

RESEARCH PAPER

Deep sequencing of prostaglandin-endoperoxide synthase (PTGE) genes reveals genetic susceptibility for cross-reactive hypersensitivity to NSAID

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Background and Purpose: Cross-reactive hypersensitivity to nonsteroidal anti-inflammatory drugs (NSAIDs) is a relatively common adverse drug event caused by two or more chemically unrelated drugs and that is attributed to inhibition of the COX activity, particularly COX-1. Several studies investigated variations in the genes coding for COX enzymes as potential risk factors. However, these studies only interrogated a few single nucleotide variations (SNVs), leaving untested most of the gene sequence.

Experimental Approach: In this study, we analysed the whole sequence of the prostaglandin-endoperoxide synthase genes, *PTGS1* and *PTGS2*, including all exons, exon-intron boundaries and both the 5' and 3' flanking regions in patients with cross-reactive hypersensitivity to NSAIDs and healthy controls. After sequencing analysis in 100 case-control pairs, we replicated the findings in 540 case-control pairs. Also, we analysed copy number variations for both *PTGS* genes.

Key Results: The most salient finding was the presence of two *PTGS1* single nucleotide variations, which are significantly more frequent in patients than in control

Abbreviations: DuP-697, 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl)thiophene; NSAIDs, non-steroidal anti-inflammatory drugs; PACERR, *PTGS2* antisense NFκB1 complex-mediated expression regulator RNA; *PTGS1*, prostaglandin-endoperoxide synthase 1 gene; *PTGS2*, prostaglandin-endoperoxide synthase 2 gene; SNVs, single nucleotide variations; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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subjects. Patients carrying these single nucleotide variations displayed a significantly and markedly lower COX-1 activity as compared to non-carriers for both heterozygous and homozygous patients.

Conclusion and Implications: Although the risk single nucleotide variations are present in a small proportion of patients, the strong association observed and the functional effect of these single nucleotide variations raise the hypothesis of genetic susceptibility to develop cross-reactive NSAID hypersensitivity in individuals with an impairment in COX-1 enzyme activity.

KEYWORDS

cyclooxygenase, hypersensitivity, NSAID, *PTGS1*, *PTGS2*

1 | INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat fever, pain and inflammation. Several drugs with different chemical structures are classified as NSAIDs and the most salient common characteristic of these drugs is their ability to inhibit COX activities. This COX inhibition is related to their therapeutic effects but also to adverse drug events related to NSAIDs use (Agundez, Blanca, Cornejo-Garcia, & Garcia-Martin, 2015; Diaz, Chepenik, Korn, Reginato, & Jimenez, 1998; Grosser, Theken, & FitzGerald, 2017; Theken et al., 2020; Vane, Bakhle, & Botting, 1998). These adverse effects include cardiovascular, renal or gastrointestinal damage as well as hypertension and usually, are dependent on the drug concentration and the level of COX-inhibition (Bjarnason et al., 2018; Khan, Andrews, & Chin-Dusting, 2019; Lovell & Ernst, 2017; Macias, Gomez Tabales, Garcia-Martin, & Agundez, 2020). Also, another common NSAID adverse event which has been linked to COX inhibition is cross-reactive NSAID hypersensitivity (Kowalski et al., 2013). More than 75% of patients with symptoms of NSAID hypersensitivity have cross-reactive NSAID hypersensitivity (Dona et al., 2011). Regarding the culprit drug in cross-reactive NSAID hypersensitivity, the most frequent were **ibuprofen**, **metamizole** (a COX-inhibitor not pertaining to the family of NSAIDs), **acetylsalicylic acid (aspirin)**, **paracetamol** and **diclofenac**. Notably, the presence of selective COX-2 inhibitors as triggering drugs was marginal (about 1% of patients), thus supporting the hypothesis of COX-1 inhibition as the main mechanism involved in cross-reactive NSAID hypersensitivity (Cahill & Boyce, 2017; Dona et al., 2018, 2019; Kowalski, Borowiec, Kurowski, & Pawliczak, 2007; Li & Laidlaw, 2019; Mastalerz et al., 2019). This hypothesis was further reinforced in a study involving more than 13,000 cases with NSAID-related hypersensitivity reactions, reporting that NSAIDs with a poor COX-selectivity, in opposition to COX-2 selective inhibitors, are the more commonly involved of hypersensitivity reactions (Bakhriansyah, Meyboom, Souverein, de Boer, & Klungel, 2019; Morales et al., 2014).

The putative mechanisms involved in cross-reactive hypersensitivity to NSAIDs are still to be clarified, but evidence suggests that

What is already known

- Cross-reactive NSAID hypersensitivity has been attributed to COX-inhibition, but the underlying mechanism remains unknown.
- Weak associations of cross-reactive NSAID hypersensitivity with arachidonic acid pathways genes have been described.

What does this study adds

- We sequenced both *PTGS* genes to obtain complete gene coverage in patients and controls.
- We identified an at-risk *PTGS1* haplotype. Such haplotype is related to diminished COX-1 activity.

What is the clinical significance

- The risk haplotype might amplify the effect of COX-1 inhibitors, leading to adverse drug events.
- *PTGS1* genetic testing shows potential for patients with family history of cross-reactive hypersensitivity to NSAIDs.

activation of the arachidonic acid pathways instead of an immunological recognition is a key factor in the etiopathogenesis of this common disorder (Laidlaw & Boyce, 2016; Stevenson & Zuraw, 2003). This explains why drugs with very different structures can be a part of a cross-reactive hypersensitivity mechanism. Because family history is a key factor in cross-reactive hypersensitivity, it could be speculated the occurrence of a genetic predisposition and several studies seeking for putative genetic factors affecting the arachidonic acid

pathways have been carried out. Some of these studies revealed a weak association that supports the hypothesis for a genetically determined alteration of the **arachidonic acid** pathways in the etiopathogenesis of cross-reactive hypersensitivity (Cornejo-Garcia et al., 2012, 2016; Plaza-Seron, Garcia-Martin, Agundez, & Ayuso, 2018; Plaza-Seron Mdel et al., 2016). However, these studies had an incomplete gene capture, pointing to isolated single nucleotide variations (SNVs), and no studies covering the whole genes have been carried out.

The approach of analysing a few SNVs per gene is clearly insufficient to establish definitive associations between genetic variability and cross hypersensitivity. Unfortunately, previous studies addressing variations in the genes coding for **COX-1** and **COX-2** enzymes (designated as *PTGS1* and *PTGS2*, respectively) in patients with cross-reactive hypersensitivity to NSAIDs did not have complete gene coverage and no, or very weak genetic associations have been identified (Agundez et al., 2009; Ayuso et al., 2015; Choi et al., 2004; Cornejo-Garcia et al., 2012; Jinnai et al., 2004; Kim & Park, 2006; Kim, Ye, Lee, & Park, 2006; Kowalski, Borowiec, Kurowski, & Pawliczak, 2007; Plaza-Seron Mdel et al., 2016; Shi et al., 2005, 2008; Shi, Misso, Duffy, Thompson, & Kedda, 2004; Yi, Cheng, Lin, Zhou, & Wang, 2016). Most common *PTGS* SNVs, as well as additional SNVs affecting the gene promoters known to disrupt transcription factor binding sites, and copy number variation in these genes have not been studied concerning the risk of developing cross-reactive hypersensitivity to NSAIDs. More than 50 *PTGS1* SNVs have been described to occur with frequencies over 0.001 (global) and higher or equal to 0.01 in any human population, and more than 20 SNVs have been related to clinical consequences (Agundez, Blanca, Cornejo-Garcia, & Garcia-Martin, 2015; Agundez, Gonzalez-Alvarez, Vega-Rodriguez, Botello, & Garcia-Martin, 2014; Lucena et al., 2019). As for *PTGS2*, more than 45 SNVs occur with the frequencies described above and more than 25 SNVs have been clinically related to diverse disorders (Agundez et al., 2014, 2015; Lucena et al., 2019).

Aiming to obtain a complete gene coverage confirming or discarding any putative genetic factor related to *PGTS* genes with the risk of developing cross-reactive hypersensitivity to NSAIDs, we sequenced the *PTGS1* and *PTGS2* genes in patients with cross-reactive hypersensitivity and in healthy subjects who tolerated NSAIDs. The study included the promoter areas, all exons, all intron-exon boundaries and the 3' flanking regions. Also, we analysed copy number variations in these genes. Besides the sequencing study, we replicated the significant findings in a large group of patients and controls by using TaqMan probes.

2 | METHODS

2.1 | Ethics statement

The study was conducted according to the principles of the Declaration of Helsinki and approved by the Ethics Committees of each

participating hospital. Written informed consent was obtained from all the participants involved in the study.

2.2 | Participants

The study included a total of 640 unrelated patients with cross-reactive hypersensitivity to NSAIDs and 640 unrelated controls who tolerated NSAIDs. All participants were Spanish Caucasian individuals, and all were recruited between 2007 and 2020 from the Allergy Services of public Hospitals integrated into the National Research Network for allergic diseases RIRAAF (Badajoz University Hospital, Málaga University Hospital, Madrid Cruz Roja Hospital, Barcelona Clinic Hospital, Madrid Infanta Leonor Hospital, Alcorcón University Hospital, and Elche University Hospital). Control individuals were selected among the staff and students. The inclusion criteria for patients included a history of episodes with more than two different NSAIDs. Details for the diagnosis, oral provocation and assignment of patients as selective or cross-intolerant are given elsewhere (Cornejo-Garcia et al., 2014; Dona et al., 2017, 2020; Kowalski et al., 2019). Those patients or control individuals with a clinical history of food allergy or positive IgE antibodies for food allergens were excluded because of the putative interaction between food allergy and NSAIDs (Cornejo-Garcia et al., 2012). The characteristics of the patients and control individuals included in the study are summarized in Table 1.

2.3 | Data analysis

The data and statistical analysis, see below, comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.4 | Sequencing analysis

We ordered a custom design to Fluidigm Service Order (South San Francisco, CA, USA) based on the coding regions of *PTGS1* and *PTGS2* genes, transcripts ENST00000362012 and ENST00000367468, respectively (GRCh37 assembly of the human genome). These primers were used to amplify a collection of 200 DNA samples using the Access Array platform (Fluidigm, CA, USA). We analysed genomic DNA from 100 patients and 100 controls, all recruited in the Badajoz University Hospital. The characteristics of participants are summarized in Table 1. The sequences studied included for both *PTGS* genes, over 2000 bp of the promoter areas, including the areas where promoter polymorphisms affecting transcription factor binding sites have been described (Agundez et al., 2014, 2015), all exons, all intron-exon boundaries, and the whole 5' untranslated regions. Table S1 shows the areas sequenced. Sequencing coverage averaged around 50x for the whole project. Coverage for the SNVs identified (Tables 2 and 3) was always over

TABLE 1 Characteristics of patients with cross-reactive hypersensitivity to NSAIDs and controls in the next-generation sequencing and in the replication studies

	NGS patients (n = 100)	NGS controls (n = 100)	Replication patients (n = 540)	Replication controls (n = 540)
Women n (%)	50 (50%)	50 (50%)	317 (58.7%)	325 (60.1%)
Age (range)	43.84 (16–77)	27.6 (20–62)	41.8 (5–92)	26.3 (19–58)
Antecedents of atopic diseases	35 (35%)	--	124 (23.0%)	--
Clinical presentation				
NECD	64 (64%)	n.a.	356 (65.9%)	n.a.
NERD	10 (10%)	n.a.	64 (11.9%)	n.a.
NIUA-anaphylaxis	18 (18%)	n.a.	40 (7.4%)	n.a.
NIUA- mixed	8 (8%)	n.a.	66 (12.2%)	n.a.
NIUA- other	--	n.a.	9 (1.7%)	n.a.
Drug involved				
AAS	67 (67%)	n.a.	467 (86.5%)	n.a.
Ibuprofen	61 (61%)	n.a.	245 (45.4%)	n.a.
Metamizole	63 (63%)	n.a.	171 (31.7%)	n.a.
Diclofenac	14 (14%)	n.a.	75 (13.9%)	n.a.
Naproxen	6 (6%)	n.a.	25 (4.6%)	n.a.
Aceclofenac	1 (1%)	n.a.	8 (1.5%)	n.a.
Piroxicam	-	n.a.	7 (1.3%)	n.a.
Indomethacin	-	n.a.	3 (0.6%)	n.a.
Meloxicam	2 (2%)	n.a.	2 (0.4%)	n.a.
Lornoxicam	-	n.a.	1 (0.2%)	n.a.
Celecoxib	-	n.a.	1 (0.2%)	n.a.

Abbreviations: AAS, acetylsalicylic acid; NECD, NSAID-exacerbated cutaneous disease; NERD, NSAID-exacerbated respiratory disease or aspirin-exacerbated respiratory disease (AERD); NGS, next generation sequencing; NIUA, NSAID-induced urticaria angioedema.

50x. Further details of the sequencing procedure and analysis are provided elsewhere (Lucena et al., 2019).

2.5 | Replication analyses

SNVs analyses were carried out in the rest of patients ($n = 540$) and controls ($n = 540$) by using TaqMan probes. The characteristics of participants are summarized in Table 1. Analyses were randomised and blinded. The SNVs selection was based on the sequencing analysis, including SNVs with different allele frequencies among patients, as compared with those observed among control individuals. Amplification and detection were carried out in triplicate in a 7500 Real-Time PCR System (Applied Biosciences, Alcobendas, Spain). All SNPs were tested using pre-designed or custom-designed TaqMan Assays (Life Sciences, Alcobendas, Madrid, Spain) as described in Table S2. As a quality control, the amplified fragments for 20 individuals carrying each genotype, when available, were sequenced. *CYP2C9* genotyping was carried out in selected patients (those carrying the *PTGS1* risk haplotype). We analysed the common *CYP2C9**2 and *CYP2C9**3 alleles. Other *CYP2C9* alleles classified as tier 1 (Pratt et al., 2019) such as *CYP2C9**5, *6, *8, and *11 were not included in the analyses because their signature SNVs, available at PharmVar website

(<https://www.pharmvar.org/gene/CYP2C9>), had extremely low frequencies in European individuals, according to public databases [Genome Aggregation Database, RRID:SCR_014964]. Genotyping was carried out as described elsewhere (Martinez et al., 2014). Copy number variations in *PTGS1* and *PTGS2* genes were assessed by using pre-designed TaqMan probes. Amplification and detection were carried out in quadruplicate in a 7500 Real-Time PCR System (Applied Biosciences, Alcobendas, Spain). Probes hybridizing exonic sequences (exon 6 for *PTGS1*, cat No. Hs05082885_cn and exon 4 for *PTGS2*, cat No. Hs01213742_cn) were selected. Details of the procedure are described elsewhere (Ayuso et al., 2013).

COX activity assays were carried out by using a Fluorescent assay (Cayman Chemical, Ann Arbor, MI, USA, Item No. 760151). In brief, to obtain about 1×10^8 cells, blood samples (20 mL) were collected from patients with selected genotypes. Leukocytes were isolated as described elsewhere (Neitzel, 1986). After centrifugation, the leukocyte precipitate was resuspended in three volumes of 100 mM Tris-HCl, 1 mM EDTA, pH 7.8, and lysed by sonication for 1 min at 4°C. The lysates were centrifuged at 10,000x g for 15 min at 4°C. The supernatants were then stored at -80°C until analyses. COX activity was determined by measuring the peroxidase activity, based on the appearance of oxidized *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm (Kulmacz & Lands, 1983). All analyses were carried

TABLE 2 PTGS1 SNPs identified by sequencing

Chromosome location (GRCh37)	SNP identification (rs number)	Area/effect	Patients (n = 100) (MAF)	Controls (n = 100) (MAF)	MAF gnomAD V2.1.1 Europeans non-Finnish	Minor allele frequency OR ^a (95% C.I.); P	Armitage's test for trend OR; P
9-125131419-G-A	rs1180299559	Promoter	0.0100	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125131480-T-C	rs10306108	Promoter	0.0500	0.0550	0.0629	0.90 (0.38–2.18); 0.823	1.11; 0.818
9-125131631-G-A	rs10306109	Promoter	0.0500	0.0550	0.0629	0.90 (0.38–2.18); 0.823	1.11; 0.818
9-125131688-T-C	rs1330344	Promoter	0.3200	0.2100	0.2164	1.77 (1.13–2.78); 0.013	1.81; 0.013
9-125132027-A-G	rs115693689	Promoter	0.0800	0.0550	0.0668	1.49 (0.68–3.31); 0.319	1.54; 0.301
9-125132028-A-C	rs114079139	Promoter	0.0250	0.0550	0.0668	0.44 (0.15–1.29); 0.126	0.43; 0.118
9-125132223-G-A	rs75993350	Promoter	0.0500	0.0550	0.0629	0.90 (0.38–2.18); 0.823	0.90; 0.818
9-125132311-A-G	rs10306110	Promoter	0.0500	0.0550	0.0629	0.90 (0.38–2.18); 0.823	0.90; 0.818
9-125132522-A-G	rs10306114	Promoter	0.0500	0.0550	0.0628	0.90 (0.38–2.18); 0.823	0.90; 0.818
9-125133479-C-T	rs1236913	Exonic Trp8Arg	0.0850	0.0650	0.0693	1.34 (0.63–2.83); 0.448	1.90; 0.461
9-125133507-C-T	rs3842787	Exonic Pro17Leu	0.0900	0.0600	0.0668	1.55 (0.73–3.31); 0.255	2.19; 0.269
9-125140074-C-T	rs147545890	Intronic	0.0100	0.0000	0.0002	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125140206-G-A	rs3842788	Splice acceptor	0.0500	0.0250	0.0250	2.05 ^a (0.69–6.12); 0.189	2.11; 0.179
9-125140287-C-T	rs3842790	Exonic synonymous	0.0100	0.0000	0.0003	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125140326-G-A	rs876567	Intronic	0.0100	0.0000	0.0031	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125140696-G-C	rs2282169	Intronic	0.1450	0.1400	0.1875	1.04 (0.59–1.83); 0.886	1.00; 0.883
9-125141239-T-C	rs12555242	Intronic	0.0300	0.0500	0.0347	0.59 (0.21–1.65); 0.308	0.59; 0.307
9-125143559-C-A	rs566847283	Intronic	0.0100	0.0000	0.0004	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125143792-C-A	rs5788	Exonic synonymous	0.1450	0.1100	0.1364	1.37 (0.76–2.48); 0.294	1.37; 0.322
9-125143902-C-T	rs199797662	Intronic	0.0100	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125143963-T-C	rs774026967	Exonic synonymous	0.0050	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125143973-C-A	rs5789	Exonic Leu237Met	0.0000	0.0005	0.0264	0.00 ^a (0.00–2.00); 0.317	0.67; 0.155
9-125144040-A-AG	rs202049266	Intronic	0.1400	0.0950	0.0001	1.55 (0.84–2.88); 0.162	1.60; 0.221
9-125145743-T-C	rs3842798	Intronic	0.1950	0.1700	0.1943	1.18 (0.71–1.97); 0.517	1.14; 0.527
9-125152621-G-A	rs5794	Exonic Val481Ile	0.0000	0.0100	0.0094	0.00 ^a (0.00–1.61); 0.157	0.67; 0.155
9-125154439-T-C	rs3842801	Intronic	0.0050	0.0000	0.0037	2.01 ^a (0.11–2.00); 0.317	1.49; 0.155
9-125154535-T-C	rs3842803	Exonic synonymous	0.0200	0.0050	0.0037	4.06 (0.45–36.66); 0.178	9.39; 0.314
9-125154955-C-CT	rs138699735	3' UTR	0.0100	0.0000	0.0016	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125155264-G-A	rs10306185	3' UTR	0.0100	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125155313-A-C	rs10306187	3' UTR	0.0250	0.0000	0.0040	2.03 ^a (0.93–2.03); 0.025	2.32; 0.024
9-125155408-A-G	rs8046	3' UTR	0.0900	0.0700	0.0605	1.31 (0.64–2.72); 0.461	1.47; 0.510
9-125155685-A-G	rs10306188	3' UTR	0.0250	0.0000	0.0040	2.03 ^a (0.93–2.03); 0.025	2.32; 0.024
9-125155786-G-T	rs10306189	3' UTR	0.0050	0.0000	0.0042	2.01 ^a (0.11–2.00); 0.317	1.49; 0.155

TABLE 2 (Continued)

Chromosome location (GRCh37)	SNP identification (rs number)	Area/effect	Patients (n = 100) (MAF)	Controls (n = 100) (MAF)	MAF gnomAD V2.1.1 Europeans non-Finnish	Minor allele frequency OR ^a (95% C.I.); P	Armitage's test for trend OR; P
9-125155789-C-G	rs10306190	3' UTR	0.0050	0.0000	0.0042	2.01 ^a (0.11–2.00); 0.317	1.49; 0.155
9-125155930-G-C	rs10306192	3' UTR	0.0050	0.0000	0.0563	2.01 ^a (0.11–2.00); 0.317	1.49; 0.155
9-125156397-C-T	rs73561092	3' UTR	0.0100	0.0050	0.0015	2.01 (0.18–22.35); 0.563	2.01; 0.562
9-125156968-A-G	rs145385879	3' UTR	0.0100	0.0000	0.0025	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125157070-C-T	rs113318139	3' UTR	0.0100	0.0000	0.0001	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125157198-C-A	rs10306194	3' UTR	0.0900	0.0200	0.1384	4.85 (1.61–14.59); 0.002	7.38; 0.006
9-125157264-G-A	rs111369216	3' UTR	0.0100	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125157307-A-G	rs140766958	3' UTR	0.0100	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125157316-G-A	rs10306196	3' UTR	0.0700	0.0650	0.0561	1.08 (0.50–2.37); 0.842	1.09; 0.999
9-125157357-G-T	rs10306197	3' UTR	0.0100	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125157672-A-C	rs10306199	3' UTR	0.0100	0.0000	0.0000	2.01 ^a (0.40–2.01); 0.157	1.49; 0.155
9-125157718-T-G	rs9233	3' UTR	0.0700	0.0650	0.0561	1.08 (0.50–2.37); 0.842	1.15; 0.854

^aWhen any of the values in the OR comparison is equal to 0, RR values are shown instead.

out by triplicate, according to the manufacturer's instructions and by using an identical amount of protein for every sample. Each well contained 120 µl 100 mM tris HCl, pH 8.0, 10 µl haem (Cayman Item No. 760116) and 10 µl DuP-697 (Cayman Item No. 760158), which is a selective inhibitor of COX-2 activity (Kargman et al., 1996), and 40 µl of lysate supernatants. Background wells were prepared as described above but using cell lysates inactivated at 100°C for 10 min. After gentle shaking, the plates were incubated for 5 min at 25°C. Then, 20 µl of the colorimetric substrate was added to each well. The reaction was started by the addition of 20 µl arachidonic acid (Cayman Item No. 760113), which yielded a final concentration of 210 µM. After gentle shaking, plates were incubated at 25°C for 5 min and the final absorbance at 590 nm was determined. Results are presented as the percentage of the mean of the COX-1 activity observed in non-carriers of the risk haplotype. The unpaired Student's t-test was used to compare the mean activities of individuals with different genotypes.

The statistical analysis was carried out by using the statistical software SPSS 19.0 for Windows (SPSS Inc. Chicago, Illinois, USA) [SPSS, RRID:SCR_002865]. Putative departures of Hardy-Weinberg equilibrium were calculated by using Haploview 4.2 software (Barrett, Fry, Maller, & Daly, 2005) [Haploview, RRID:SCR_003076]. Statistical analyses were undertaken only for studies where each group size was at least $n = 5$, with the exception of the next-generation sequencing analysis, where SNVs with very low frequencies were identified, and Figure 2 where only two individuals homozygous for the risk haplotype were analysed. Outliers were included in data analysis and presentation. The association between SNVs and the risk of developing hypersensitivity was estimated by odds ratio (OR) with 95% confidence interval (CI). The gene-dose effect was analysed by means of the Cochran-Armitage test for trend. Haplotype reconstruction was carried out by using PHASE (Stephens, Smith, & Donnelly, 2001). The reconstructed haplotypes were used for risk analyses related to inferred phenotypes. Sensitivity, specificity, and number needed to genotype were calculated as described elsewhere (Tonk, Gurwitz, Maitland-van der Zee, & Janssens, 2017). Linkage analyses for SNVs were carried out by using the online LDproxy Tool (<https://ldlink.nci.nih.gov/?tab=ldproxy>) in Iberian population from Spain. The putative effects of SNVs on miRNA binding sequences were analysed by using the online miRBase (Kozomara & Griffiths-Jones, 2011). Differences were considered to be significant when P values were under 0.05. Potential confounders considered were gender, age, and clinical presentation. Adjustments for multiple analyses were performed by using the False Discovery Rate correction. Statistical analyses for COX-1 activities were done using measurements as independent values (i.e. not treating technical replicates as independent values).

2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to

TABLE 3 PTGS2 SNPs identified by sequencing

Chromosome location (GRCh37)	SNP identification (rs number)	Area/effect	Patients (n = 100) (MAF)	Controls (n = 100) (MAF)	MAF gnomAD V2.1.1 Europeans non-Finnish	Minor allele frequency OR ^a (95% C.I.); P	Armitage's test for trend OR; P
1-186651296-T-C	rs4648250	Promoter	0.0450	0.0150	0.0071	3.09 (0.83–11.60); 0.079	3.09; 0.079
1-186651083-T-G	rs689462	Promoter	0.0250	0.0400	0.0218	0.62 (0.20–1.90); 0.398	0.62; 0.398
1-186651032-A-G	rs115215683	Promoter	0.0100	0.0000	0.0001	2.01 ^a (0.40–2.01); 0.157	1.49; 0.156
1-186650877-A-C	rs689464	Promoter	0.0300	0.0000	0.0000	2.03 ^a (1.04–2.03); 0.014	2.61; 0.014
1-186650876-T-TAC	rs760197562	Promoter	0.0000	0.0050	0.0039	0.00 ^a (0.00–1.90); 0.317	0.82; 0.317
1-186650846-T-C	rs689465	Promoter	0.1800	0.2350	0.1234	0.72 (0.44–1.16); 0.176	0.84; 0.274
1-186650751-T-C	rs689466	Promoter	0.1600	0.2150	0.1782	0.70 (0.42–1.15); 0.159	0.67; 0.165
1-186650321-C-G	rs20417	Promoter	0.1600	0.1900	0.1455	0.81 (0.48–1.36); 0.430	0.72; 0.118
1-186649618-G-C	rs20424	Promoter	0.0050	0.0200	0.0132	0.25 (0.03–2.22); 0.178	0.25; 0.177
1-186648197-C-G	rs5277	Exonic synonymous	0.1850	0.1800	0.1604	1.03 (0.62–1.72); 0.897	1.18; 0.049
1-186645927-C-T	rs2066826	Intronic	0.1300	0.1050	0.1308	1.27 (0.69–2.35); 0.438	1.29; 0.470
1-186645078-A-G	rs5279	Exonic synonymous	0.0100	0.0050	0.0001	2.01 (0.18–22.35); 0.563	2.01; 0.562
1-186643238-C-G	rs4648290	3' UTR	0.0200	0.0100	0.0118	2.02 (0.37–11.16); 0.411	2.02; 0.411
1-186643058-A-G	rs5275	3' UTR	0.2000	0.2050	0.3333	0.97 (0.60–1.58); 0.901	0.98; 0.911
1-186642987-C-T	rs5276	3' UTR	0.0100	0.0150	0.0003	0.66 (0.11–4.01); 0.653	0.66; 0.653
1-186642429-G-A	rs2206593	3' UTR	0.0750	0.0500	0.0615	1.54 (0.68–3.52); 0.302	1.96; 0.242
1-186641682-T-C	rs4648298	3' UTR	0.0000	0.0050	0.0222	0.00 ^a (0.00–1.90); 0.317	0.82; 0.316
1-186641626-A-G	rs2853805	3' UTR	0.0500	0.0050	0.0000	10.48 (1.33–82.61); 0.006	13.5; 0.011
1-186641273-A-C	rs689467	3' UTR	0.0250	0.0250	0.0637	1.00 (0.29–3.51); 1.000	1.00; 1.000
1-186641265-C-T	rs689468	3' UTR	0.0000	0.0050	0.0208	0.00 ^a (0.00–1.90); 0.317	0.82; 0.317
1-186641197-C-T	rs689469	3' UTR	0.0000	0.0100	0.0209	0.00 ^a (0.00–1.61); 0.157	0.67; 0.156

^aWhen any of the values in the OR comparison is equal to 0, RR values are shown instead.

PHARMACOLOGY <http://www.guidetopharmacology.org> and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Identification of SNVs U7sing next-generation sequencing

In this phase, we identified in 200 individuals (100 patients and 100 control subjects), 45 SNVs in the *PTGS1* gene. Table 2 shows the allele frequencies in patients and controls as well as the allele frequencies described in the Genome Aggregation Database (gnomAD) online resource. All SNVs were at Hardy-Weinberg's equilibrium. It is to be noted that all the missense SNVs previously described for the *PTGS1* gene were identified in the sequencing analysis with two exceptions:- the SNV rs186089151 (9-125152522-G-A), which causes the amino acid substitutions Val488Met and the SNV rs5794 (9-125152621-G-A), which causes the amino acid substitution Val481Ile (Agundez, Blanca, Cornejo-Garcia, & Garcia-Martin, 2015). The absence of these missense SNVs in the sequencing study was expected as their allele frequencies are extremely low (in both cases <0.05) in the population studied. We compared the allele frequencies between patients and control individuals, and we identified four SNV with different allele frequencies. One of the SNVs, designated as rs1330344, was located in the promoter area and it affects a transcription factor binding site for globin transcription factor 1 (GATA1) and caudal type homeo box transcription factor 1 (CDX1) (Agundez, Gonzalez-Alvarez, Vega-Rodriguez, Botello, & Garcia-Martin, 2014). In addition, three additional *PTGS1* SNVs located in the 3' untranslated area (3' UTR) displayed different frequencies when comparing patients and control subjects, namely, rs10306187, rs1036188 and rs10306194.

Table 3 shows the *PTGS2* SNVs identified in the sequencing study. We identified 21 SNVs, none of which was missense. This is consistent with the extremely low frequency that missense *PTGS2* SNV present in the population studied (Agundez, Blanca, Cornejo-Garcia, & Garcia-Martin, 2015). One SNV located in the promoter area, designated as rs689464, presented significant differences in the allele frequencies when comparing patients and controls, although no changes in transcription factor bindings sites have been identified in this area (Agundez, Gonzalez-Alvarez, Vega-Rodriguez, Botello, & Garcia-Martin, 2014). One additional SNV, designated as rs2853805 and located at the 3' UTR presented statistically significant differences between patients and controls.

3.2 | Replication phase

To replicate the exploratory findings obtained in the sequencing study analyses, we analysed all *PTGS1* and *PTGS2* SNVs with *P* values ≤0.5 in Tables 2 and 3, in DNA samples from 540 patients and 540 controls. Also, we included in the analyses all nonsynonymous SNVs identified in

the sequencing study. The replication phase was carried out by using TaqMan genotyping probes as described in the methods section. Results are summarized as pooled analyses of the whole group of 640 patients and 640 controls in Table 4 and Figure 1. A strong statistical association was identified for two 3' UTR SNVs in the *PTGS1* gene (rs10306187 and rs10306188) and a weak association was observed for a *PTGS2* SNV located in the gene promoter (rs4648250). The differences remained significant after adjustment by gender and after False Discovery Rate corrections for multiple testing only for the two *PTGS1* gene variants (Table 4). These SNVs were identified in 36 patients with NSAIDs hypersensitivity (18 women and 18 men) and eight control subjects. Table 5 shows the characteristics of the 36 patients carrying either of the risk SNVs. These SNVs were not associated with a particular hypersensitivity clinical presentation, or with a specific NSAID except for ibuprofen: The SNVs were significantly over-represented among patients with hypersensitivity to ibuprofen as compared with patients with hypersensitivity to other NSAIDs (OR = 2.28; *P* < 0.05) (Table S3). It is to be noted that the two *PTGS1* SNVs are at strong linkage disequilibrium in the Iberian population from Spain, (*D'* = 1.0; *P* < 0.05), thus indicating that the *PTGS1* haplotype rs10306187C + rs10306188G has a significantly increased frequency among patients with cross-reactive hypersensitivity to NSAIDs.

Since cytochrome P450 CYP2C9 plays a major role in the metabolism of some of the NSAIDs involved in cross-reactive hypersensitivity (Agundez, Garcia-Martin, & Martinez, 2009; Theken et al., 2020) we analysed the signature SNVs for the common CYP2C9*2 and CYP2C9*3 alleles in all the patients carrying the *PTGS1* risk haplotype. For these patients, allele frequencies were 0.166 for CYP2C9*2 and 0.056 for CYP2C9*3. Because of the known impact of CYP2C9 genotypes on ibuprofen metabolism (Garcia-Martin, Martinez, Tabares, Frias, & Agundez, 2004), we analysed separately the CYP2C9 allele frequencies in patients with hypersensitivity to ibuprofen and the *PTGS* risk haplotype. In this subgroup, the allele frequencies were 0.166 for CYP2C9*2 and 0.083 for CYP2C9*3. These frequencies are similar to those reported in healthy Spanish individuals with no antecedents of NSAID hypersensitivity (Martinez et al., 2014).

Copy number variation analyses for *PTGS1* and *PTGS2* genes were carried out in 430 patients and 515 controls due to DNA shortage. Two copies of the *PTGS1* and the *PTGS2* genes were present in all participants (patients and controls) with two exceptions:- one patient, a 42 years man who presented with NSAID-exacerbated cutaneous disease with **naproxen**, had a single copy of the *PTGS1* gene and another patient, a 26 years woman who presented with NSAID-induced urticaria angioedema-anaphylaxis with metamizole, had a single copy of the *PTGS2* gene. All control individuals had two copies of both *PTGS* genes and, therefore, the copy number variations analyses were not informative in this study.

3.3 | Functional analysis

Since both, the sequencing and the replication studies identified a strong association of two 3' UTR SNVs (rs10306187 and

TABLE 4 *PTGS1* and *PTGS2* SNPs in the whole study group

Gene; chromosome location	SNP identification (rs number)	Location, effect	Known clinical association	Patients (MAF)	Controls (MAF)	Allele frequency OR (95% CI); P	Test for trend OR; P	P values after FDR adjustment
<i>PTGS1</i> ; 9:125131688	rs130344 C/T	Promoter	Yes	0.2297	0.2250	1.03 (0.85–1.24); 0.777	1.17; 0.785	0.855
<i>PTGS1</i> ; 9:125133479	rs1236913 T/C	Exonic Trp8Arg	Yes	0.0651	0.0641	1.02 (0.74–1.40); 0.911	1.24; 0.911	0.911
<i>PTGS1</i> ; 9:125133507	rs3842787 C/T	Exonic Pro17Leu	Yes	0.0664	0.0547	1.23 (0.89–1.70); 0.214	1.20; 0.227	0.589
<i>PTGS1</i> ; 9:125143973	rs5789 C/A	Exonic Leu237Met	Yes	0.0280	0.0352	0.79 (0.51–1.24); 0.303	0.79; 0.295	0.610
<i>PTGS1</i> ; 9:125152621	rs5794 G/A	Exonic Val481Ile	Yes	0.0110	0.0148	0.74 (0.37–1.48); 0.388	0.73; 0.385	0.771
<i>PTGS1</i> ; 9:125155313	rs10306187 A/C	3' UTR	No	0.0211	0.0063	3.44 (1.56–7.60); 0.00118	4.80; 0.00330	0.007
<i>PTGS1</i> ; 9:125155685	rs10306188 A/G	3' UTR	No	0.0313	0.0063	5.14 (2.40–11.04); 0.00000029	7.18; 0.00003	0.000003
<i>PTGS1</i> ; 9:125157198	rs10306194 C/A	3' UTR	No	0.1876	0.1945	0.96 (0.79–1.17); 0.656	0.98; 0.670	0.854
<i>PTGS2</i> ; 1:186651296	rs4648250 T/C	Promoter	No	0.0273	0.0141	1.97 (1.11–3.49); 0.019	2.00; 0.0174	0.070
<i>PTGS2</i> ; 1:186650884	rs689464 CA/–	Promoter	No	0.0430	0.0508	0.84 (0.58–1.21); 0.354	1.05; 0.359	0.610
<i>PTGS2</i> ; 1:186641626	rs2853805 G/A	3' UTR	No	0.0344	0.0367	0.94 (0.62–1.42); 0.755	0.93; 0.750	0.855

Note: Clinical associations are described in detail elsewhere (Agundez, Blanca, Cornejo-García, & García-Martín, 2015). Abbreviations: FRD, false discovery rate.

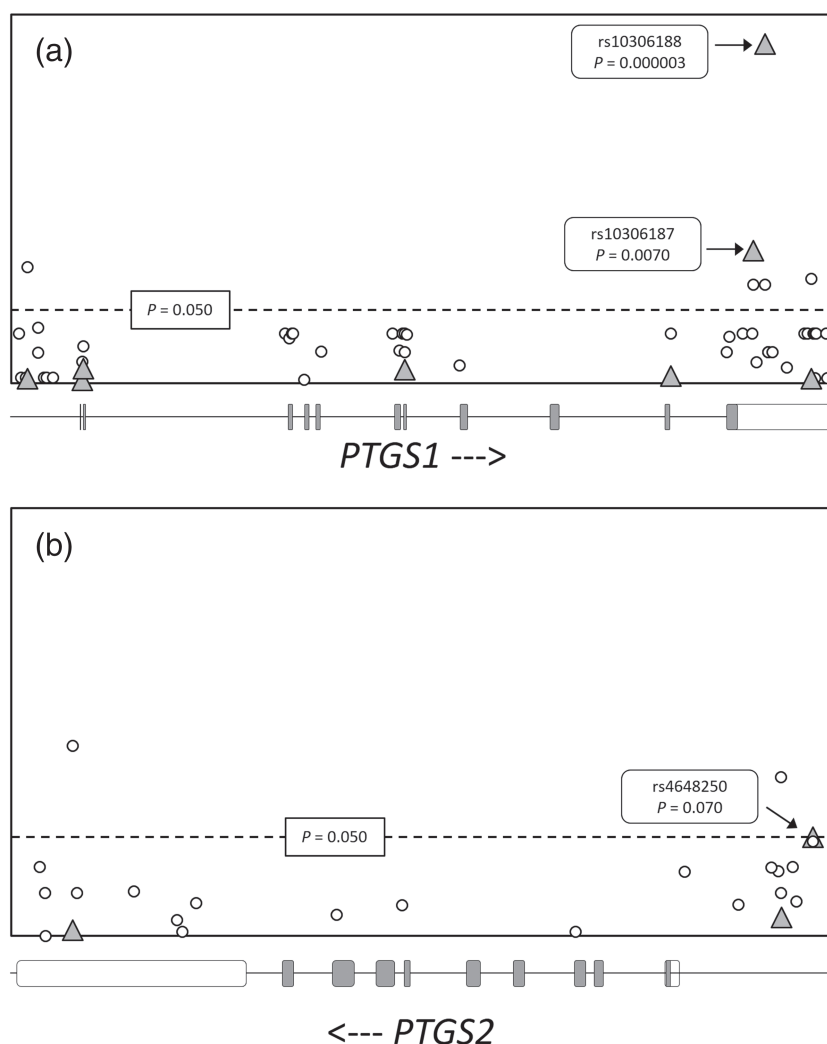
rs10306188) in the *PTGS1* gene with the risk of developing cross-reactive hypersensitivity to NSAIDs (Table 4), we determined the functional impact of these SNVs in samples from patients carrying these SNVs and in patients lacking these SNVs. To that end, we collected blood samples from 21 patients, 12 of which did not carry any of the SNVs, seven patients carried both SNVs in heterozygosity, and two patients carried both SNVs in homozygosity. To determine COX-1 activity we added a specific COX-2 inhibitor (DuP-697) provided with the COX Activity Assay. All measurements were carried out in triplicate. Table S4 and Figure 2 shows the enzyme activities (arbitrary units) where 100% represent the average activity in patients carrying no SNVs (mean $\pm$ SD = 100.00 $\pm$ 51.74). Patients with both SNVs in heterozygosity had average activities = 37.34 $\pm$ 12.98, and patients with both SNVs in homozygosity had average activities = 22.91 $\pm$ 4.18; unpaired Student's *t*-test analyses revealed statistically significant differences between carriers and non-carriers, although the data corresponding to homozygous individuals should be considered as preliminary since only two subjects were analysed (Figure 2), thus indicating that the *PTGS1* risk haplotype is unambiguously related to a functional decrease in COX-1 enzyme activity, and suggesting a gene-dose effect.

3.4 | In silico analyses

We analysed the linkage of the SNVs present in the risk *PTGS1* haplotype with other functional *PTGS1* SNVs as described in the Methods section. Table S5 shows the *PTGS1* SNVs linked to the rs10306187 SNV. In the Iberian population in Spain, there is a linkage with three synonymous SNVs, namely, rs3842802, rs3842803 and rs75358267. Only one of these SNVs, rs3842803, was identified in the sequencing analysis, although no significant differences in the allele frequencies between patients and controls were identified. Table S6 shows the *PTGS1* SNVs linked to the rs10306188 SNV. There is a linkage with three synonymous SNVs, namely, rs3842802, rs3842803, and rs5788. Again, no significant differences in the rs5788 allele frequencies between patients and controls were identified as shown in Table 2. This aside, no functional SNVs are linked to the rs10306187 or rs10306188 SNVs in the whole *PTGS1* gene. Table S7 shows the SNVs linked to the *PTGS2* rs4648250 SNV. Such *PTGS2* SNV does not show any major linkage with functional SNVs in the *PTGS2* gene. This SNV is located outside the *PTGS2* gene area, and there is another gene, designated as P50-associated COX-2 extragenic RNA (*PACERR*), located between the rs4648250 SNV and the *PTGS2* gene.

Since the SNVs involved in the *PTGS1* risk haplotype are located in the 3' untranslated region, we investigated whether the mutated areas were located within miRNA binding sequences, as described under Methods. The mutated sequence for the rs10306187 SNV increases the binding score of the cgr-mir-29a from a score equal to 65 in the wild-type sequence to a score equal to 74 in the mutated sequence. The mutated sequence for the rs10306188 SNV sequence creates a novel miRNA binding site for dre-mir-196a, which is not present at all in the wild-type sequence.

FIGURE 1 Summary of the genetics findings. For both panels, x-axes refers to the gene location; y-axes represent the P values. Open circles represent the associations identified in the next-generation sequencing (NGS) study. Filled triangles represent the associations identified in the replication analyses. The dashed lines represent the $P = 0.050$ threshold. Single nucleotide variations (SNVs) with significant associations are situated above dashed lines. (a) SNVs identified in the *PTGS1* gene analyses. (b) SNVs identified in the *PTGS2* gene analyses



4 | DISCUSSION

Cross-reactive NSAID hypersensitivity is a relatively common adverse drug event with clinical consequences that vary from mild symptoms to anaphylaxis (Table 1). In a large cohort composed of more than 60,000 patients who received NSAIDs, more than 1000 patients experienced adverse drug events, and about one in five cases presented with hypersensitivity (Blumenthal et al., 2017). Among these, the majority of patients experienced cross-reactive NSAID hypersensitivity (Dona et al., 2011). This places NSAIDs among the leading causes of hypersensitivity reactions to drugs (Dona et al., 2016).

Cross-reactive NSAID hypersensitivity occurs with completely different chemical structures such as acetylsalicylic acid, ibuprofen or metamizole (Table 1). These drugs, however, share their therapeutic target as all are potent inhibitors of COX activities (COX) and it has been proposed that the mechanism underlying cross-reactive NSAID hypersensitivity is mediated by inhibition of the COX-1 enzyme (Laidlaw & Boyce, 2016; Stevenson & Zuraw, 2003). Indeed, the drugs that are commonly involved in cross-reactive hypersensitivity are predominantly COX-1 inhibitors (Antman, DeMets, & Loscalzo, 2005; Ayuso et al., 2014; Cryer & Feldman, 1998; Pierre et al., 2007). COX

selectivity is related to the presentation of cross-reactive NSAID hypersensitivity, with COX-1 inhibitors being commonly involved in these reactions (Bakhriansyah, Meyboom, Souverein, de Boer, & Klungel, 2019), whereas COX-2 specific inhibitors are well tolerated in patients with cross-reactive NSAID hypersensitivity (Weberschock, Muller, Boehncke, & Boehncke, 2007), in agreement with the low frequency of cases related to COX-2 inhibitors in this study (Table 1).

It has been proposed that cross-reactive NSAID hypersensitivity is related to dose-dependent inhibition of COX-1 (Blumenthal et al., 2017; Kong, Teuber, & Gershwin, 2007; Palmer, 2005). This hypothesis is in agreement with the findings obtained in the present study. We have identified a haplotype in the *PTGS1* gene which is over-represented in patients with cross-reactive NSAID hypersensitivity, and that is related to severely decreased COX-1 enzyme activity. The statistical association with the risk of developing cross-reactive NSAID hypersensitivity remains highly significant after testing for multiple comparisons, was present both in the discovery and in the replication phases of this study, and has a plausible functional association.

As shown in Figure 2, the risk haplotype is related to a relevant decrease of COX-1 activity both, in heterozygous and in homozygous

TABLE 5 Characteristics of patients with NSAID-induced hypersensitivity who carried either of the risk PTGS1 3' UTR SNVs

Patient ID	Age (years)	Gender	Clinical presentation	AAS	Ibuprofen	Metamizole	Diclofenac	Other NSAID	Genotype rs10306187	Genotype rs10306188
1	48	Man	ANAPHYLAXIS		+				A/C	A/G
2	58	Woman	ANAPHYLAXIS		+	+			A/C	A/G
3	29	Man	MIXED	+	+				A/A	A/G
4	47	Man	MIXED	+				+	A/A	A/G
5	24	Woman	MIXED	+	+			+	A/C	A/A
6	14	Man	MIXED	+	+				A/C	A/G
7	35	Woman	MIXED	+		+			C/C	G/G
8	16	Man	MIXED	+	+				A/A	A/G
9	48	Man	NECD	+	+	+		+	A/A	A/G
10	79	Woman	NECD	+		+	+		A/A	A/G
11	62	Man	NECD	+			+	+	A/A	A/G
12	58	Woman	NECD		+	+		+	A/A	A/G
13	16	Woman	NECD		+	+		+	A/A	A/G
14	38	Man	NECD	+	+				A/A	A/G
15	29	Woman	NECD	+	+				A/A	A/G
16	31	Woman	NECD	+	+				A/A	A/G
17	29	Woman	NECD	+	+				A/C	A/A
18	41	Woman	NECD	+	+				A/C	A/G
19	50	Man	NECD	+				+	A/C	A/G
20	33	Man	NECD	+				+	A/C	A/G
21	37	Woman	NECD	+		+		+	A/C	A/G
22	25	Woman	NECD	+	+	+		+	A/C	A/G
23	35	Man	NECD	+	+				A/C	A/G
24	42	Man	NECD	+				+	A/C	A/G
25	23	Woman	NECD		+			+	A/C	A/G
26	35	Woman	NECD	+		+		+	A/C	A/G
27	27	Man	NECD	+			+	+	A/C	A/G
28	32	Man	NECD	+	+				A/C	A/G
29	44	Woman	NECD		+	+		+	A/C	A/G
30	53	Man	NECD	+		+			A/C	A/G
35	44	Man	NECD	+	+		+		C/C	G/G
32	16	Man	NECD	+		+			C/C	G/G

TABLE 5 (Continued)

Patient ID	Age (years)	Gender	Clinical presentation	AAS	Ibuprofen	Metamizole	Diclofenac	Other NSAID	Genotype rs10306187	Genotype rs10306188
33	54	Woman	NECD	+	+				C/C	G/G
34	53	Woman	NERD	+	+				A/A	G/G
35	36	Man	NERD	+	+	+		+	A/A	G/G
36	35	Woman	NERD	+	+	+	+	+	A/C	A/G
TOTAL		18 woman (50%)		30 (83.3%)	24 (66.6%)	15 (41.7%)	5 (13.9%)	18 (50.0%)		

Abbreviations: AAs, acetylsalicylic acid; NECD, NSAID-exacerbated cutaneous disease; NERD, NSAID-exacerbated respiratory disease

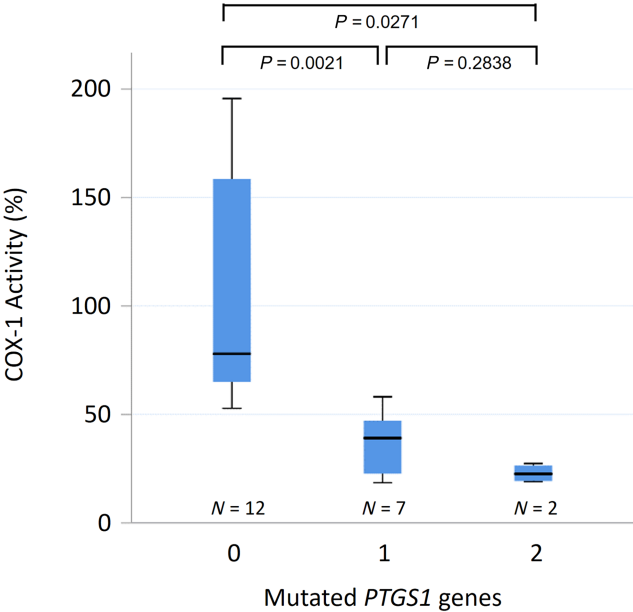


FIGURE 2 COX-1 activity according the number of *PTGS1* genes with the risk haplotype. Results are expressed as the percentage of the mean activity for all individuals with the wild-type genotype (rs10306187A + rs10306188A). Mutated genes correspond to the haplotype rs10306187C + rs10306188G. *P* values for intergroup comparisons were calculated by using the unpaired Student's *t*-test

individuals. An association of functional *PTGS1* functional gene variants with NSAID-related drug-induced liver injury has been reported recently (Lucena et al., 2019). Thus suggesting that genetically determined COX-1 activity might modify the risk of developing adverse drug reactions caused by COX-inhibitors. It could be speculated that individuals with decreased basal COX-1 activity might be at increased risk of presenting adverse drug events with mechanisms based on COX-1 inhibition.

Regarding the mechanism by which the risk *PTGS1* haplotype causes decreased COX-1 activity, it could be speculated that one or both SNVs implicated in the risk haplotype might be linked with other functional *PTGS1* SNVs. However, linkage analyses (Tables S5 and S6) suggest that the risk of developing cross-reactive hypersensitivity to NSAIDs should not be attributable to linkage with other SNVs. As for the *PTGS2* rs4648250 SNV, which is located in the gene promoter, such *PTGS2* SNV does not affect any known transcription factor binding site (Agundez, Gonzalez-Alvarez, Vega-Rodriguez, Botello, & Garcia-Martin, 2014) and no major linkage with functional SNVs in the *PTGS2* gene is present (Table S7). In addition, no clinical or functional associations for this SNV have been described so far. This, together with the weak statistical association in the replication analysis (Table 3, Figure 1), indicates that such *PTGS2* SNV has little relevance in the risk of developing cross-reactive hypersensitivity to NSAIDs.

When we analysed whether the SNVs involved in the *PTGS1* risk haplotype affect miRNA binding sequences, such analyses revealed that both SNVs altered miRNA binding sequences. Therefore, it could be speculated that gene variations in miRNA binding sequences might be related to the decrease in the COX-1 enzyme activity shown in

Figure 2, but further studies are required to gain ground on the specific mechanisms causing the observed decrease of enzyme activity.

Among the most common culprit drugs (Table 1), aspirin, ibuprofen, naproxen and metamizole triggered the reaction in the vast majority of patients. These drugs cause a potent COX-1 inhibition (Angiolillo & Weisman, 2017; Campos et al., 1999; Park & Bavry, 2014). Other drugs involved in a minority of patients were diclofenac and aceclofenac. These drugs are considered as predominant COX-2 inhibitors, although they can also inhibit COX-1 (Altman, Bosch, Brune, Patrignani, & Young, 2015; Hinz et al., 2003). Therefore, the culprit drugs in this study are consistent with the hypothesis that cross-reactive hypersensitivity is related to COX-1 inhibition.

The present study has some limitations and some unanswered questions remain. Regarding limitations, although the sample size is high, we cannot rule out that SNVs with very low allele frequencies could have been overlooked in the sequencing analyses. For that reason, we included in the replication analyses all nonsynonymous SNVs for both, *PTGS1* and *PTGS2* genes. Another limitation is that only a few patients (5.6%) carry the risk haplotype and, therefore, this genetic factor can only explain a small percentage of cases. As shown in Table S3, the sensitivity and specificity of the genetic associations are not adequate to test the presence of the *PTGS1* 3' UTR SNVs in the general population, but might be acceptable for testing among patients with a positive family history of cross-reactive hypersensitivity to NSAIDs.

Among open questions, it is to be noted the presence of five patients with cross-hypersensitivity to the COX-2 inhibitor diclofenac among patients with the risk haplotype (Table 5). Nevertheless, all these patients are positive for two or more COX-1 inhibitors and therefore COX-1 inhibition seems to be a relevant factor in these patients. Moreover, the safety profile of diclofenac is worse than that of celecoxib or etoricoxib, regarding adverse gastrointestinal events, which are related to COX-1 inhibition (Bjarnason et al., 2018; Walker, 2018). Therefore, despite being predominantly a COX-2 inhibitor, diclofenac has the ability to inhibit COX-1 and it does not have the safety profile of a typical selective COX-2 inhibitor.

In summary, we identified a haplotype that is strongly associated with the risk of developing cross-reactive NSAID hypersensitivity. The strength of the association and the functional effect of the risk haplotype provide proof of mechanism. The findings in this study raise the hypothesis of increased risk of developing cross-reactive NSAID hypersensitivity in individuals with a genetically determined impairment in COX-1 enzyme activity. It is conceivable that NSAIDs-induced COX-1 inhibition would add to the effect of SNVs causing decreased COX-1 activity and, therefore, that carriers of the risk *PTGS1* haplotype would be more prone to developing adverse drug reactions when receiving COX-1 inhibitors. By turn, we did not identify any genetic association with *PTGS2* SNVs, which is consistent with the fact that COX-2 inhibitors are well tolerated among patients with cross-reactive NSAID hypersensitivity. This study raises the question of whether *PTGS1* genetic variability could be a relevant susceptibility factor for other common NSAID-related adverse drug events linked to COX-inhibition (Ho et al., 2018; Khan, Andrews, & Chin-Dusting, 2019; Macias, Gomez Tabales, Garcia-Martin, &

Agundez, 2020; Radi & Khan, 2019; Sostres, Gargallo, & Lanás, 2013). One study in this regard has been already carried out with positive findings (Lucena et al., 2019), and additional studies are underway.

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AUTHOR CONTRIBUTIONS

All authors have made substantial contributions as follows: E.G.M. and J.A.G.A. did the study design. Manuscript drafting was carried out by E.G.M. and J.A.G.A. Genotyping analyses were performed by G.E., E.G.M., and J.A.G.A. Patient recruitment and clinical evaluation were carried out by all authors. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST

Nothing to disclose.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design and Analysis](#) and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

ETHICS APPROVAL STATEMENT

The study was conducted according to the principles of the Declaration of Helsinki and approved by the Ethics Committees of each participating hospital. Written informed consent was obtained from all the participants involved in the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Supplemental Table S1. Details of the areas sequenced.

Gene	Area sequenced	Corresponds to
PTGS1	Eight overlapping amplicons between 125131159 and 125133582	Gene promoter (2,200 bps), exons 1 and 2
	A single amplicon spanning from 125139990 to 125140373	Exon 3
	Two overlapping amplicons between 125140658 and 125141272	Exons 4 and 5
	Two overlapping amplicons between 125143496 and 125144089	Exons 6 and 7
	A single amplicon spanning from 125145716 to 125146077	Exon 8
	A single amplicon spanning from 125148658 to 125149039	Exon 9
	A single amplicon spanning from 125152354 to 125152694	Exon 10
	Thirteen overlapping amplicons between 125154403 and 125158017	Exon 11 and 5' untranslated area (complete)
PTGS2	Eight overlapping amplicons between 186649312 and 186651605	Gene promoter (2,046 bps), exon 1
	Two overlapping amplicons between 186648095 and 186648609	Exons 2 and 3
	A single amplicon spanning from 186647338 to 186647602	Exon 4
	A single amplicon spanning from 186646708 to 186647025	Exon 5
	Two overlapping amplicons between 186645535 and 186646096	Exons 6 and 7
	Two overlapping amplicons between 186644963 and 186645381	Exon 8
	A single amplicon spanning from 186644338 to 186644721	Exon 9
	Sixteen overlapping amplicons between 186640825 and 186643926	Exon 10 and 5' untranslated area (complete)

All exons sequenced in this study included the flanking regions.

Supplemental Table S2. TaqMan genotyping assays used in this study.

Gene	rs identification number	Assay identification
PTGS1	rs1330344 C/T	C__7584368_1_
	rs1236913 T/C	C__7584364_20
	rs3842787 C/T	C__30252576_20
	rs5789 C/A	C__11722595_20
	rs5794 G/A	C__29693876_10
	rs10306187 A/C	C__30361102_10
	rs10306188 A/G	C__29693878_20
	rs10306194 C/A	C__2742154_10
PTGS2	rs4648250 T/C	C__31274571_10
	rs689464 CA/-	Custom TaqMan Assay
	rs2853805 G/A	Custom TaqMan Assay
CYP2C9	rs1799853 C/T	C__25625805_10
	rs1057910 A/C	C__27104892_10

Supplemental Table S3. Comparison of the characteristics of patients with NSAID-induced hypersensitivity who carried either of the risk *PTGS1* 3' UTR SNVs vs. non-carrier patients.

	Carriers (positive to clinical presentation or drug)	Carriers (negative to clinical presentation or drug)	Non-Carriers (positive to clinical presentation or drug)	Non-carriers (negative to clinical presentation or drug)	OR (95% C.I.), <i>P</i>	Sensitivity	Specificity	Number needed to genotype
Clinical Presentation								
NECD	25	11	395	209	1.20 (0.58- 2.49), 0.620	0.050	0.950	8731
NERD	3	33	71	533	0.68 (0.20- 2.28), 0.533	0.041	0.942	520
Anaphylaxis	2	34	56	548	0.58 (0.14- 2.46), 0.451	0.034	0.942	478
Mixed	6	30	68	536	1.57 (0.63- 3.93), 0.325	0.081	0.947	329
Total	30	108	590	1826	1.03 (0.70- 1.52), 0.875	0.048	0.944	690
Drug involved								
AAS	30	6	504	100	0.99 (0.40- 2.45), 0.986	0.056	0.943	16107
Ibuprofen	24	12	282	322	2.28 (1.12- 4.65), 0.020	0.078	0.964	89
Metamizole	15	21	219	385	1.26 (0.63- 2.49), 0.513	0.064	0.948	329
Diclofenac	5	31	84	520	1.00 (0.38- 2.64), 0.998	0.056	0.944	96640
Total	74	70	1089	1327	1.29 (0.92- 1.80), 0.139	0.064	0.950	282

Supplemental Table S4. Characteristics of patients with NSAID-induced hypersensitivity who participated in the functional determination of COX-1 activities

Gender	Genotype rs10306187	Genotype rs10306188	COX-1 activity % (mean of triplicate measurements). Determination 1	COX-1 activity % (mean of triplicate measurements) Determination 2
Woman	A/A	A/A	70.03	73.01
Woman	A/A	A/A	193.83	160.00
Man	A/A	A/A	176.26	195.62
Woman	A/A	A/A	60.35	69.56
Woman	A/A	A/A	63.10	73.61
Man	A/A	A/A	109.7	98.96
Woman	A/A	A/A	55.07	67.36
Man	A/A	A/A	126.19	157.10
Man	A/A	A/A	65.60	64.37
Woman	A/A	A/A	52.82	53.77
Woman	A/A	A/A	183.43	194.98
Man	A/A	A/A	85.57	82.36
Man	A/C	A/G	50.63	42.30
Woman	A/C	A/G	20.55	21.99
Man	A/C	A/G	42.96	47.13
Woman	A/C	A/G	18.52	22.70
Woman	A/C	A/G	38.12	40.15
Woman	A/C	A/G	30.71	34.83
Man	A/C	A/G	58.16	54.12
Man	C/C	G/G	27.36	19.11
Woman	C/C	G/G	19.59	25.57

Two determinations were carried out in different days.

Samples are ordered by genotypes.

100% is the average for all samples with genotypes A/A for both SNVs.

Supplemental Table S5. PTGS1 SNVs strongly linked to the SNV rs10306187.

SNV designation	Allele frequency	D'	R ²	Effect
rs377685188	0.0047	1.0	1.0	NA
rs143071963	0.0047	1.0	1.0	NA
rs143633959	0.0047	1.0	1.0	NA
rs139797615	0.0047	1.0	1.0	NA
rs139026062	0.0047	1.0	1.0	NA
rs149444094	0.0047	1.0	1.0	NA
rs79809051	0.0047	1.0	1.0	NA
rs190949088	0.0047	1.0	1.0	NA
rs113222500	0.0047	1.0	1.0	NA
rs73557072	0.0047	1.0	1.0	NA
rs147330098	0.0047	1.0	1.0	NA
rs113200425	0.0047	1.0	1.0	NA
rs113819014	0.0047	1.0	1.0	NA
rs77664851	0.0047	1.0	1.0	NA
rs73563130	0.0047	1.0	1.0	NA
rs116346618	0.0047	1.0	1.0	NA
rs77676149	0.0047	1.0	1.0	NA
rs79440518	0.0047	1.0	1.0	NA
rs10306138	0.0047	1.0	1.0	NA
rs73563107	0.0047	1.0	1.0	NA
rs113922275	0.0047	1.0	1.0	NA
rs113155182	0.0047	1.0	1.0	NA
rs78094806	0.0047	1.0	1.0	NA
rs73561102	0.0047	1.0	1.0	NA
rs9299283	0.0047	1.0	1.0	NA
rs140073806	0.0047	1.0	1.0	NA
rs10306168	0.0047	1.0	1.0	NA
rs10306169	0.0047	1.0	1.0	NA
rs59533310	0.0047	1.0	1.0	NA
rs28660644	0.0047	1.0	1.0	NA
rs28569123	0.0047	1.0	1.0	NA
rs112688842	0.0047	1.0	1.0	NA
rs73561056	0.0047	1.0	1.0	NA
rs73561059	0.0047	1.0	1.0	NA
rs10306170	0.0047	1.0	1.0	NA
rs10306171	0.0047	1.0	1.0	NA
rs10306172	0.0047	1.0	1.0	NA
rs10306174	0.0047	1.0	1.0	NA
rs10306179	0.0047	1.0	1.0	NA
rs7874014	0.0047	1.0	1.0	NA
rs7874531	0.0047	1.0	1.0	NA

rs7863496	0.0047	1.0	1.0	NA
rs7848265	0.0047	1.0	1.0	NA
rs111889184	0.0047	1.0	1.0	NA
rs7848086	0.0047	1.0	1.0	NA
rs73561092	0.0047	1.0	1.0	NA
rs10306182	0.0047	1.0	1.0	NA
rs3842801	0.0047	1.0	1.0	NA
rs3842802	0.0047	1.0	1.0	synonymous
rs3842803	0.0047	1.0	1.0	synonymous
rs10306190	0.0047	1.0	1.0	NA
rs10306189	0.0047	1.0	1.0	NA
rs10306183	0.0047	1.0	1.0	NA
rs10306188	0.0047	1.0	1.0	NA
rs145951466	0.0093	1.0	0.4977	NA
rs375645507	0.0093	1.0	0.4977	NA
rs114696773	0.0093	1.0	0.4977	NA
rs75358267	0.0093	1.0	0.4977	synonymous
rs79723201	0.0093	1.0	0.4977	NA
rs16911935	0.0093	1.0	0.4977	NA
rs7032147	0.0093	1.0	0.4977	NA
rs7031700	0.0093	1.0	0.4977	NA
rs114930573	0.0093	1.0	0.4977	NA
rs150072724	0.0093	1.0	0.4977	NA
rs369360062	0.0093	1.0	0.4977	NA
rs193003182	0.0093	1.0	0.4977	NA
rs187803051	0.0093	1.0	0.4977	NA
rs368892261	0.0093	1.0	0.4977	NA
rs7045223	0.0093	1.0	0.4977	NA
rs7029024	0.0093	1.0	0.4977	NA
rs7042205	0.0093	1.0	0.4977	NA
rs7025445	0.0093	1.0	0.4977	NA
rs7025637	0.0093	1.0	0.4977	NA
rs183763522	0.0093	1.0	0.4977	NA
rs79403056	0.0093	1.0	0.4977	NA
rs115709283	0.0093	1.0	0.4977	NA
rs142654200	0.0093	1.0	0.4977	NA
rs114981517	0.0093	1.0	0.4977	NA
rs138331678	0.0093	1.0	0.4977	NA
rs142144550	0.0093	1.0	0.4977	NA
rs79476396	0.0093	1.0	0.4977	NA

Linkage was determined by using LDproxy Tool (<https://ldlink.nci.nih.gov/?tab=ldproxy>) in IBS (Iberian population from Spain).

Supplemental Table S6. PTGS1 SNVs strongly linked to the SNV rs10306188.

SNV designation	Allele frequency	D'	R ²	Effect
rs77676149	0.0047	1.0	1.0	NA
rs10306125	0.0093	1.0	0.4977	NA
rs10306126	0.0093	1.0	0.4977	NA
rs35871335	0.014	1.0	0.3302	NA
rs12000670	0.0093	1.0	0.4977	NA
rs12004533	0.0093	1.0	0.4977	NA
rs12004534	0.0093	1.0	0.4977	NA
rs1213265	0.0748	1.0	0.0581	NA
rs1213266	0.0841	1.0	0.0511	NA
rs79440518	0.0047	1.0	1.0	NA
rs10306135	0.1262	1.0	0.0325	NA
rs57350981	0.229	1.0	0.0158	NA
rs7866582	0.1449	1.0	0.0277	NA
rs35119072	0.1308	1.0	0.0312	NA
rs35324743	0.1308	1.0	0.0312	NA
rs10306138	0.0047	1.0	1.0	NA
rs2282169	0.1542	1.0	0.0258	NA
rs10306141	0.1075	1.0	0.039	NA
rs10306143	0.1075	1.0	0.039	NA
rs7026055	0.1121	1.0	0.0372	NA
rs60185476	0.1075	1.0	0.039	NA
rs10306145	0.1121	1.0	0.0372	NA
rs10306146	0.1121	1.0	0.0372	NA
rs10306148	0.1121	1.0	0.0372	NA
rs5788	0.1121	1.0	0.0372	synonymous
rs3215925	0.1121	1.0	0.0372	NA
rs113922275	0.0047	1.0	1.0	NA
rs10306149	0.1121	1.0	0.0372	NA
rs4273915	0.1308	1.0	0.0312	NA
rs113155182	0.0047	1.0	1.0	NA
rs4240474	0.1121	1.0	0.0372	NA
rs3842798	0.1495	1.0	0.0267	NA
rs78094806	0.0047	1.0	1.0	NA
rs4836885	0.1121	1.0	0.0372	NA
rs4836886	0.1121	1.0	0.0372	NA
rs4836887	0.1121	1.0	0.0372	NA
rs4836888	0.1121	1.0	0.0372	NA
rs4836889	0.1121	1.0	0.0372	NA
rs4836890	0.1121	1.0	0.0372	NA
rs9299279	0.1121	1.0	0.0372	NA
rs9299280	0.1121	1.0	0.0372	NA

rs9299281	0.1121	1.0	0.0372	NA
rs9299282	0.1121	1.0	0.0372	NA
rs9299283	0.0047	1.0	1.0	NA
rs72444571	0.1822	1.0	0.0211	NA
rs6478565	0.1308	1.0	0.0312	NA
rs10306163	0.1495	1.0	0.0267	NA
rs10306168	0.0047	1.0	1.0	NA
rs10306169	0.0047	1.0	1.0	NA
rs12551233	0.0421	1.0	0.1069	NA
rs59533310	0.0047	1.0	1.0	NA
rs11313763	0.1869	1.0	0.0204	NA
rs28660644	0.0047	1.0	1.0	NA
rs12238478	0.0421	1.0	0.1069	NA
rs28569123	0.0047	1.0	1.0	NA
rs112688842	0.0047	1.0	1.0	NA
rs73561056	0.0047	1.0	1.0	NA
rs73561059	0.0047	1.0	1.0	NA
rs12238505	0.0421	1.0	0.1069	NA
rs10306170	0.0047	1.0	1.0	NA
rs10306171	0.0047	1.0	1.0	NA
rs10306172	0.0047	1.0	1.0	NA
rs10306174	0.0047	1.0	1.0	NA
rs10306176	0.0234	1.0	0.1962	NA
rs10306179	0.0047	1.0	1.0	NA
rs7874014	0.0047	1.0	1.0	NA
rs7874531	0.0047	1.0	1.0	NA
rs7863496	0.0047	1.0	1.0	NA
rs7848265	0.0047	1.0	1.0	NA
rs111889184	0.0047	1.0	1.0	NA
rs7848086	0.0047	1.0	1.0	NA
rs5900521	0.1355	1.0	0.0299	NA
rs12005866	0.0234	1.0	0.1962	NA
rs10306182	0.0047	1.0	1.0	NA
rs3842801	0.0047	1.0	1.0	NA
rs3842802	0.0047	1.0	1.0	synonymous
rs3842803	0.0047	1.0	1.0	synonymous
rs10306183	0.0047	1.0	1.0	NA
rs10306187	0.0047	1.0	1.0	NA
rs8046	0.0421	1.0	0.1069	NA
rs10306189	0.0047	1.0	1.0	NA
rs10306190	0.0047	1.0	1.0	NA
rs73561092	0.0047	1.0	1.0	NA
rs10306202	0.0421	1.0	0.1069	NA
rs10985631	0.0467	1.0	0.0958	NA

Linkage was determined by using LDproxy Tool (<https://ldlink.nci.nih.gov/?tab=ldproxy>) in IBS (Iberian population from Spain).

Supplemental Table S7. PTGS2 SNVs strongly linked to the SNV rs4648250.

SNV designation	Allele frequency	D'	R2	Effect
rs20417	0.1495	1.0	0.0267	NA
rs689465	0.1075	1.0	0.039	NA
rs10911904	0.1028	1.0	0.041	NA
rs10911905	0.1075	1.0	0.039	NA

Linkage was determined by using LDproxy Tool (<https://ldlink.nci.nih.gov/?tab=ldproxy>) in IBS (Iberian population from Spain).