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Targeted next-generation sequencing panel to investigate antiplatelet adverse reactions in acute coronary syndrome patients undergoing percutaneous coronary intervention with stenting

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ABSTRACT

Antiplatelet therapy, the gold standard of care for patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI), is one of the therapeutic approaches most associated with the development of adverse drug reactions (ADRs). Although numerous studies have shown that pharmacological intervention based on a limited number of high-evidence variants (primarily *CYP2C19*2 and *3*) can reduce the incidence of major adverse cardiovascular events (MACEs), ADRs still occur at variable rates (10.1 % in our case) despite personalized therapy.

This study aimed to identify novel genetic variants associated with the endpoint of MACEs 12 months after PCI by designing and analyzing a targeted gene panel. We sequenced 244 ACS-PCI-stent patients (109 with event and 135 without event) and 99 controls without structural cardiovascular disease and performed an association analysis to search for unexpected genetic variants.

No single nucleotide polymorphisms reached genomic significance after correction, but three novel variants, including *ABCA1* (rs2472434), *KLB* (rs17618244), and *ZNF335* (rs3827066), may play a role in MACEs in ACS patients. These genetic variants are involved in regulating high-density lipoprotein levels and cholesterol deposition, and as they are regulatory variants, they may affect the expression of nearby lipid metabolism-related genes. Our findings suggest new targets (both at the gene and pathway levels) that may increase susceptibility to MACEs, but further research is needed to clarify the role and impact of the identified variants before these findings can be incorporated into the therapeutic decision-making process.

Abbreviations: *ABCA1*, ATP Binding Cassette Subfamily A Member 1 gene; *ABCB1*, ATP Binding Cassette Subfamily B Member 1 gene; ACS, acute coronary syndrome; ADR, adverse drug reaction; AMI, acute myocardial infarction; ASA, acetylsalicylic acid; BA, bile acid; CV, cardiovascular; *CYP2C19*, cytochrome P450 Family 2 Subfamily C Member 19 gene; DAPT, dual antiplatelet therapy; FGFR, fibroblast growth factor receptor; GWAS, genome-wide association studies; HDL, high-density lipoproteins; *KLB*, Klotho Beta gene; LD, linkage disequilibrium; LDL, low-density lipoprotein; LOF, loss-of-function; MACE, major adverse cardiovascular event; MAF, minority allele frequency; NGS, next generation sequencing; PCI, percutaneous coronary intervention; PD, pharmacodynamics; PGx, pharmacogenomics; PharmGKB, pharmacogenomics knowledgebase; PK, pharmacokinetics; SNP, single nucleotide polymorphisms; VIP, very important pharmacogenes; WES, whole exome sequencing; WGS, whole genome sequencing; *ZNF335*, Zinc finger protein 335 gene.

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1. Introduction

Giving the same dose of the same drug to patients with a common pathology usually means that not all patients will respond in the same way. There is often a subset of patients who experience therapeutic failure and may develop adverse drug reactions (ADRs). These ADRs are currently a major clinical concern due to their significant impact on morbidity, mortality and economic costs. Although clinical trials involve thousands of patients to assess drug efficacy and safety, it is difficult to predict an individual patient's response to a given drug or the potential ADRs that may occur in subpopulations not accounted for in the trial. Causes of inter-individual heterogeneity in drug response include environmental, clinical (e.g., gender, age, disease severity, drug-drug interactions, and adherence), and genetic factors. Increasing evidence suggests that genetic variations in drug-metabolizing enzymes, drug transporters, and drug targets can significantly affect drug pharmacokinetics (PK) and pharmacodynamics (PD) and contribute to drug-induced ADRs [1–3].

Dual antiplatelet therapy (DAPT), consisting of a cyclooxygenase-1 inhibitor (acetylsalicylic acid, ASA) and a $P2Y_{12}$ inhibitor (clopidogrel, prasugrel or ticagrelor), is the standard of care for acute coronary syndrome (ACS) patients undergoing percutaneous coronary intervention (PCI) with stent implantation to prevent subsequent major adverse cardiovascular events (MACEs). Although clinical trials in ACS patients undergoing PCI have shown superior efficacy of prasugrel and ticagrelor in reducing ischaemic events, clopidogrel remains widely used in routine clinical practice (elderly patients (≥75 years), those with low bodyweight (<60 kg), and those at high risk of bleeding) [4,5].

Clopidogrel is a prodrug that, once absorbed by an intestinal efflux pump (MDR1, encoded by *ABCB1* gene), requires biotransformation by cytochrome P450 (CYP) enzymes, particularly CYP2C19, to generate its active metabolite. It binds specifically and irreversibly to platelet surface $P2Y_{12}$ purinergic receptors, thereby inhibiting ADP-mediated platelet activation and aggregation (see Fig. 1). A major drawback of its use is the heterogeneity of individual pharmacological response to clopidogrel, sometimes leading to therapeutic failure, with up to 10 % of patients experiencing recurrent ischemic events at 12 months despite DAPT. Several mechanisms have been proposed, such as differences in drug absorption, affinity of $P2Y_{12}$ receptors for its active metabolite, and variability in intrinsic signaling pathways, which may be affected by genetic polymorphisms, and which could explain the significant variability in clopidogrel response [6–8].

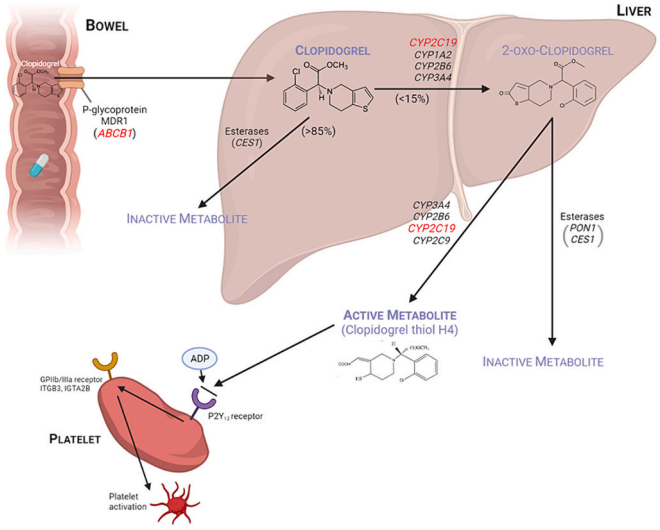

Fig. 1. Clopidogrel absorption, metabolism and mechanism of action.
Abbreviations: *ABCB1*, ATP Binding Cassette Subfamily B Member 1; ADP, adenosine diphosphate; *CES1*, carboxylesterase 1; *CYP1A2*, Cytochrome P450 Family 1 Subfamily A Member 2; *CYP2B6*, Cytochrome P450 Family 2 Subfamily B Member 6; *CYP2C19*, Cytochrome P450 Family 2 Subfamily C Member 19; *CYP2C9*, Cytochrome P450 Family 2 Subfamily C Member 9; *CYP3A4*, Cytochrome P450 Family 3 Subfamily A Member 4; *ITGA2B*, Integrin Subunit Alpha 2b; *ITGB3*, Integrin Subunit Beta 3; MDR1, multidrug resistance protein 1; *PON1*, paraoxonase 1.

Pharmacogenomics (PGx) examines the role of genetic variation in drug response and identifies biomarkers that help physicians personalize drug treatment and minimize side effects. Despite the multiple polymorphisms identified, variability in clopidogrel PK/PD profile has been consistently associated with genetic variation in CYP2C19 enzyme, a key determinant in both metabolic steps of clopidogrel conversion to active metabolite. The presence of loss-of-function (LOF) alleles in the *CYP2C19* gene has been associated with reduced clopidogrel metabolism and formation of its active metabolite, resulting in reduced antiplatelet effects and increased risk of atherothrombotic events [1,9]. In contrast, antiplatelet bioavailability is limited by the P-glycoprotein transporter (encoded by *ABCB1*). Several polymorphisms, especially c.3435C>T, have been suggested to decrease drug absorption and increase the risk of MACEs in ACS-PCI patients. However, conflicting and inconclusive results on the effect of these polymorphisms on clopidogrel response have limited their use in clinical practice [10,11]. Accordingly, numerous randomized trials such as PHARMCLO [12], TAILOR-PCI [13], and IAC-PCI [14] have shown that personalized antiplatelet therapy based on genotype of a limited number of candidate variants (mainly *CYP2C19*2*, **17*, and *ABCB1 C3435T*) improves patient outcomes. In this regard, we conducted a clinical trial to evaluate the possibility of implementing *CYP2C19/ABCB1* genotype-guided antiplatelet therapy prescription in an attempt to reduce MACEs rates in ACS-PCI stent patients [15]. However, as in the aforementioned studies, we continue to observe a MACEs rate (10 % in our population), the causes of which we are unable to explain. Therefore, since recurrent MACEs occur despite preventive genotyping, prescribing based on known LOF *CYP2C19* alleles alone may not be sufficient to improve clinical outcomes. In addition, studies suggest that LOF *CYP2C19* alleles account for only 12 % of the variability in clopidogrel antiplatelet efficacy, highlighting the need for identification of additional genetic variants to better predict variable responses to clopidogrel and provide more effective PGx-guided antiplatelet therapy in clinical practice [9,16].

Targeted gene panels have demonstrated advantages over whole exome/genome sequencing (WES/WGS) in terms of speed, cost, coverage, and sensitivity, providing fertile ground for future PGx applications in precision medicine for rare variants that may be missed in genome-wide association studies (GWAS), and may contribute significantly to inter-individual variation in drug response [17]. Therefore, this work aimed to design a custom-target next-generation sequencing (NGS) panel to deepen our understanding of drug targets and to discover potential PGx markers that could be used to predict and explain individual drug response. Specifically, we used an association approach to investigate whether MACEs can be genotypically predicted despite genotype-guided therapy, whether they are caused by drug transport/metabolism enzyme variants, or instead by variants directly related to disease onset.

2. Methods

2.1. Customized NGS panel design

The selection of candidate variants for our customized NGS panel design was performed as follows. We included two sets of genes and single nucleotide polymorphisms (SNPs); the first well-defined group corresponds to genes involved in the metabolic pathways of the most commonly used antiplatelet agents in ACS-PCI patients (clopidogrel, prasugrel and ticagrelor), as well as the most relevant pharmacogenes to date (Very Important Pharmacogenes, VIP), according to the PharmGKB

database [18]. The second group includes "cardiovascular (CV) disease onset" genes, involved in the molecular mechanisms of atherosclerosis, vessel angiogenesis, lipid metabolism, clotting, fibrinolysis, and arterial thrombosis. The latter group represents the largest set of genes reported in GWAS (literature, GWAS catalog, ClinVar) with a high association degree (p-value $<5.0 \times 10^{-8}$). A more detailed description of the entire panel design process is provided in the *Supplementary Data*.

2.2. Study patients

The current study is a continuation of a previous clinical trial conducted by our group from 2012 to 2016 [15,19], where the target population was patients with ACS undergoing PCI with stenting and indication for antiplatelet therapy (in addition to an indefinite course of ASA) with a follow-up period of 12 months. Two groups were considered; in the intervention group, patients carrying *CYP2C19* LOF alleles and/or with *ABCB1 C3435T* homozygous mutant genotype (*TT*) received prasugrel or ticagrelor as antiplatelet therapy, and the remaining patients with normal *CYP2C19* and *ABCB1* gene function received clopidogrel. In the non-intervention group, patients were treated mainly with clopidogrel. Primary efficacy endpoint was the composite of ACS, CV death or stroke within 12 months after PCI. Secondary endpoints were the rate of definite stent thrombosis and the need for urgent revascularization unrelated to stent thrombosis. Safety endpoints included major or minor TIMI (Thrombolysis In Myocardial Infarction) bleeding not related to coronary artery bypass grafting. The study [15,19] concluded that the *CYP2C19/ABCB1* genotype-guided strategy in the choice of antiplatelet therapy was able to reduce MACEs and bleeding rates during the 12 months after PCI compared to a non-guided strategy in ACS-PCI-stent patients. However, the primary endpoint occurred in 32 patients (10.1 %) in the intervention group and in 59 patients (14.1 %) in the non-intervention group (HR 0.63, 95 % CI (0.41–0.97), $p = 0.037$).

From these previous results arose our interest in conducting the current study, we wanted to identify new genetic variants associated with MACEs occurrence during the follow-up period despite PGx-guided antiplatelet therapy.

Based on the previous results of our group, we decided to structure the new study population for the investigation of adverse effects of antiplatelet therapy into three well-characterized groups:

- o A cohort of ACS patients undergoing PCI on antiplatelet therapy (whether or not guided by genetic testing), who suffered MACEs at 1-year follow-up (G1).
- o A cohort of ACS patients undergoing PCI on PGx-guided antiplatelet therapy who did not experience MACEs (G2).
- o A healthy population cohort without ACS or antiplatelet therapy (control group) (G3).

Further details of the inclusion and exclusion criteria of study groups are summarized in Fig. 2.

Patients included in the ACS-PCI-stent cohort (G1 and G2) were recruited as part of a clinical trial between 2012 and 2016 [15,19]; and patients included in the control cohort (G3) were recruited throughout 2022 and 2023, both at the San Cecilio University Hospital of Granada (Spain). The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Granada (Spain) "CEIM/CEI Provincial de Granada". In addition, all samples were managed as a private sample collection (n°0004153), registered in the National Biobank Registry of the Carlos III Institute (project RD09/0076/00148). All participants signed a written informed consent. Phenotypic data were available from the previous study and the medical record of each new patient has been carefully reviewed to collect all clinical and demographic information. All these data have been summarized in a single comprehensive database.

ACS patients undergoing PCI cohort (already recruited)	Healthy population without ischemic cardiopathy (to recruit in this study)
INCLUSION CRITERIA (all the following)	
▪ Patients ≥ 18 years old with diagnostic of ACS with/without segment ST elevation or unstable angina ▪ Performed PCI with stent implantation ▪ Treated with clopidogrel, prasugrel or ticagrelor for 12 months ▪ Signed informed consent to participate in the study	▪ Patients ≥ 18 years old without previous familiar or personal history of structural cardiovascular disease confirmed by ECG, stress test, echocardiogram or/and coronariography ▪ Signed informed consent to participate in the study
EXCLUSION CRITERIA (any of the following)	
▪ Patients requiring oral anticoagulation ▪ Presenting contraindication for taking acetylsalicylic acid, clopidogrel, prasugrel or ticagrelor ▪ With a high risk of bleeding	▪ Previous clinical history or previous data from image tests (ECG, echocardiogram, cardiac MRI or myocardial gammagraphy) suggesting the presence of previous myocardial infarction ▪ Previous history of taking clopidogrel, prasugrel or ticagrelor

Fig. 2. Enrolment criteria.
Abbreviations: ACS, Acute Coronary Syndrome; ECG, electrocardiogram; MRI, magnetic resonance imaging; PCI, Percutaneous Coronary Intervention.

2.3. Sample collection, library preparation and sequencing

Genomic DNA was extracted from a total of 343 buccal swabs collected from each participant following the method of *Freeman* et al. [20] with modifications of *Gómez-Martín* et al. [21] The quality and concentration of genomic DNA were determined using Nanodrop and Qubit (Thermo Fisher Scientific, Waltham, MA, USA).

An amount of 350 ng of isolated DNA was used to prepare sequencing libraries using the KAPA HyperPlus kit (Roche, Basel, Switzerland) with ultrasonic fragmentation according to the manufacturer's instructions. Library fragment size was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Quantitative analysis of the individual libraries was performed with the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) to ensure equimolar pooling of all sample libraries for future sequencing. Targeted gene enrichment was performed with the NimbleGen SeqCap EZ Library Prep kit (Roche, Basel, Switzerland), which hybridized to the custom capture probe for subsequent sequencing.

Sequencing was performed on the Illumina NextSeq 500 platform using the NextSeq 500/500 High Output v2.5 kit (150 cycles) (Illumina, San Diego, CA, USA). Libraries were sequenced paired-end at 75pb sequence length, giving us 2–5 million reads per sample, which we considered adequate for the detection of germline variants.

2.4. Data analysis

Data were analyzed in R version 4.2.2. Descriptive analysis of the main demographic and clinical characteristics, as well as the characteristics of the treatment prescribed after hospital admission, were performed with the tableone package (version 0.13.2).

We then performed the association analysis between the different groups. Sequencing of our custom panel provided us with the sequence file of each sample (FASTQ), the quality of which was validated using the FASTQC software. Reads were aligned against the GRCh37 assembly using the BWA-MEM aligner. Variant identification was performed using bcftools v1.4; these variants were filtered by quality (QUAL>30), functionally annotated, and their effects predicted using SNPNexus software.

Prior to statistical analysis, genotype data were subjected to a standard quality control procedure and preprocessed for imputation. Data

was filtered based on the following parameters: sample call rate < 95 %, genotype call rate < 98 %, autosomal heterozygosity rate ± 3 standard deviations from the mean, duplicate and monomorphic markers, samples that were not in Hardy-Weinberg equilibrium (HWE with a *p*-value of 0.000001). The low minority allele frequency (MAF) and linkage disequilibrium (LD) filters were omitted because the study regions were delimited, and a large number of variants were lost. By not applying these filters, a larger number of variants are retained, which affects the retained samples, the imputation process, and the subsequent statistical tests. Imputation was performed using the Michigan Imputation Server (Haplotype Reference Consortium reference panel).

Once the variants were identified and annotated, and after applying the different filters, the GENESIS v2.30.0 package (in R) was used to perform the relationship, ancestry and differential analyses. Samples with a kinship relationship ("kinship coefficient threshold" > 0.04) or ancestry detected by principal component analysis were eliminated. Phenotype-association testing by using post-imputation genotype probabilities was performed under an additive genetic effect model using the frequentist likelihood score method. The covariates age and gender, along with PC1 and PC2, were entered directly into the model for all comparisons.

3. Results

3.1. Patients and treatment characteristics

Baseline characteristics of the patients are shown in Table 1. Of the 343 patients recruited, 111 (32.4 %) were women (all female ACS patients were postmenopausal), the mean age was 65.9 ± 11.1 years, and almost all were Caucasian. Regarding CV risk factors, 214 patients (62.4 %) had hypertension, 201 (58.6 %) had dyslipidaemia, 111 (32.4 %) had diabetes, 87 (25.4 %) were smokers, and 93 (27.1 %) had CV history at enrollment.

According to the proposed groups for this study, we divided our population into ACS patients undergoing PCI-stent with an event, G1 (31.8 %), ACS-PCI-stent patients without an event, G2 (39.3 %), and controls without structural CV disease, G3 (28.9 %). Characteristics of thrombotic and haemorrhagic events within the G1 group are detailed in the *Supplementary Data*. We found that, among ACS patients, the presence of any type of previous CV history was associated with the incidence of subsequent CV events (47.7 % vs 30.4 %, $p = 0.008$). Furthermore, higher prevalence of smoking and diabetes was observed in ACS patients (with and without event) than controls ($p < 0.001$ for both).

After hospital admission, all patients were prescribed a DAPT, including an antiplatelet drug (guided or not by genetic testing) and ASA. Additionally, 203 patients (83.2 %) were prescribed β-blockers, 217 patients (88.9 %) antihypertensives (ACEI or ARA-II), and 19 patients (7.8 %) diuretics. Statins for hypercholesterolemia were prescribed to 229 patients (93.8 %). In addition, 221 patients (90.5 %) were prescribed a stomach protector (see Table 2).

Regarding the prescription of antiplatelet agents, 169 patients (69.3

Table 2
Characteristics of the treatment prescribed to patients upon admission.

	All (*N* = 244)	G1 (N = 109)	G2 (N = 135)	p-value
Acetylsalicylic acid	244 (100)	109 (100)	135 (100)	1.000
Antiplatelet therapy				
Clopidogrel	169 (69.3)	82 (75.2)	87 (64.4)	
Prasugrel	73 (29.9)	25 (22.9)	48 (35.6)	**0.035**
Ticagrelor	2 (0.8)	2 (1.8)	0 (0.0)	
DAPT duration				
1 month	14 (5.7)	11 (10.1)	3 (2.2)	
3 months	2 (0.8)	2 (1.8)	0 (0.0)	**0.006**
6 months	2 (0.8)	2 (1.8)	0 (0.0)	
12 months or more	226 (92.6)	94 (86.2)	132 (97.8)	
B-blockers	203 (83.2)	92 (84.4)	111 (82.2)	0.779
Antihypertensive therapy				
ACEI	176 (72.1)	77 (70.6)	99 (73.3)	0.747
ARA-II	41 (16.8)	20 (18.3)	21 (15.6)	0.683
Antialdosteronics	19 (7.8)	11 (10.1)	8 (5.9)	0.334
Statins	229 (93,8)			
Atorvastatin	159 (65.2)	75 (68.8)	84 (62.2)	
Simvastatin	55 (22.5)	24 (22.0)	31 (23.0)	0.575
Rosuvastatin	15 (6.1)	5 (4.6)	10 (7.4)	
Stomach protector	221 (90.5)			
Pantoprazole	178 (73.0)	71 (65.1)	107 (79.3)	
Omeoprazole	15 (6.1)	7 (6.4)	8 (5.9)	**0.003**
Ranitidine	24 (9.8)	20 (18.3)	4 (3.0)	
Others	4 (1.6)	3 (2.7)	1 (0.7)	

Values are shown as n (%). Statistically significant results are shown in bold ($p < 0.05$). Abbreviations: G1, group 1 of ACS patients with secondary event; G2, group 2 of ACS patients without secondary event; DAPT, dual antiplatelet therapy; ACEI, angiotensin-converting enzyme inhibitors; ARA-II, angiotensin II receptor antagonists.

Table 1
Main demographic and clinical characteristics of all study participants analyzed in the gene panel.

	All (*N* = 343)	G1 (*N* = 109)	G2 (*N* = 135)	*p*-value	G3 (*N* = 99)	*p*-value
Age	65.9 (11.1)	66.8 (11.4)	64.8 (12.3)	0.202	66.4 (8.8)	0.339
Gender						
Male	232 (67.6)	78 (71.6)	101 (74.8)	0.670	53 (53.5)	**0.002**
Female	111 (32.4)	31(28.4)	34 (25.2)		46 (46.5)	
BMI	28.6 (4.6)	28.9 (4.6)	28.4 (4.5)	0.369	NA	NA
Ethnic origin						
Caucasian	335 (97.7)	105 (96.3)	132 (97.8)		98 (99.0)	
Gypsy	6 (1.7)	3 (2.8)	3 (2.2)	0.517	0 (0.0)	0.423
Moroccan	2 (0.6)	1 (0.9)	0 (0.0)		1 (1.0)	
Cv history*	93 (27.1)	52 (47.7)	41 (30.4)	**0.008**	0	**< 0.001**
Cv risk factors						
Hypertension	214 (62.4)	75 (68.8)	79 (58.5)	0.128	60 (60.6)	0.233
Dyslipidaemia	201 (58.6)	70 (64.2)	77 (57.0)	0.313	54 (54.5)	0.329
Smoking	87 (25.4)	30 (27.5)	47 (34.8)	0.280	10 (10.1)	**< 0.001**
Diabetes mellitus	111 (32.4)	50 (45.9)	50 (37.0)	0.206	11 (11.1)	**< 0.001**
Renal failure	13 (3.8)	6 (5.5)	7 (5.2)	1.000	0 (0.0)	0.064

Values are shown as n (%); for age and BMI, they are shown as mean (standard deviation). Statistically significant results are shown in bold ($p < 0.05$). Abbreviations: G1, group 1 of ACS patients with secondary event; G2, group 2 of ACS patients without secondary event; G3, group 3 of controls without Cv disease; BMI, body mass index; Cv, cardiovascular; NA, not applicable.

* This variable refers to having had one or more of the following: previous angina, previous acute myocardial infarction, previous peripheral arterial disease, and/or previous stroke.

%) were prescribed clopidogrel, 73 (29.9 %) prasugrel, and only 2 (0.8 %) ticagrelor. ACS patients who experienced a secondary event (G1) were more likely to be prescribed clopidogrel (75.2 %) and less likely to be prescribed prasugrel (22.9 %) than those who did not experience an adverse event (G2) (64.4 % and 35.6 %, respectively). Furthermore, 13.8 % of patients in the G1 group who experienced MACEs were treated with DAPT for <12 months due to a benefit/risk issue or the need for triple antiplatelet therapy, compared to 97.8 % of patients in the G2 group who completed one year of DAPT. The prescription of the remaining drugs was similar between the two groups, except in the case of stomach protectors, where there was a higher prescription of ranitidine (18.3 % in G1 vs. 3 % in G2) and a lower prescription of pantoprazole (65.1 % in G1 vs. 79.3 % in G2) when comparing individuals who experienced an adverse event to those who did not.

3.2. Association analysis of single variants and genes with MACEs at one-year follow-up

We performed an association analysis comparing ACS-PCI-stent patients who experienced a secondary event after antiplatelet therapy (group G1) with patients with the same condition who did not experience such adverse events (group G2). For the MACEs-related phenotype, we observed that after applying the appropriate correction, no loci from the single SNP analyses reached genome-wide significance cut-off (p-value $< 5.0 \times 10^{-8}$) in models adjusted for age, gender, and principal components, but we found some interesting loci with a rather low p-value (lowest p-value $= 1.0 \times 10^{-04}$) (Fig. 3).

The ten most significant associations obtained from our personalized gene panel that are in some way related to 12-month MACEs are shown in Table 3. The most significant SNP was rs2472434 at *ABCA1* (A>C, MAF = 0.28). Carriers of the alternative *C* allele had higher MACEs occurrence during the 12 months of follow-up compared to non-carriers (0.35 vs. 0.19; β, −0.92, $p = 1.0 \times 10^{-04}$). Rs2472434 has a high LD with the rs2472433 locus ($r^2 = 0.97$ and |D|' = 1.00) and with the rs2472378 locus ($r^2 = 0.87$ and |D|' = 1.00) in the Iberian population. Similarly, these two SNPs also contributed significantly to MACEs (rs2472433: β, −0.78; $p = 1.4 \times 10^{-03}$ and rs2472378: β, −0.75; $p = 2.0 \times 10^{-03}$). Previously published GWAS [22] have identified variants in this gene that, in combination with other SNPs, are independent predictors of variability in antiplatelet response to clopidogrel.

Another important association was the rs17618244 locus in *KLB* (G>A, MAF = 0.19), with the *G* allele increasing the occurrence of MACEs (β, 0.83, $p = 3.4 \times 10^{-04}$). This gene has been reported to be involved in the fibroblast growth factor receptor signaling pathway and contributes to transcriptional repression of cholesterol 7 alpha hydroxylase (CYP7A1) [23].

Among the major SNPs, we found rs3827066 at *ZNF335* (C>T, MAF = 0.16), where carriers of the alternative allele had lower MACEs incidence during the year of follow-up compared to non-carriers (β, 0.87; $p = 6.0 \times 10^{-04}$). This gene is associated with total cholesterol measurement, and a recently published GWAS [24] identified rs3827066 in *PCIF1-ZNF335-MMP9* as a novel locus for coronary artery disease (CAD) (OR = 1.04).

4. Discussion

As genotype-guided pharmacotherapies advance in the clinical setting, NGS technologies offer great utility in simultaneously and unbiasedly analyzing large numbers of genes potentially relevant to ADRs. In fact, researchers already have PGx panels available to analyze individual response to certain drugs, including antiplatelet agents, as well as CV disease panels. However, the risk prediction of potential ADRs by identifying the genes underlying them remains a major challenge in disease management [25–27].

To our knowledge, our study is the first to design and analyze a targeted gene panel containing most of the clinically relevant PGx loci and a large proportion of loci associated with treatment-related MACEs to search for novel candidate variants involved in variable antiplatelet response in ACS patients receiving DAPT after PCI. While we did not find any variants that reached the genome-wide significance threshold after applying the appropriate correction, we observed several interesting suggestive associations (lowest p-value $= 1.0 \times 10^{-04}$). To further investigate the functional evidence for SNPs contributing to MACEs at 12 months, we searched PubMed and previously published Gene Expression Omnibus (GEO) databases for relevant information referring to SNP-containing genes and CV disease.

ABCA1 encodes an integral membrane protein that belongs to the ABC transporter superfamily. Although ABC transporters have been widely studied in diseases such as cancer and multidrug resistance, their role in the pathogenesis of CV disease is just beginning to be elucidated. In particular, *ABCA1* is thought to play an important role in the development of atherosclerosis and hypertension, as it not only has catalytic functions in the formation of high-density lipoproteins (HDL), but also functions as a cellular transporter of cholesterol and lipid efflux in vascular endothelial cells and macrophages (Fig. 4) [28]. HDL has been suggested to be cardioprotective as it plays an important role in reverse cholesterol transport, which facilitates cholesterol removal from peripheral tissues, including atherosclerotic plaques, and subsequent hepatic elimination, thereby limiting plaque formation and progression [29]. In humans, mutations in *ABCA1* can lead to cholesterol deposition in tissue macrophages, resulting in low HDL levels and prevalent atherosclerosis. Indeed, approximately 10 % of individuals with very low serum HDL levels have been reported to carry certain *ABCA1* variants, underscoring the importance of this gene in HDL homeostasis

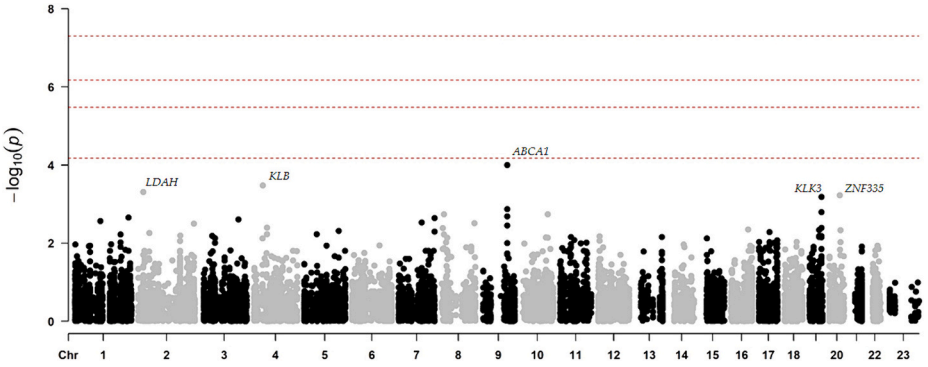

Fig. 3. Manhattan plot based on the results of the association study.
The chromosomal position is on the x-axis and the -log10 of the association p-value is on the y-axis. The four significance levels considered are indicated in dotted lines: genome-wide significance level, −log10($5e^{-8}$); 95 % confidence, −log10(0.05/N°rs); 90 % confidence, −log10(0.1/N°rs), and "suggestive", −log10(1/N°rs). Genes with the most significant SNPs associated with MACEs at 1 year follow-up are labelled.

Table 3
Top ten SNPs identified by custom gene panel, adjusted for age, sex and principal components for association with MACEs.

SNP	Chr	Position*	Ref	Alt	Location	Gene	G1_EAF	G2_EAF	MAF**	Beta	SD	p-value
rs2472434	9	107,623,249	A	C	intronic	*ABCA1*	0.35	0.19	0.28	−0.92	0.24	0.0001014
rs17618244	4	39,448,529	G	A	exonic	*KLB*	0.17	0.31	0.19	0.83	0.23	0.0003360
rs114193458	2	20,989,039	C	T	intronic	*LDAH*	0.000	0.037	0.005	2.44	0.70	0.0004903
rs3827066	20	44,586,023	C	T	intronic	*ZNF335*	0.12	0.23	0.16	0.87	0.25	0.0005986
rs2659122	19	51,363,026	C	T	UTR3	*KLK3*	0.63	0.75	0.75	0.72	0.21	0.0006588
rs2472433	9	107,623,326	C	T	intronic	*ABCA1*	0.31	0.18	0.27	−0.78	0.24	0.0013565
rs2455069	19	51,728,641	A	G	exonic	*CD33*	0.50	0.36	0.42	−0.62	0.20	0.0016215
rs35141404	10	112,404,302	G	A	exonic	*RBM20*	0.25	0.15	0.15	−0.77	0.25	0.0018202
rs2409653	8	10,677,792	T	C	intronic	*PINX1*	0.04	0.11	0.05	1.14	0.37	0.0018295
rs2472378	9	107,623,570	G	T	intronic	*ABCA1*	0.30	0.18	0.27	−0.75	0.24	0.0020637

Abbreviations: SNP, single nucleotide polymorphism; Chr, chromosome; Ref, reference allele; Alt, alternative allele; G1_EAF, effect allele frequency (alternative allele) for Group 1 of ACS-PCI-stent patients with event; G2_EAF, effect allele frequency (alternative allele) for Group 2 of ACS-PCI-stent patients without event; MAF, minor allele frequency; Beta, beta coefficient corresponding to the effect size measure; SD, standard deviation.

* Genomic position is according GRCh37/hg19 assembly.

** MAF was obtained from the Genome Aggregation (gnomAD) - Exomes Database report for Europeans.

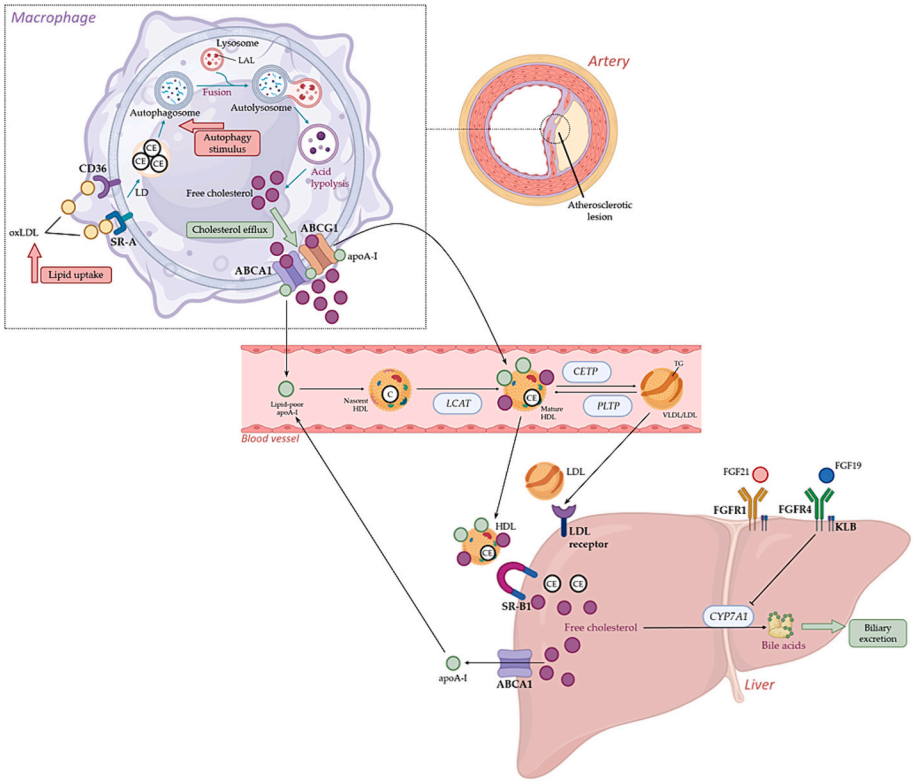

Fig. 4. Molecular mechanisms of reverse cholesterol transport (RCT).
RCT involves the transport of cholesterol from peripheral cells (including macrophage foam cells in atherosclerotic plaques) back to the liver for further excretion and thus plays an important role in reducing atherosclerosis. Cholesterol-loaded macrophages in the arterial wall deliver intracellular free cholesterol via ATP-binding cassette transporters (ABCA1 and ABCG1) to the cell membrane and to extracellular acceptors (cholesterol efflux), thereby preventing foam cell formation. While ABCA1 preferentially lipidates small HDL particles, namely apoA-I to form nascent HDL, ABCG1 stimulates the net efflux of cholesterol into larger HDL, but not into lipid-poor apoA-I. Lipid-poor apoA-I is also synthesized in the liver or intestine and secreted into plasma via hepatic or intestinal ABCA1. In the circulation, apoA-I interacts with phospholipids to form nascent pre-β-HDL. Following cholesterol transfer to HDL particles, the next step in HDL biology is the esterification of the acquired cholesterol by lecithin-cholesterol acyltransferase (LCAT) to form cholesterol ester (CE), resulting in mature HDL. In humans (but not in mice), CE in the core of mature HDL can be either 1) transferred by cholesterol ester transfer protein (CETP) to triglyceride-rich lipoproteins (VLDL and LDL) for elimination via hepatic clearance by LDLR, or 2) selectively taken up via SR-B1, which acts as a hepatic receptor for CE on HDL. Once in the liver, CE is hydrolyzed and free cholesterol is converted to bile acids (BAs), mainly by the neutral (classical) CYP7A1 pathway. BAs pass through the bile duct into the intestine where they are excreted (5 %) or re-circulated (95 %) [33–35].
Abbreviations: ABCG1, ATP-Binding Cassette G1 Transporter; ABCA1, ATP-Binding Cassette A1 Transporter; apoA-I, apolipoprotein A-I; C, cholesterol; CE, cholesterol ester; *CETP*, Cholesteryl Ester Transfer Protein; *CYP7A1*, Cytochrome P450 Family 7 Subfamily A Member 1; FGF19, Fibroblast growth factor 19; FGF21, Fibroblast growth factor 21; FGFR1, Fibroblast growth factor receptor 1; FGFR4, Fibroblast growth factor receptor 4; HDL, high-density lipoprotein; KLB, Klotho Beta; LD, lipid droplet; LAL, lysosomal acid lipase; *LCAT*, Lecithin-Cholesterol Acyltransferase; oxLDL, oxidized-low density lipoprotein; *PLTP*, Phospholipid Transfer Protein; SR-A, scavenger receptor class A; SR-B1, scavenger receptor class B type I; TG, triglycerides; VLDL/LDL, very low/low density lipoprotein.

[28,30]. Our study found that rs2472434, an intronic variant located in *ABCA1* and in LD with rs2472433 and rs2472378 variants, increased the risk of MACEs. Several common SNPs have been identified in non-coding regions of *ABCA1* (e.g., in the promoter, intron 1, and 5′ untranslated region) that may have an impact on the proper regulation of *ABCA1* expression and the severity of atherosclerosis [31]. Consistent with these findings, a transcriptional analysis by *Suresh* et al. [32] revealed that *ABCA1* was downregulated in the transcriptome of acute myocardial infarction (AMI) patients who developed recurrent events. Their results showed that changes in the cholesterol transport pathway during the initial presentation of AMI are associated with increased disease severity. However, to better understand the mechanism by which these identified SNPs may influence *ABCA1* expression, it will be necessary to reconstitute them in vitro and evaluate their functionality at basal levels and in response to various regulatory stimuli.

The β-klotho (*KLB*) gene encodes a transmembrane protein that acts as a cofactor to form complexes with fibroblast growth factor receptors (FGFR), resulting in a high-affinity receptor for the endocrine hormones FGF21 and FGF19. FGF21, produced mainly in the liver and adipose tissue, has been described to exert various metabolic effects on glucose and lipid metabolism in an endocrine manner by interacting with the FGFR1-KLB complex. Although cardiac secretion of FGF21 is lower than hepatic secretion, cardiac cells secrete abundant FGF21 in response to cardiac stress. The presence of FGFR1c, β-klotho, and FGF21 in the heart suggests that in cardiomyocytes, FGF21 may participate in cardioprotection in a paracrine manner by activating MAPK signaling through FGFR1c activation with β-klotho as cofactor [36]. Interestingly, a recent study showed that FGF21 exerts an anticoagulant effect by inhibiting Factor VII (FVII) expression and activity, and an antiplatelet effect by inhibiting platelet activation and improving fibrinolysis [37]. On the other hand, several investigations have revealed that FGF19 plays a key role in inhibiting de novo bile acid (BA) synthesis in the liver. Through the FGFR4-KLB complex, FGF19 potently suppresses cholesterol 7a-hydroxylase (CYP7A1) mRNA levels (Fig. 4). Indeed, Fgf15, Fgfr4, and Klb knockout animals share a dysregulated BA metabolism, whereas exogenous FGF19 administration fails to suppress *CYP7A1* in Fgfr4 and Klb knockout animals [38]. Several studies have reported *KLB* variants associated with disease or phenotypic outcomes and analyzed their effects at the protein level. For example, *Panera* et al. [23] demonstrated that the rs17618244 G>A missense variant (Arg728Gln) leads to decreased *KLB* expression in hepatocytes (plasma KLB levels were lower in patients carrying the minor *A* allele), resulting in increased intracellular lipid accumulation, whereas the ancestral *G* allele exerts a protective effect against lipid overload, lipotoxicity, and inflammation. In our study, carriers of the *G* allele of rs17618244 had a higher rate of MACEs during 12-month follow-up than non-carriers. Consistent with these results, some functional analyses have shown that the *KLB* Arg728 variant (*G* allele) reduces protein stability compared to *KLB* Gln728 (*A* allele), which could weaken FGF19 binding and signaling. Weakened FGF19 signaling leads to increased *CYP7A1* expression and consequently to increased hepatic BA synthesis [39]. *Desai* et al. [40] reported that abnormally elevated BAs levels reduce fatty acid oxidation in cardiomyocytes and can cause cardiac dysfunction and cardiomyopathy in mice, suggesting cardiotoxicity of these BAs. This correlates with recent studies confirming that AMI is inextricably linked to cholesterol metabolism regulated by BAs [41]. All this suggests that the reduced KLB stability caused by the *KLB* Arg728 variant (*G* allele) may similarly weaken FGF21 binding and signaling, thereby increasing serum FGF levels associated with established CV risk factors and severity of coronary artery disease [42]. For example, in the context of metabolic dysregulation, elevated serum FGF21 levels correlate with several cardiac pathologies and are used as prognostic indicators of cardiac dysfunction [36]. Combining all this evidence, we suggest that KLB disruption may lead to cardiac dysfunction, which may explain the mechanism underlying MACEs. However, the effect of this missense variant on KLB protein function and its impact on the pathways discussed requires further functional analysis.

ZNF335 encodes a zinc finger protein that regulates gene transcription by recruiting histone methyltransferase complexes and coactivators or directly binding to gene promoters. *ZNF335* is widely expressed in human tissues and has multiple functions, including roles in neuronal development and T-cell maturation. Recently, it has been suggested that reduced Zfp335 function in mice results in decreased plasma cholesterol levels and attenuated low-density lipoprotein (LDL) response to statins. However, further investigation is needed to determine which genes may be transcriptionally regulated by *ZNF335* and the mechanism by which *ZNF335* affects plasma cholesterol phenotypes [43]. In our study, carriers of the *C* allele of the rs3827066 variant experienced more MACEs during the 12-month follow-up than non-carriers. The SNP is located within an intron of the *ZNF335* gene, but the surrounding 100 kb region contains other genes such as *PLTP*, *PCIF1*, *NEURL2*, and *MMP9* [44]. Thus, it is possible that this SNP influences phenotypic variation through altered gene expression. Previous research by *LeBlanc* et al. [45] and *Jones* et al. [46] has shown that the rs3827066 genetic variant affects *PLTP* (phospholipid transfer protein) gene expression (eQTL effects in adipose and aortic tissues), which is known to play an important role in cholesterol uptake from peripheral cells and tissues, and transport to the liver for degradation and excretion (cholesterol efflux). PLTP transfers phospholipids from triglyceride-rich lipoproteins to HDL (Fig. 4). This is consistent with previous work in mice showing that *Pltp* overexpression results in higher HDL cholesterol, whereas targeted deletion results in lower HDL cholesterol [47]. Similarly, other authors have found that induced *Pltp* overexpression in mice results in an increase in atherogenic lipid profile, atherosclerotic lesion area, and plaque instability. However, there is no literature suggesting a possible association between the *ZNF335* gene and ACS. While the rs3827066 locus (near *PCIF1/MMP9/ZNF335*) has been reported as a potential abdominal aortic aneurysm-specific risk locus not associated with other CV disorders [46], a recent GWAS by *Van der Harst* et al. [24] identified this SNP as a novel CAD locus (OR = 1.04, $p = 4.4 \times 10^{-9}$). Thus, evidence suggests that this locus may significantly influence gene expression and, in combination with *PLTP*, likely exerts its pathogenic effects by altering lipid metabolism and consequently vascular wall inflammation and extracellular matrix composition, similar to the *SORT1* locus [44].

To summarize, the SNPs associated with MACEs in our population are primarily related to lipid metabolism, a significant risk factor for developing ACS. Specifically, they contribute to the reduction of HDL levels, a key player in the protection against atherothrombosis by intervening in several regulatory mechanisms, such as cholesterol efflux capacity, antioxidant, antithrombotic and anti-inflammatory activity. In addition, HDL has been recognized to play a role in increasing platelet reactivity after discontinuation of $P2Y_{12}$ receptor antagonists. In particular, the rebound phenomenon after discontinuing clopidogrel in patients receiving DAPT has been associated with altered lipid profile. Loss of $P2Y_{12}$ receptor blockade may represent a significant prothrombotic and proinflammatory impulse, particularly in patients at increased CV risk, such as those with low HDL. However, the relationships between HDL levels, platelet aggregability and associated MACEs are complex and remain to be elucidated [48,49]. According to *Đukanović* et al. [48] low HDL levels may indirectly contribute to increased platelet aggregability by increasing the uptake of oxidized LDL (oxLDL) in inflammatory adipocytes through the PPARγ/CD36 pathway. Thus, in HDL deficiency, more oxLDL reaches platelets, so the mechanism described increases their activation. On the other hand, highly oxidized HDL appears to exert a dose-dependent prothrombotic and proinflammatory effect through the CD36 receptor, and blocking its binding to CD36 attenuates platelet stimulation. These hypotheses are supported by the fact that patients with lower HDL levels show greater changes in inflammatory markers released by activated platelets after discontinuation of clopidogrel and cessation of its antithrombotic effect. Indeed, the bidirectional interaction between inflammation and lipids is considered a hallmark of atherosclerosis, and further studies are needed

to clarify the role of the identified gene variants in determining susceptibility to MACEs and whether this is due to gene overlap between these two interacting pathophysiological arms of atherogenesis [45].

The main limitation of our study was the sample size, which is a common challenge in PGx research. It can be difficult to enroll enough participants to achieve the statistical power needed to detect associations. PGx studies are often limited to people with a given condition, only a small fraction of which take take the drug of interest, and an even smaller fraction of which could develop a given ADR [50]. In fact, a review of 23 PGx GWAS conducted between 2007 and 2010 [51] showed that drug response GWAS had an average of 570 participants and ADR GWAS had a case/control ratio of 70/120. Estimates for 2015 to 2020 showed that these numbers are increasing, as conducting PGx studies in the context of randomized clinical trials or international consortia (e.g., PGRN-RIKEN), facilitates recruitment of large cohorts and replication of results [50,52].

Another limitation was panel size and, ultimately, the number of variants identified. However, an advantage of our targeted panel for clinical practice is that it not only focuses on cardiac and metabolic genes with evidence, rather than the entire exome, but also extends 300 bp upstream and downstream of positions of interest. This customized design allows coding regions to be analyzed as well as non-coding variants that may be relevant but unnoticed by WES. The fact that 96.4 % of the associated genetic polymorphisms identified in PGx GWAS reside in non-coding regions underscores the importance of regulatory variants in drug response. However, regulatory variants are rarely included in PGx studies, despite the knowledge that regulatory elements have far-reaching structural and functional effects on genomic regions or downstream products of the gene(s) they regulate [53].

Finally, we conducted a retrospective observational study focused only on the study of genetic variants not previously described to be associated with the occurrence of MACEs in ACS patients undergoing PCI, and did not include complementary approaches to assess the effects of the identified variants. The inclusion in our study of assays measuring plasmatic factors of endothelial activation/inflammation, lipid levels and/or platelet reactivity would have been useful to confirm the hypotheses generated from the sequencing results. However, our initial hypothesis was based on the possibility that variants potentially associated with MACEs occurrence were related to the metabolism of prescribed antiplatelet drugs or predisposition to structural cardiovascular disease. In fact, this hypothesis guided the design of the personalized gene panel used for the analysis proposed in this work. Nevertheless, preliminary results showed that the variants with the highest trend to significance are involved in alterations of lipid metabolism, suggesting a more relevant role of this process in the occurrence of MACEs. Therefore, the findings of the current study highlight the need for future research that complements genetic analysis with measurements of blood lipid levels. Thus, the integration of these parameters would provide us with a more complete picture of the relationships between genetic variants and the risk of MACEs and would help us to clarify the true role of the identified variants in lipid metabolism.

5. Conclusion

We present the first customized gene panel to perform a large-scale association analysis of both PGx and CV disease-related variants in ACS patients treated with DAPT after PCI. We have identified potential SNPs, genes, and biological pathways associated with MACEs risk, and found that these genetic variants may affect HDL levels or, as regulatory variants, may alter the expression of nearby lipid metabolism-related genes. These results should be interpreted with caution given the limitations of the study, and further research is needed to elucidate the possible mechanisms of these candidate genes in individual MACE susceptibility.

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CRediT authorship contribution statement

Alba Antúnez-Rodríguez: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sonia García-Rodríguez:** Writing – review & editing, Methodology, Data curation. **Ana Pozo-Agundo:** Writing – review & editing, Data curation. **Jesús Gabriel Sánchez-Ramos:** Validation, Methodology. **Eduardo Moreno-Escobar:** Validation, Methodology. **José Matías Triviño-Juárez:** Writing – review & editing, Validation, Methodology. **Luis Javier Martínez-González:** Writing – review & editing, Validation, Supervision, Conceptualization. **Cristina Lucía Dávila-Fajardo:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2024.109060.

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