# *HLA-DRB1*16:01-DQB1*05:02* is a novel genetic risk factor for flupirtine-induced liver injury

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**_Objective_ Flupirtine is a nonopioid analgesic with regulatory approval in a number of European countries. Because of the risk of serious liver injury, its use is now limited to short-term pain management. We aimed to identify genetic risk factors for flupirtine-related drug-induced liver injury (DILI) as these are unknown.**

**_Materials and methods_ Six flupirtine-related DILI patients from Germany were included in a genome-wide association study (GWAS) involving a further 614 European cases of DILI because of other drugs and 10 588 population controls. DILI was diagnosed by causality assessment and expert review. Human leucocyte antigen (HLA) and single nucleotide polymorphism genotypes were imputed from the GWAS data, with direct HLA typing performed on selected cases to validate HLA predictions. Four replication cases that were unavailable for the GWAS were genotyped by direct HLA typing, yielding an overall total of 10 flupirtine DILI cases.**

**_Results_ In the six flupirtine DILI cases included in the GWAS, we found a significant enrichment of the _DRB1*16:01-DQB1*05:02_ haplotype compared with the controls (minor allele frequency cases 0.25 and minor allele frequency controls 0.013; $P=1.4\times10^{-5}$). We estimated an odds ratio for haplotype carriers of 18.7 (95% confidence interval 2.5–140.5, $P=0.002$) using population-specific HLA control data. The result was replicated in four additional cases, also with a haplotype frequency of 0.25. In the combined cohort (six GWAS plus four replication cases), the haplotype was also significant (odds ratio 18.7, 95% confidence interval 4.31–81.42, $P=6.7\times10^{-5}$).**

**_Conclusion_ We identified a novel HLA class II association for DILI, confirming the important contribution of HLA genotype towards the risk of DILI generally. _Pharmacogenetics and Genomics_ 00:000–000 Copyright © 2016 Wolters Kluwer Health, Inc. All rights reserved.**

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## Introduction

Flupirtine is a nonopioid analgesic that appears to act as a selective neuronal potassium channel-opening agent [1]. It was approved for use in Germany and Austria in 1984 and subsequently in a further 10 European Union countries, but it has been used particularly widely in Germany [2,3]. Flupirtine has been implicated in a number of cases of serious liver injury including those leading to acute liver failure and fatality reported through spontaneous adverse drug reaction (ADR) reporting in Germany [3]; on the basis of a review by European Medicines Agency's Pharmacovigilance Risk Assessment Committee, the drug is now recommended by German regulators only for short-term use in cases where other analgesics such as nonsteroidal anti-inflammatory drugs are contraindicated [4]. The precise incidence of liver injury in those treated with the drug remains slightly unclear, with estimates ranging from 31% of patients exposed to at least 6 weeks of treatment in a clinical trial showing some increase in liver enzymes [5] down to 0.8 in 10 000 on the basis of ADR reports in the German

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health authority (BfArM) database [3]. In a hospital-based case–control study from Berlin, flupirtine was the drug with the strongest association with acute DILI, with an odds ratio (OR) of 40 [95% confidence interval (CI) 5.5–856.9] [6]. The liver injury pattern observed is predominantly hepatocellular and liver biopsies have shown perivenular necrosis with mild-to-moderate lymphocytic infiltration [2]. In addition, cases of acute liver failure and death from flupirtine have been included in the German spontaneous reporting system [4].

The metabolism of flupirtine is complex, but does not involve the cytochromes P450 to any significant extent. The parent drug is subject to *N*-glucuronidation and may also be hydrolysed by esterases, followed by *N*-acetylation to form an active metabolite D13223 [7]. Flupirtine and D13223 are also oxidized by peroxidases, generating reactive quinone imines, which can be conjugated with glutathione [7]. In a recent in-vivo study, *NAT2*, *UGT1A1* and *GSTP1* genotypes did not affect the metabolic profile, but some evidence was obtained that the *GSTP1* genotype for rs1695 and the rarer rs1138272, which are both associated with amino acid changes, affected oral bioavailability [8].

To gain further insights into genetic factors affecting susceptibility to flupirtine-induced liver injury, we analysed six cases that were recruited as part of a larger multidrug genome-wide association study (GWAS) on drug-induced liver injury (DILI) because of a variety of different drugs. We report an association for flupirtine-related injury with a specific class II human leucocyte antigen (HLA) haplotype and describe the confirmation and replication of this finding in four additional cases.

## Materials and methods

### DILI cases

Recruitment was performed as part of the larger International DILI Consortium (iDILIC) study. We recruited 11 individuals (10 women and one man, mean age at date of DILI was 55 years) who were suspected of having suffered flupirtine-related DILI, but had recovered following drug withdrawal. The cases were identified by the German spontaneous reporting database. All participants provided written informed consent and the study was approved by the Ethics Committee of the Medical Faculty, University of Kiel. All cases provided a 10 ml blood sample for DNA preparation. Clinical data were collected from hospital or medical practice records. Only 10 cases who were classified on causality assessment as having at least possible flupiritine-related DILI were included in the study [9]. Causality assessment was performed using the Council for International Organizations of Medical Science scale, also called the Roussel Uclaf Causality Assessment Method (RUCAM) [10], and by an expert review from two hepatologists. The pattern of liver injury was classified according to the International Consensus Meeting Criteria [10]. The discovery portion of the analysis was a GWAS that utilized the six initially available flupirtine DILI cases together with an additional 614 DILI cases that had also passed expert adjudication, but related to other drugs, with genotyping performed on the Infinium HumanCoreExome BeadChip (Illumina, San Diego, California, USA). Imputed HLA genotypes were later confirmed where possible by direct HLA typing. A further four flupirtine DILI cases became available later and were HLA typed using a sequence-based assay. These served as the replication cohort to confirm the HLA association for flupirtine-related DILI identified in the discovery phase.

### European controls

As DILI has a very low prevalence, we used general population samples as study controls. The validity of this approach in studies on ADRs has been discussed in detail previously [11,12]. We selected 10 588 ethnicity-matched White controls from several available sources: the Welcome Trust Case Control Consortium (*http://www.wtccc.org.uk*), POPulation REference Sample collection [11], the PGX40001 clinical study [13], the dbGAP study phs000346.v1 [14], the hypergenes cohort (*http://www.hypergenes.eu*), the National Spanish DNA Bank cohort (*http://www.bancoadn.org/*), the Swedish Twin Registry cohort (*http://ki.se/en/research/the-swedish-twin-registry*) and the Italian Penicillin Tolerant Cohort. As the hypergenes dataset contains only autonomic chromosomes, the sex chromosomes were not considered in the analysis. Information on the genotyping platform used by each of the control cohorts is reported in Supplementary Table S1 (Supplemental digital content 1, *http://links.lww.com/FPC/A989*). The number of controls chosen was based on the power calculations reported in a previous study [12] but also to ensure appropriate ethnic matching for the entire DILI cohort of 620 cases who were recruited from a range of both Northern and Southern European countries.

### Quality checks for each genotyped cohort

Quality checks (QC) were performed at both single marker and patient levels before performing the single nucleotide polymorphism (SNP) imputation. Any marker that did not fulfil the following criteria was excluded from analysis: (i) genotype call rate in the batch of patients greater than 95%, (ii) missing genotype rate greater than 5% and (iii) *P*-value for Hardy–Weinberg equilibrium greater than $10^{-7}$ in controls (if applicable). Any patient who did not fulfil the following criteria was excluded from analysis: (i) missing genotype rate less than 0.05 among the SNPs that passed QC; (ii) not a sample duplicate or closely related on the basis of estimated identity-by-descent using PLINK v 1.07.

### HLA genotyping

DNA was prepared as described previously [15]. High-resolution genotyping of HLA-A, B, C, DRB1, DQA1

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and DQB1 was performed on all cases by Histogenetics (Ossining, New York, USA). Sequencing data files were analysed using Histogenetics' proprietary analysis software for HLA genotype calling (Histomatcher and HistoMagic). Allele assignments are based on the IMGT/HLA Database release, version 2.21.0, dated April 2008 (*http://www.ebi.ac.uk/imgt/hla/*).

### SNP imputation

For each cohort, SNPs and samples with poor-quality data were pruned before the imputation to avoid false positives (See Supplementary Material, Supplemental digital content 1, *http://links.lww.com/FPC/A989*). The imputation was performed in batches by dividing the cohorts according to genotyping platform. For each batch, we first phased the data by SHAPEIT (version v2.r727) to increase the accuracy of the imputation [16]. Then, imputation was carried out using IMPUTE2 (version 3) [17] with data from the 1000 Genomes Project (release v3) [16] as the reference panel. We used an ethnic mixed panel to improve the quality of the imputation [18]. We retained imputed genotypes with: (i) posterior probability greater than 0.9 in each genotyping batch, (ii) no significant difference in missingness between cases and controls ($\chi^2$-test, $P>0.0001$), (iii) no significant deviation from Hardy–Weinberg equilibrium ($P>0.0001$), (iv) no missing data at a frequency greater than 5% in each single genotyping batch, (v) info score greater than 0.8 in each genotyping batch and (vi) minor allele frequency in the 1000 Genomes Project greater than or equal to 0.01. Batch effects for imputed SNPs were corrected by testing for an association between ethnically matched controls typed by different platforms (using logistic regression). SNPs with association *P*-values less than 0.005 were excluded from the analysis.

### HLA imputation

For each cohort, HLA alleles were inferred using HIBAG [19] using the reference predictor panels specific for the genotyping chip.

### Statistical analysis

The effect of population structure was assessed through principal components analysis using the smartPCA program from the EIGENSTRAT package (version 3.0) [20]. The statistical association of each genomic variant was determined by logistic regression using the first seven significant principal components as covariates under an additive model. Association analyses were carried out using PLINK 1.07 [21]. We set the GWAS genome-wide significance *P*-value threshold to $5\times10^{-8}$ to correct for multiple testing. In total, we imputed 217 HLA alleles in the overall European cohort. We set the major histocompatibility complex (MHC)-region-wide significance *P*-value threshold for the HLA allele association to $2.3\times10^{-4}$ to correct for multiple testing (Bonferroni correction). Fisher's exact test was used to test the HLA association when data from the allele frequencies repository (*http://www.allelefrequencies.net*) were analysed. The number of carriers was estimated on the basis of the reported HLA allele frequency assuming Hardy–Weinberg equilibrium. The analyses were carried out using R (version 3.0.2) [22].

## Results

### Clinical characteristics of the cases

A summary of the clinical characteristics is presented in Table 1. The six cases used in the discovery GWAS analysis were recruited first and passed adjudication with RUCAM scores of between 3 and 8 (indicating a 'possible' or a 'probable' causal relationship). For one of these cases, the causal drug was ambiguous, with identical RUCAM scores for flupirtine and diclofenac. For the other five cases, flupirtine was found to be the sole cause of DILI. An additional five cases became available later. The RUCAM scores for these varied between 2 and 7. A single case showing a score of 2 was eliminated from the study, leaving four cases available for replication genotyping.

### GWAS findings and HLA allele assignment by imputation

Principal component analysis confirmed the self-reported ethnicity for the cases and divided the controls into four major clusters (Fig. S1, Supplemental digital content 1, *http://links.lww.com/FPC/A989*). No genome-wide significant signal for flupirtine cases was detected in the GWAS (Supplementary Fig. S2, Supplemental digital content 1, *http://links.lww.com/FPC/A989*). Findings for other cases have been described elsewhere [23]. Table 2 summarizes the imputed *DRB1* and *DQB1* genotypes for all the flupirtine cases that passed adjudication. Three out of the six cases included in the GWAS were predicted to be heterozygous for two class II HLA alleles *DRB1*16:01* and *DQB1*05:02*, showing higher allele frequency in cases (0.25) compared with the controls (0.013 for *DRB1*16:01* and 0.017 for *DQB1*05:02*). These two HLA alleles have a similar frequency in the controls and are in linkage disequilibrium ($r^2=0.8$). Their combination forms a well-known haplotype observed in a range of populations worldwide (*http://www.allelefrequencies.net*). It was therefore probable that all three cases would be heterozygous for the HLA class II haplotype *DRB1*16:01-DQB1*05:02*, yielding a frequency of 0.25 for the haplotype. Interestingly, the single DILI case (case 5) for which causal drug assignment was ambiguous was predicted to be negative for the haplotype.

The OR of the haplotype *DRB1*16:01-DQB1*05:02* in cases versus controls was 45 (95% CI 8.0–251.3, $P=1.4\times10^{-5}$). rs137931178 was the SNP with the lowest *P*-value in the MHC region (OR = 79, 95% CI

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**Table 1 Clinical characteristics of the patients**

| Number | Study phase | Age/sex | DILI phenotype | RUCAM score | Time on flupirtine prior DILI | Exposure to other hepatotoxic drugs | Peak ALT (U/l) |
|---|---|---|---|---|---|---|---|
| 1 | Discovery phase (GWAS) | 57/F | Hepatocellular | 5 (Possible) | 180 | No | 2460 |
| 2 | Discovery phase (GWAS) | 49/F | Hepatocellular | 7 (Probable) | 43 | No | 800 |
| 3 | Discovery phase (GWAS) | 40/F | Hepatocellular | 3 (Possible) | 164 | No | 868 |
| 4 | Discovery phase (GWAS) | 70/F | Hepatocellular | 6 (Probable) | 30 | No | 725 |
| 5 | Discovery phase (GWAS) | 77/M | Hepatocellular | 8 (Probable) | 15 | Diclofenac | 1856 |
| 6 | Discovery phase (GWAS) | 43/F | Hepatocellular | 7 (Probable) | 25 | No | 955 |
| 7 | Replication phase | 57/F | Hepatocellular | 4 (Possible) | 41 | No | 834 |
| 8 | Replication phase | 78/F | Hepatocellular | 4 (Possible) | 40 | Metamizole | 591 |
| 9 | Not included | 29/F | Hepatocellular | 2 (Unlikely) | 92 | Oxycodon | 477 |
| 10 | Replication phase | 52/F | Hepatocellular | 3 (Possible) | 20 | No | 2042 |
| 11 | Replication phase | 55/F | Hepatocellular | 7 (Probable) | 60 | No | 2190 |

ALT, alanine aminotransferase; DILI, drug-induced liver injury; GWAS, genome-wide association study; RUCAM, Roussel Uclaf Causality Assessment Method.

**Table 2 HLA *DRB1* and *DQB1* genotypes in cases showing possible or higher causality**

| Number | Type of case | Genotyping method | DRB1 genotype | | DQB1 genotype | |
|---|---|---|---|---|---|---|
| 1 | GWAS | Imputation and direct typing | 11:04:01 | 16:01:01 | 03:01:01 | 05:02:01 |
| 2 | GWAS | Imputation | 01:01 | 03:01 | 02:01 | 05:01 |
| 3 | GWAS | Imputation and direct typing | 04:04:01 | 16:01:01 | 03:02:01 | 05:02:01 |
| 4 | GWAS | Imputation | 11:01 | 16:01 | 03:01 | 05:02 |
| 5 | GWAS | Imputation | 14:01 | 15:01 | 05:03 | 06:02 |
| 6 | GWAS | Imputation | 07:01 | 13:01 | 03:03 | 06:03 |
| 7 | Replication | Direct typing | 04:04:01 | 07:01:01G | 02:01:01G | 03:02:01G |
| 8 | Replication | Direct typing | 07:01:01G | 16:01:01 | 03:03:02 | 05:02:01 |
| 10 | Replication | Direct typing | 03:01:01G | 04:08:01 | 02:01:01 | 03:04:01 |
| 11 | Replication | Direct typing | 04:01:01 | 16:01:01 | 03:01:01 | 05:02:01 |

Where direct typing was performed, six digit genotypes are shown if available. Imputed genotypes were to four digits only.
GWAS, genome-wide association study; HLA human leucocyte antigen.

11.72–533.7, $P = 7.2 \times 10^{-6}$) and correlated with these HLA alleles in the controls ($r^2$ of 0.7 with *DRB1*16:01*). The frequency of the imputed haplotype *DRB1*16:01-DQB1*05:02* in 616 European nonflupirtine DILI cases from the iDILIC cohort was found to be 0.015, which was comparable with the frequency of this haplotype in the overall controls (0.013), suggesting that the apparent association is specific to flupirtine-related DILI.

Haplotype frequency data are not available for any large general European or German cohorts, but there are detailed data on individual allele frequencies available (*http://www.allelefrequencies.net*). The frequency of *DRB1*16:01* in the largest European group (*USA NMDP European Caucasian cohort*, $n = 1\,242\,890$ individuals) was 0.014 and confirmed our estimate of 0.013 for European controls. To further assess the significance of the candidate association with *DRB1*16:01*, we used population-specific frequency data for *DRB1*16:01* only in Germans (*German pop 8*, $n = 39\,689$), where *DRB1*16:01* occurred at a frequency of 0.026. On the basis of carriage of the *DRB1*16:01* allele, the population-corrected OR for flupirtine DILI was 18.7 (95% CI 2.5–140.5, $P = 0.002$).

### Confirmation of the imputed HLA genotypes and replication of the association

We used sequence-based HLA typing to profile the four replication cases along with two of the six cases from the discovery GWAS phase. The imputed HLA genotypes were confirmed for two of the three GWAS cases predicted to carry *DRB1*16:01-DQB1*05:02*. No DNA from the third positive case and the cases predicted to be negative for the *DRB1*16:01* haplotype was available for the additional typing. As summarized in Table 2, two of the four replication cases were heterozygous for *DRB1*16:01* and *DQB1*05:02*, yielding an allele frequency of 0.25 for both alleles in line with the GWAS findings. This suggested an overall frequency of 0.25 for the haplotype among the 10 confirmed DILI cases, with 50% of all cases heterozygous carriers of the risk haplotype. In the combined cohort, we estimated the German-specific OR for carriage of *DRB1*16:01* to be 18.74 (95% CI 4.31–81.42). After correction for multiple comparisons for the entire MHC region, the association remained statistically significant (uncorrected *P*-value $6.7 \times 10^{-5}$).

### Genotypes for polymorphisms relevant to flupirtine metabolism

*NAT2*, *UGT1A1* and *GSTP1* are involved in flupirtine metabolism [8], and these genes are also subject to common polymorphisms with well-established phenotypic effects. *NAT2* (rs1801280, rs1799930, rs1799931) and *GSTP1* (rs1695) genotypes relevant to phenotype were available from the GWAS data. Three cases (50% of the group) were predicted to be NAT2 slow acetylators with genotypes of *5/*6 (two cases) and *5/*5 (one case). The other three samples were fast acetylators with genotypes of *4/*5 (two samples) and *4/*4 (one sample).

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This frequency of *NAT2* slow acetylators is not significantly different from that in Europeans generally, which has been estimated to be 57% from genome sequencing [24]. Three cases were heterozygous for *GSTP1* rs1695, with the other three homozygous wild type for this allele. The frequency of this polymorphism in our Europeans controls (0.34) was higher than that in cases (0.25), but it was not significantly different (OR = 0.7, 95% CI 0.2–2.7, $P = 0.6$). For UGT1A1, genotype data for the well-characterized TA repeat polymorphism that can be used to predict the decreased UGT1A1 expression that gives rise to Gilbert's syndrome were not available in our cases. However, genotypes for rs4124874, which is a tag SNP for the most common haplotype associated with normal UGT1A1 activity [25], were available. We found that the frequency of this SNP in the cases was not significantly different from the controls (OR = 0.64, 95% CI 0.18–2.24, $P = 0.49$).

## Discussion

We found an association of *DRB1*16:01-DQB1*05:02* with flupirtine-related DILI, with carriers of this haplotype being at a 19-fold higher risk of developing a clinically significant ADR. The finding that HLA alleles confer an increased risk is consistent with previous reports on DILI because of a number of other drugs [26] as well as previous clinical and histological reports of immune-mediated toxicity with moderate lymphocytic infiltration and extensive perivenular necrosis observed on liver biopsy in some flupirtine DILI cases [2,5]. This indicates that the increased risk of DILI because of flupirtine in *DRB1*16:01-DQB1*05:02* carriers is likely to involve an inappropriate T cell response within the liver. Both HLA class I and II associations with DILI because of other drugs have been reported previously, although the current study is the first report of an association with *DRB1*16:01-DQB1*05:02*. A class II haplotype that includes *DRB1*15:01* has been shown to be associated with both coamoxiclav and lumiracoxib DILI in previous studies [27,28]. There is homology between *DRB1*15:01* and *DRB1*16:01*, with both being members of the DR2 serotype group. However, despite showing sequence homology, the alleles have distinct disease associations. For example, *DRB1*15:01* is protective against type I diabetes, but this protective effect does not extend to *DRB1*16:01* [29], whereas *DRB1*15:01* is a well-established susceptibility factor for multiple sclerosis, but *DRB1*16:01* is not a risk factor [30].

So far, HLA associations for DILI with a predominantly hepatocellular phenotype have been found with class II but not class I alleles (e.g. lapatinib [31], ximelagatran [32], lumiracoxib [28]) and the new findings for flupirtine are in line with this. However, certain cholestatic/mixed reactions to single drugs such as flucloxacillin [33] and ticlopidine [34] involve HLA class I alleles. The risk of DILI involving the antimicrobial coamoxiclav appears to be higher in individuals expressing particular HLA class I and/or class II alleles, but this more complex association appears to involve inappropriate T cell responses to both drugs [35] and the phenotype, although most frequently cholestatic or mixed, is sometimes hepatocellular [36]. The underlying mechanism by which inappropriate T cell reactions are triggered in DILI reactions remains unclear, but recent in-vitro studies on T cell responses in DILI cases because of flucloxacillin and coamoxiclav [35, 37] suggest that a hapten mechanism is more likely than a direct interaction by the drug with the HLA protein of the type that appears to occur in abacavir hypersensitivity [38]. Further similar investigations on the mechanism involved in the apparently exclusively HLA class II-associated flupirtine DILI would be interesting.

Specific HLA alleles have been identified as strong risk factors for the development of ADRs in studies involving small numbers of cases, but the current study appears to have involved the smallest discovery cohort to date, with only six cases. Some previous studies showing HLA associations with DILI involved only slightly larger numbers of cases, for example 22 cases for the ticlopidine DILI association with *HLA-A*33:03* [34] and 35 cases in the original report of the association of *HLA-DRB1*15:01* with coamoxiclav DILI [39]. These associations were also with relatively rare HLA alleles. In the current study, the very low population frequency of the 'at risk' haplotype combined with the relatively large effect size were important factors in our ability to detect a significant genetic association when only a small number of cases were available. The subsequent availability of replication cases was a further advantage. It remains possible that HLA associations relevant to other ADRs may be detectable when similarly small numbers of cases are available.

No other genetic risk factors with high ORs were detected in the GWAS. The metabolic pathway of flupirtine is now well understood and it has been suggested that quinone imine intermediates could contribute towards the liver toxicity [8]. In particular, it was suggested previously that the well-established polymorphisms in NAT2, UGT1A1 and GSTP1 could influence levels of toxic metabolites, but a study in healthy volunteers failed to detect statistically significant differences in pharmacokinetics between the various genotype groups, except for a small difference for GSTP1 [8]. Our study confirmed that the frequencies of relevant polymorphisms from the GWAS data were similar to those observed in European populations generally; thus, these genotypes do not appear to be risk factors. However, in view of the small numbers of cases studied, the findings need to be treated with caution.

It should be noted that only a very small proportion of the total risk of DILI will relate to the HLA genotype described in the current study, but factors such as age

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older than 60 years and female sex also appear to contribute towards risk. The age and sex distribution of the cases in the current study together with the DILI phenotype are in agreement with previous reports on DILI because of other drugs [40]. Age older than 60 years has been described as a risk factor for flucloxacillin DILI, where there is also a strong association with the HLA genotype [33]. Flupirtine DILI appears to manifest relatively early in the course of therapy, with 31% of patients treated for at least 6 weeks showing an increase in alanine aminotransferase and/or aspartate aminotransferase in excess of three-fold above ULN [5]. Another study, however, reported elevated 'liver enzymes/bilirubin' in three of 105 patients treated with the drug for 5–7 days [41]. In line with some patients showing toxicity after relatively short exposures, the mean length of exposure in the current study was 65 days, but the overall exposure length ranged from 15 to 180 days. However, this duration was above the recommended maximum treatment period of 14 days [4] in all cases.

In summary, we have found a strong association between flupirtine DILI and the *DRB1*16:01-DQB1*05:02* haplotype, adding further evidence to the involvement of the adaptive immune system in the pathogenesis of DILI.

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### Conflicts of interest

There are no conflicts of interest.

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