frontiers in Genetics

CASE REPORT
published: 28 February 2019
doi: 10.3389/fgene.2019.00134

# Next-Generation Sequencing of *PTGS* Genes Reveals an Increased Frequency of Non-synonymous Variants Among Patients With NSAID-Induced Liver Injury

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***Specialty section:***
*This article was submitted to Pharmacogenetics and Pharmacogenomics, a section of the journal Frontiers in Genetics*

***Received:*** *19 November 2018*
***Accepted:*** *08 February 2019*
***Published:*** *28 February 2019*

***Citation:***
*Lucena MI, García-Martín E, Daly AK, Blanca M, Andrade RJ and Agúndez JAG (2019) Next-Generation Sequencing of PTGS Genes Reveals an Increased Frequency of Non-synonymous Variants Among Patients With NSAID-Induced Liver Injury. Front. Genet. 10:134. doi: 10.3389/fgene.2019.00134*

**Purpose:** The etiopathogenesis of drug-induced liver injury (DILI) is still far from being elucidated. This study aims to the study of genetic variations in DILI, related to the drug target, and specifically in the genes coding for the cyclooxygenase enzymes.

**Methods:** By using Next-generation Sequencing we analyzed the genes coding for COX enzymes (*PTGS1* and *PTGS2*) in 113 individuals, 13 of which were patients with DILI caused by COX-inhibitors.

**Results:** The key findings of the study are the increased frequency, among DILI patients, of SNPs causing alterations in transcription factor binding sites and non-synonymous *PTGS* gene variants, as compared to control subjects. Moreover, the association with non-synonymous SNPs was exclusive of DILI patients with late-onset (50 days or more) $Pc < 0.001$ as compared to DILI patients with early onset, or with control subjects.

**Conclusions:** Our findings suggest an interaction of long-term exposure to COX inhibitors combined with functional variants of the COX enzymes in the risk of developing DILI. This is a novel observation that might have been overlooked by previous genetic studies on DILI because of the limited coverage of PTGS genes in exome chips.

**Keywords: PTGS1, PTGS2, next generation sequencing, drug-induced liver injury, COX1, COX2**

## BACKGROUND

Although drug-induced liver injury (DILI) is a rare adverse drug event, it is often life-threatening because of the risk of developing acute liver failure. The mechanisms underlying DILI risk are not well understood and hence, the search for biomarkers of DILI risk is a major research field that aims to identify markers that could be used as both proof of the mechanisms involved and of the risk factors that can be used for DILI prediction, as has already been done with many pharmacogenomics biomarkers (Lucena et al., 2008, 2010; Agúndez, 2009; Agundez et al., 2009, 2011; Andrade et al., 2009; Robles-Diaz et al., 2016; Nicoletti et al., 2017). There are presently several

independent hypotheses to explain idiosyncratic DILI, but none of these is able to explain all the circumstances in which DILI occurs.

Some genetic biomarkers for DILI either mechanistically-based using a case-control strategy or with a GWAs/exome sequencing approaches have been identified [for a review, see (Robles-Diaz et al., 2016)]. However, the involvement of genetic changes in DILI risk (for instance HLA risk alleles) has been documented for only a few drugs (Kaliyaperumal et al., 2018). On the other hand, case-control genotyping studies, GWAS and exome sequencing have important limitations because only some SNPs are tested, and most of the target sequence is not checked. To overcome this problem, deep sequencing comprising whole genes is necessary.

In this study, we analyzed the potential effect of mutations in cyclooxygenase genes (*PTGS1* and *PTGS2*) on DILI risk related to NSAIDs. From a mechanistic point of view, such a risk could be related to genetic alteration in the arachidonic acid pathways, which are closely related to inflammation. On the other hand, adverse drug events for drugs acting on the COX enzymes (that is, COX inhibitors) may be more likely if COX activity is altered because of genetic variations. For this reason, we analyzed patients who developed DILI after the administration of COX-inhibitors and healthy individuals who tolerated COX-inhibitors.

## CASE PRESENTATION

Thirteen patients (8 women and 5 men) who experienced DILI caused by COX inhibitors and 100 individuals who tolerated COX-inhibitors at standard doses were included in this study. The culprit drug for DILI and clinical details of patients are shown in **Table 1.** Gender-matched control individuals who tolerated COX-inhibitors (62 women and 38 men) individuals were recruited among staff and medical students of the Hospitals and the Universities participating in this study. Individuals which were considered as healthy after medical examination, to exclude pre-existing disorders and history of adverse events after the use of COX-inhibitors, were asked to participate and over 95% of these agreed to do so. We selected consecutive control subjects matched with patients for drug exposure: Fifty control subjects who have received ibuprofen within the previous month to sample collection, 20 who received diclofenac, 10 indomethacin, 10 naproxen, and 10 rofecoxib. These frequencies match with the frequencies for the DILI patients, except that no control subject received nimesulide since this drug was discontinued from the Spanish market due to liver safety. Both patients and controls were Caucasian Spanish individuals. Written informed consent for participation in this case report was obtained from all participants. The protocol for this study was in accordance with the Declaration of Helsinki and its subsequent revisions and was approved by the respective Ethics Committees of the participating Hospitals.

**Abbreviations:** COX, Cyclooxygenase, prostaglandin-endoperoxide synthase; NSAID, Non-steroidal anti-inflammatory drug; DILI, Drug-induced liver injury; GWAS, Genome-wide association study; SNP, Single nucleotide polymorphism; HLA, Human leukocyte antigen; PTGS1, Prostaglandin-Endoperoxide Synthase 1; PTGS2, Prostaglandin-Endoperoxide Synthase 2.

## DESCRIPTION OF LABORATORY INVESTIGATIONS AND DIAGNOSTIC TESTS

To achieve complete gene capture, we sequenced all exons, intron-exon boundaries as well as the 5′ and 3′ flanking regions for both genes. Referred to the GRCh37 assembly of the human genome, the sequences studied were the following: *PTGS1*: Chromosome 9:125.131.159 to 125.158.017; *PTGS2*: Chromosome 1:186.640.825 to 186.651.605. Partially overlapping amplicons with a size lower than 400 bp were designed. A total of 62 CS1/CS2 tagged primer pairs were synthesized and used to amplify 113 DNA samples using the Access Array platform (Fluidigm). During amplification, samples were labeled with standard MID barcodes designed for the FLX454 sequencing system. After amplification and MID-labeling, individual amplicon libraries were analyzed using a Bioanalyzer 2100 (Agilent) and bioanalyzer traces were used to estimate the amplicon concentration for each sample. Samples were then pooled, and libraries were purified by SPRI using Ampure beads to remove all possible traces of small molecules, primers, primer-dimers, or any other contaminants. The pooled library was again quantified and titrated so that a final amount of 1.95E+10 molecules with an enrichment percentage of 7% was loaded on a Pico Titer Plate (Roche) for a 200-cycle titanium-based sequencing run, made on FLX-454 equipment. Reads were processed using an amplicon processing pipeline and sff files were used for further analyses. Coverage averaged around 50x for the whole project. Coverage for the SNPs identified (shown in **Supplemental Table 1**) was always over 50x. Sequencing reads were de-multiplexed and aligned using the Amplicon Variant Analyzer software v2.8 (Roche) so that reads for each particular sample- target region combination were analyzed in search of variants. Details of the amplification and sequencing primers are available in **Supplemental Table 1**.

The putative effect on the non-synonymous variants identified *in silico* was assessed by using the Sorting Tolerant form Intolerant (SIFT) and Polymorphism Phenotyping (PolyPhen) scores as shown in the 1,000 genomes website for every SNP, as well as the online application MutationAssessor (http://mutationassessor.org/r3/).

## RESULTS

The sequencing results (summarized in **Table 2**) reveal that *PTGS* genes are well conserved. Although dozens of *PTGS1* and *PTGS2* single nucleotide polymorphisms (SNPs) have been described to occur in Caucasian populations (see Agúndez et al., 2015), our findings show that most of these SNPs were not identified, or were extremely rare, in this cohort.

**TABLE 1 |** Demographic and clinical characteristics of 13 patients with NSAIDs-induced idiosyncratic liver injury.

| N° | Culprit drug | Age/ Sex | Indication | Daily dose (mg) | Duration treatment (days) | Time to onset (days) | Associated medications | Clinical presentation | Type of liver injury | TB (xULN) | ALT (xULN) | ALP (xULN) | Severity index | Outcome | CIOMS score |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Diclofenac | 76/F | Lumbar Pain | 50 | 93 | 30 | Levothyroxine Metamizole | Jaundice ASMA 1/80 | HC | 2.9 | 24 | 2.6 | Moderate | Resolution 221 days | Highly probable |
| 2 | Nimesulide | 62/F | Osteoarthritis | 200 | 30 | 30 | None | Jaundice Eosinophilia | HC | 24 | 98 | 2.5 | Moderate | Resolution 90 days | Probable |
| 3 | Nimesulide | 61/F | Osteoarthritis | 200 | 62 | 62 | Fosinopril Torasemide | Jaundice | HC | 15 | 30 | 1.7 | Moderate, hospitalized | Resolution 126 days | Highly probable |
| 4 | Indometacin | 54/F | Osteoarthritis | 100 | 11 | 8 | Tramadol Tetrazepam Dexamethasone | Elevated liver enzymes | Mix | 1 | 16 | 3.6 | Mild | Resolution 44 days | Highly probable |
| 5 | Ibuprofen | 64/F | Pain | 600 | 17 | 10 | Enalapril Insulin | Jaundice ASMA 1/40 | Mix | 9 | 5.9 | 1.7 | Moderate, hospitalized | Resolution 180 days | Highly probable |
| 6 | Naproxen | 65/M | Pain | 1,000 | 44 | 48 | Tetrazepam Ranitidine Heparin Bisoprolol Metamizole | Jaundice | Mix | 4.5 | 9.7 | 3.4 | Moderate, hospitalized | Lost of follow-up | Highly probable |
| 7 | Ibuprofen | 18/M | Pain | 600 | 1 | 2 | None | Jaundice Lymphopenia | Mix | 7.9 | 5.5 | 2.6 | Moderate, hospitalized | Lost of follow-up | Highly probable |
| 8 | Rofecoxib | 82/M | Osteoarthritis | 12.5 | 60 | 59 | Nimodipine Omeprazole Paracetamol Troxerutin Insulin Vitamins Folic Acid | Lymphopenia | Chol | 2.6 | 4.9 | 4.4 | Moderate | Resolution 94 days | Highly probable |
| 9 | Ibuprofen | 57/M | Pain | 1,200 | 31 | 50 | Glibenclamide | Elevated liver enzymes | HC | 1 | 24 | 0.9 | Mild | Lost of follow-up | Highly probable |
| 10 | Nimesulide | 59/F | Osteoarthritis | 200 | 25 | 466 | Atenolol Captopril Hydrochlorothiazide Insulin Metformin | Eosinophilia | Chol | 1.2 | 1.2 | 5 | Moderate, hospitalized | Resolution 284 days | Highly probable |
| 11 | Ibuprofen | 43/M | Pain | 1,200 | 8 | 8 | Metronidazole | Jaundice | HC | 1.9 | 8.2 | 1.3 | Mild, hospitalized | Resolution 308 | Probable |
| 12 | Diclofenac | 80/F | Pain | 75 | 171 | 171 | Telmisartan Allopurinol Glibenclamide Ebastine Troxerutin | Jaundice | HC | 4.9 | 38 | 1.9 | Moderate, hospitalized | Resolution 348 | Highly probable |
| 13 | Ibuprofen | 41/F | Rheumatoid arthritis | 600 | 3 | 7 | Diclofenac Paracetamol Diazepam Fluoxetine | Elevated liver enzymes | HC | 1.6 | 14 | 1.8 | Mild | Resolution 31 | Probable |

*F, Female; M, Male; ASMA, anti-smooth muscle autoantibody; HC, Hepatocellular, Mix, mixed; Chol, cholestatic; TB, total bilirubin; ALT, alanine aminotransferase; ALP, alkaline phosphatase; ULN, upper limit of normal. Severity index, Mild, elevated ALT/ALP meeting DILI criteria with total bilirubin <2 mg/dL; Moderate, elevated ALT/ALP with total bilirubin ≥ 2 g/dL; Severe, elevated ALT/ALP and one of the following, ascites, encephalopathy, international normalization ratio >1,5 and/or other organ failure considered to be due to DILI; Fatal, death or transplantation due to DILI.*

**TABLE 2 |** *PTGS1* and *PTGS2* variant sequences identified in the study group.

| Coordinate GRCh37.p13 (GCA_000001405.14) | rs ID | N° case | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Effect | MAF DILI | MAF control | MAF IBS | MAF AFR | MAF AMR | MAF EAS | MAF EUR | MAF SAS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PTGS1 (COX-1)** | | | | | | | | | | | | | | | | | | | | | | | | |
| 9:125131480 | rs10306108 | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | UGV | 0.077 | 0.055 | 0.06 | 0.13 | 0.03 | 0.00 | 0.07 | 0.01 |
| 9:125131631 | rs10306109 | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | UGV | 0.077 | 0.055 | 0.06 | 0.13 | 0.03 | 0.00 | 0.07 | 0.01 |
| 9:125131688 | rs1330344 | | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | UGV | 0.154 | 0.210 | 0.24 | 0.52 | 0.21 | 0.42 | 0.20 | 0.39 |
| 9:125131832 | rs10306225 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 0 | 0 | UGV | 0.154 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 |
| 9:125132027 | rs115693689 | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | UGV | 0.038 | 0.055 | 0.07 | 0.13 | 0.04 | 0.05 | 0.08 | 0.02 |
| 9:125132028 | rs114079139 | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | UGV | 0.038 | 0.055 | 0.07 | 0.13 | 0.04 | 0.05 | 0.08 | 0.02 |
| 9:125132069 | rs77676149 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | UGV | 0.077 | 0.000 | 0.00 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 |
| 9:125132223 | rs75993350 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | UGV | 0.038 | 0.055 | 0.06 | 0.13 | 0.03 | 0.00 | 0.07 | 0.01 |
| 9:125132311 | rs10306110 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | UGV | 0.038 | 0.055 | 0.06 | 0.13 | 0.03 | 0.00 | 0.07 | 0.01 |
| 9:125132522 | rs10306114 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | UGV | 0.038 | 0.055 | 0.06 | 0.13 | 0.03 | 0.00 | 0.07 | 0.01 |
| 9:125132909 | rs10306115 | | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 5′RRV | 0.077 | 0.000 | 0.00 | 0.07 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9:125133479 | rs1236913 | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | MSV[a] | 0.115 | 0.065 | 0.07 | 0.01 | 0.07 | 0.01 | 0.06 | 0.18 |
| 9:125133507 | rs3842787 | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | MSV[b] | 0.077 | 0.060 | 0.06 | 0.15 | 0.03 | 0.00 | 0.07 | 0.01 |
| 9:125140206 | rs3842788 | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | SV | 0.038 | 0.025 | 0.02 | 0.32 | 0.03 | 0.07 | 0.04 | 0.04 |
| 9:125140287 | rs3842790 | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | SV | 0.038 | 0.000 | 0.00 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9:125140696 | rs2282169 | | 0 | 1 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | IV | 0.192 | 0.140 | 0.15 | 0.60 | 0.28 | 0.11 | 0.19 | 0.23 |
| 9:125140823 | rs5787 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | MSV[c] | 0.077 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9:125141239 | rs12555242 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | IV | 0.077 | 0.070 | 0.02 | 0.00 | 0.03 | 0.05 | 0.02 | 0.08 |
| 9:125143707 | rs3842792 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | MSV[d] | 0.038 | 0.000 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9:125143792 | rs5788 | | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | SV | 0.115 | 0.110 | 0.11 | 0.69 | 0.24 | 0.04 | 0.14 | 0.10 |
| 9:125143882 | rs3842794 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | IV | 0.077 | 0.000 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9:125144040 | rs3215925 | | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | IV | 0.115 | 0.095 | 0.11 | 0.67 | 0.24 | 0.04 | 0.14 | 0.10 |
| 9:125145743 | rs3842798 | | 0 | 1 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | IV | 0.231 | 0.170 | 0.15 | 0.76 | 0.30 | 0.11 | 0.20 | 0.23 |
| 9:125155408 | rs8046 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 3′UTRV | 0.038 | 0.000 | 0.04 | 0.51 | 0.10 | 0.09 | 0.07 | 0.14 |
| 9:125155930 | rs10306192 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 3′UTRV | 0.077 | 0.000 | 0.04 | 0.01 | 0.05 | 0.09 | 0.06 | 0.13 |
| 9:125156374 | rs199981440 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 3′UTRV | 0.077 | 0.000 | – | – | – | – | – | – |
| 9:125157198 | rs10306194 | | 0 | 0 | 1 | 0 | 1 | 0 | 2 | 0 | 0 | 1 | 2 | 0 | 0 | 3′UTRV | 0.269 | 0.175 | 0.20 | 0.02 | 0.11 | 0.04 | 0.15 | 0.14 |
| 9:125157316 | rs10306196 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 3′UTRV | 0.038 | 0.065 | 0.04 | 0.01 | 0.05 | 0.09 | 0.06 | 0.13 |
| 9:125157357 | rs10306197 | | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 3′UTRV | 0.077 | 0.000 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9:125157672 | rs10306199 | | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.077 | 0.000 | 0.00 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9:125157718 | rs9233 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 3′UTRV | 0.038 | 0.065 | 0.04 | 0.01 | 0.05 | 0.09 | 0.06 | 0.14 |
| **PTGS2 (COX-2)** | | | | | | | | | | | | | | | | | | | | | | | | |
| 1:186640853 | rs4648304 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.000 | 0.00 | 0.10 | 0.01 | 0.00 | 0.00 | 0.00 |
| 1:186641058 | rs689470 | | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.077 | 0.000 | 0.05 | 0.50 | 0.05 | 0.01 | 0.03 | 0.07 |
| 1:186641265 | rs689468 | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.005 | 0.03 | 0.00 | 0.01 | 0.01 | 0.02 | 0.03 |
| 1:186641273 | rs689467 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.025 | 0.07 | 0.00 | 0.03 | 0.00 | 0.05 | 0.03 |

*(Continued)*

**TABLE 2** | Continued

| Coordinate GRCh37.p13 (GCA_000001405.14) | rs ID | N° case | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Effect | MAF DILI | MAF control | MAF IBS | MAF AFR | MAF AMR | MAF EAS | MAF EUR | MAF SAS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1:186641577 | rs4648299 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186641682 | rs4648298 | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.005 | 0.03 | 0.00 | 0.01 | 0.00 | 0.02 | 0.03 |
| 1:186642059 | rs4648297 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186642429 | rs2206593 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.050 | 0.06 | 0.00 | 0.05 | 0.00 | 0.09 | 0.06 |
| 1:186642856 | rs4648292 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 3′UTRV | 0.077 | 0.000 | 0.00 | 0.08 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186642987 | rs5276 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.015 | 0.00 | 0.18 | 0.01 | 0.00 | 0.00 | 0.00 |
| 1:186643058 | rs5275 | | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 3′UTRV | 0.269 | 0.205 | 0.33 | 0.64 | 0.37 | 0.20 | 0.31 | 0.40 |
| 1:186643204 | rs36233646 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3′UTRV | 0.077 | 0.000 | 0.02 | 0.47 | 0.04 | 0.00 | 0.01 | 0.04 |
| 1:186643238 | rs4648290 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 3′UTRV | 0.038 | 0.010 | 0.00 | 0.00 | 0.01 | 0.00 | 0.01 | 0.00 |
| 1:186643803 | rs4648288 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | SV | 0.038 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186645078 | rs5279 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | SV | 0.038 | 0.005 | 0.00 | 0.08 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186645669 | rs5278 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | SV | 0.038 | 0.000 | 0.00 | 0.08 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186645927 | rs2066826 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | IV | 0.038 | 0.105 | 0.12 | 0.36 | 0.19 | 0.04 | 0.12 | 0.16 |
| 1:186646005 | rs3218622 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | MSV[e] | 0.038 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186647418 | rs4648265 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | SV | 0.038 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186648157 | rs2066823 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | IV | 0.038 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186648197 | rs5277 | | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 2 | 1 | IV | 0.346 | 0.180 | 0.15 | 0.01 | 0.11 | 0.04 | 0.18 | 0.06 |
| 1:186650163 | rs20419 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | RRV | 0.038 | 0.000 | 0.00 | 0.10 | 0.01 | 0.00 | 0.00 | 0.00 |
| 1:186650214 | rs148416467 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | RRV | 0.077 | 0.000 | 0.15 | 0.08 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1:186650321 | rs20417 | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | RRV | 0.154 | 0.190 | 0.00 | 0.35 | 0.21 | 0.04 | 0.15 | 0.19 |
| 1:186650688 | rs20415 | | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | RRV | 0.038 | 0.000 | 0.15 | 0.10 | 0.01 | 0.00 | 0.00 | 0.00 |
| 1:186650751 | rs689466 | | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | RRV | 0.077 | 0.215 | 0.15 | 0.08 | 0.26 | 0.48 | 0.19 | 0.13 |
| 1:186650846 | rs689465 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | RRV | 0.154 | 0.235 | 0.11 | 0.16 | 0.18 | 0.05 | 0.13 | 0.16 |
| 1:186650857 | rs4648253 | | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | RRV | 0.038 | 0.000 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| 1:186650877 | rs72366725 | | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | RRV | 0.038 | 0.005 | 0.05 | 0.27 | 0.04 | 0.00 | 0.03 | 0.03 |
| 1:186651296 | rs4648250 | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | RRV | 0.077 | 0.015 | 0.00 | 0.00 | 0.07 | 0.03 | 0.01 | 0.07 |
| 1:186651571 | unknown | | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | RRV | 0.038 | 0.000 | – | – | – | – | – | – |

*MAF, Minor allele frequency referred to the GRCh37 assembly as shown in the 1,000 genomes website http://phase3browser.1000genomes.org/Homo_sapiens/Info/Index. 0, Non-mutated; 1, heterozygous; 2, Homozygous for the minor allele; UGV, Upstream gene variant; RRV, Regulatory region variant.; MSV, Missense variant; SV, Synonymous variant; IV, INtron variant; UTRV, Untranslated region variant; Populations, IBS correspond to the Iberian Populations in Spain (a subpopulation of Europeans); AFR, Africans; AMR, Ad mixed americans; EAS, East asians; EUR, Europeans; SAS, South asians, as described in detail in the website http://phase3browser.1000genomes.org/Help/Faq?id=328. Predicted consequences for missense variants:*

[a] *Nonsynonymous (W8R), SIFT score = 0.85 (tolerated, low confidence), PolyPhen score = 0 (unknown), Mutation Assessor = neutral.*

[b] *Nonsynonymous (P17L), SIFT score = 1.00 (tolerated, low confidence), PolyPhen score = 0 (unknown), Mutation Assessor = low impact.*

[c] *Nonsynonymous (R108Q), SIFT score = 0.12 (tolerated), PolyPhen score = 0.21 (benign), Mutation Assessor = medium impact.*

[d] *Nonsynonymous (K185T), SIFT score = 0.36 (tolerated), PolyPhen score = 0.007 (benign), Mutation Assessor = neutral.*

[e] *Nonsynonymous (R228H), SIFT score = 1.00 (tolerated), PolyPhen score = 0.002 (benign), Mutation Assessor = neutral.*

**TABLE 3 |** Detailed genotype distribution for relevant SNPs.

| Coordinate GRCh37.p13 (GCA_000001405.14) | rs ID | Effect | Patients with late-onset DILI *N* = 5 (Non carriers / heterozygous/ homozygous); MAF | Rest of DILI patients *N* = 8 (Non carriers / heterozygous/ homozygous); MAF | Control individuals *N* = 100 (Non carriers / heterozygous/ homozygous); MAF |
|---|---|---|---|---|---|
| 9:125131832 | rs10306225 | UGV | 3/0/2; 0.400 | 8/0/0; 0.000 | 100/0/0; 0.000 |
| 9:125133479 | rs1236913 | MSV | 2/3/0; 0.300 | 8/0/0; 0.000 | 88/11/1; 0.065 |
| 9:125133507 | rs3842787 | MSV | 3/2/0; 0.200 | 8/0/0; 0.000 | 89/10/1; 0.060 |
| 9:125140823 | rs5787 | MSV | 4/0/1; 0.200 | 8/0/0; 0.000 | 100/0/0; 0.000 |
| 9:125143707 | rs3842792 | MSV | 4/1/0; 0.100 | 8/0/0; 0.000 | 100/0/0; 0.000 |
| 1:186646005 | rs3218622 | MSV | 4/1/0; 0.100 | 8/0/0; 0.000 | 100/0/0; 0.000 |

*MAF, Minor allele frequency; UGV, Upstream gene variant; MSV, Missense variant.*

**TABLE 4 |** Haplotype analysis.

| Haplotype frequencies | rs10306225 | rs1236913 | rs3842787 | rs5787 | rs3842792 | rs3218622 | Frequency (Total) | Frequency (late-onset DILI cases) | Frequency (controls) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A | T | C | G | A | C | 0.9048 | 0.300 | 0.935 |
| 2 | A | C | T | G | A | C | 0.0619 | 0.100 | 0.060 |
| 3 | T | T | C | A | A | C | 0.0095 | 0.200 | NA |
| 4 | A | C | C | G | A | C | 0.0048 | NA | 0.005 |
| 5 | A | T | C | G | A | T | 0.0048 | 0.100 | NA |
| 6 | T | C | C | G | A | C | 0.0048 | 0.100 | NA |
| 7 | T | T | C | G | A | C | 0.0048 | 0.100 | NA |
| 8 | T | T | C | G | A | C | 0.0048 | 0.100 | NA |
| 9 | T | T | C | G | A | C | 0.0048 | 0.100 | NA |
| **Haplotype association with late-onset DILI** | **rs10306225** | **rs1236913** | **rs3842787** | **rs5787** | **rs3842792** | **rs3218622** | **Frequency (Total)** | **OR (95% CI)** | ***P*-value** |
| 1 | A | T | C | G | A | C | 0.9048 | 1.00 | — |
| 2 | A | C | T | G | A | C | 0.0619 | 0.09 (0.01–1.43) | 0.0910 |
| Rare haplotypes | * | * | * | * | * | * | 0.0333 | 0.00 (0.00–0.09) | 0.0024 |

*Global haplotype association $p < 0.0001$.*
*NA, not applicable; *any nucleotide.*

Interestingly, most of the *PTGS1* and *PTGS2* SNPs included in the Illumina human exome chip or human core exome chip (Urban et al., 2012) are also absent in this study group. This raises doubts about the coverage of exome chips to identify genetic associations related to *PTGS1* and *PTGS2* genes.

In the whole population study, we identified 31 single nucleotide polymorphisms (SNPs) for *PTGS1*, including four non-synonymous SNPs. For *PTGS2* we identified 31 SNPs including one non-synonymous. We observed an increased frequency of *PTGS1* and *PTGS2* mutations among DILI patients, as compared to that observed in control individuals. Most of the SNPs identified in patients were rare among control individuals and were rare also according to the 1,000 genomes database (as shown in **Table 2**). All patients but one (case 1 in **Table 2**) had mutations at the *PTGS1* gene and all patients but one (case 5 in **Table 2**) had mutations at the *PTGS2* gene. **Table 3** summarizes the comparison of relevant SNPs across patients with late-onset DILI, the rest of DILI patients and control individuals.

## DISCUSSION OF THE UNDERLYING PATHOPHYSIOLOGY AND THE NOVELTY OR SIGNIFICANCE OF THE CASE

The most remarkable findings in this study are the presence among DILI patients of SNPs causing alterations in transcription factor binding sites such as the *PTGS1* SNP rs10306225 (Agundez et al., 2014), and the *PTGS2* SNPs rs4648253, rs689466, and rs20417, as well as non-synonymous SNPs such as *PTGS1* rs1236913 (W 8 R), rs3842787 (P 17 L), rs5787 (R 108 Q), rs3842792 (K 185 T), and *PTGS2* rs3218622 (R 228 H). These missense variants are extremely rare among European individuals (Agúndez et al., 2015). The putative effects of the most relevant SNPs shown in **Table 3** have been revised elsewhere (Agúndez et al., 2015). In brief, besides the rs10306225 SNP, which is a promoter variant that causes a modification in a CDX1 binding site (Agundez et al., 2014), the rest of SNPs are non-synonymous. According to functional predictions and functional analyses (reviewed in Agúndez et al., 2015) the SNPs rs1236913, rs3842787 have a little functional effect,

although clinical associations for these SNPs with urticaria induced by NSAIDs (Cornejo-Garcia et al., 2012) and myocardial infarction/stroke (Lee et al., 2008; Lemaitre et al., 2009; Gao et al., 2014), respectively, have been proposed. The functional effect of the rs5787 SNP is unknown, although functional prediction suggests a mild functional impact (see **Table 2**), rs3842792 SNP is predicted as functional (**Table 2**), but *in vitro* findings suggest reduced functionality (Lee et al., 2007), and no functional impact for the *PTGS2* SNP rs3218622 has been described.

No particular association of missense SNPs with culprit drug, age, gender, clinical presentation, type of liver injury, and severity of the disease was identified. However, as shown in **Table 1**, there is heterogeneity in the duration of treatment before DILI onset. This heterogeneity, rather than being a weakness, is a strong point in this study because it allowed discriminating the frequencies of PTGS gene variations in DILI patients with late and short-term onset. All the five DILI patients with the longest times to DILI onset (50 or more days; patients n° 3, 8, 9, 10, 12 in **Table 1**) had missense variants, and no patient with shorter time to DILI onset had such missense variants. The intergroup comparison values for carriers of any non-synonymous *PTGS* variants were as follows: Patients with late DILI onset (50 or more days) vs. the rest of DILI patients ($P < 0.001$). Patients with late DILI onset vs. control individuals ($P < 0.001$). By turn, no significant differences for carriers of non-synonymous *PTGS* variants were observed among patients with DILI onset shorter than 50 days and control subjects ($P = 0.325$). Haplotype analyses (**Table 4**), and linkage disequilibrium (LD) analyses (**Supplemental Table 2**), show that the risk is due to the presence of rare haplotypes (containing missense variants) in the group of patients with late-onset DILI, but it is not due to LD variations for these variants. The strong association observed in this report, although it is based in five cases only, suggests a relationship of non-synonymous *PTGS* gene variations with DILI onset after long-term NSAID therapy. This is a novel observation that has not been raised by previous studies. Although the putative role of *PTGS* gene variations has been explored using the Illumina human exome chip or human core exome chip, it is of note that chip coverage was very limited for *PTGS* genes (Urban et al., 2012). By turn, this study has complete coverage thus allowing the identification of, as yet, disregarded SNPs. Another relevant difference with most DILI genetic studies is that in this report we stratified patients according to the time to onset. It cannot be ruled out heterogeneity in the etiopathogenesis of DILI, and it is conceivable that the mechanisms involved in DILI with a late onset might be different from those involved in immediate or short-latency reactions. This study, albeit with the inherent limitations of statistical power that case reports have, reinforces the view that a complete gene coverage and a detailed phenotype stratification of DILI patients could be essential to gain strength in further genetic association studies.

## AUTHOR CONTRIBUTIONS

ML and EG-M participated in the design of the study, in data acquisition, and in critical revision for important intellectual content. AD, MB, and RA participated in the analysis and interpretation of the data and critical revision for important intellectual content. JA participated in the conception, design, data analysis and interpretation, the drafting of the manuscript and critical revision for important intellectual content. All authors approved the final version of the manuscript and all agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

## FUNDING

Financed in part by grants PI12/00241, PI12/00378, PI12/00324, PI15/00303, and RETICS RD16/0006/0004 from Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Spain, and IB16170, GR18145 from Junta de Extremadura, Spain. Financed in part with FEDER funds from the European Union.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00134/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.