axes and acetylator status groups or genotypes as binomial variable or as categorical variable having 44 genotype or rapid/intermediate as baseline groups. The meta-analysis *P* value was calculated using METAL with the default approach that combines *P* value and direction of effect, weighted according to sample size.³² Significance was defined using a Bonferroni threshold of $P < 0.01$ considering four comparisons.

Candidate gene analysis

For analysis of additional candidate genes, we selected four genes previously proposed to have a role in the pathogenesis of INH-related DILI, including *CYP2E1*, *CES2*, *CES1*, and *PXR/NR1I2*. We extracted all variants belonging to each gene from GnomAD (<https://gnomad.broadinstitute.org/>). We performed an association analysis by Plink version 1.07.³¹ Significance was defined using a Bonferroni threshold of $P < 0.003$ considering 16 multiple comparisons.

RESULTS

Cases and controls

Clinical characteristics of the 55 DILI cases from India and 70 European cases are summarized in **Table 1**. The total Indian DILI cohort was enriched in cases with severe liver injury with 22% of all patients progressing to acute liver failure, transplantation, or death. The majority of the European DILI cases met the definition of moderate to severe DILI used in our previous European DILI studies.²¹ Nine European patients (13%) suffered liver failure, underwent liver transplant, or died (**Table 1**).

As controls for the Indian cases, we used 1,199 ethnically matched individuals (105 patients treated for TB by similar regimens who did not develop DILI and 104 healthy adults recruited for this study, 356 from the 1000 Genome Project phase III dataset, and 634 from BioMe dataset (**Figure S1**)). As European controls, we used 10,397 ethnically matched individuals (**Figure S2**).

A schematic summary of the overall study is provided in **Figure 1**.

GWAS analysis

The case-control GWAS in the European cohort showed one marker that passed the significance threshold (rs117491755, OR = 4.37, 95%

Table 1 Clinical phenotype for the Indian and European cases

Groups	Indian cohort	European cohort
Clinical characteristics		
Number of cases	55	70
Gender (F/M)	28/27	37/33
Age, years (mean, SD)	40 (16.1)	55.1 (13.9)
Time to onset from first drug exposure, days (mean, SD)	46.8 (55.1)	55.2 (45.8)
Pattern of DILI		
Cholestatic	18% (10)	5.7% (4)
Hepatocellular	59% (32)	77.1% (54)
Mixed	22% (12)	10.0% (7)
Unknown	1% (1)	7.1% (5)
Causal drug		
Isoniazid and rifampicin		1.4% (1)
Isoniazid, rifampicin, and pyrazinamide		21.4% (15)
Isoniazid, rifampicin, pyrazinamide, and ethambutol	100% (55)	15.7% (11)
Isoniazid		61.43% (43)
Severity		
Mild	0	11% (8)
Moderate	78% (43)	73% (51)
Severe/fatal	22% (12)	13% (9)
Not reported	0	3% (2)
Causality score (CIOMS/RUCAM)		
3–5 (possible)	6% (3)	18% (13)
6–8 (probable)	60% (33)	60% (42)
> 8 (highly probable)	34% (19)	21% (15)
Genotyping platform		
1 M Illumina Duo (% , N)		39% (27)
Infinium Core Exome (% , N)	100% (55)	50% (35)
Multi-Ethnic Genotyping Array Consortium (% , N)		11% (8)

DILI, drug-induced liver injury; RUCAM, Roussel Uclaf Causality Assessment Method.

confidence interval (CI) 2.702–7.061, $P = 1.8 \times 10^{-9}$; allele frequency (AF)_{cases} = 0.143; AF_{controls} = 0.037; **Figure 2a**). The rs117491755 is an intronic SNP in *ASTN2*. For the Indian cohort, there were no genomewide significant associations between DILI and imputed or genotyped variants and rs117491755 did not pass quality control checks in the imputed dataset (**Figure 2b**). Trans-ethnic meta-analysis between the two ethnic groups analyzed 4,900,532 shared markers between the two cohorts. None of the variants in the meta-analysis reached genomewide significance (**Figure 2c**).

HLA analysis

In the European cohort, we identified two MHC significant signals, *HLA-C*12:02* (OR = 6.43, 95% CI 2.53–16.37, $P = 9.4 \times 10^{-5}$; **Table 2** and **Table S1**) and *HLA-B*52:01* (OR = 6.39; 95% CI

2.25–16.29, $P = 1.0 \times 10^{-4}$; **Table 2**). All carriers presented with both alleles. The *B*52:01-C*12:02* haplotype conferred a significant increase in DILI risk of almost sevenfold ($P = 7.8 \times 10^{-5}$; **Table S2**), although the overall haplotype frequency is low even in the cases. The alleles were mainly associated with DILI cases due to drug combinations compared with INH alone (**Table S3**). Slightly different HLA results were observed for Indian cases. These cases showed an enrichment in class II HLA risk alleles with *HLA-DQA1*03:01* (OR = 2.60, 95% CI 1.54–4.38, $P = 0.0003$; **Table 2**) as the most significantly associated allele, although not passing the MHC multiple correction threshold. Indian cases also showed a nominal enrichment of *HLA-B*52:01* and *HLA-C*12:02* alleles compared with controls (**Table S2**). In line with the data from Europeans, the haplotype conferred a 1.45-fold increase in risk of DILI ($P = 0.04$; **Tables 2** and **S2**). In meta-analysis *HLA-B*52:01* showed an MHC-significant association (OR = 2.67, 95% CI 1.63–4.37, $P = 9.4 \times 10^{-5}$; **Table 3**).

Candidate gene analysis

NAT2 genotypes. The frequency of the *NAT2* alleles in both cohorts are summarized in **Table 3**. *NAT2* allele frequencies for our cohorts were similar to frequencies reported previously with Indians showing a high frequency of the *NAT2*6* allele in the control group compared with Europeans, whereas in the European controls, *NAT2*5* was the most common allele.³⁷ We tested association between each allele and DILI correcting for population stratification (**Table 4**). We found that *NAT2*5* was the most under-represented *NAT2* allele in both European and Indian cases, passing the Bonferroni correction for the number of the predicted *NAT2* alleles ($P_{\text{Meta-analysis}} = 0.01$; **Table 4**). *NAT2*6* was significantly enriched in Indian cases ($P = 0.01$). Severe Indian cases ($n = 12$) showed a slightly higher frequency for *NAT2*6* (AF = 0.54) and lower frequency of *NAT2*5* (AF = 0.11) compared with the overall cases, with *NAT2*5* statistically significant as a protective factor compared with controls (OR = 0.30, 95% CI 0.08–0.94, $P = 0.05$). Among Europeans, INH alone cases ($n = 43$) showed a significantly decreased frequency for *NAT2*5* (AF = 0.34, $P = 0.04$; **Table 4**). The group of European severe cases ($n = 9$) was too small to detect significant trends.

We then assigned *NAT2* genotypes for each individual and classified them in four acetylator status groups. We evaluated if there was an enrichment of any of the groups in our cases compared with controls. We found that ultraslow acetylators (those carrying homozygous or compound heterozygous genotypes for *NAT2*6* and *NAT2*7*) were significantly enriched in cases compared with controls ($P_{\text{Meta-analysis}} = 0.004$; **Table 5**). This enrichment was not significant in a multivariable regression model for which we combined rapid and intermediate acetylators as the baseline group ($P_{\text{European}} = 0.08$ and $P_{\text{Indian}} = 0.1$). Examining the enrichment of single genotype groups, the only set significantly associated with DILI by meta-analysis was *NAT2*6/NAT2*7* ($P = 0.002$), which was also significant in Europeans alone ($P = 0.01$; **Table S4**).

Other candidate gene analysis. We extracted variants located in selected candidate genes relevant to INH metabolism (*CYP2E1*,

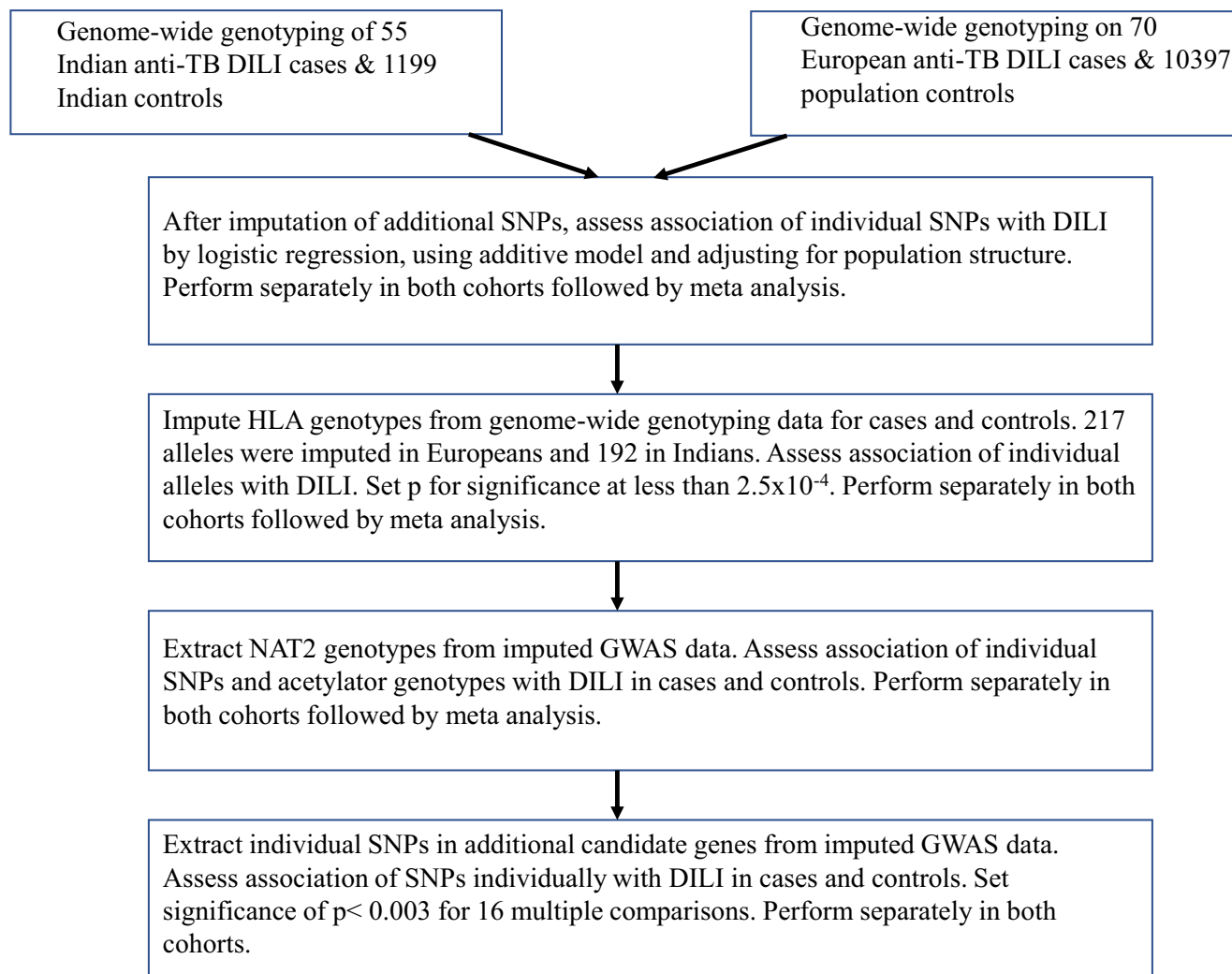


Figure 1 Schematic summary of the study including details on the number of cases and controls and analysis steps undertaken. DILI, drug-induced liver injury; GWAS, genomewide association study; SNP, single nucleotide polymorphism; TB, tuberculosis. [Colour figure can be viewed at wileyonlinelibrary.com]

CES2, *CES1*, and *PXR/NR1I2*) from GnomAD (<https://gnomad.broadinstitute.org>) for a total of 16 imputed/genotyped SNPs in European (*CYP2E1* $n = 9$; *CES2* $n = 2$; *CES1* $n = 0$; and *PXR/NR1I2* $n = 5$) 14 in Indians (*CYP2E1* $n = 5$; *CES2* $n = 3$; *CES1* $n = 4$; and *PXR/NR1I2* $n = 2$). Because imputation is based on ethnicities and genotyping platforms, the SNPs available for analysis in the candidate genes were different for the two groups. No variants were significantly associated with DILI (Table S5).

Frequency of rs2476601 in *PTPN22* that has been previously associated with DILI due to several different drugs,²⁹ was not increased in European DILI cases ($AF_{cases} = 0.10$, $P = 0.40$) and INH alone cases ($AF_{cases} = 0.12$, $P = 0.15$) but was marginally increased in the Indian cases ($OR = 3.8$, 95% CI 1.06–13.83, $P = 0.04$, $AF_{cases} = 0.03$ and $AF_{controls} = 0.01$).

DISCUSSION

Despite a relatively large number of published studies, the genetic basis for susceptibility to DILI due to anti-TB drugs, including INH, remains poorly understood compared with DILI caused by

certain other drugs, such as flucloxacillin and amoxicillin-clavulanate. There are a number of reasons for this, including (i) the complexity of the phenotype (both mild and more serious cases of DILI due to anti-TB drugs are common); (ii) the fact that the standard treatment typically involves a combination of four different drugs; and (iii) both TB as a disease and DILI induced by anti-TB drug treatment are more common in developing countries where relevant genetic polymorphisms may show differing frequencies than in Europeans where DILI has been studied more extensively, making worldwide comparisons difficult. Furthermore, in some countries where concomitant infection with TB and HIV is more common, assessment of causality as to whether the DILI is due to the anti-HIV drugs, anti-TB drugs, or both, is often unclear.

Similar to the findings in two earlier GWAS involving Europeans,^{20,21} we failed to detect genomewide significance when we undertook a GWAS in the Indian population. This is in contrast to the recent GWAS performed on a Thai population, where genomewide significance was seen for *NAT2*,¹⁹ but is more consistent with a separate GWAS performed in Ethiopians, which also failed

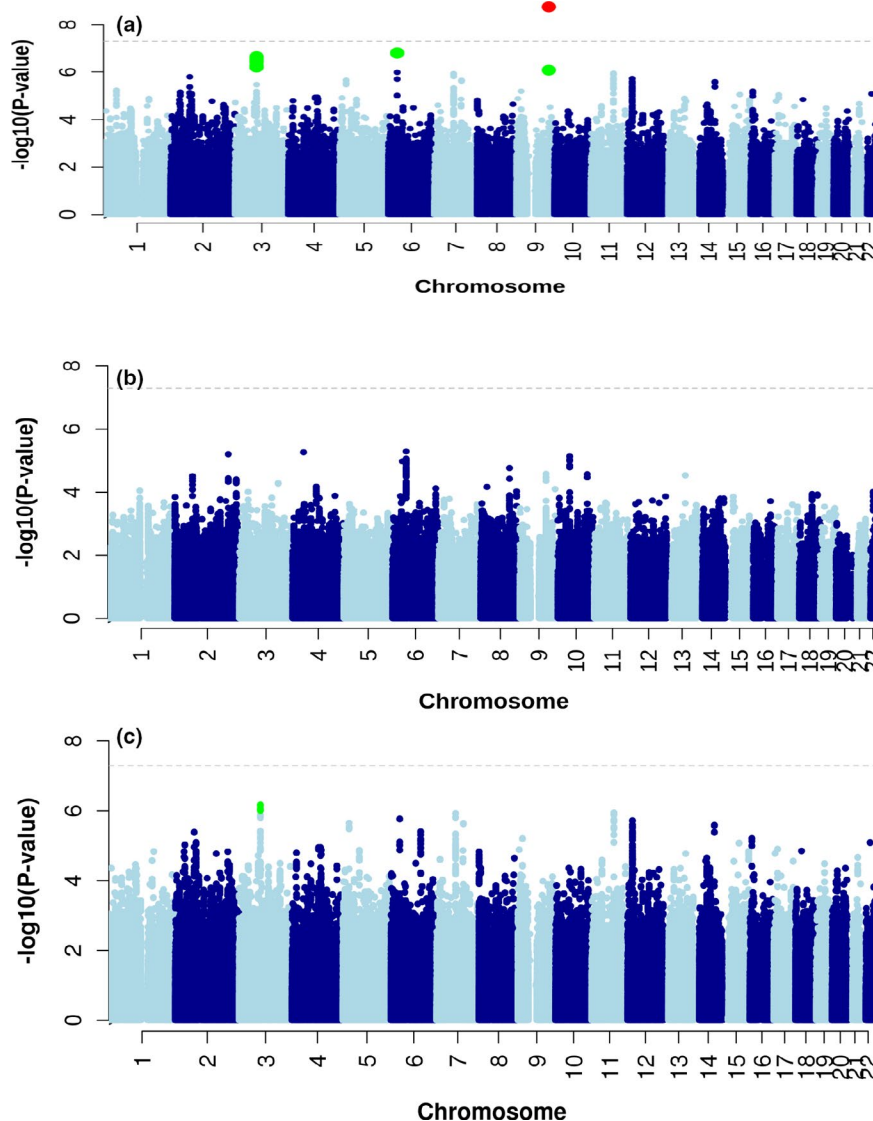


Figure 2 Manhattan plots displaying the association results of the genetic association analyses. (a) Manhattan plot showing the summary statistics for the European genomewide association study (GWAS); (b) Manhattan plot showing the summary statistics for the Indian GWAS; (c) Manhattan plot showing the summary statistics for the meta-analysis. The overall significance level was set at 5×10^{-8} . Single nucleotide polymorphisms in green have a significance level $< 5 \times 10^{-6}$.

to observe genomewide significant signals,³⁸ although the Ethiopian patients were also undergoing HIV treatments that can cause DILI. In our enlarged European population, which includes the previously studied cases^{20,21} as well as eight new cases,²⁹ we did see one genomewide significant signal in an intronic SNP in *ASTN2*. This gene product appears to affect synaptic strength by trafficking and degradation of surface proteins.³⁹ The relevance to DILI is not immediately clear and this signal was not seen in the Indian population. Without a positive replication, we have to consider this signal as either a false-positive or putative until further patients have been studied.

Although the current study and several previous reports failed to detect strong HLA associations, we found some evidence that a rare *HLA-C*12:02 HLA-B*52:01* haplotype, which has been recently reported to be a risk factor for Crohn's disease in Asians, might contribute to risk of anti-TB drug DILI in some individuals.⁴⁰ The effect of the haplotype seems to be consistent across

both cohorts and the association passes the Bonferroni correction based on the number of imputed alleles. Meta-analysis showed that the effect of the *B*52:01* allele alone was more significant than for *C*12:02*. The number of cases positive for the risk allele was low but importantly both cohorts showed this association and that there is an already reported association for the haplotype with an autoimmune disease.⁴⁰ In view of the small number of cases positive for the "at risk" haplotype and the absence of a signal in the INH only cases, it is possible that the HLA signal may reflect DILI induced by one of the other anti-TB drugs, possibly pyrazinamide.

A previous HLA class II typing study in an Indian population reported that absence of *HLA-DQA1*01:02* and presence of *HLA-DQB1*02:01* were risk factors for DILI due to anti-TB drugs.²⁶ However, in the current study, the most significant findings for class II were increased frequencies of *HLA-DQA1*01:03* and *HLA-DQA1*03:01*, although we did not observe this in

Table 2 The most significant HLA associations for European and Indian cohorts

Markers	OR	95% CI	P value	AF Cases	AF Controls	AF reference dataset
European associations						
<i>HLA-C*12:02</i>	6.43	2.526–16.37	0.00009	0.04	0.006	0.009
<i>HLA-B*52:01</i>	6.40	2.511–16.29	0.0001	0.04	0.007	0.009
<i>HLA-DRB1*15:02</i>	6.36	2.489–16.25	0.0001	0.04	0.006	0.007
Indian associations						
<i>HLA-DQA1*03:01</i>	2.60	1.53–4.38	0.00035	0.15	0.06	0.09
<i>HLA-DPB1*01:01</i>	3.24	1.58–6.61	0.0013	0.09	0.02	0.02
<i>HLA-DPB1*03:01</i>	3.60	1.58–8.19	0.0023	0.07	0.02	0.05
<i>HLA-DRB1*04:06</i>	8.48	2.02–35.52	0.00346	0.02	0.00	0.00
<i>HLA-DQA1*01:03</i>	1.85	1.21–2.84	0.0047	0.27	0.14	0.14

95% CI, confident interval of the odd ratio; AF Cases, allele frequency in cases; AF Controls, allele frequency in controls; AF reference dataset, allele frequency calculated based on the number of carriers estimated from all cohorts belonging to a particular geographic region reported in www.allelefrequencies.net; OR, odds ratio of a multivariate regression model correcting for population stratification.

Europeans. We also saw no association with B*57 alleles but, as discussed previously, we consider that this particular association may relate to a combination of anti-HIV and anti-TB drug treatment,²⁸ which we would not expect to see confirmed in the current study.

The *PTPN22* variant rs2476601 has recently been found to be an additional risk factor for some forms of DILI showing HLA associations.²⁹ We therefore also evaluated the role of this variant in our patients with anti-TB-related DILI, but did not find a significant association. However, the allele frequency of rs2476601 in South Asian populations is consistently lower compared with European populations (1% vs. 10%, as reported in GnomAD) so our ability to detect any association was also limited.

In view of very limited genomewide or near genomewide significant signals in the GWAS, we proceeded with additional candidate gene analysis using the GWAS data, focusing on *NAT2*, given the extensive literature which has demonstrated that *NAT2* genotype and phenotype is a risk factor for INH-induced DILI. Three meta-analyses on *NAT2* as a DILI risk factor, which include studies published up to 2017 together with two recent large studies, appear to be the most informative to use for comparison with the current study.^{13–15,19,41} In general, most previous studies on *NAT2* genotype as a risk factor have examined all slow acetylators in comparison with either homozygous wildtypes (who are often now classified as the fast acetylator group without inclusion of heterozygotes) or both homozygous wildtypes and those heterozygous for one variant allele only (the traditional fast acetylator group). In some studies, heterozygotes are designated as intermediate acetylators and were analyzed separately.

There is, however, increasing data available that indicates that *NAT2*5*, which is common in Europeans, South Asians, and

Africans, but not in East Asians, is not a true “slow acetylator” allele with the gene product retaining some enzyme activity, whereas the enzymes encoded by both *NAT2*6* and *NAT2*7* are associated with no activity.^{36,42,43} In line with this, a recent study of South African Zulus examined levels of INH and certain metabolites in relation to *NAT2* genotype in patients undergoing treatment with INH and did not find a significant difference in drug and metabolite levels when comparing *NAT2*5* homozygotes or heterozygotes with those homozygous for two rapid acetylator alleles. *NAT2*6* and *NAT2*7* alleles were not detected in this population.⁴⁴ One relatively small study of phenotype-genotype relationships in healthy Swedish volunteers used INH for phenotype determination and reported a higher metabolic ratio for *NAT2*5* homozygotes compared with *NAT2*6* homozygotes.⁴⁵

The *NAT2* genotype distribution among the DILI cases in the current study indicates a protective effect for the *NAT2*5* allele with *NAT2*4* “neutral” whereas an increased risk was seen for *NAT2*6* with the combined “ultraslow” group also showing a statistically significant increased risk. This is in agreement with a meta-analysis reporting an increased risk for *NAT2*6* and *NAT2*7*.⁴³ A recent study performed in Singapore also suggests that the risk for INH-related DILI is from *NAT2*6* and *7 only.⁴¹ This could also explain the recent genomewide significance reported for *NAT2* variants in Thailand¹⁹ because *NAT2*5* is rarely seen in this East Asian population. One of the earliest reports on *NAT2* genotype as a risk factor for INH-related DILI was performed in Taiwan.⁴⁶ This study found a small number of individuals positive for *NAT2*5* but hepatotoxicity was seen almost entirely in those carrying at least one *NAT2*6* or *NAT2*7* allele. The biological

Table 3 HLA alleles meta-analysis association results

Marker	Direction of effect	OR	95% CI	PV _m	HetPV
<i>HLA-B*52:01</i>	Concordant	2.67	1.63–4.37	9.4×10^{-5}	0.03
<i>HLA-C*12:02</i>	Concordant	2.31	1.41–3.75	0.0008	0.01
<i>HLA-DQA1*01:03</i>	Concordant	1.75	1.24–2.45	0.0013	0.66

95% CI, confidence interval of the odds ratio; HetPV, heterogeneity P value; OR, odds ratio of a multivariate regression model correcting for population stratification; PV_m, meta-analysis P value.

Table 4 Frequency of NAT2 alleles in case and controls of European and Indian cohorts

Allele	Europeans (70 cases)										Indians (55 cases)					Meta-analysis				
	Control freq	Case freq	INH only	INH comb	OR	95% CI	P value	Control freq	Case freq	OR	95% CI	P value	Effect dir	OR	95% CI	P _m	Het P			
NAT2*4	0.23	0.24	0.29	0.14	1.06	0.71–1.58	0.75	0.22	0.16	0.66	0.39–1.11	0.12	N/P	0.77	0.61–0.98	0.81	0.1			
NAT2*5	0.46	0.37	0.34	0.41	0.7	0.49–0.98	0.04	0.33	0.25	0.68	0.43–1.07	0.1	C	0.69	0.57–0.83	0.01	0.43			
NAT2*6	0.29	0.35	0.31	0.41	1.3	0.91–1.85	0.14	0.37	0.5	1.77	1.18–2.65	0.01	C	1.42	0.97–2.08	0.03	0.05			
NAT2*7	0.02	0.04	0.04	0.04	1.88	0.81–4.34	0.14	0.08	0.08	1.09	0.55–2.17	0.79	C	1.21	0.61–2.38	0.14	0.82			

C, concordant; Freq, allele frequency; HetP, heterogeneity P value; Effect dir, effect direction; INH comb, INH combination; INH, isoniazid; NAT2, N-acetyltransferase 2; N/P, null/positive; OR, odds ratio of a multivariate regression model correcting for population stratification; P, multinomial P value; P_m, meta-analysis P value.

Table 5 Frequency of NAT2 acetylator status genotypes in European and Indian case/control cohorts and their association in a multivariate regression model

Acetylator status	Europeans (70 cases)										Indians (55 cases)					Meta-analysis				
	Control freq	Case freq	OR	95% CI	P value	Control freq	Case freq	OR	95% CI	P value	Effect dir	OR	95% CI	P _m	Het P					
Rapid	0.05	0.06	1.18	0.44–3.28	0.73	0.06	0.02	0.29	0.03–2.20	0.23	D	0.38	0.22–0.65	0.1	0.75					
Intermediate	0.35	0.36	1.02	0.62–1.66	0.93	0.34	0.29	0.8	0.43–1.45	0.45	D	0.9	0.64–1.26	0.95	0.21					
Slow	0.49	0.4	0.6	0.41–1.08	0.1	0.41	0.4	0.93	0.53–1.64	0.82	C	0.68	0.52–0.88	0.87	0.46					
Ultra Slow	0.1	0.19	2.03	1.10–3.72	0.02	0.19	0.29	1.78	0.98–3.39	0.06	C	1.89	0.84–4.22	0.004	0.3					

We considered (a) in rapid group *4/*4 genotypes; (b) in intermediate group *4/*5, *4/*6, and *4/*7 genotypes; (c) in slow group *5/*5, *5/*6, and *5/*7 genotypes; (d) in ultra-slow *6/*6, *7/*6, and *7/*7 genotypes.

C, concordant; D, discordant; Freq, allele frequency; HetP, heterogeneity P value; Effect dir, effect direction; OR, odds ratio of a multivariate regression model correcting for population stratification; P, multinomial P value; P_m, meta-analysis P value.

basis for this complex association with *NAT2* genotype is not completely clear but *6 and *7 carriers may be at increased risk of toxicity due to higher levels of the parent drug undergoing metabolism by alternative routes to toxic intermediates, such as hydrazine or possibly by accumulation of the acetylhydrazine metabolite, which also may be converted to hydrazine.⁹ INH-related DILI was reported to be more common among East Asians compared with white Europeans and African Americans in early population studies.^{47,48} This could reflect the higher frequency of the *NAT2**6 and *NAT2**7 alleles in these populations⁴⁶ compared with those reported for Europeans,⁴⁵ despite the overall average higher acetylation activity seen in East Asians. It also remains possible that the recent GWAS findings reported for a Thai population¹⁹ showing significance for *NAT2* are not directly comparable to the current study as the liver enzyme elevation thresholds for participation in that study were considerably lower than in the current study.

We also studied four additional genes potentially relevant to INH disposition in detail in both cohorts to see if any evidence for trends toward genome-wide significance could be detected. These were chosen on the basis of direct relevance to the INH metabolic pathway⁹ and either encode enzymes (CYP2E1, CES1, and CES2) or transcription regulators with a role in regulation of gene expression (PXR/NR1I2, which regulates CES expression). Our findings were entirely negative. We believe this is not too surprising and is generally consistent with reports in the existing literature of no significance or small effects.^{24,49} Larger studies might enable the detection of smaller effects than was feasible in the current study.

A limitation of this study and most others on DILI due to anti-TB drugs is that in addition to INH, the other drugs used in treatment, especially pyrazinamide, can also cause DILI. All cases in the Indian cohort were related to combination anti-TB drug therapy, whereas a significant proportion of DILI cases in the European cohort were attributable to INH monotherapy so the two cohorts are not identical in terms of drug treatment. This is an important limitation but the results obtained for the two cohorts, especially for *NAT2* genotype and to some extent for *HLA* genotype, are still comparable. Alternative regimens not involving INH show slightly lower incidence of hepatotoxicity but DILI may still occur⁵⁰ and although it may be possible to determine which drug causes DILI in a particular patient, this requires a series of individual drug rechallenges, which is difficult to perform and was not feasible in the current study.

In conclusion, we have obtained some evidence that certain *NAT2* alleles increase the risk of DILI in patients receiving INH-containing anti-TB drug regimens. The two cohorts we studied were large compared with most previous studies with a well-defined phenotype but were still small compared with GWAS on more common diseases where relatively small but significant effects can be detected by use of very large cohorts. There is a need for additional studies involving either larger cases numbers or additional meta-analyses to better understand the underlying risk factors for DILI due to INH and other anti-TB drugs.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

ACKNOWLEDGMENTS

The authors are extremely grateful to Arthur Holden of ISAEC for his support. We are grateful to Julia Patch, Julian Leathart, and Julian Arbuckle for technical help, study management, and assistance with recruitment on the iDILIC study, and to Daniele Cusi (Hypergenes), Patrik K. Magnusson (Swedish Twin Registry), and Javier Martin (Spanish DNA bank) for provision of control data. M.P. thanks the MRC Centre for Drug Safety Science for infrastructure support. We thank the Drug Induced Liver Injury Network (DILIN) for providing its data included in this paper, which is now publicly available in dbGAP. The DILIN was not involved in data analyses, manuscript preparation, or its review. The views expressed are those of the authors and not necessarily those of the National Health Service (NHS), the National Institute for Health Research (NIHR), or the UK Department of Health.

FUNDING

The genome-wide association study and iDILIC case enrollment and sample collection in Europe and India was funded by the International Serious Adverse Events Consortium with (Phase 2) membership support from Abbott, Amgen, Daiichi-Sankyo, GlaxoSmithKline, Merck, Novartis, Pfizer, Roche, Sanofi-Aventis, Takeda, and the Wellcome Trust. This is a summary of independent research partly (the DILIGEN and iDILIC sample collection) funded by the National Institute for Health Research (NIHR) Nottingham Digestive Diseases Biomedical Research Unit and NIHR Nottingham Biomedical Research Centre (BRC-1215-20003) at the Nottingham University Hospitals NHS Trust and University of Nottingham. G.P.A. is the gastrointestinal and liver disorder theme lead for the NIHR Nottingham BRC (Reference no: BRC-1215-20003). The Spanish DILI Registry (R.J.A. and M.I.L.) is partly funded by the Spanish Medicine Agency, Fondo Europeo de Desarrollo Regional - FEDER (P10-CTS-6470, FIS PI12/00378, PI16/01748). CIBERehd is funded by Instituto de Salud Carlos III. The Swedish case collection (SWEDEGENE) (M.W.) has received support from the Swedish Medical Products Agency, the Swedish Society of Medicine (2008-21619), Swedish Research Council (Medicine 521-2011-2440 and 521-2014-3370), and Swedish Heart and Lung Foundation (20120557). The Swedish Twin Registry, which provided control data is managed by Karolinska Institutet and receives funding through the Swedish Research Council under the grant no. 2017-00641. M.P. is an NIHR Emeritus Senior Investigator. J.I.G., E.B., M.I.L., R.A., A.K.D. and G.P.A. are supported by IMI2 TransBioLine Project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICTS OF INTEREST

M.P. has received grants from the MRC Clinical Pharmacology Training Scheme (joint funding by MRC and Roche, UCB, Eli Lilly and Novartis), grants for a Joint PhD studentship funded by EPSRC and Astra Zeneca, and educational grants from Bristol Myers Squibb and UCB, but all these grants were in relation to other work outside of the subject area covered in this paper. P.N. is an employee of Sema.⁴ G.P.A. has served as an advisory board member for Medicines and Healthcare Products Regulatory Agency, Department of Health and Social Care, UK, Pfizer and Glaxo SmithKline; he has been a consultant to Amryt Pharmaceuticals and Astra Zeneca. All other authors report no competing interests for this work.

AUTHOR CONTRIBUTIONS

G.P.A. and A.K.D. designed the research. G.P.A., H.D., A.G., C.E.E., E.B., M.W., D.L., L.I., P.B.W., A.-H.M.-Z., R.J.A., M.I.L., M.P., R.V., J.I.G., and A.K.D. performed the research. P.N., S.Z., G.P.A., and A.K.D. analyzed the data. P.N., G.P.A., and A.K.D. wrote the manuscript.

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