

# Impact of Insulin Receptor Substrate-1 Genotypes on Platelet Reactivity and Cardiovascular Outcomes in Patients With Type 2 Diabetes Mellitus and Coronary Artery Disease

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## Objectives

The aim of this study was to assess the association between genetic variants of the insulin receptor substrate (IRS)-1 gene, platelet function, and long-term outcomes in patients with type 2 diabetes mellitus (DM) and stable coronary artery disease while on aspirin and clopidogrel therapy.

## Background

The effects of pharmacogenetic determinants on platelet function and cardiovascular outcomes in type DM patients are unknown.

## Methods

The association between IRS-1 genetic variants, platelet function, and the risk of major adverse cardiac events (MACE) at 2 years was assessed in 187 patients with type 2 DM and stable coronary artery disease on maintenance aspirin and clopidogrel therapy.

## Results

Seven tag single nucleotide polymorphisms were selected. Individuals with high platelet reactivity were more frequent among carriers of the C allele (GC and CC genotypes; approximately 20% of population) of the rs956115 marker (44.4% vs. 20.5%; odds ratio: 3.1, 95% confidence interval [CI]: 1.44 to 6.67;  $p = 0.006$ ). These patients were at higher risk of MACE (28.0% vs. 10.9%; hazard ratio: 2.90, 95% CI: 1.38 to 6.11;  $p = 0.005$ ). The C allele carriers of the rs956115 marker were more commonly associated with a hyperreactive platelet phenotype. This was confirmed in an external validation cohort of patients with type 2 DM but not in an external validation cohort of patients without DM. Carriers of the C allele of the rs956115 marker also had a significantly higher risk of MACE compared with non-carriers (30.6% vs. 11.4%; hazard ratio: 2.88, 95% CI: 1.35 to 6.14;  $p = 0.006$ ).

## Conclusions

Type 2 DM patients who are carriers of the C allele of the rs956115 marker of the IRS-1 gene have a hyperreactive platelet phenotype and increased risk of MACE. (J Am Coll Cardiol 2011;58:30-9) © 2011 by the American College of Cardiology Foundation

Dual antiplatelet therapy with aspirin and clopidogrel is the recommended treatment for patients with acute coronary syndromes (ACS) and in those undergoing percutaneous

coronary interventions (PCI) (1,2). Numerous investigations have shown a broad variability in interindividual response to antiplatelet therapy, and patients with high

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on-treatment platelet reactivity (HPR) have an increased risk of ischemic events (3,4). Patients with diabetes mellitus (DM) have a greater prevalence of HPR compared with non-DM, which might explain their overall enhanced risk of developing atherothrombotic complications (5–8). However, heterogeneous antiplatelet drug effects might also be observed among patients with DM, and platelet function profiling even within this high-risk cohort identifies subjects at a greater risk of recurrent ischemic events (9). The mechanisms leading to variable antiplatelet drug response profiles in patients with DM are not fully elucidated. Although there is growing evidence that single nucleotide polymorphisms (SNPs) might modulate antiplatelet drug effects (10), whether these might explain the heterogeneity in response profiles and clinical outcomes selectively in patients with DM remain unexplored.

Human platelets are targets of insulin effects that are mediated by the insulin receptor substrate (IRS)-1 (8,11). In healthy volunteers, insulin interferes with calcium increases induced by adenosine diphosphate (ADP)-P2Y<sub>1</sub> contact through G<sub>i</sub> activity and, thereby, with P2Y<sub>12</sub>-mediated suppression of cyclic adenosine monophosphate (cAMP) (11). However, platelets from patients with type 2 DM have lost responsiveness to insulin, leading to increased P2Y<sub>12</sub>-mediated suppression of cAMP and decreased antiplatelet drug effects (12). Importantly, studies performed in subjects without DM or in a pre-DM status have shown that gene sequence variations of IRS-1 are associated with the functional activity of this receptor (13) as well as being a risk factor for coronary artery disease (14). However, whether IRS-1 genotypes are associated with variations in antiplatelet drug response profiles and whether these might impact clinical outcomes in patients with DM is unknown. To address this issue we evaluated whether IRS-1 genotypes were associated with platelet function profiles and cardiovascular outcomes.

## Methods

**Study population.** Blood samples for platelet function analyses and genotyping were collected from a total of 208 medically treated (with oral hypoglycemic agents and/or insulin) patients with type 2 DM and stable coronary artery disease from November 2003 to March 2007. To avoid stratification of the sample due to ethnicity, only Caucasian patients homogeneous for ethnic background were included. All patients (primary cohort as well as 2 external validation cohorts) were from the central regions of Spain. To be eligible, patients with type 2 DM (>18 years of age) needed to have undergone PCI and been receiving aspirin and clopidogrel therapy for 6 to 9 months in the absence of cardiovascular events during this period. Type 2 DM was defined according to the World Health Organization Report (15). All patients were recruited from the outpatient clinic of our hospital as part of their routine follow-up after PCI. Aspirin (100 mg/day) was used indefinitely, and

clopidogrel (75 mg/day) was prescribed for 12 months after coronary revascularization. Blood sampling was not performed if 1 of the following exclusionary criteria was present: 1) use of antiplatelet agents other than aspirin and clopidogrel; 2) use of oral anticoagulants; 3) occurrence of an acute cardiovascular event during the interval between PCI and blood sampling; 4) impaired glucose tolerance without pharmacologic treatment, gestational diabetes, or transient hyperglycemia; 5) platelet count <125,000/mm<sup>3</sup>; 6) hematocrit <25%; 7) creatinine levels >2.5 mg/dl; or 8) hepatic enzymes (alanine aminotransferase or aspartate aminotransferase) twice the upper normal limit.

Patients meeting study eligibility criteria were followed for 24 months, and clinical events were recorded. Follow-up was performed by means of telephone contacts every 6 months and clinic visits on a yearly basis. Patients with nonvaluable pharmacodynamic assessments were excluded from the final analysis. The primary outcome measure was a composite of cardiovascular death, ACS leading to hospital stay, and nonfatal stroke. Such major adverse cardiovascular events (MACE) were defined according to definitions proposed by the American College of Cardiology (see the Online Appendix for complete description) (16). The treating physicians and investigators who adjudicated the clinical endpoints were blinded to the results of the pharmacodynamic and genotype assessments.

After our initial investigation to define the prevalence and functional impact of IRS-1 genotypes in our main study cohort, validation assessments were performed to replicate the pharmacodynamic findings associated with the carrier status of the C allele of the rs956115 marker, which emerged from the main cohort to be associated with a hyperreactive platelet phenotype. In particular, a separate external cohort of patients with type 2 DM (n = 52) undergoing elective PCI was identified to confirm the pharmacodynamic impact of this marker in the acute phase of clopidogrel therapy. All patients were taking aspirin therapy and received a 600-mg loading dose of clopidogrel at the time of intervention; pharmacodynamic assessments were performed at hospital discharge. Furthermore, to determine whether or not the pharmacogenetic findings were specific to patients with DM, a pharmacodynamic assessment was also extended to a cohort of patients without DM (n = 90). Similarly, to study subjects from the main

## Abbreviations and Acronyms

<b>ACS</b>	= acute coronary syndrome
<b>ADP</b>	= adenosine diphosphate
<b>CI</b>	= confidence interval
<b>CYP</b>	= cytochrome P450
<b>DM</b>	= diabetes mellitus
<b>HbA1C</b>	= hemoglobin A1C
<b>HPR</b>	= high platelet reactivity
<b>HR</b>	= hazard ratio
<b>IRS</b>	= insulin receptor substrate
<b>LD</b>	= linkage disequilibrium
<b>MACE</b>	= major adverse cardiovascular event(s)
<b>OR</b>	= odds ratio
<b>PCI</b>	= percutaneous coronary intervention
<b>ROC</b>	= receiver-operator characteristic
<b>SNP</b>	= single nucleotide polymorphism

cohort, these patients were in their steady state phase of the same doses of aspirin and clopidogrel therapy. A flow diagram of the main study cohort and separate external validation cohorts is provided in the Online Appendix (Online Fig. 1).

The study complied with the Declaration of Helsinki and was approved by the Ethical Committee of the San Carlos University Hospital, and all patients gave their informed consent.

**Pharmacodynamic assessments.** Pharmacodynamic effects were assessed with light transmittance aggregometry according to standard protocols, as previously described (see the Online Appendix for complete description) (5,6,9). Maximum platelet aggregation was measured after stimuli with ADP (20  $\mu$ mol/l) to assess purinergic mediated platelet function. High platelet reactivity was defined as the upper quartile of ADP-induced aggregation, as previously described (9,17,18). To define nonpurinergic mediated platelet function, platelet aggregation after collagen (6  $\mu$ g/ml) stimuli was also performed (6,9).

**Genotyping and haplotype association analyses.** Genomic deoxyribonucleic acid was extracted from peripheral-blood leucocytes with standard salting-out procedures. The selection of the tag SNPs of IRS-1 gene was performed with GEVALT 2.0 software (GEnotype Visualization and ALgorithmic Tool) (19–21). The SNP genotype data for Utah residents with ancestry from northern and western Europe (CEU) population were downloaded from HapMap Project Browser, submitting a 100-kilobase pair region as a query (chr2: 227,290,450..227,390,449; release April 2007). Because rs1801278 has been extensively investigated in the published data, this was force-included in the list of tag SNPs identified (13,14). The 7 tag SNPs gave an estimated prediction value of 97.6% for the IRS-1 genomic region investigated. Genotyping was performed with FRET (Fluorescent Resonance Energy Transfer) Probes technology on the LightCycler 2.0 instrument (Roche Diagnostics, Basel, Switzerland) and TaqMan SNP Genotyping assays on and Applied Biosystems StepOnePlus instrument (Applied Biosystems, Foster City, California) (see the Online Appendix for complete description). Lewontin's  $D'$  and the square of correlation coefficient  $r$  between 2 markers were used as a measure of linkage disequilibrium (LD) of all marker pairs (22). The LD haplotype block structure was identified with GEVALT 2.0 according to the gerbil algorithm (20). Haplotype association analyses were performed on the entire haplotype region (i.e., including the 7 tag SNPs) and on subregions defined by LD blocks (23).

**Statistical analysis.** Continuous variables were analyzed for a normal distribution with the Kolmogorov-Smirnov test and presented as mean  $\pm$  SD or median and interquartile range, as appropriate. Normally distributed variables were analyzed with Student  $t$  tests, whereas the Mann-Whitney  $U$  test was used for comparisons of non-normally distributed variables. Categorical variables are presented as frequencies and percentages and were compared with the

Table 1

Baseline Demographic Data and Clinical Characteristics According to Post-Treatment Platelet Reactivity Status

Variable	HPR (n = 47)	No HPR (n = 140)	p Value
Age (yrs)	64 $\pm$ 10	67 $\pm$ 10	0.15
Male	31 (66)	101 (72)	0.54
Risk factors/past medical history			
Insulin-treated diabetes	20 (43)	41 (29)	0.13
HbA1C	7.4 $\pm$ 1.3	7.1 $\pm$ 1.1	0.18
Smoking	9 (19)	19 (14)	0.49
Hyperlipidemia	30 (64)	106 (76)	0.16
Hypertension	31 (66)	91 (65)	0.95
Body mass index (kg/m <sup>2</sup> )	28.3 $\pm$ 4.1	28.8 $\pm$ 3.7	0.44
Obesity (body mass index $\geq$ 30 kg/m <sup>2</sup> )	19 (40)	52 (37)	0.82
Prior cerebrovascular event	0	6 (4)	0.34
Peripheral vascular disease	4 (9)	16 (11)	0.79
Chronic renal dysfunction	10 (21)	37 (26)	0.61
Left ventricular dysfunction	7 (15)	27 (19)	0.65
Prior myocardial infarction	31 (66)	75 (54)	0.19
Prior CABG	1 (2)	3 (2)	1.00
Multivessel CAD	34 (72)	99 (71)	0.98
Treatment*			
Beta-blockers	29 (62)	105 (75)	0.12
Calcium-channel blockers	9 (19)	40 (29)	0.28
Nitrates	21 (45)	59 (42)	0.89
ACE inhibitors/ARB	24 (51)	78 (56)	0.70
Statins	39 (83)	110 (79)	0.92
CYP3A4-metabolizing statin	32 (82)	95 (86)	0.88
Non-CYP3A4-metabolizing statin	7 (18)	15 (14)	0.61
Proton pump inhibitors	39 (83)	108 (77)	0.52

Values are n (%) or mean  $\pm$  SD. \*Aspirin and clopidogrel were used in all (100%) patients. There were no differences in length of clopidogrel therapy between high on-treatment platelet reactivity (HPR) and non-HPR groups (total duration: 12 months; post platelet function assessment: 4.78  $\pm$  1.49 months vs. 4.63  $\pm$  1.50 months,  $p$  = 0.53). Among noninsulin-treated diabetic subjects, there were no differences in type of oral hypoglycemic agents used between HPR and non-HPR groups.

ACE = angiotensin-converting enzyme; ARB = angiotensin II receptor blockers; CABG = coronary artery bypass grafting; CAD = coronary artery disease; CYP = cytochrome P450; HbA1C = hemoglobin A1C.

use of chi-square test or Fisher exact test where appropriate. Receiver-operator characteristic (ROC) analyses were performed for an exploratory evaluation of the optimal cutoff value of ADP- and/or collagen-induced platelet aggregation for predicting MACE in our study population (9,24). Spearman's rank correlation was used to examine the correlation between profiles of platelet reactivity and glycemic control, defined by hemoglobin A1C (HbA1C) levels. Interaction among genotype, insulin resistance (defined by homeostatic model assessment; see Online Appendix for description), and HPR was also determined. Rates of the primary endpoint are expressed as Kaplan-Meier estimates at 24 months and compared with log rank testing. Univariable and multivariable Cox proportional hazards regression models were used to assess unadjusted and adjusted risk of the combined cardiovascular endpoint associated with HPR and genotype. Demographic, clinical, and laboratory variables provided in Table 1 were entered in the Cox model for the multivariable analysis, and those that were not significant at  $p$  < 0.10 were removed by a backward stepwise

elimination. With this method, chronic renal insufficiency was identified as the only significant predictor associated with the primary endpoint. The HPR and genotype were added as independent categorical variables in the model, including chronic renal insufficiency as variable for statistical adjustment. The assumption of proportional hazard was checked with time-dependent covariates and was found to be reasonable. First order interactions were evaluated. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated. Odds ratios (ORs) are provided for the association between ADP and/or collagen-induced aggregation above the upper quartile with the genotype. A  $p$  value  $<0.05$  was considered statistically significant for all the tests mentioned in the preceding text. Statistical analysis was performed with SPSS software (version 14.0, SPSS, Inc., Chicago, Illinois).

Hardy-Weinberg equilibrium was evaluated for each tag SNP, and markers were rejected if they violated Hardy-Weinberg equilibrium with a threshold of  $p < 0.01$ . Bonferroni correction was applied to adjust the nominal significance level of the association test of HPR status with each of the 7 tag SNPs ( $p = 0.05/7 = 0.007$ ). A multivariable logistic regression analysis, including the genotype along with all the covariates that might impact the degree of platelet aggregation, was performed to assess the adjusted OR for ADP and/or collagen-induced aggregation above the upper quartile, associated with the genotype. The ADP and/or collagen-induced aggregation above the upper quartile were treated as a dependent variable, and age, sex, body mass index, diabetes status (insulin- or noninsulin-treated), hyperlipidemia, hypertension, smoking, HbA1C, renal insufficiency, and concomitant medications were included into the statistical model as covariates. All probability values reported are 2-sided, and a value of  $p < 0.05$  was considered to be significant. The SNPs showing significant associations ( $p < 0.05$ ) were then tested for recessive or dominant model (i.e., grouping the heterozygotes together with homozygotes for the major allele or for the minor allele, according with the model).

Generalized linear models were used to assess haplotype associations while adjusting for the effects of nongenetic cofactors. The null hypothesis of no haplotype effects was tested by standard methods that compare the deviances of the model including or not including genetic data (global test). The significance of the effect of each individual haplotype was also tested (individual haplotype test). Association of individual haplotypes was considered significant when both  $p$  values of global and at least 1 of the individual haplotype tests were below a threshold value ( $p < 0.05$ ). The effect of each haplotype was assumed to be additive (i.e., a linear increase in log-odds). Haplotypes whose estimated frequency was  $<0.01$  were grouped into “rare” haplotypes and then treated as a single haplotype. Haplotype association analyses were computed with the R software (R Foundation for Statistical Computing, Vienna, Austria) and the library “haplo.stats” (25).

We estimated that this study has enough statistical power ( $\beta = 0.80$ ) to detect an association between HPR (defined as ADP-induced platelet aggregation  $\geq 64\%$ ) and an SNP at the significance level of 0.05 under the hypothesis that the risk allele has a frequency of 10% and is in absolute LD ( $D' = 1$ ) with the causative variant and that the OR of the carrier of the risk allele versus noncarrier is  $\geq 2.8$ . The number of patients determined to be included in the validation samples was approximately one-third of the number of patients included in the main cohort, as previously established (26).

## Results

**Characteristics of the patients and platelet reactivity.** Of the 208 patients enrolled in the main cohort of the present study, pharmacodynamic and genotype assessments were both available in 187 (89.9%), who were therefore considered for the present analysis. The remaining 21 patients (10.1%) were excluded, due to inability to measure platelet aggregation for reasons including hemolysis, low platelet-rich-plasma platelet counts ( $<150,000/\mu\text{l}$ ), and instability of tracings. In the overall study population, ADP-induced platelet aggregation was  $55 \pm 15\%$  and followed a normal bell-shaped distribution indicative of a heterogeneous response profile. The ADP-induced platelet aggregation quartile cut points for the 25th, 50th, and 75th percentiles of the study population were 45.0%, 55.0%, and 64.0%. The HPR was defined as ADP-induced platelet aggregation  $\geq 64\%$ . Baseline demographic data and clinical characteristics of patients with ( $n = 47$ ) and without ( $n = 140$ ) HPR are provided in Table 1. Insulin-treated diabetic subjects were more frequent in the HPR group, although no statistically significant differences were found. Also, there were no significant differences between groups for all other variables. In the overall population, collagen-induced aggregation was  $45 \pm 19\%$ . Quartile cut points for the 25th, 50th, and 75th percentiles were 33.0%, 46.0%, and 59.0%, respectively. Collagen-induced aggregation was  $58 \pm 15\%$  versus  $41 \pm 18\%$  in patients with and without HPR defined with ADP stimuli, respectively ( $p < 0.0001$ ). Among patients with HPR, 55.3% had collagen-induced aggregation above the 75th percentile.

**IRS-1 genotypes and platelet reactivity.** Seven tag SNPs (rs11683087, rs2251692, rs1801278, rs1801123, rs6725330, rs1896832, rs956115) with an estimated prediction value of 97.6% were selected. None of these 7 SNPs showed deviation from Hardy-Weinberg equilibrium, and their frequencies were similar to those reported in the Utah residents with ancestry from northern and western Europe population. Table 2 summarizes marker information and the observed genotype frequencies for the 7 tag SNPs assessed. Of the 7 tag SNPs, only the rs956115 marker showed a significant association with HPR. Individuals with HPR were more frequent among carriers of the C allele (GC and CC genotypes) of the rs956115 marker (44.4% vs.



**Table 2** Tag SNPs From the IRS-1 Gene Region and Genotype Frequency

Tag SNP	Position	Alleles	Genotypes		
		Major (M)/Minor (m)	MM	Mm	mm
rs11683087	227294850	A/G	145 (77.5)	41 (21.9)	1 (0.5)
rs2251692	227298024	G/A	129 (69.0)	53 (28.3)	5 (2.7)
rs1801278	227368788	G/A	109 (58.3)	59 (31.5)	19 (10.2)
rs1801123	227369287	A/G	153 (81.8)	31 (16.6)	3 (1.6)
rs6725330	227375101	A/G	147 (78.6)	37 (19.8)	3 (1.6)
rs1896832	227380730	A/G	164 (87.7)	21 (11.2)	2 (1.1)
rs956115	227382808	G/C	151 (80.7)	33 (17.6)	3 (1.6)

Values are n (%), unless otherwise indicated. Position = position (nucleotides) on chromosome 2 according to single nucleotide polymorphism database (dbSNP) (Map to Genome Build:36.3).

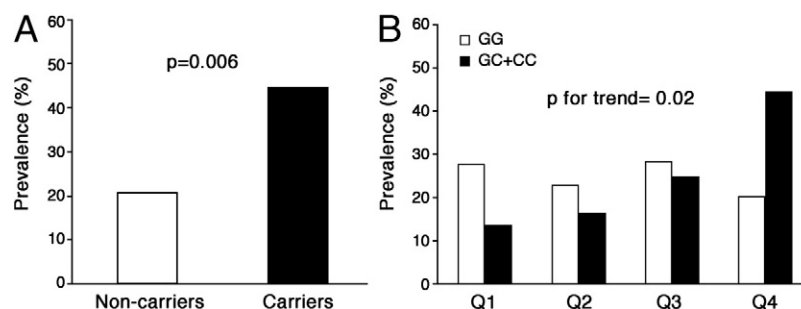
IRS = insulin receptor substrate; MM = homozygote for major (most frequent) allele M; Mm = heterozygote; mm = homozygote for minor allele m.

20.5%; OR 3.1, 95% CI: 1.44 to 6.67;  $p = 0.006$ ) (Fig. 1A), which remained statistically significant when applying the Bonferroni correction ( $p < 0.007$  required for significance). This association was also confirmed in an adjusted regression analysis (adjusted OR: 3.56, 95% CI: 1.51 to 8.38;  $p = 0.004$ ). The prevalence of C allele carriers of the rs956115 marker increased across quartile distribution of ADP-induced aggregation (Fig. 1B). There was no correlation between ADP- ( $r = 0.092$ ,  $p = 0.210$ ) and collagen-induced ( $r = 0.094$ ,  $p = 0.200$ ) platelet reactivity and HbA1C levels. Furthermore, there was no interaction between C carrier status of the rs956115 marker and insulin resistance in determining HPR ( $p$  for interaction = 0.419). Proportions of patients with HPR after collagen and ADP- and collagen-induced stimuli are reported in Table 3.

In the external validation cohort of patients with type 2 DM, C allele carriers of the rs956115 marker represented 19.2% of the patient population. These patients were treated with a 600-mg loading dose of clopidogrel, resulting in more suppressed platelet reactivity compared with patients from the main cohort who were receiving maintenance therapy. The ADP- and collagen-induced platelet

aggregation values after clopidogrel loading dose administration were  $30.7 \pm 29.5\%$  and  $25.9 \pm 24.2\%$ , respectively. Proportions of patients with HPR after ADP, collagen, and both ADP- and collagen-induced stimuli were significantly greater among carriers of the C allele of the rs956115 marker (Table 3). In the external validation cohort of patients without DM in their steady state phase of dual antiplatelet therapy, C allele carriers of the rs956115 marker represented 31.1% of the patient population. The ADP- and collagen-induced platelet aggregation were  $53.2 \pm 16.5\%$  and  $41.0 \pm 19.7\%$ , respectively. Although there was a greater prevalence of patients with HPR among patient who were carriers of the C allele of the rs956115 marker, this was not statistically significant (Table 3).

**IRS-1 haplotypes and platelet reactivity.** The IRS-1 gene haplotypes were inferred from the 7 tag SNPs (Online Table 7). Haplotype association tests were conducted on the primary sample (208 individuals) in which there was no significant association between any of these inferred haplotypes and the phenotypes investigated in this study (data not shown). Three haplotype LD blocks were identified in the genotyped sample for the IRS-1 gene region: block-1 (rs11683087-rs2251692), block-2 (rs1801278-rs1801123-rs6725330), and block-3 (rs1896832-rs956115) (see Online Table 8 for frequencies, Online Fig. 2 for pairwise LD structure, and Online Table 9 for LD measures). Haplotype LD block analyses showed no significant associations for block-1 or block-2 with any of the phenotypes investigated. There was a significant association between haplotypes of LD block-3 (rs1896832-rs956115) and HPR (global  $p = 0.036$ ); a trend was observed with ADP- and collagen-induced aggregation above the upper quartiles (global  $p = 0.09$ ). Haplotype rs1896832-A/rs956115-C showed a significant association with HPR (adjusted OR: 2.63; CI: 1.26 to 5.48;  $p = 0.01$ ) and ADP- and collagen-induced aggregation above the upper quartiles (adjusted OR: 2.36; CI: 1.02 to 5.47;  $p = 0.045$ ).



**Figure 1** Prevalence of High On-Treatment Platelet Reactivity According to rs956115 Genotypes of the IRS-1 Gene

Carriers of the C allele (GC and CC genotypes; solid bars) of the rs956115 genotype of the insulin receptor substrate-1 (IRS-1) gene have a greater prevalence of high on-treatment platelet reactivity compared with noncarriers (GG genotype; open bars) (A). The prevalence of carriers of the C allele increases across quartile (Q) distribution of platelet reactivity (B).

**Table 3** Prevalence of Patients With HPR Among Carriers and Noncarriers of the C Allele of the rs956115 Marker

Primary Cohort					
Patients With HPR	Total (n = 187)	Carriers (n = 36)	Noncarriers (n = 151)	p Value*	OR (95% CI)
After ADP	47 (25.1)	16 (44.4)	31 (20.5)	0.006	3.10 (1.44–6.67)
After collagen	47 (25.1)	14 (38.9)	33 (21.9)	0.058	2.28 (1.05–4.93)
After ADP and collagen	26 (13.9)	10 (27.8)	16 (10.6)	0.01	3.25 (1.33–7.93)

Validation Cohort of Patients With DM					
	Total (n = 52)	Carriers (n = 10)	Noncarriers (n = 42)	p Value*	
After ADP	4 (7.7)	3 (30.0)	1 (2.4)	0.02	17.6 (1.59–193.88)
After collagen	9 (17.3)	6 (60.0)	3 (7.1)	0.001	19.5 (3.47–109.57)
After ADP and collagen	2 (3.8)	2 (20.0)	0	0.03	—

Validation Cohort of Patients Without DM					
	Total (n = 90)	Carriers (n = 28)	Noncarriers (n = 62)	p Value*	
After ADP	23 (25.6)	10 (35.7)	13 (21.0)	0.22	2.09 (0.78–5.61)
After collagen	18 (20.0)	8 (28.6)	10 (16.1)	0.28	2.08 (0.72–6.02)
After ADP and collagen	14 (15.6)	6 (21.4)	8 (12.9)	0.35	1.84 (0.57–5.92)

Values are n (%). \*The p values are calculated with the use of chi-square test or Fisher exact test as appropriate.

ADP = adenosine-diphosphate; CI = confidence interval; DM = diabetes mellitus; HPR = high platelet reactivity; OR = odds ratio.

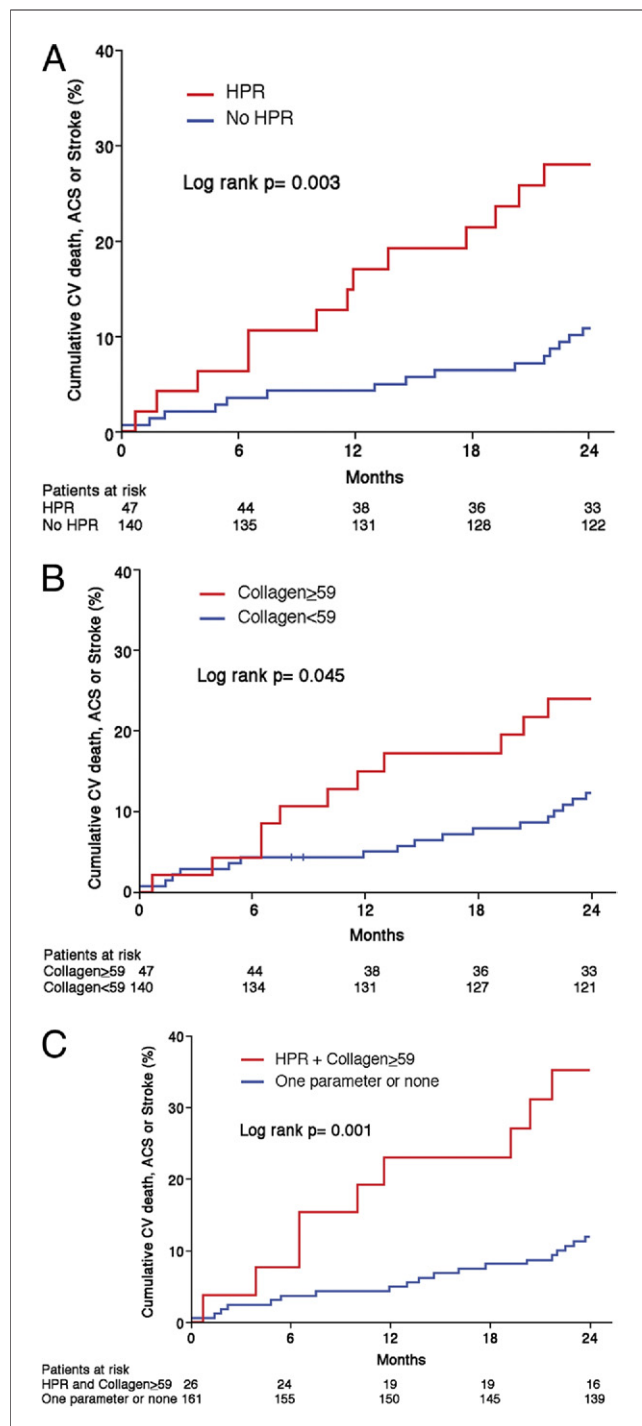
**Platelet reactivity and clinical outcomes.** Major adverse cardiovascular events occurred in a total of 28 patients (15%) during the 24-month follow-up period. Major adverse cardiovascular events were largely driven by ACS requiring hospital stay (n = 26; 93%); 2 patients experienced a cardiovascular death and a nonfatal ischemic stroke. There were a total of 4 noncardiac deaths. Patients with HPR were at significantly higher risk of MACE (28.0% vs. 10.9%; HR: 2.90, 95% CI: 1.38 to 6.11; p = 0.005) (Fig. 2). A significant association between HPR and MACE was confirmed in the multivariable analysis (adjusted HR: 3.10, 95% CI: 1.47 to 6.52; p = 0.003). In patients with HPR, a trend toward a higher risk of MACE was observed during clopidogrel treatment (6.4% vs. 2.1%; p = 0.16), whereas differences in outcomes were statistically significant after its withdrawal (26.8% vs. 9.8%; p = 0.01). A higher risk of MACE was seen in patients in the upper quartile of collagen-induced aggregation (23.9% vs. 12.3%; HR: 2.13, 95% CI: 1.00 to 4.55; p = 0.051; adjusted HR: 2.18, 95% CI: 1.02 to 4.67; p = 0.044) and in patients with both ADP- and collagen-induced aggregation above the upper quartiles (35.2% vs. 11.9%; HR: 3.49, 95% CI: 1.58 to 7.71; p = 0.002; adjusted HR: 3.71, 95% CI: 1.67 to 8.25; p = 0.001) (Fig. 2). The upper quartiles of ADP- and collagen-induced platelet aggregation were found to be the best predictors of MACE in the ROC analyses.

**IRS-1 genotypes and clinical outcomes.** Carriers of the C allele of the rs956115 marker had a significantly higher risk of MACE (30.6% vs. 11.4%; HR: 2.88, 95% CI: 1.35 to 6.14; p = 0.006) (Fig. 3). Carriers of the C allele had a nonsignificant increase in MACE while receiving clopidogrel treatment (5.6% vs. 2.6%; p = 0.39). Major adverse cardiovascular events increased over time after clopidogrel

withdrawal in C allele carriers (31.2% vs. 9.9%; p = 0.005) (see Online Fig. 3 for landmark analysis). In the multivariable analysis, the C allele of the rs956115 marker showed to be an independent predictor of MACE both in the model not including (adjusted HR: 3.11, 95% CI: 1.45 to 6.68; p = 0.004) and in that including HPR (adjusted HR: 2.31, 95% CI: 1.03 to 5.19; p = 0.04) as a covariate. There were no differences in baseline demographic data and clinical characteristics of patients with (n = 36) and without (n = 151) the C allele of the rs956115 marker (Online Table 10). There was no interaction according to insulin usage on HPR (p for interaction = 0.58) and MACE (p for interaction = 0.82) (Online Table 11). There was a significant association between haplotypes of LD block-3 (rs1896832-rs956115) and MACE (global p = 0.028). Haplotype rs1896832-A/rs956115-C showed a significant association with MACE (adjusted OR: 3.0, 95% CI: 1.36 to 6.7; p = 0.007).

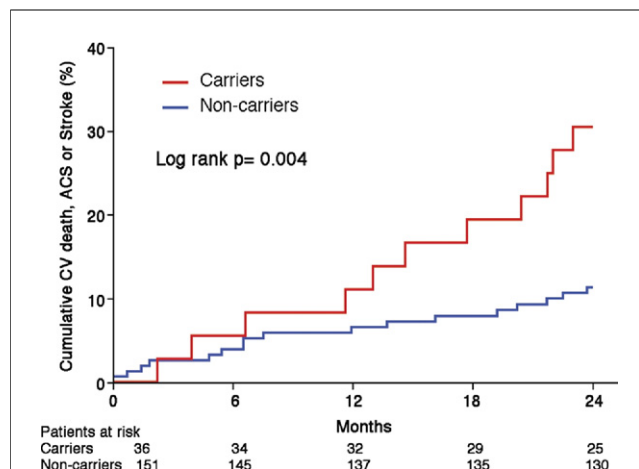
## Discussion

This is the first study to evaluate the impact of gene sequence variations on antiplatelet drug effects and clinical outcomes in patients with DM. In particular, the results of the present study demonstrate that, in patients with type 2 DM and stable coronary artery disease, gene sequence variations of IRS-1—namely C allele carriers of the rs956115 polymorphism (observed in approximately 20% of patients)—associate independently with a hyperreactive platelet phenotype and enhanced long-term cardiovascular risk. These findings not only provide further insights on pharmacogenetic modulation of antiplatelet drug effects but also provide a genetic explanation as to why variable clinical



**Figure 2** Association Between Profiles of Platelet Reactivity and Major Adverse Cardiac Events

Among 187 type 2 diabetic patients who were classified as having high on-treatment platelet reactivity (HPR) after adenosine diphosphate (ADP) (A), collagen (B), or both (C) stimuli, the rate of major adverse ischemic events (composite of cardiovascular [CV] death, acute coronary syndrome [ACS], or stroke) was greater compared with those without (ADP: 28.0% vs. 10.9%, hazard ratio [HR]: 2.90, 95% confidence interval [CI]: 1.38 to 6.11; collagen: 23.9% vs. 12.3%, HR: 2.13, 95% CI: 1.00 to 4.55; ADP+collagen: 35.2% vs. 11.9%, HR: 3.49, 95% CI: 1.58 to 7.71).



**Figure 3** Association Between rs956115 Genotype Status and Major Adverse Cardiac Events

Among 187 type 2 diabetic patients who were classified as carriers (GC and GG genotypes) or noncarriers (GG genotype) of the rs956115 genotype of the insulin receptor substrate-1 (IRS-1) gene, the rate of major adverse ischemic events (composite of CV death, ACS, or stroke) was 30.6% among carriers as compared with 11.4% among noncarriers (HR: 2.88, 95% CI: 1.35 to 6.14). Abbreviations as in Figure 2.

outcomes might occur within a population, such as those with DM homogeneous for baseline risk profile.

Variability in individual response to antiplatelet therapy is an emerging clinical entity (3,4). The mechanisms leading to antiplatelet drug response variability are not fully established and are likely multifactorial (3,4). Pharmacogenetics has recently emerged as a field that tries to explain this phenomenon (10). Recent findings have shown genetic targets modulating pharmacokinetic profiles of clopidogrel through its metabolism by the cytochrome P450 (CYP) enzymatic system to have a major role on its pharmacodynamic effects (27–32). This might explain why recent studies have shown that gene sequence variations of CYP2C19 are associated with an increased risk of adverse events in clopidogrel-treated patients (30–35). However, gene sequence variations of CYP2C19 contribute to only approximately 12% of the interindividual response profile to clopidogrel (32), and these findings cannot be extrapolated to patients with DM who have specific aberrations in their platelet function compared with patients without DM, leading to differences in pharmacodynamic profiles that are ultimately determinants of thrombotic mediated processes (5–8). In fact, in vitro and ex vivo studies have shown that reduced pharmacodynamic effects of antiplatelet agents in patients with DM are attributed to upregulation of platelet signaling pathways (5–9,12), suggesting the potential modulating role of genetic determinants of “downstream” (e.g., platelet membrane receptors) mediators of platelet reactivity. Although glycemic control is known to be associated with platelet reactivity through various mechanisms, including glycation of platelet surface proteins (36), in our study this was not observed—likely because patients in our study

had good glycemic control and limited variability in HbA1C levels, as also shown in prior investigations (5,6). The impact of downstream genetic determinants are supported by our study findings in which IRS-1 genotypes were associated with a hyperreactive platelet phenotype in patients with type 2 DM but not in those without this metabolic disorder. These pharmacodynamic effects were confirmed irrespective of whether patients were in the acute phase of treatment after a high loading dose regimen or in the maintenance phase of dual antiplatelet therapy. A high loading dose of clopidogrel in patients undergoing PCI leads to enhanced platelet inhibitory effects with a broader range of variability compared with patients in their long-term maintenance of standard dosing (3), as also shown in this study. More variable profiles of platelet reactivity enables better identification of whether there are specific factors associated with poor response. This might explain why the ORs of having a hyperreactive platelet phenotype among carriers of the C allele of the rs956115 marker were higher in the acute phase of therapy compared with the maintenance phase.

The present study further supports the prognostic implications associated with a hyperreactive platelet phenotype (3,4). Of note, the magnitude of effect on clinical outcomes (approximately 3-fold increase in MACE) observed in our study was of the same extent or even greater than that observed in recent CYP2C19 studies (31–35). It should be underscored that the latter were performed in ACS patients, many undergoing PCI, in whom most events occurred early and survival curves paralleled over time, suggesting a prognostic role of CYP2C19 gene variants for early but not late events. In contrast, in our analysis, we studied stable patients—in a period remote from when recurrent events most commonly occur (6 to 9 months after PCI)—and showed that survival curves diverge over time. Previous studies, performed primarily in subjects without DM or in pre-DM states, have shown functional polymorphisms of the IRS-1 gene to modulate insulin sensitivity (13) as well as to be a risk factor for coronary artery disease (14). In our study population of patients with type 2 DM, however, we did not find any interaction among the rs956115 marker, degree of insulin resistance, and platelet reactivity. Because the rs956115 polymorphism is located in the 5' region of the IRS-1 gene, this does not affect amino acid coding and does not directly affect protein function. Therefore, our findings might be due to a linkage with other SNPs in exons (resulting in functional polymorphism) or in regulatory regions (affecting the expression of IRS-1 gene). The complexity of intraplatelet signaling and the potential for interplay with other pathways that derives from IRS-1 suggest that, although levels of insulin sensitivity remain a contributor to platelet function profiles, many other mechanisms might be involved in determining a hyper-reactive platelet phenotype as a consequence of a dysfunction of IRS-1-mediated signaling. Because the rs956115 C allele was an independent predictor of clinical outcomes after

adjustment for potential confounders (with and without HPR as a covariate), other unknown reasons that are not entirely linked to HPR might be implied and warrant further investigation.

Although loss of responsiveness to insulin via IRS-1 has shown to be associated with upregulation of P2Y<sub>12</sub> signaling, it cannot be excluded that this might also affect other platelet signaling pathways, commonly upregulated in platelets from patients with DM (12,37). In fact, IRS-1 is a major tyrosine phosphorylated substrate for the insulin receptor acting as a multisite docking protein to several Src homology 2 domains containing proteins, such as the regulatory subunits of phosphatidylinositol 3-kinase (PI3K)—which are key in multiple platelet activation processes (13). This is in line with the fact that patients with HPR to ADP frequently have HPR to non-purinergic stimuli (i.e., collagen), indicative of an overall hyper-reactive platelet phenotype, which might also be a better predictor of adverse outcomes, as also suggested by this study (9,38,39). This might contribute to the elevated prevalence of reduced aspirin-induced antiplatelet effects when measured by cyclooxygenase-1 nonspecific assays in patients with DM (6,40–42). Furthermore, patients with DM presenting with a hyper-reactive platelet phenotype have been shown to have a marked increase in platelet reactivity after clopidogrel withdrawal (43). Overall, these findings might explain why our long-term survival curves continue to diverge over time, particularly while patients were only taking aspirin therapy. Whether prolonging clopidogrel therapy in patients defined to be at higher risk on the basis of our laboratory findings would have led to improved clinical outcomes cannot be extrapolated from this study. It might be hypothesized that patients who are type 2 DM carriers of the C allele of the IRS-1 rs956115 tag SNP, who our study demonstrated to have a hyper-reactive platelet phenotype and worse outcomes, might benefit from more potent antithrombotic regimens. These might include high-dose clopidogrel (44), triple therapy (aspirin, clopidogrel, and cilostazol) (45), or novel and more potent P2Y<sub>12</sub> receptor antagonists (46,47). Among the latter, prasugrel has been shown to be associated with better clinical outcomes, particularly in patients with DM (48). However, atherothrombotic event rates continue to be high in patients with DM, which might be attributed to upregulation of other pivotal platelet signaling pathways triggering thrombosis, suggesting the need for antiplatelet agents that are able to block these alternative pathways (46,49).

In summary, heterogeneous antiplatelet drug effects are observed in type 2 DM patients, and patients with HPR have a greater risk of recurrent events. The C allele of the rs956115 polymorphism of IRS-1, observed in approximately 20% of patients, is independently associated with HPR and enhanced long-term cardiovascular risk. These findings might explain why in clinical practice, although type 2 DM represents per se a high-risk cohort, some patients have worse outcomes than others and might war-



rant more aggressive antithrombotic treatment. Our observations provide further insights on how pharmacogenetic analyses might identify patients with type 2 DM at different cardiovascular risk, suggesting the need for personalized treatment strategies in these patients.

**Study limitations.** Several cutoff values of HPR have been defined in the published data, although these might vary according to the specific population under investigation or timing from an acute event, among many other variables (50). Because this study selectively investigated a population with DM in a period remote from their PCI for which there is limited data on cutoff values of HPR, in agreement with prior investigations, we considered a ROC analysis to define the value with the highest sensitivity and highest specificity in our study population (50). Furthermore, it might be argued that, although marker rs956115 is in LD with rs1896832, only rs956115 showed a significant association with outcome measures. This can be explained by the degree of LD between the 2 markers. In fact, although the C allele of rs956115 is fully associated with the A allele of rs1896832, the opposite is not true, because most of the A alleles of marker rs1896832 are not associated with the C allele of marker rs956115 (approximately 88%). This is because alleles at 2 different markers have different frequencies and thus not the same as in the case of an absolute LD. Because in the association analysis of marker rs1896832 only a proportion of A alleles has a different effect compared with the other G alleles (those in linkage with the C allele of marker rs956115), a larger sample of individuals as reported by others would be required to detect a significant effect of allele A (51). An independent validation would also allow a better estimation of allelic frequencies of the IRS-1 gene, which in our study showed some differences, likely attributable to the sample size, in patients with and without DM. Although the advantage of using a study population without very strong LD between SNP to map causal variants might be a good strategy to identify portions of genes implicated in the susceptibility of the phenotypes of interest, indeed sequencing of the entire gene and promoter region is the definitive approach to identify all the important sequence variants. Ultimately, in the logistic regression for statistical adjustment, the potential for overfitting of the model may not be excluded, because the selection of the variables was based on clinical judgment by entering all the covariates that might impact the degree of platelet aggregation.

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**Key Words:** antiplatelet therapy ■ diabetes mellitus ■ gene ■ platelet function.

## APPENDIX

For supplementary methods, figures, and tables, please see the online version of this article.