High on-treatment platelet reactivity by ADP and increased risk of MACE in good clopidogrel metabolizers.

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ORIGINAL ARTICLE

High on-treatment platelet reactivity by ADP and increased risk of MACE in good clopidogrel metabolizers

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Abstract
High on-treatment platelet reactivity (HPR) by ADP, which primarily reflects the effect of thienopyridines, has been found to be an independent predictor of ischemic events in patients with acute coronary syndrome (ACS) on dual antiplatelet therapy. CYP2C19*2 is associated with HPR by ADP. The aim of our study was to evaluate if high on-clopidogrel platelet reactivity (HPR) by ADP is associated with an increased risk of major adverse coronary events (MACE) after ACS independent of CYP2C19*2 allele, i.e. whether genotyping patients for CYP2C19*2 polymorphism is sufficient to identify those to be switched to novel antiplatelets. A total of 1187 patients were included (CYP2C19 *1/*1 $n = 892$; *1/*2 $n = 264$; *2/*2 $n = 31$); 76 MACE (CV death and non-fatal MI) were recorded in non-carriers of CYP2C19*2 (8.5%) and 39 in carriers of CYP2C19*2 (13.2%). At the landmark analysis in the first 6 months, HPR by ADP and CYP2C19*2 allele were both significantly and independently associated with MACE [HPR by ADP: HR = 2.0 (95% CI 1.2–3.4), $p = 0.01$; CYP2C19*2 allele: HR = 2.3 (95% CI 1.3–3.9), $p = 0.003$]. At the land mark analysis from 7 to 12 months, only HPR by ADP remained significantly associated with the risk of MACE [HPR by ADP: HR = 2.7 (95% CI 1.4–5.3), $p = 0.003$; CYP2C19*2: HR = 0.8 (95% CI 0.2–1.1), p = ns]. CYP2C19*2 allele and HPR by ADP are both independently associated with an increased risk of MACE in the first 6 months after ACS. HPR by ADP is associated with an increased risk until 12 months of follow-up. Therefore, both phenotype and genotype are clinically relevant for the evaluation of the antiplatelet effect of clopidogrel and for the prognostic stratification of ACS patients.

Keywords: *High on-treatment platelet reactivity, cardiovascular death, non-fatal myocardial infarction, clopidogrel, CYP2C19*2 polymoprhism, acute coronary syndrome*

Introduction

High on-treatment platelet reactivity (HPR) by ADP, which primarily reflects the effect of thienopyridines, has been found to be an independent predictor of ischemic events in patients with acute coronary syndrome (ACS) on dual antiplatelet therapy [1–13]. Clopidogrel is a prodrug and, among the different CYP450 isoenzymes involved in its metabolism, CYP2C19 has an important role in its conversion to the active metabolite [14]. Recently, a single nucleotide polymorphism (CYP2C19*2) has been associated with the inactivation of the enzyme and an impaired metabolism of clopidogrel [14]. The CYP2C19*2 loss-of-function polymorphism is a G681A nucleotide substitution that introduces a splicing defect resulting in a truncated, non-functional protein responsible for the poor metabolizer phenotype [15]. Different groups have contemporarily demonstrated that CYP2C19*2 is associated with HPR by ADP and is a *per se* determinant of the occurrence of major adverse cardiac events (MACE) in percutaneous coronary intervention (PCI) patients on clopidogrel treatment [16–23].

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On the other hand, available data demonstrate that HPR by ADP is associated with clinical and biological characteristics, such as advanced age, diabetes, cardiac failure, the entity of platelet turnover and of inflammatory state [24–26].

Searching for a good tool to identify patients at high risk of recurrences who might need the new, more potent, antiplatelet agents such as prasugrel and ticagrelor, clinicians have the possibility to investigate the phenotype – HPR by ADP – or genotype – CYP2C19*2 polymorphism. The utility of genetic tests to identify differences in CYP2C19 function has been highlighted by the 'boxed warning' issued of the Food and Drug Administration advising healthcare professionals to consider use of other antiplatelet medications or alternative dosing strategies for clopidogrel in these patients [27].

The aim of our study was to evaluate if HPR by ADP is associated with MACE even in the absence of CYP2C19*2 allele, i.e. whether genotyping patients for CYP2C19*2 polymorphism is sufficient to identify those with platelet hyperreactivity to be switched to novel antiplatelet drugs.

Materials and methods

Study population

Patients with ACS who underwent PCI and with an anticipated non-adherence to dual antiplatelet treatment for at least 6 months were considered eligible for the study. Informed written consent was obtained from all patients, and the study was approved by the local Ethical Review Board.

PCI and antiplatelet management

All interventions were performed according to the current standard guidelines, and the type of stent implanted and the use of IIb/IIIa inhibitors were at the discretion of the operator. All patients received one clopidogrel loading dose of 600 mg followed by a daily dose of 75 mg. All patients received unfractioned heparin 70 IU/Kg during the procedure and acetylsalicylic acid i.v. 500 mg followed by a daily dose of 100–325 mg by oral route according to the current guidelines for PCI.

*CYP2C19*2 polymorphism*

Genomic DNA was extracted from whole blood specimens with the use of GeneCatcher™ kit (Invitrogen) automated on the Tecan® Freedom EVO® platform (Tecan) according to the manufacturers' recommendations. Genotyping for *2 variant of CYP2C19 (rs4244285) was performed with the use of an allelic discrimination assay and the detection system – ABI prism 7900HT Sequence Detection System (Applied Biosystems). Allele definition follows the nomenclature of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee (www.cypalleles.ki.se).

Platelet reactivity assessment

Venous blood samples anticoagulated with 0.109 M sodium citrate (ratio 9:1) were taken from each patient within 24 h from 600 mg clopidogrel loading. For patients receiving in the catheterization laboratory both the loading dose of clopidogrel and a IIb/IIIa inhibitor, blood samples were obtained 6 days after while the patients were on the 75 mg maintenance dose of clopidogrel.

Platelet-rich plasma, obtained by centrifuging whole blood for 10 min at 200 g, was stimulated with 10 μmol ADP (Mascia Brunelli, Milan, Italy) and aggregation was assessed using a APACT 4 light transmission aggregometer (LTA; Helena Laboratories, Milan, Italy) as previously reported [28].

Data collection and follow-up

All data were prospectively collected and entered in a central database. Clinical follow-up information was obtained by contacting all patients at 12 months, and source documents of potential events were obtained.

Endpoints: (1) cardiovascular (CV) death, defined as death in the presence of ACS, significant cardiac arrhythmia or refractory cardiac heart failure; (2) non-fatal myocardial infarction (MI) (a rise in serum troponin I and/or an increase in creatine kinase MB isoenzyme at least twice the upper normal limits with at least one of the following: acute onset of prolonged ($\geq$20 min) typical ischemic chest pain; ST-segment elevation of at least 1 mm in two or more contiguous electrocardiographic leads or ST-depression of $\geq$0.5 mm in $\geq$2 contiguous leads).

Statistical analysis

Continuous variables are presented as median (range) or mean and SD as appropriate. Categorical data are reported as frequencies. Differences in continuous variables were compared by Student's *t*-test or Mann–Whitney *U* test, as appropriate. Dichotomous variables were compared by χ^2 test or Fisher's Exact test, as appropriate.

A receiver operating characteristic (ROC) curve analysis was used to determine the ability of 10 μmol ADP to distinguish between patients with and without post-discharge events after PCI. Cumulative survival curves for both patients with and without HPR (as defined by 10 μmol ADP LTA $\geq$55% [29]) were constructed by the Kaplan–Meier (KM) method in patients who are carriers and

non-carriers of CYP2C19*2 allele. A landmark analysis was computed by the KM method for CV death and non-fatal MI from 0 to 6 months and from 7 to 12 months. We selected the 6-month point as a landmark according to the prespecified exclusion criterion of anticipated non-adherence to dual antiplatelet treatment for at least 6 months. Univariate and multivariate regression analyses were used, respectively, to identify risk factors for clinical end points and to adjust for potential confounders (CV risk factors, renal failure, left ventricular ejection fraction (LVEF) <40%, multivessel disease, total stent length, bifurcation lesions, number of lesions treated and type of stent (BMS or DES), use of GpIIb/IIIa inhibitors and aspirin dosage). McNamar test was used to compare specificity between different aggregometry tests. A significant level was defined when $p < 0.05$. All analysis was performed using SPSS 10.0 (SPSS Inc., Chicago, IL).

Results

A total of 1218 patients with ACS were recruited. Among these patients, 1187 were included in this analysis, while 31 were excluded for the following reasons: anticipated non-adherence to dual antiplatelet treatment, refusal to participate and non-availability of platelet function tests because of thrombocytopenia (Figure 1); 1187 (882 M/305 F) patients with ACS undergoing PCI with stent implantation were included. Genotype distribution of CYP2C19*2 polymorphism was as follows: *1/*1 $n = 892$; *1/*2 $n = 264$; and *2/*2 $n = 31$ (Hardy–Weinberg equilibrium: $\chi^2 = 2.095$, $p = 0.35$). As expected, LTA by 10 μmol ADP was significantly higher in patients who are carriers of *2 allele with respect to the others: 52.5% ± 19.9% vs. 46.8% ± 21.7%, $p < 0.0001$.

Baseline characteristics are given in Tables I and II. The 1-year follow-up rate was 100%. One hundred and fifteen MACE were recorded: 76 events in *non-carriers* of CYP2C19*2 (8.5%) and 39 events in *carriers* of CYP2C19*2 (13.2%). Ten micromol ADP LTA values were significantly higher in patients with the subsequent occurrence of CV death and non-fatal MI in both groups (Tables I and II). Clinical characteristics according to the occurrence of CV deaths and non-fatal MI in *carriers* and

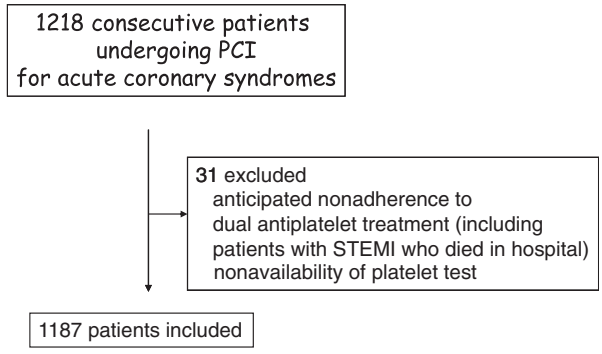

Figure 1. Study flow.

Table I. Clinical characteristics of patients investigated – non-carriers of CYP2C19*2.

	Overall group ($n = 892$)	12-month follow-up MACE ($n = 76$)	12-month follow-up No MACE ($n = 816$)	p[a]
Age, years	69 (32–93)	69 (52–90)	69 (32–93)	0.006
Male gender, *n* (%)	672 (75.3)	54 (71)	618 (75.7)	0.2
Diabetes, *n* (%)	219 (24.6)	21 (27.6)	198 (24.3)	0.1
Smoking, *n* (%)	327 (36.7)	29 (38.2)	298 (36.5)	0.3
Hypertension, *n* (%)	580 (65.0)	48 (63.2)	532 (65.2)	0.4
Dyslipidemia, *n* (%)	483 (54.1)	41 (53.9)	442 (54.2)	0.4
LVEF <40%, *n* (%)	233 (36.1)	24 (31.6)	209 (25.6)	0.01
Renal failure[b], *n* (%)	80 (9.0)	7 (9.2)	73 (8.9)	0.4
STEMI, *n* (%)	318 (35.7)	29 (38.1)	289 (35.4)	0.4
ACE-inhibitors, *n* (%)	598 (67.0)	52 (68.4)	554 (67.9)	0.9
Beta-blockers, *n* (%)	357 (40.0)	31 (40.7)	334 (40.9)	0.8
Statins, *n* (%)	562 (63)	48 (63.1)	514 (62.9)	0.8
Pump inhibitors, *n* (%)	829 (93)	70 (92.1)	759 (93)	0.8
Glycoprotein IIb/IIIa, *n* (%)	357 (40)	29 (38.1)	326 (39.9)	0.5
Vessels treated, *n*	1328	155	1173	–
Drug eluting stent, *n* (%)	156 (17.5)	16 (21)	142 (17.4)	0.2
Bifurcation lesion, *n* (%)	366 (41)	31 (40.7)	335 (41)	0.6
Total stent length (mm)	34.5 ± 22.3	33.6 ± 22.6	34.5 ± 22.3	0.4
LTA by 10 μmol ADP	46.8 ± 21.7	55.8 ± 21.4	45.9 ± 21.5	0.001

Notes: MACE = CV death and non-fatal MI.
[a]MACE *vs.* no MACE and [b]renal insufficiency defined by creatinine levels above 2.0 mg/dl.

Table II. Clinical characteristics of patients investigated – carriers of CYP2C19*2 (*1/*2 + *2/*2).

	Overall group ($n = 295$)	12-month follow-up MACE ($n = 39$)	12-month follow-up No MACE ($n = 256$)	p[a]
Age, years	69 (29–94)	71 (29–85)	69 (36–94)	0.001
Male gender, *n* (%)	216 (73.2)	30 (76.9)	183 (71.4)	0.2
Diabetes, *n* (%)	66 (22.3)	13 (33.3)	53 (20.7)	0.1
Smoking, *n* (%)	108 (36.6)	16 (41)	92 (35.9)	0.3
Hypertension, *n* (%)	197 (66.7)	28 (71.7)	169 (66)	0.4
Dyslipidemia, *n* (%)	163 (55.2)	20 (51.2)	143 (55.8)	0.4
LVEF <40%, *n* (%)	75 (25.4)	12 (30.1)	63 (24.6)	0.05
Renal failure[b], *n* (%)	26 (8.8)	3 (7.6)	23 (8.9)	0.4
STEMI, *n* (%)	101 (34.2)	13 (33.3)	88 (34.4)	0.2
ACE-inhibitors, *n* (%)	195 (66)	27 (69.2)	168 (65.6)	0.8
Beta-blockers, *n* (%)	114 (38.6)	16 (41)	98 (38.2)	0.5
Statins, *n* (%)	191 (64.7)	25 (64.1)	166 (64.8)	0.4
Pump inhibitors, *n* (%)	285 (96.6)	36 (92.3)	249 (97.2)	0.3
Glycoprotein IIb/IIIa, *n* (%)	115 (38.9)	15 (38.4)	100 (39.0)	0.8
Vessels treated, *n*	439	79	360	–
Drug eluting stent, *n* (%)	54 (18.3)	8 (20.5)	46 (17.9)	0.1
Bifurcation lesion, *n* (%)	94 (31.8)	14 (35.8)	80 (31.2)	0.8
Total stent length (mm)	35.6 ± 26.9	33.6 ± 22.6	34.5 ± 22.3	0.4
LTA by 10 μmol ADP	52.5 ± 19.9	61.6 ± 17.6	51.1 ± 19.9	0.001

Note: As in notes of Table I.

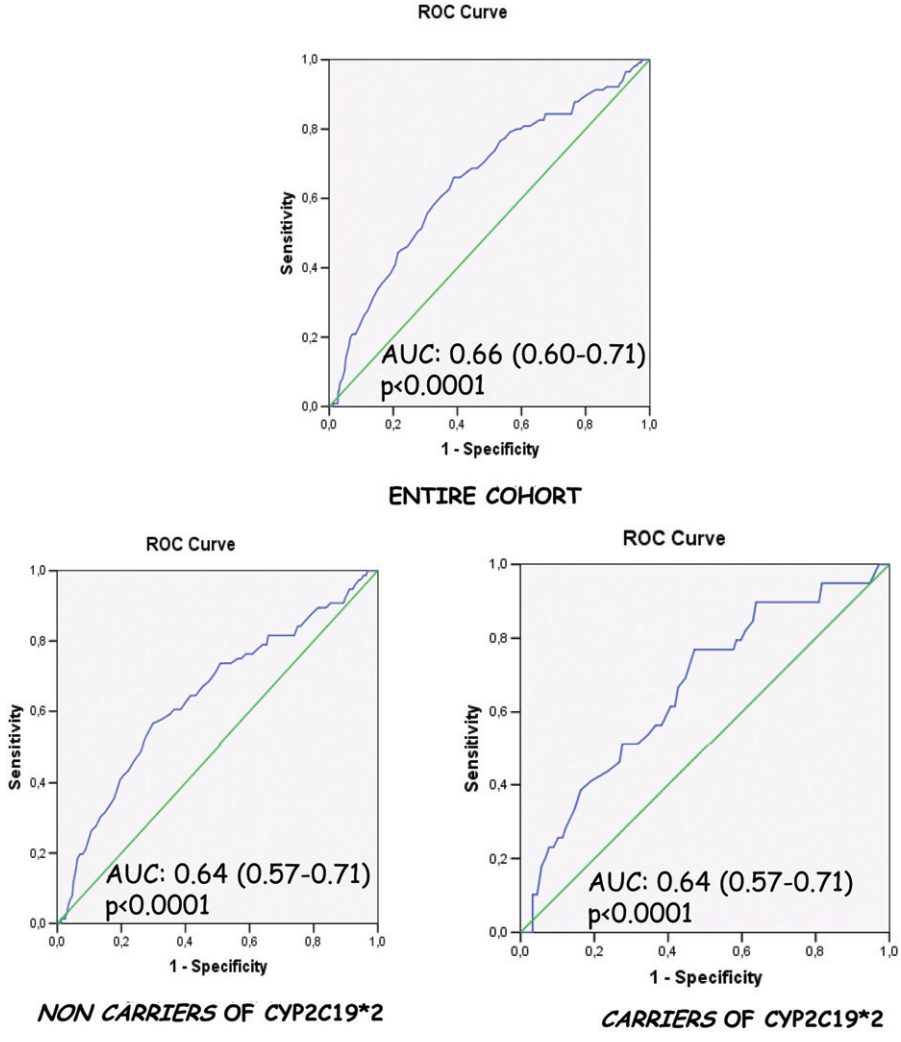

Figure 2. ROC curve for LTA by 10 μmol ADP.

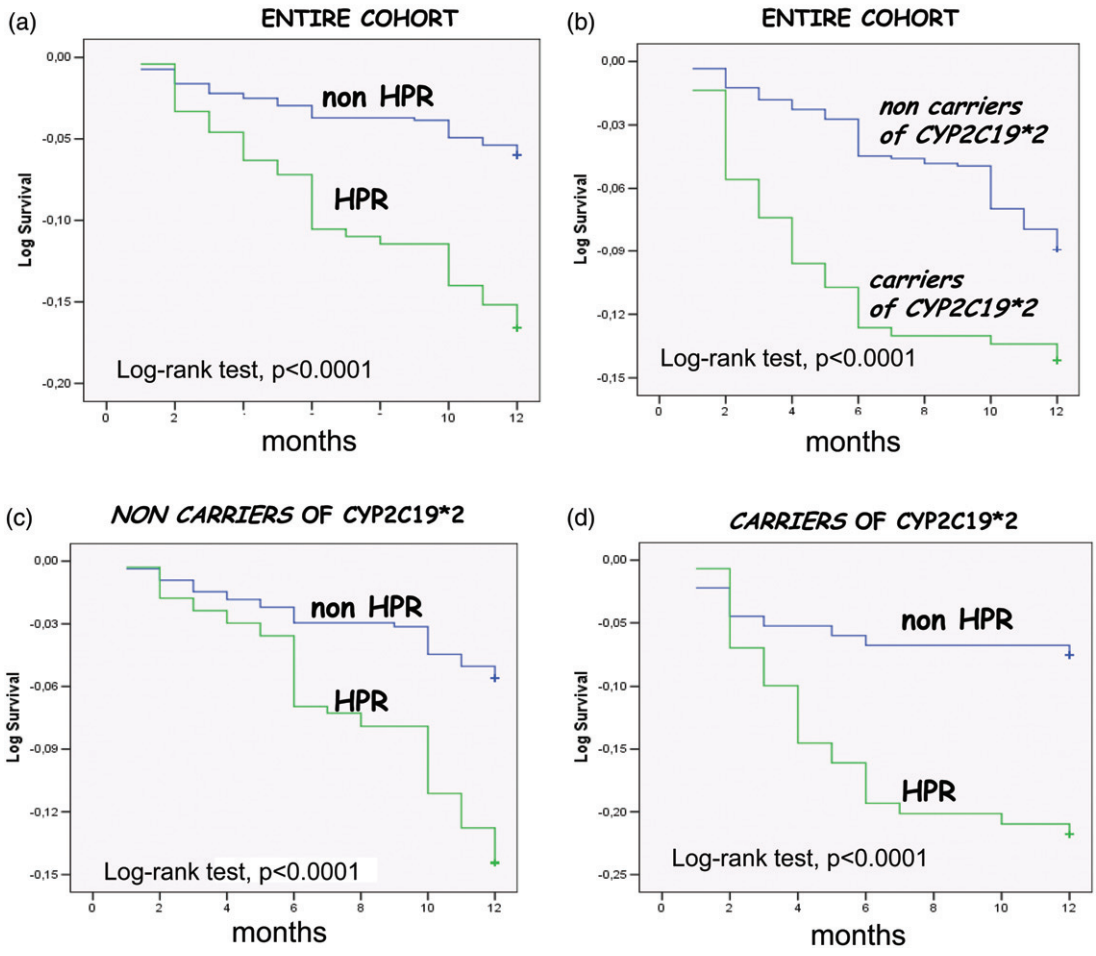

Figure 3. KM survival curves for CV death and non-fatal MI.

non-carriers of CYP2C19*2 are reported in Tables I and II.

ROC curve analysis demonstrated that 10 μmol ADP LTA was able to distinguish between patients with and without subsequent CV death and non-fatal MI at 12-month follow-up both in *carriers* and *non-carriers* of CYP2C19*2 (Area under the curve 0.66 (0.57–0.75), $p < 0.001$ and 0.64 (0.57–0.71), $p < 0.001$, respectively) (Figure 2).

The event-free survival curves for CV death and non-fatal MI according to *2 allele carriers vs. non-carriers are shown in Figure 3(b) (10 μmol ADP LTA in CYP2C19 *1/*1 = 46.8% ± 21.7%; *1/*2 = 51.3% ± 19.6%; and *2/*2 = 63.2% ± 19.3%). In Figure 3(a), (c), and (d), KM curves according to HPR vs. non-HPR both in carriers and non-carriers of CYP2C19*2 allele are reported.

The landmark analysis (0–6 months and 7–12 months) showed that the differences between HPR and non-HPR groups in CV death and non-fatal MI emerged both in the short-term follow-up (Figure 4) as well as later on (Figure 5). On the other hand, carriers of CYP2C19*2 were associated with a significant higher risk until 6 months (Figure 4), but not between 6 and 12 months (Figure 5).

Patients with LTA by ADP ≥55%, which we previously demonstrated to be the optimal cut-off for detection of ischemic recurrences [29], were significantly older, more likely to be female, diabetic and with reduced LVEF with respect to patients without RPR (Table III).

At the land mark analysis in the first 6 months (0–6), HPR by ADP and CYP2C19*2 allele were both significantly and independently associated with a higher risk of CV death and non-fatal MI after adjustment for CV risk factors, renal failure, reduced ejection fraction, multivessel disease, total stent length, bifurcation lesions, number of lesions treated, type of stent and use of GpIIb/IIIa inhibitors [HPR by ADP: HR = 2.0 (95% CI 1.2–3.4), $p = 0.01$; CYP2C19*2 allele: HR = 2.3 (95% CI 1.3–3.9), $p = 0.003$]. The presence of both – HPR by ADP and CYP2C19*2 allele – was associated with a higher risk: HR = 2.7 (1.5–5.1), $p < 0.001$.

At the land mark analysis from 7 to 12 months, only HPR by ADP remained significantly associated

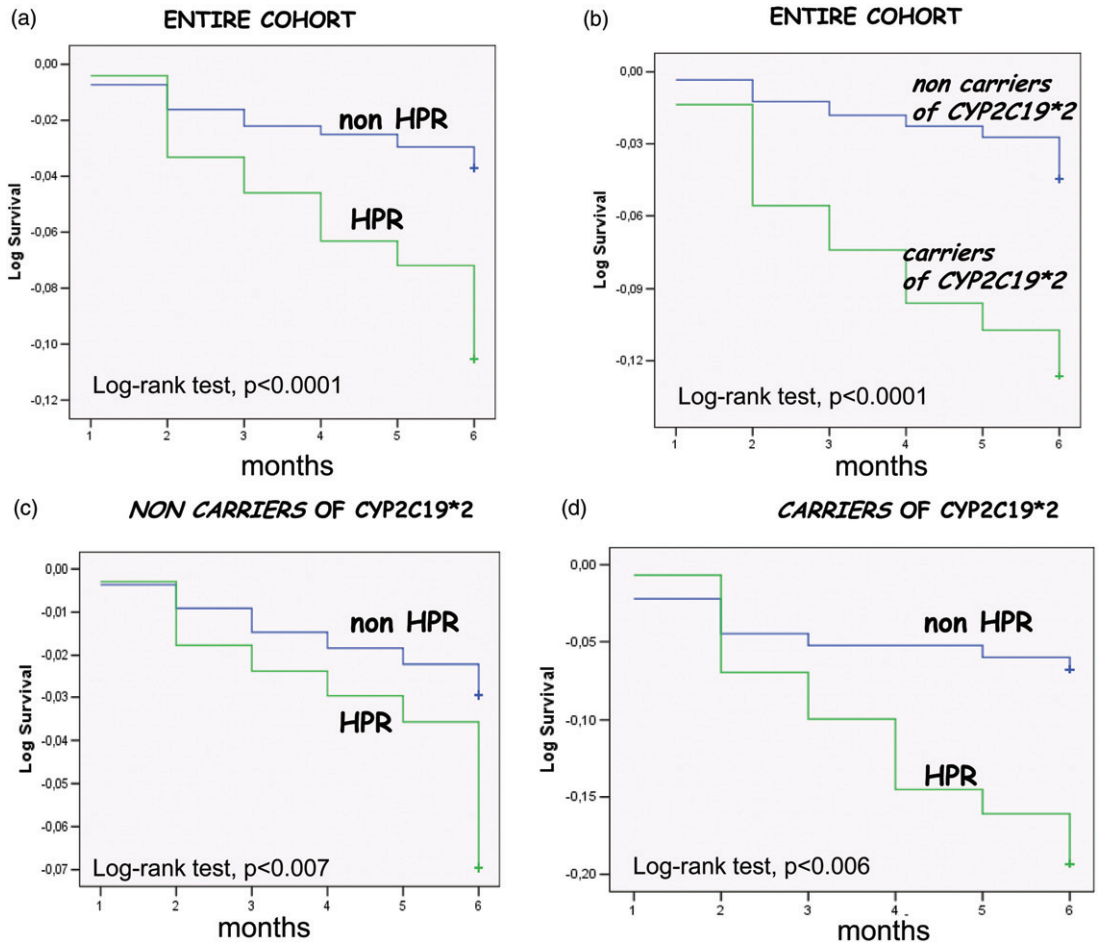

Figure 4. KM landmark analysis survival curves for CV death and non-fatal MI (0–6 months).

with the risk of MACE at the Cox regression analysis adjusted for CV risk factors, renal failure, reduced ejection fraction, multivessel disease, total stent length, bifurcation lesions, number of lesions treated, type of stent and use of GpIIb/IIIa inhibitors [HPR by ADP: HR = 2.7 (95% CI 1.4–5.3), $p = 0.003$; CYP2C19*2: HR = 0.8 (95% CI 0.2–1.1), p = ns].

Discussion

In this analysis, we demonstrate that HPR by ADP is a risk factor for clinical recurrences in ACS patients on dual antiplatelet treatment, even in the absence of CYP2C19*2 allele.

The history of the 'resistance' to clopidogrel moves from the studies which found an association between HPR by ADP and risk of vascular complications, i.e. stent thrombosis, CV death and non-fatal MI [1–16]. Subsequently, different researchers, including our group, studied the possible genetic determinants of response to clopidogrel focusing on the presence of polymorphisms in genes coding for CYP450 enzymes [17]. It has been detected that CYP2C19*2 polymorphism is associated with HPR by ADP and it is *per se* associated with vascular risk [18–24]. Furthermore, we found that both HPR by ADP and CYP2C19*2 polymorphism were independently associated with the occurrence of stent thrombosis and CV death [23] at a 6-month follow-up.

Indeed, both genotype – CYP2C19*2 allele – and phenotype – HPR by ADP – are available in order to identify patients who might need a more potent platelet inhibition, potentially by the new antiplatelet drugs such as prasugrel or ticagrelor.

The question to be answered is if the issue of platelet hyperreactivity on treatment is a phenomenon to be restricted, in the clinical practice, to the problem of the metabolism of clopidogrel. The recent ''boxed warning'' of FDA underlines the importance of CYP2C19*2 polymorphism for the choice of alternative drugs to clopidogrel [27].

For this purpose, we have decided to evaluate the risk of clinical recurrences according to HPR by ADP in carriers and non-carriers of

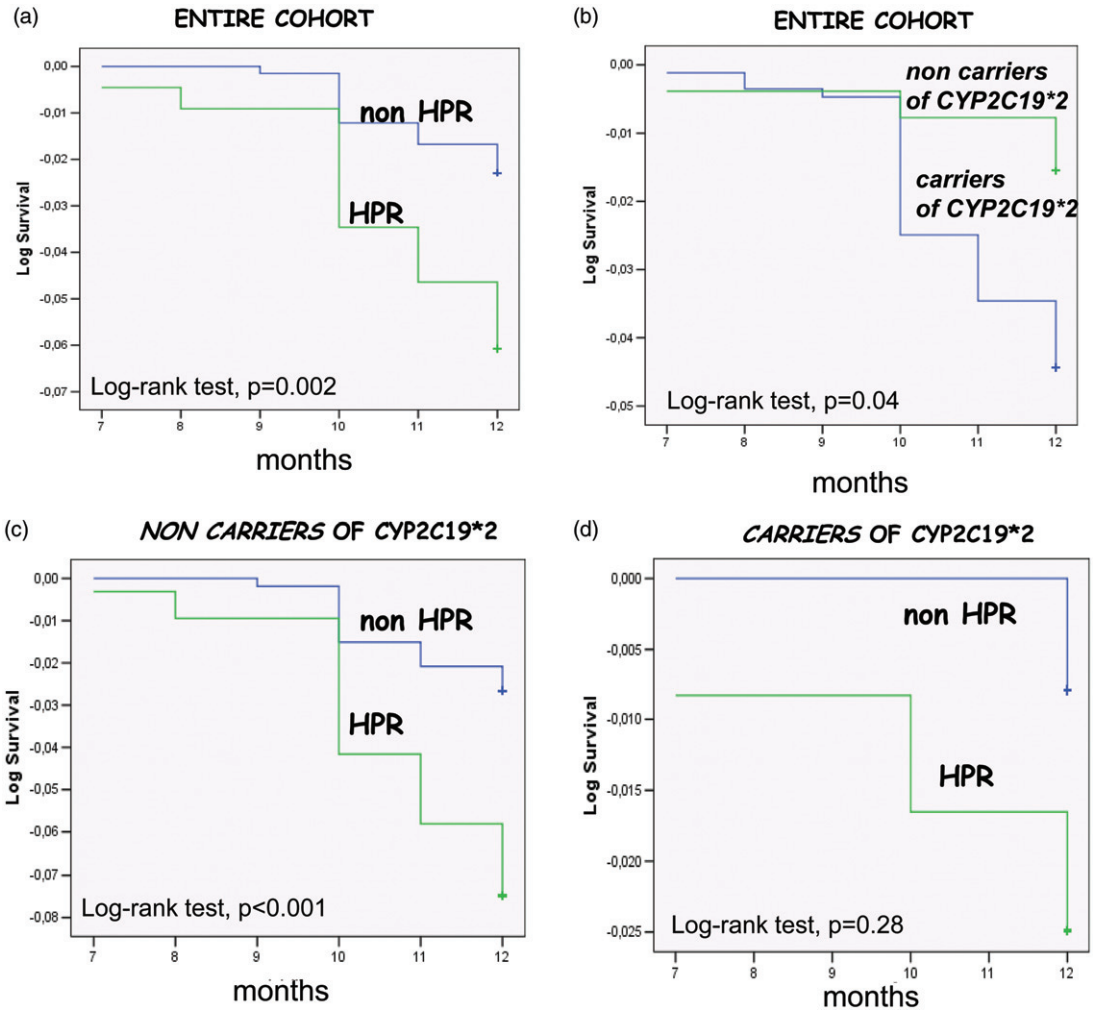

Figure 5. KM landmark analysis survival curves for CV death and non-fatal MI (7–12 months).

Table III. Clinical characteristics according to HPR by ADP (10 μmol ADP LTA ≥55%).

	Non-carriers of CYP2C19*2			Carriers of CYP2C19*2		
	HPR by ADP ($n=342$)	No HPR by ADP ($n=550$)	p	HPR by ADP ($n=144$)	No HPR by ADP ($n=151$)	p
Age, years	69 (46–93)	69 (32–93)	<0.05	73 (29–90)	67 (36–94)	<0.005
Male gender, *n* (%)	250 (73)	422 (76.7)	<0.05	104 (72.2)	112 (74.1)	<0.05
Diabetes, *n* (%)	90 (26.3)	129 (23.5)	<0.001	40 (27.7)	26 (17.2)	<0.0001
Smoking, *n* (%)	120 (35.1)	207 (37.6)	0.4	55 (38.2)	53 (35.1)	0.04
Hypertension, *n* (%)	240 (70.2)	340 (61.8)	0.5	98 (68)	99 (65.5)	0.3
Dyslipidemia, *n* (%)	171 (50)	312 (56.7)	0.2	75 (52.1)	88 (58.3)	0.4
LVEF <40%, *n* (%)	91 (26.6)	142 (25.8)	<0.001	38 (26.4)	37 (24.5)	<0.05
Renal failure[a], *n* (%)	23 (6.7)	57 (10.4)	0.4	10 (6.9)	16 (10.6)	0.4
STEMI, *n* (%)	104 (30.4)	176 (32)	<0.001	36 (25)	65 (43)	<0.001
ACE-inhibitors, *n* (%)	229 (66.9)	374 (68)	0.3	96 (66.6)	99 (65.6)	0.2
Beta-blockers, *n* (%)	140 (40.9)	225 (40.9)	0.3	59 (40.9)	55 (36.4)	0.5
Statins, *n* (%)	215 (62.8)	346 (62.9)	0.1	91 (63.2)	100 (66.2)	0.09
Pump inhibitors, *n* (%)	318 (92.9)	511 (92.9)	0.2	136 (94.4)	149 (98.6)	0.07
Glycoprotein IIb/IIIa, *n* (%)	140 (40.9)	214 (38.9)	0.9	59 (40.9)	56 (37)	0.8
Vessels treated, *n*	514	814	–	216	223	–
Drug eluting stent, *n* (%)	74 (21.6)	83 (15)	<0.01	32 (22.2)	22 (14.5)	<0.005
Bifurcation lesion, *n* (%)	145 (42.3)	221 (40.2)	0.2	52 (36.1)	42 (27.8)	0.2
Total stent length (mm)	35.5 ± 21.6	33.8 ± 22.7	<0.01	39.1 ± 29.9	31.9 ± 23.5	<0.001

CYP2C19*2 allele. If the problem of HPR by ADP is only associated with clopidogrel metabolism, we should find that HPR in the absence of CYP2C19*2 allele is not associated with an increased risk of recurrences.

Conversely, our data demonstrate that the presence of CYP2C19*2 allele confers a higher risk in the first months after the acute event, and HPR by ADP also later on. These data are in line with those obtained in the meta-analysis by Mega et al. [23], in which the authors failed to find a significant association between CYP2C19*2 allele and MACE in landmark analyses from 31 days until the end of follow-up.

HPR on treatment is a complex phenomenon for which pharmacogenetic accounts only in part.

In other words, these findings demonstrate that the entity of platelet inhibition on treatment is a prognostic factor in ACS patients, independent of the variability associated with clopidogrel metabolism.

Accordingly, both genotype and phenotype are clinically relevant:

(1) Genotyping patients for CYP2C19*2 allele allows us to identify patients who immediately need an alternative drug to clopidogrel, independent of the clinical characteristics and clinical manifestation of the disease.
(2) Phenotyping patients for the entity of platelet inhibition by ADP allows us to obtain an information about a factor which maintains its prognostic role until 12 months after the acute event. Available evidence tells us that we have to obtain a platelet inhibition which might be around 55% to reduce the risk of ischemic recurrences.

The main limitation of our study is the lack of information on the occurrence of stent thrombosis.

Furthermore, this study includes only a genetic determinant of HPR by ADP. A number of genetic polymorphisms are under evaluation to determine their possible role in affecting the entity of platelet inhibition on treatment. Nevertheless, till now, only CYP2C19*2 allele has been consistently associated with the intermediate phenotype 'HPR by ADP' and with a significantly increased vascular risk [18–23].

Furthermore, we are aware that platelet reactivity at the time of ACS may be affected by a number of clinical and laboratory parameters, including the high level of the inflammatory state and the increased platelet turnover [24–26]. Consequently, a percentage of patients with platelet hyperreactivity in the acute state might subsequently return to an adequate platelet inhibition. The issue of the durability of HPR remains to be solved by ad hoc studies. Nevertheless, our results underline the concept that antiplatelet therapy, driven by the determination of the phenotype HPR, might be modulated not only with respect to the type of the disease, but also with respect to the different phases of the same disease (acute vs. chronic phase) suggesting the need of checking the persistence of HRP over the time.

In conclusion, we found that HPR by ADP is associated with an increased risk of 12-month follow-up MACE even in the absence of the genetic polymorphism – CYP2C19*2 - associated with a reduced metabolism of clopidogrel. Therefore, both phenotype and genotype are clinically relevant for the evaluation of the antiplatelet effect of clopidogrel and for the prognostic stratification of ACS patients.

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