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PHARMACOGENETICS OF CLOPIDOGREL IN ACUTE CORONARY SYNDROMES

"FARMACOGENETICA DEL CLOPIDOGREL NEL TRATTAMENTODELLE SINDROMI CORONARICHE ACUTE"

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TABLE OF CONTENTS

Abstract

Background:

The antiplatelet agent clopidogrel is an effective drug for the prevention of thrombotic events in patients with acute coronary syndromes, and is therefore one of the most frequently prescribed drugs worldwide. Accumulating data suggest that the response to clopidogrel is characterised by significant inter-patient variability in the degree of platelet inhibition and the risk of cardiovascular events. Recent research findings have highlighted the role of genetic variations in determining antiplatelet response variability, and this has aroused interest in genotyping all thienopyridine-eligible patients in order to identify those who would be at increased risk of harm if treated with clopidogrel.

This study tested the hypothesis that selecting antiplatelet therapy for patients with acute coronary syndromes (ACS) on the basis of a combination of genetic and clinical characteristics would lead to better clinical outcomes in comparison with the standard of care which bases the selection on clinical characteristics alone.

Methods:

Consecutive patients hospitalised for ACS were randomly assigned to the standard or the pharmacogenomic arm, which included the genotyping of ABCB1, CYP2C19*2 and CYP2C19*17 by means of an ST Q3 system. In the pharmacogenomic group, clopidogrel, prasugrel or ticagrelor were selected on the basis of an algorithm that considered clinical variables and genetic findings made available within 70 minutes at each patient's bedside. All of the patients were followed-up for 12 ± 1 months for the occurrence of the primary composite end-point of cardiovascular death and the first

occurrence of non-fatal myocardial infarction, non-fatal stroke and BARC 3 to 5-defined major bleeding.

Results:

After the enrolment of 888 patients, the study was prematurely stopped. Clopidogrel was used more frequently in the standard arm (50.7% *vs* 43.3%), ticagrelor more frequently in the pharmacogenomic arm (42.6% *vs* 32.7%; P=0.02), whereas prasugrel was equally used in both arms. The primary end-point occurred in 71 patients (15.9%) in the pharmacogenomic arm and in 114 (25.9%) in the standard of care arm (HR 0.58; 95% CI 0.43 to 0.78; P<0.001).

Conclusion:

A personalised approach to the selection of antiplatelet therapy for ACS patients leads to a clinically meaningful reduction in the composite end-point of ischemic and bleeding events.

The trial has been registered at www.clinicaltrials.gov with the identifier NCT03347435 “Pharmacogenetics of clopidogrel in patients with acute coronary syndromes”.

Introduction

Dual antiplatelet therapy with aspirin and a P2Y12 receptor antagonist is the mainstay of the treatment of acute coronary syndromes. Although the international guidelines [1-4] strongly suggest front-line treatment with prasugrel or ticagrelor, clopidogrel remains the preferred P2Y12 receptor antagonist in real world clinical practice. The use of more potent antiplatelet drugs involves a fundamental trade-off between decreasing the risk of ischemia and increasing the risk of bleeding. For this reason, clopidogrel is frequently chosen in the real world, but, when balancing ischemic/bleeding risks, only the clinical characteristics of individual patients are taken into account.

It has been clearly shown that dual antiplatelet pathway inhibition offers synergistic benefits in preventing thrombus formation, but not all patients benefit to the same extent. Up to 10% of those taking clopidogrel experience a recurrent ischemic event during the first year after ACS, 1-3% experience subacute stent thrombosis after PCI probably due to a poor drug response, and about 1.5% experience major bleeding mainly due to an enhanced response.[5] It has been reported that, despite adequate treatment, about 30% of patients continue to show the high degree of platelet reactivity that is central to the development of atherothrombotic complications and poorer clinical outcomes.[6]

Limitations of current antiplatelet therapy

It is known that the combined inhibition of ADP P2Y12 receptors and the TXA2 pathway provide more comprehensively inhibits platelet activation and leads to the

greater inhibition of platelet-mediated thrombosis than blocking the formation of TXA2 alone.[13]

Evidence that the clopidogrel and aspirin combination markedly enhances antithrombotic efficacy originally came from studies in rabbits[14] and *ex vivo* experimental models.[15] On the basis of these data, it was possible to speculate that such combined treatment would provide substantial protection against platelet aggregation leading to thrombotic occlusion at sites of endothelial injuries and against coronary artery stenosis in humans. The reduction in the risk of ischemic events observed in clininical trials has led to dual antiplatelet therapy becoming the standard of care in cardiovascular patients.[2-5] In the CURE trial, randomisation to clopidogrel was associated with a 20% reduction in the relative risk of cardiovascular death, stroke or myocardial infarction (MI) in comparison with standard therapy, including aspirin alone. This benefit was observed soon after the administration of the clopidogrel loading dose, and was sustained during long-term treatment. Furthermore, in the subset of patients who underwent PCI, those who were randomised to clopidogrel showed a 31% reduction in the combined risk of cardiovascular death or MI (P=0.002).[16,17] Clopidogrel is therefore indicated for reducing atherothrombotic events in patients with ACS and those undergonig PCI.[17]

However, despite its impressive benefits in large and diverse groups of patients, there is increasing evidence that the clinical efficacy of clopidogrel has a number of pharmacodynamic and pharmacokinetic limitations. It has been shown that platelets can still be activated and aggregated even though the P2Y12 ADP receptor- and TxA2-related activation pathways are blocked by the current standard of care treatment.[18] As multiple pathways contribute to platelet activation, it can be

hypothesised that new therapies targeting other activation pathways could contribute to the greater inhibition of platelet- mediated thrombosis. Secondly, clopidogrel inhibition involves only about 40% of circulating platelets.[19,20] Furthermore, clopidogrel is an irreversible P2Y12 receptor inhibitor and its effect on circulating platelets lasts approximately 8-10 days, which may be associated with an increased risk of hemorrhagic complications particularly in patients requiring urgent coronary artery by-pass grafting or other surgery. In the CURE trial, despite the greater ischemic protection, the patients randomised to receive aspirin plus clopidogrel experienced more major bleeding events than those treated with aspirin alone (3.7% *vs* 2.7%; p= 0.001).[16] Furthermore, the CHARISMA trial showed that clopidogrel was not more beneficial than aspirin alone in a large population of patients at high risk for atherothrombotic events: although there was a suggestion of a benefit in patients with symptomatic atherothrombosis (6.9% *vs* 7.9%; RR 0.88, 95% CI 0.77-0.99; p= 0.046), there was also a suggestion of harm in patients with multiple risk factors (6.6% *vs* 5.5%; RR 1.2, 95% CI 0.91-1.59; p= 0.20) and a trend towards a significant increase in the rate of moderate bleeding in the clopidogrel group.[21]

The efficacy of clopidogrel is also limited by some of its pharmacokinetic characteristics. First of all, it is a pro-drug that requires two-stage activation by specific hepatic cytochrome P450 enzymes (particularly CYP2C19 and CYP3A4). Secondly, approximately 85% of clopidogrel is inactivated *in vivo* by esterases, and only 15% is converted to the active metabolite. Thirdly, because of its metabolic activation, clopidogrel has a slow onset of action, which is clearly disavantageous during urgent PCIs. Finally, a number of studies have shown that not all patients respond to clopidogrel in the same way, and this inter- and intraindividual variability

means that a considerable number of patients may experience little or no antiplatelet effect.[6]

Clopidogrel response variability

The variability of responses to clopidogrel (as measured by means of platelet aggregation in response to ADP) was first described in 2003 by Gurbel *et al*.[22] They showed that the platelet inhibitory response to the standard clopidogrel dose regimen for coronary stenting is patient specific, variable and follows a normal distribution; moreover, the level of platelet reactivity critically depends on pretreatment reactivity, and so patients with the greatest pretreatment platelet reactivity have the least antithrombotic protestion. Since then, various definitions of clopidogrel responsiveness have been used, particularly the degree of inhibition of platelet aggregation (defined as the percentage decrease in aggregation values before and after treatment), and recent new data[23-26] suggest that clopidogrel responsiveness should be considered a continuous rather than a dichotomous parameter.

A number of studies have investigated the clinical implications of the variability of individual responses to clopidogrel. In particular, it has been shown that it is significantly related to a high risk of stent thrombosis and ischemic events during follow-up in patients with stable angina and in those with ACS.[27-31] Matetzky *et al*. found that 25% of STEMI patients are clopidogrel resistant, and that this is associated with a higher risk of recurrent cardiovascular events over a 6-month follow-up period.[29] Ajzenberg *et al*. demonstrated that patients with subacute stent thrombosis show increased platelet aggregation in comparison with control subjects receiving dual antiplatelet therapy,[30] and the CREST study of Gurbel *et al*. showed

that high post-treatment platelet reactivity was a risk factor for subacute stent thrombosis.[31]

Non-genetic factors of variability

The mechanisms leading to the variability in clopidogrel responsiveness are not fully known and are likely to be multifactorial, including patient non-compliance, inappropriate dosing or underdosing, variability in intestinal drug absorption and drug-drug interactions.[32] Moreover, high pretreatment levels of platelet reactivity, which are frequently observed in patients with an increased body mass index and diabetes mellitus, may contribute to reducing clopidogrel-induced antiplatelet effects. It has been shown that patients with type 2 diabetes are much more likely show an impaired platelet response to clopidogrel than non-diabetic patients, and that insulin resistance is associated with increased platelet reactivity. Likewise, obesity (which is highly prevalent in diabetic patients) independently impairs the response to antiplatelet agents. Finally, high levels of pro-inflammatory mediators (common in diabetics) has been associated with increased platelet activation.[33]

Differences in individual clopidogrel absorption and it the levels of its active metabolite may also be responsible for the variability in responses. It has been reported that patients ages $\geq$75 years show a higher incidence of residual platelet activity when treated with clopidogrel than younger patients, probably because their reduced cytochrome oxidase activity reduces the amount of the active metabolite.[32] It has also been hypothesised that drug enhancers or inhibitors of CYP 450 isoenzymes may interfere with the conversion of clopidogrel into its active metabolite, leading to reduced antiplatelet effects.

Some reports suggest that lipophilic statins such as atorvastatin and simvastatin (which are metabolised by CYP3A4) reduce clopidogrel-induced antiplatelet effects;[34-37] however, these findings have not been replicated in larger studies that did not find any clinical or biological interaction between statins and clopidogrel.[38-40]

Concerns have revcently been raised about the clinical impact of the interaction between clopidogrel and proton pump inhibitors (PPIs).[41-46] The findings of *ex vivo* biological studies suggest that PPIs, especially omeprazole, may decrease the antiplatelet effect of clopidogrel by inhibiting hepatic cytochrome P450 2C19, and therefore the conversion of clopidogrel into its active metabolite, as a result of competition for the same substrate.[47] However, although there is a mechanistic and pharmacodynamic basis underlying the interaction between PPIs and clopidogrel, its clinical significance is still unclear. Observational studies have found that the use of clopidogrel plus PPIs increases the risk of death and rehospitalisation for ACS in comparison with the use of clopidogrel without PPIs (OR 1.25, 95% CI 1.11-1.41),[48] although these data was not confirmed in the *post hoc* analysis of the TRITON TIMI-38 trial involving a large population of patients with ACS. Despite the observed attenuation of the *in vitro* antiplatelet effect of clopidogrel, the use of a PPI was not independently associated with an increased risk of adverse clinical outcomes after adjusting for potential confunders and the propensity to be treated with a PPI (HR 0.94, 95% CI 0.80-1.11).[49] Moreover, the negative effect of PPIs on the antiplatelet action of clopidogrel did not lead to worse clinical outcomes in the COGENT trial, the first randomized phase III trial comparing a combination of 20 mg omeprazole and clopidogrel with placebo and clopidogrel in patients requiring clopidogrel for at least 12 months. After only 133 days of follow-up (due to the early termination of the trial

when the sponsor declared bankruptcy), the patients assigned to omeprazole had experienced fewer upper gastrointestinal clinical events but there was no difference in the clinical endpoints of cardiovascular death and MI between the two groups (HR 1.02, 95% CI 0.70-1.51).[50]

The clinical impact of the interaction between clopidogrel and PPIs is still a subject of intense debate and ongoing research. A recent meta-analysis has shown that clopidogrel-treated patients taking a PPI were at increased risk of MACE (OR 1.41, 95% CI 1.34-1.48) and mortality (OR 1.18; 95% CI 1.07-1.30) in comparison with non-users, although the impact of PPI use was significantly affected by the baseline cardiovascular risk as it was significant only in high-risk patients.[51]

Genetic basis of clopidogrel response variability

There is growing evidence that genetic polymorphisms affecting clopidogrel's pharmacokinetics and pharmacodynamics may influence response variability, and could play a pivotal role in determining individual susceptibility to the drug. A number of polymorphisms in the genes encoding efflux pump P-glycoprotein, CYP 450 enzyme isoforms and platelet components have been investigated[52], and the main findings are summarised below.

ABCB1

The gut absorption of clopidogrel is opposed by the efflux pump P-glycoprotein encoded by the ABCB1 gene. It has been hypothesised that variations in the ABCB1 gene may affect responses to clopidogrel and clinical outcomes as a result of increased function. However, the data conscerning this genetic variant are still

partial, and clinical studies have led to mixed results. Taubert *et al*. found that, in comparison with non-carriers (CC genotype), the bioavailability of clopidogrel was significantly reduced in patients with one (CT genotype) or two copies (TT genotype) of the ABCB1 C3435T polymorphism;[53] Spiewak *et al*. showed that ACS patients who were 3435T homozygotes were more likely to have an impaired platelet response to clopidogrel;[54] finally, the recent pharmacogenetic analysis of the TRITON-TIMI 38 population by Mega *et al*. indicated that clopidogrel-treated ABCB1 3435 homozygous patients (TT) have lower concentrations of the active drug metabolite, show reduced platelet inhibition, and are more likely to experience an adverse clinical outcome (OR 1.72, 95% CI 1.38-2.82) than heterozygous or wild-type patients (CT/CC). When the ABCB1 3435C-T genotype was considered in the context of CYP2C19 reduced-function allele status, both variants were significant independent predictors of cardiovascular death, MI or stroke, and variants in the two genes offered independent and complementary information concerning cardiovascular risk.[25] In contrast with these findings, the FAST-MI investigators reported that 3435CT heterozygous patients also had an increased relative risk of an adverse clinical outcome,[55] and both Wallentin *et al*. and Tiroch *et al*. found that carrying the 3435T allele homozygously or heterozygously had no effect on clinical endpoints.[56,57]

CYP2C19

It is known that variability in the catalytic activity of the hepatic CYP system affects the conversion of clopidogrel into its active metabolite, thus further attenuating the drug's pharmacodynamic action. It has been recently demonstrated that this

variability may be due to genetic alterations in genes encoding for constituent parts of the hepatic CYP system. CYP450 genetic variants may reduce or enhance enzymatic function and interfere with the production of clopidogrel's active metabolite and its antiplatelet effects[58,59].

The active metabolite of clopidogrel, which irreversibly blocks the platelet ADP receptor P2Y12, arises as a result of complex biochemical reactions involving a number of different cytochromes (CYP2C19, CYP1A2, CYP2B6, CYP3A4/5, CYP2B6 and CYP2C9), with CYP2C19 playing a key role. The CYP2C19 enzyme is involved in both of the metabolic steps of clopidogrel bioactivation, and contributes an estimated 45% of the generated 2-oxo-clopidogrel, and 21% of its conversion to the active metabolite. For this reason, research has concentrated on the potential impact of its genetic polymorphisms on the activity of enzyme and subsequent drug metabolism.

All of the CYP2C19 polymorphism data suggest that enzymatic activity and clopidogrel-induced platelet inhibition are reduced in carriers of loss-of-function alleles, whereas carriers of gain-of-function alleles show high levels of CYP activity and therefore greater platelet inhibition[23,24,55, 60-64]. Twenty-five polymorphic variants of the CYP2C19 gene have so far been identified, and their frequency varies among different ethnic groups. One of the most common is called CYP2C19*2 and has an allelic frequency of 15-30%. This poorly metabolising polymorphism causes a splicing defect and the complete loss of enzyme activity, and accounts for the majority of poor metabolisers in Europe (in Asia, *CY*P2C19*3 also contributes to this phenotype). Other rarer alleles causing deficient metabolism are *C*YP*2C*19*4, *5, *6, *7 and *8, whereas *C*YP2*C*19*17 leads to increased enzyme activity and is found with a

frequency of 18-27% in European populations and 1.3% in Asians.[65] On the basis of these data, patients can be classified into categories of metabolising phenotypes using the established common-consensus star allele nomenclature. Patients carrying two “normal” alleles (i.e. *1/*1) are “extensive metabolisers”; those with one reduced-function allele (e.g. *1/*2) are “intermediate metabolizers”; those carrying two reduced-function alleles (e.g. *2/*2 or *1/*3) are “poor metabolisers”; and those carrying one or two increased-function alleles (i.e. *1/*17 or *17/*17) are “ultrarapid-metabolisers”.

Pharmacokinetic studies have shown that the metabolisation of clopidogrel to its active thiol metabolite is diminished in carriers of the CYP2C19 loss-of-function alleles,[66,67] and platelet function studies have found that these alleles are associated with higher levels of platelet aggregation after clopidogrel treatment.[68] In particular, Hulot *et al*. found that platelet aggregation in the presence of 10 M ADP decreased gradually during treatment with clopidogrel 75 mg once daily in wild-type (*1/*1) young healthy male volunteers, but did not change in carriers of at least one loss-of-function allele (*1/*2)[69]. In an elegant study published in the New England Journal of Medicine, Mega *et al*. clearly showed that genetic variants of CYP2C19 are associated with differences in the bioavailability of clopidogrel’s active metabolite, its antiplatelet effects, and clinical outcomes. Clopidogrel-treated healthy carriers of at least one *CY*P2C19 reduced-function allele showed a relative reduction of 32.4% in plasma exposure to the active metabolite of clopidogrel, and an absolute reduction in maximal platelet aggregation that was 9% less than that seen in non-carriers. Moreover, among the clopidogrel-treated subjects in TRITON–TIMI 38 population, the carriers of a CYP2C19 loss-of-function allele showed a relative 53% increase in

the composite primary efficacy outcome of the risk of death from cardiovascular causes, myocardial infarction or stroke (HR 1.53; 95% CI 1.07-2.19; p = 0.01) and a 3-fold increase in the risk of stent thrombosis (HR 3.09; 95% CI 1.19- 8.00; p = 0.02).[25]

Among the acute myocardial infarction patients receiving clopidogrel in the FAST-MI study, those carring CYP2C19 loss-of-function alleles experienced more subsequent cardiovascular events than those who were not, and this effect was particularly marked among the patients undergoing PCI. Patients carrying any two CYP2C19 loss-of-function alleles (*2, *3, *4, or *5) were more likely to experience death, recurrent MI or stroke than those with none (21.5 *vs* 13.3%; adjusted HR, 1.98; 95% CI 1.10-3.58), and those who underwent PCI were 3.58 times more likely to experience cardiovascular events than those with none (95% CI 1.71-7.51); on the contray, patients with one CYP2C19 loss-of-function allele were not at any increased risk in comparison with those who had no CYP2C19 variant allele.[55]

The CYP2C19*2 polymorphism also greatly affected the risk of recurrent thrombotic coronary events in a population of young MI patients receiving prolonged clopidogrel treatment.[60]

Clinical data concerning the relevance of CYP2C19*2 carrier status are also available in the setting of coronary stenting. Sibbing *et al*. studied a large population of patients undergoing coronary stent placement after pretreatment with clopidogrel and found that carriers of at least one CYP2C19*2 allele were at significantly higher risk of definite stent thrombosis than non-carriers: the incidence of stent thrombosis was approximately three times higher in the CYP2C19*2 carriers (*1/*2 or *2/*2). The authors also reported a significant gene-dose effect, with homozygous allele carriers

at highest risk[61]. In a very recent case-control study, Harmsze *et al*. found that carriers of the CYP2C19*2 or CYP2C19*3 loss-of-function alleles respectively had a 1.7- and 2.4-fold increased risk of developing stent thrombosis after PCI.[62]

In contrast with these findings, the recently published large-scale analysis of the CURE trial population showed that the presence of loss-of-function alleles in patients with ACS was not associated with an increased risk of major cardiovascular events and there was no significant difference in the effects of clopidogrel treatment on clinical outcomes when the patients were stratified on the basis of their metaboliser phenotype. [71]

However, a recent meta-analysis of 9685 ACS patients involved in nine clopidogrel pharmacogenetic studies (including 91.5% undergoing PCI) by Mega *et al*. found a significantly increased risk of the composite endpoint of cardiovascular death, MI or stroke in carriers of one or two loss-of function alleles (HR 1.55, 95% CI 1.11-2.17; HR 1.76, 95% CI 1.24-2.50).[63] The strongest association between carriers of the CYP2C19 genotype and adverse cardiovascular outcomes therefore seems to be limited to patients with ACS undergoing PCI with stenting, and a reasonable explanation for the controversial data of the CURE population analysis may be that PCI with stenting was less extensively used (14.5%).

There are only few data concerning the relationship between an aggravated response to clopidogrel and bleeding events. It has recently been found that the novel allelic variant CYP2C19*17, which is characterised by a mutation that increases the transcription of CYP2C19, induces more rapid substrate metabolisation of substrates. Sibbing *et al*. reported that CYP2C19*17 carrier status is significantly associated with an enhanced response to clopidogrel treatment and a 4-fold increase

in the risk of bleeding events, and has no protective effect on the occurrence of ischemic events[64] .

PON 1

A genome-wide association study performed to identify markers of clopidogrel response in 429 healthy Amish subjects administered a 300 mg oral loading dose of clopidogrel followed by 75 mg daily for six days. Interestingly, the strongest association with a diminished clopidogrel response was found for the *rs12777823* polymorphism, which is in linkage disequilibrium with *CYP2C19*2* . However, platelet response to clopidogrel was not entirely accounted by CYP2C19 status and the CYP2C19*2 genotype explained only 12% of the variation, thus suggesting that other genetic variants might be important.[24] Bouman *et al*. used *in vitro* metabolomic profiling techniques to identify paraoxonase-1 (PON1) as the crucial enzyme for clopidogrel bioactivation, with its common Q192R polymorphism determining the rate of active metabolite formation. In comparison with subjects with the *PON1* RR192 or QR192 genotype, QQ192 homozygous subjects were at considerably higher risk of stent thrombosis (OR 3.6; 95% CI 1.6-7.9; *P* = 0.003), less plasma PON1 activity, lower plasma concentrations of the active metabolite, and a lower degree of platelet inhibition. In this study, the PON1 Q192R polymorphism explained 72.5% of the variability in ADP-stimulated platelet aggregation after clopidogrel administration, and is therefore likely to be the primary predictor of clopidogrel response.[26]

P2Y12

Studies have also assessed genetic variations in the gene encoding the P2Y12 receptor. It has been found that some genetic polymorphisms called haplotype H2 are strongly associated with increased ADP-induced platelet aggregation in healthy volunteers[72,73]. However, their clinical impact was not confirmed in the FAST-MI registry, the only published study evaluating this hypothesis in patients with MI,[55] and so these findings should be considered only exploratory.

Clinical implications

In March 2010, the American Food and Drug Administration (FDA) issued a black-box warning noting that carriers of two reduced-function CYP2C19 alleles show a diminished response to standard doses of clopidogrel.[74] It also pointed out that tests are available to identify patients with genetic polymorphisms, for whom alternative treatment strategies should be considered. The FDA did not explicitly recommend CYP2C19 genetic testing in patients prescribed clopidogrel, and did not offer any specific guidance on drug dosing in carriers of the CYP2C19 variant allele, thus leaving it uncertain as to how the warning should be translated into clinical practice.

In July 2010, the American College of Cardiology Foundation (ACCF) and the American Heart Association (AHA) published a Clinical Alert in response to the FDA's black box warning on clopidogrel[75] stating that the evidence base is insufficient to recommend routine genetic testing, but it may be considered before starting clopidogrel therapy in patients believed to be at moderate or high risk of a poor outcome. The impact of genotype-guided algorithms for the use of clopidogrel on clinical outcomes has not yet been adequately tested in prospective controlled trials,

and few data are available concerning alternative clopidogrel dosing strategies.[76-79] Some investigators have tried to determine whether a different loading dose can help patients carrying loss-of-function genotypes. In a substudy of the PRINC trial, a higher loading dose of clopidogrel improved platelet inhibition as measured by the VerifyNow analyser in carriers of loss-of-function alleles,[78] and a higher maintenance dose for one week helped maintain platelet inhibition. Bonello *et al*. also reported that repeated loading doses of clopidogrel could help most patients carrying the *CYP2C19*2* allele to overcome high platelet reactivity during treatment as measured by the VASP assay.[79] However, neither study investigated long- term clinical outcomes. Current evidence based on the results of the CURRENT-OASIS 7 and GRAVITAS trials does not support the use of high-dose clopidogrel. The CURRENT-OASIS 7 trial found no benefit in doubling the clopidogrel dose (HR 0.94; 95% CI 0.83-1.06; P = 0.30) and an increase in bleeding rates with high-dose clopidogrel (HR 1.24; 95% CI 1.05-1.46; P = 0.01).[80] The GRAVITAS trial, did not find any benefit from a double dose of clopidogrel in terms of cardiovascular outcomes or stent thrombosis in patients with high residual reactivity identified by a single platelet function test after PCI.[81,82] In conclusion, although many physicians have been using high-dose clopidogrel as a default strategy in non-responders, it can now be said that this is not the best solution and alternative treatment strategies should be sought.

Over the last few years, two new antiplatelet agents have been developed with the aim of overcoming limitations of clopidogrel. Prasugrel, a third-generation thienopyridine, irreversibly binds the ADP P2Y12 receptor and provides more rapid and more potent platelet inhibition than standard doses of clopidogrel as a result of

more efficient metabolism,[8] and ticagrelor, a new oral reversible non-thienopyridine platelet inhibitor, directly binds the ADP P2Y12 receptor, does not require metabolic activation and, in comparison with clopidogrel, provides greater platelet inhibition with a more rapid onset.[7]

Interestingly, the impact of CYP2C19 alleles and the ABCB1 genotype do not significantly affect pharmacological or clinical outcomes in patients treated with prasugrel or ticagrelor. The pharmacogenetic analysis of the TRITON-TIMI 38 population found that CYP2C19 and ABCB1 genetic variants had no significant effect on the pharmacokinetic, pharmacodynamic or clinical outcomes of patients taking prasugrel,[25] and the genetic substudy of the PLATO trial clearly showed that ticagrelor is more effective than clopidogrel in treating ACS regardless of CYP2C19 polymorphisms.[56]

The higher benefit/risk ratio of the new drugs seems to be particularly relevant in patients at higher risk of responding poorly to clopidogrel, but is not conclusive for patients who do not carry any CYP2C19 loss-of-function alleles. There is no planned head-to-head comparison with regard to genotype data, but the currently available evidence suggests that clopidogrel may remain the drug of choice in terms of benefit/risk and benefit/cost ratios for the homozygous CYP2C19 wild-type patients who represent the majority of treated patients, whereas the new drugs may be preferred in patients carrying the two loss-of-function variant alleles of CYP2C19.

Genotype-directed decisions regarding the antiplatelet agent to use in a specific patient also have a considerable economic impact when the costs of equally efficacious medications are very different, and this is particularly worth considering given the generic availability of clopidogrel and the consequent reduction in its cost.

In order to avoid wasting economic resources, clopidogrel should therefore continue to be administered to patients who do not carry deleterious CYP2C19 mutations. Bearing this in mind, it is clear that if genetic testing allows the use of a less expensive generic antiplatelet therapy, the tests will essentially pay for themselves.

Knowing that certain genetic profiles preclude or enhance the effects of clopidogrel may be critical for clinicians. For this reason, targeting clopidogrel therapy towards sensitive and away from resistant patients by means of genetic testing may become a reasonable treatment strategy. This will also have a significant pharmacoeconomic impact if a one-time test can be used in individual patients in order to determine clopidogrel sensitivity and thus allow its effective use as an alternative to the newer drugs in this class. The current clinical question is whether knowing the genetic make-up of one CYP enzyme provides enough information to identify patients who fall outside clopidogrel's therapeutic window and justify the use of alternative treatment. It has been estimated that 83% of the individual variance in clopidogrel responses is attributable to genetic effects, but the gene variants so far investigated explain only a small proportion of the variability.[83] There is growing evidence that genotyping for CYP2C19 alleles alone does not capture all of the genetic variability in the pharmacodynamic, pharmacokinetic or clinical responses to clopidogrel, and variants in other genes, such as ABCB1 or PON 1, seem to be important.[84-86] Moreover, the response to clopidogrel is unclear in the case of some CYP2C19 genotypes, such as reduced-function alleles in the presence of *17. The only study to assess the combined effect of *17 and reduced-function alleles directly found a gradient of effect on the pharmacodynamic response to clopidogrel. [65]

The benefits of DNA testing may be limited by the following considerations. As stated above, the CYP2C19*2 allele only explains 12% of the variation in platelet reactivity[24] and, although a number of commercial assays do also check for other loss-of-function alleles, few detect the CYP2C19*17 allele and none the ABCB1 and PON 1 variants. The predictive capacity of genotyping is also generally weak. Hochholzer *et al*. found that CYP2C19*2 carrier status was only 45% sensitive and 75% specific for detecting a high level of residual platelet activity as 53.3% of the CYP2C19*2 homozygous patients showed normal platelet reactivity, and 22.4% of the CYP2C19*1 homozygous subjects showed impaired reactivity.[69] Finally, the impact of genotype-guided algorithms for the use of clopidogrel on clinical outcomes has not yet been adequately tested in prospective controlled trials. Only when there are clinical data to support the hypothesis that genotype-guided therapy reduces the rate of ischemic and bleeding events in comparison with the standard approach will it be possible to justify the use of genetic testing in all potential patients. When that happens, genotype-guided antiplatelet therapy will also be available in the field of cardiovascular medicine.

STUDY BACKGROUND

Antiplatelet therapy is the cornerstone of medical treatment for patients experiencing an acute coronary syndrome (ACS). As a synergistic antiplatelet effect can be obtained by simultaneously inhibiting thromboxane-A2 and adenosine diphosphate P_2Y_{12} platelet receptors, the current standard of care includes dual antiplatelet therapy with aspirin, and one of the three currently available ADP P_2Y_{12} inhibitors: clopidogrel, prasugrel or ticagrelor. [1-6]

Clopidogrel has been the standard of care for nearly a decade, making it the second best-selling drug in the world. However, its clinical efficacy is limited by highly variable patient responses and the fact that it is associated with an increased risk of the re-occurrence of ischemic events. [7-13]

Recent research has highlighted the role of CYP enzyme genetic variations in determining the variability of antiplatelet responses to clopidogrel, and shown a clear relationship between lower levels of clopidogrel's active metabolite, reduced platelet inhibition, and a higher rate of major adverse cardiovascular (CV) events. [14-39] All of the CYP2C19 polymorphism data suggest that enzymatic activity and clopidogrel-induced platelet inhibition are reduced in carriers of the loss-of-function CYP2C19*2 allele (which is carried by nearly 30% of people of Western European ancestry and as many as 50% of those of Asian descent), [14,16,23,28-30,32,34-40] whereas carriers of the gain-of- function CYP2C19*17 allele show high levels of CYP activity and therefore greater platelet inhibition.[24,31,32]

The impact of loss-of-function alleles on clinical outcomes was clearly shown by the findings of the *post hoc* analysis of the TRITON-TIMI-38 trial. Among the clopidogrel-treated patients, the carriers of a CYP2C19 loss-of-function allele showed a relative

53% increase in the composite primary efficacy outcome of the risk of death due to CV causes, myocardial infarction (MI) or stroke (HR 1.53; 95% CI 1.07-2.19; p = 0.01). The trial also showed that heterozygous and homozygous carriers of the loss-of-function allele respectively experienced a 3-fold and 5-fold increase in stent thrombosis.[14] Two large meta-analyses confirmed the consistency of these data by showing that one copy of the CYP2C19*2 allele is associated with a significantly increased risk of major adverse CV events (HR 1.55) and stent thrombosis (HR 2.67). [30,40]

There are only some data concerning the relationship between a worse response to clopidogrel and bleeding events. It has been found that the novel allelic variant CYP2C19*17, which is characterised by a mutation that increases the transcription of CYP2C19, induces more rapid substrate metabolisation.[24,31,32] Sibbing *et al*. found that CYP2C19*17 carrier status is significantly associated with an enhanced response to clopidogrel treatment and a 4-fold increase in the risk of bleeding, and has no protective effect on the occurrence of ischemic events.[31]

Variations in the genes regulating clopidogrel absorption, such as ABCB1, may also affect the variability of the response to clopidogrel and clinical outcomes.[16,21,23] The bioavailability of clopidogrel is significantly reduced in carriers of the ABCB1 3435 polymorphism, and homozygous patients (TT) show an increased risk of adverse CV outcomes during treatment with clopidogrel in comparison with heterozygous patients (CT) and wild-type carriers (CC).[16] Patients who are carriers of a CYP2C19 reduced-function allele and/or are homozygous for ABCB1 3435 show an absolute 7.3% increase in the risk of death due to CV causes, MI or stroke in comparison with those carrying neither variant. [16]

On the basis of the above findings, the US Food and Drug Administration revised the product labelling of clopidogrel to include information about the availability of tests designed to identify patients with genetic polymorphisms, and highlighted the fact that healthcare providers should use alternative antiplatelet drugs for patients at increased risk of harm.[41,42] However, as it did not explicitly recommend CYP2C19 genetic testing in patients prescribed clopidogrel or offer any specific guidance on drug dosing in carriers of the CYP2C19 variant allele, it was not clear how the warning should be translated into clinical practice. [43-48]

With the aim of overcoming the limitations of clopidogrel, two new antiplatelet agents (prasugrel and ticagrelor) have been developed and approved for use in ACS patients over the last few years.[1-4] Interestingly, CYP2C19 alleles and the ABCB1 genotype do not significantly affect pharmacological or clinical outcomes in patients treated with either of these drugs. The pharmacogenetic analyses of the TRITON-TIMI-38 population found that the CYP2C19 and ABCB1 genetic variants had no significant effect on the pharmacokinetic, pharmacodynamic or clinical outcomes of patients taking prasugrel,[16] and the genetic substudy of the PLATO trial clearly showed that ticagrelor is more effective than clopidogrel in treating ACS regardless of CYP2C19 polymorphisms.[23]

The greater antiplatelet activity of these agents in comparison with clopidogrel reduced the rate of MI and CV death in the TRITON-TIMI-38 and PLATO trials, thus leading to a change in the ACS medical treatment paradigm in Europe.[3,4] The revised European guidelines downgraded clopidogrel to patients who cannot receive prasugrel or ticagrelor, which were both clearly recommended for all patients with ACS (Recommendation Class I, Evidence Level B). [1,2]

However, the guidelines do not mention some important limitations indicating that not all real-world patients with ACS are eligible for treatment with the new antiplatelet agents. First of all, the findings of theTRITON-TIMI-38 only relate to patients undergoing angioplasty because the trial excluded those for whom a conservative strategy or surgery was planned. The results revealed that prasugrel led to a 30% increase in the overall risk of bleeding (HR 1.32, 95% CI 1.03-1.68, p=0.03), and a small increase in the occurrence of fatal bleeding, both of which must be taken into account when individualising treatment.[3] The *post hoc* TRITON-TIMI-38 analyses identified three specific subgroups characterised by a reduced net clinical benefit driven by the excess bleeding risk.[3] In clinical practice, prasugrel is therefore contraindicated in patients with a history of stroke or transient ischemic attack (TIA), and caution is needed when it is used in patients with a low body weight or those aged >75 years. This evidence does not support the widespread use of prasugrel in everyday clinical practice. [49-52]

The PLATO findings are more generally applicable because the study included all categories of ACS patients regardless of whether it was planned to manage them invasively or non-invasively,[4] and showed that ticagrelor also seems to be burdened by a greater risk of bleeding than that associated with clopidogrel. Although CABG-related bleeding event rates were similar in the two treatment groups (HR 0.95, 95% CI 0.85-1.06, p=0.32), the rate of CABG-unrelated major bleeding was higher in the ticagrelor group (HR 1.19, 95% CI 1.02-1.38, p=0.03).[4] Ticagrelor is therefore contraindicated in patients with active pathological bleeding and those with a history of intracranial hemorrhage, and needs to be used with caution in all patients at increased risk of bleeding. Moreover, the mandated twice daily dosing of ticagrelor

and its frequently observed side effect of dyspnea are other factors that limit its general use.[49-52] Recent observational studies have shown that ticagrelor is prematurely discontinued by approximately 14% of patients. [53,54] It is therefore becoming increasingly clear that up to one-third of patients with ACS are not eligible for treatment with ticagrelor in the real world.

On the basis of the prasugrel and ticagrelor findings, and the increasing complexity and comorbidities of ACS patients, clopidogrel is still anything but obsolete and continues to be the mainstay of treatment. Clinicians should therefore consider all three antiplatelet agents in order to ensure that the right drug is given to the right patient. The appropriate selection of antiplatelet treatment has so far only been guided by the patients' phenotype, but it is possible to hypothesise that the addition of genotype-tailored strategies could aid in personalising the therapeutic approach to patients with ACS.

The aim of this study is to test the hypothesis that adding genotype data to clinical variables when making decisions concerning dual antiplatelet therapy improves the clinical outcomes of patients with ACS.

METHODS

STUDY DESIGN

The study is a two-armed, single blind, randomised controlled trial designed to test the hypothesis that adding genotype data to clinical variables when making decisions concerning dual antiplatelet therapy will improve the clinical outcomes of patients with acute coronary syndrome. The study was designed by the authors and approved by the institutional review board at Parma and at each participating clinical center.

STUDY POPULATION AND RANDOMISATION

From June 2013 through February 2015, 889 consecutive patients hospitalised because of an acute coronary syndrome were enrolled: about 50% of the patient population have experienced a non-ST-segment elevated acute coronary syndrome (NSTE-ACS) and 50% an ST-segment elevation acute coronary syndrome (STE-ACS). The diagnosis of ACS with or without ST-segment elevation were defined by the presence of at least two of the following criteria:

-ischemic symptoms at rest lasting > 20 minutes;

-electrocardiographic changes with ST-segment elevation or depression of at least 1 mm in two contiguous leads;

-cardiac biomarker (TnI/T or CK-MB) levels above the 99th percentile of the upper reference limit.

INCLUSION CRITERIA

- Diagnosis of ACS (STE-ACS or NSTE-ACS) during the index hospitalisation
- Age >18 years
- Ability to sign the informed consent form
- Ability to attend scheduled visits

EXCLUSION CRITERIA

- Cognitive or other causes of an inability to provide informed consent or follow study procedures
- Any contraindication to the use of ADP P2Y12 inhibitors

- Life expectancy <1 year
- Thrombolytic therapy within the previous 24 hours
- Known ABCB1, CYP2C19 *2 orCYP2C19 *17 genotype

Immediately after the diagnosis of myocardial infarction, the patients were randomly assigned to the strategy of choosing an ADP P_2Y_{12} inhibitor on the basis of clinical variables plus genotype data or on the basis of clinical variables alone. All participants gave written informed consent before taking part in the trial.

An automatic telephone randomisation system was used to assign the patients to treatment in a 1:1 ratio based on a centralised list. The randomisation list was stratified by diagnosis (ST-segment elevation *vs* all the rest) and by centre using the SAS PLAN procedure (SAS Institute Inc., Cary, North Carolina). The investigators had to digitise the patient's date of birth, diagnosis and site number by means of an interacting voice system in order to obtain the assigned allocation. The data were confirmed by e-mail and the print-out was included in the patient's case report form. The eligible patients were therefore randomised to undergo genetic testing for CYP2C19*2 (10q24.1-q24.3; rs4244285), CYP2C19*17 (10q24.1-q24.3; rs12248560) and ABCB1 3435 (7q21.1; rs1045642), or not.

GENOTYPING

Blood samples were taken from all of the patients for DNA analyses. The conventional genotyping methods so far used for diagnostic purposes will not be used in this study because appropriate labs may not be readily available and the processing time is prohibitive. The genotyping were carried out using the ST Q3

system, a compact platform enabling the classical laboratory analysis of DNA by means of real-time PCR that has been developed by ST Microelectronics and the researchers at Cardiology Division of Parma Hospital. The Q3 is designed as a low entry-cost portable system for foolproof use by unskilled personnel as a point-of-care instrument. The reaction takes place in a disposable Lab-on-Chip, which is pre-functionalised with all the required reagents. The user only has to add the sample through a simple loading and then the disposable device self-seals, providing results in approximately 70 minutes.

Briefly, about 10 μL of whole blood and a disposable Lab-on-a-Chip will be inserted into the instrument, which makes use of a TaqMan® Sample-to-SNP™ kit (Life Technlogies) for DNA release starting from whole blood. The DNA will subsequently be amplified by means of three drug metabolism assays (Life Technologies), each of which is specific for the SNPs under investigation. After switching on the instrument and launching the dedicated Q3 controlling software, the user will be guided through the easy operational steps by the software interface and dedicated videos. The reaction will last approximately 30 minutes, after which the software will display the SNP score and the patient's clopidogrel metaboliser status. All of the results will be automatically saved in a .pdf report file in order to ensure accurate data collection.

STUDY PROCEDURE AND FOLLOW-UP

The patients randomised to the pharmacogenomic arm received one of the P2Y12 receptor antagonists (clopidogrel, prasugrel or ticagrelor) on the basis of genotype (Figure 1) in addition to clinical variables. It is important to underline that the genetic algorithm was designed to consider always the three genes simultaneously, but the

ultimate decision on antiplatelet therapy selection was made taking into account also the clinical characteristics and was left to the discretion of the prescribers.

Unless medically contraindicated, it was suggested to administer clopidogrel to patients whose genetic test results indicate they are:

1)ABCB1 3435 wild type or heterozygous, CYP2C19*2 and CYP2C19*17 both wild type;

2)ABCB1 3435 wild type or heterozygous, CYP2C19*2 homozygous or heterozygous and CYP2C19*17 homozygous.

Because of the high risk of bleeding, it was suggested to use prasugrel or ticagrelor to patients whose genetic test results indicate they are:

1)ABCB1 3435 wild type or heterozygous, CYP2C19*2 wild type and CYP2C19*17 homozygous.

Because of the high ischemic risk, it was suggested to use prasugrel or ticagrelor to patients whose genetic test results indicate they are:

1)ABCB1 3435 homozygous if any CYP2C19*2 and CYP2C19*17 genotype;

2)ABCB1 3435 wild type or heterozygous, CYP2C19*2 wild type and CYP2C19*17 homozygous or heterozygous;

3) ABCB1 3435 wild type or heterozygous, CYP2C19*2 heterozygous if any CYP2C19*17 genotype;

4) ABCB1 3435 wild type or heterozygous, CYP2C19*2 homozygous and CYP2C19*17 wild type or heterozygous.

The clinical variables that were used to individualise the choice of P2Y12 receptor antagonist were: age, weight, ischemic risk, bleeding risk, diabetes, prior history of

stroke/TIA or intracranial bleeding, history of bleeding, active bleeding, anemia, chronic kidney disease.

Given the TRITON-TIMI-38 trial findings and exclusion criteria, prasugrel was not prescribed for 1) patients whose coronary anatomy was unknown; 2) those at high bleeding risk (particularly those with a history of stroke or TIA, those aged >75 years, and those with a body weight of <60 kg); 3) those receiving fibrinolytic therapy within the previous 24 hours; 4) those with active internal bleeding; 5) those with severe liver disease; 6) those taking oral anticoagulant therapy that cannot be stopped; 7) those with known clinically important thrombocytopenia.

Given the PLATO trial exclusion criteria, ticagrelor was contraindicated in: 1) patients with active pathological bleeding; 2) those with a history of intracranial bleeding; 3) those requiring dialysis, 4) those taking oral anticoagulant therapy that cannot be stopped; 5) those with known clinically important thrombocytopenia; 6) those receiving fibrinolytic therapy within the previous 24 hours; 7) those taking concomitant therapy with strong CYP3A inhibitors or inducers.

The patients were followed up for 12 months by means of outpatient visits after one, six and 12 months.

Education and on-site training

This was a multicentre study funded by the Emilia Romagna Region (Project Identification Number: E35E09000880002) and performed at the Cardiological Divisions of the four participating centres in Emilia-Romagna (Italy). Project implementation was preceded by a preliminary 2-year phase during which the engineers, clinicians, biotechnologists and geneticists working at the Cardiology

Division of Parma University Hospital and at ST Microelectronics developed, produced and validated the Q3 instrument, which provides genetic information directly and immediately at a patient's bedside.

All of the investigators were trained in the study procedures (DNA extraction, genotyping, randomisation, e-CRF data entry) during the Investigators' Meeting that took place at Parma Hospital in June 2013. Before the beginning of the study, an Initiation Visit was performed at each participating centre during which, a multidisciplinary team of cardiologists and geneticists delivered the study materials to each local investigator (DNA extraction system, pipettes (10-200 µL, 100-1000 µL), aerosol-resistant pipette tips, ST lab-on-chips, ST Q3 instrument and a PC with Q3 Software installed) and flow-charts showing DNA isolation, genotyping and randomisation. The expert team also provided to the installation and funzionalisation of each instrument, as well as the supervision of investigators during simulations of DNA isolation and genotyping simulations, in order to consolidate their newly-acquired skills.

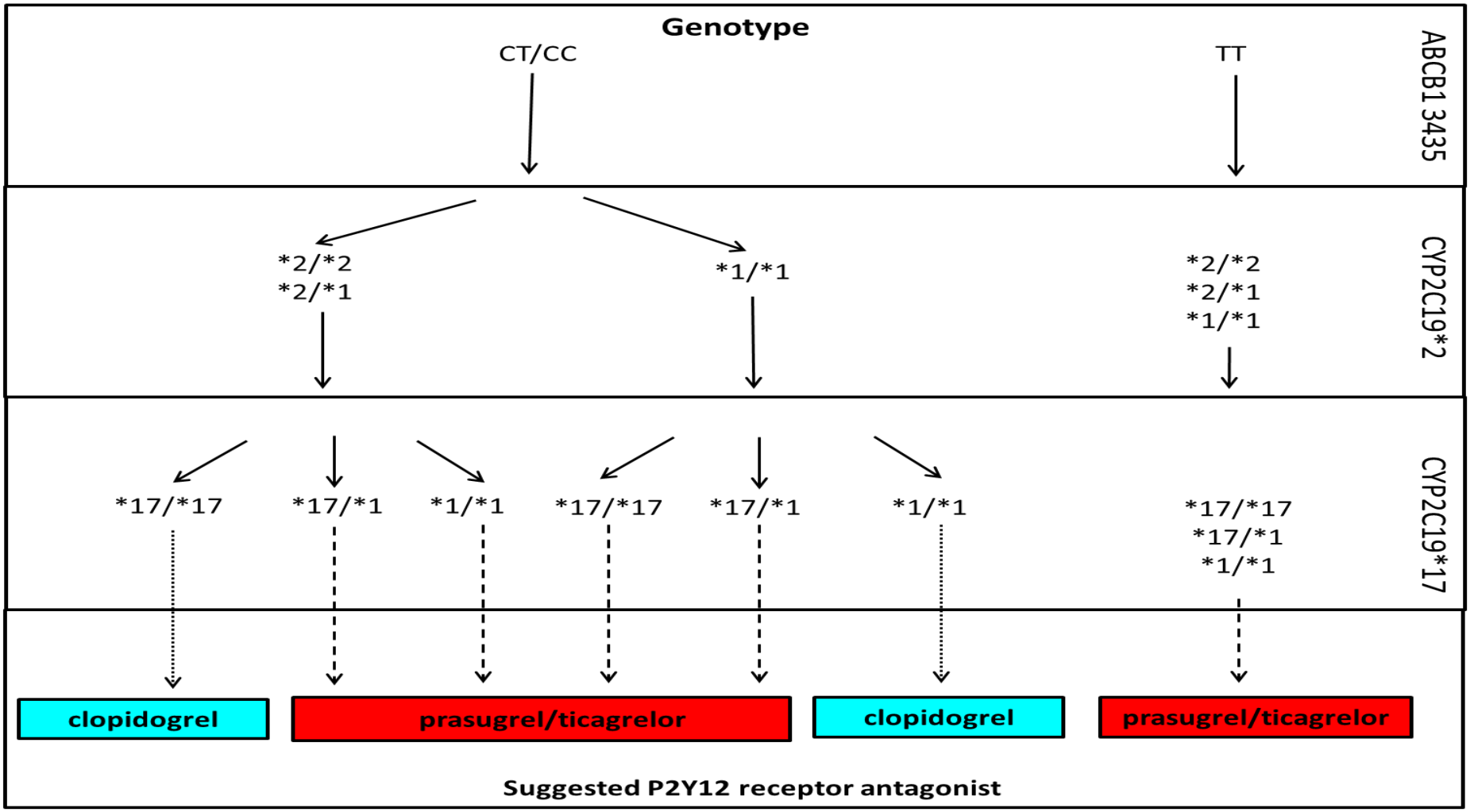
Genotype
CT/CC
TT
ABCB1 3435
*2/*2
*2/*1
*1/*1
*2/*2
*2/*1
*1/*1
CYP2C19*2
*17/*17
*17/*1
*1/*1
*17/*17
*17/*1
*1/*1
*17/*17
*17/*1
*1/*1
CYP2C19*17
clopidogrel
prasugrel/ticagrelor
clopidogrel
prasugrel/ticagrelor
Suggested P2Y12 receptor antagonist

Figure 1 legend. Genetic algorithm for the suggested P2Y12 antagonists. In ABCB1 homozygous patients it is suggested to administer prasugrel or ticagrelor regardless of the CYP2C19*2 and CYP2C19*17 genotype. In ABCB1 heterozygous or wild type patients it is suggested to administer clopidogrel if they are wild type for both CYP2C19*2 and CYP2C19*17 or if they are CYP2C19*2 homozygous or heterozygous and CYP2C19*17 wild type. For the all of the remaining CYP2C19*2 and CYP2C19*17 genotype combinations, it is suggested to administer prasugrel or ticagrelor.

STUDY OUTCOMES

The clinical events were identified by means of standard follow-up visits after one, six and 12 months or on the basis of telephone contacts using a common data collection form. When an event was recorded, the investigators provided source documents to the Clinical Event Committee. The cause of death was attributed by checking the death certificate.

All of the events were adjudicated by an independent Clinical Event Committee of two cardiologists who were unaware of the patients' randomisation. In the case of disagreement, the opinion of a third cardiologist was required.

The primary end-point was the composite of cardiovascular death and the first occurrence of non-fatal myocardial infarction, non-fatal stroke and BARC 3 to 5-defined major bleeding within 12 months of randomisation.

The secondary end-points were the composite of the primary end-point plus the occurrence of definite or probable stent thrombosis; the composite of the first occurrence of ischemic end-points (cardiovascular death, non-fatal myocardial

infarction and non-fatal stroke) and the composite of the first occurrence of the bleeding end-points namely BARC 3 to 5-defined major bleeding events.

End-point definitions

Cardiovascular death

All deaths reported were recorded and adjudicated. Cardiovascular death was defined on the basis of the patient's death certificate and included sudden cardiac death, death due to acute myocardial infarction, death due to heart failure, death due to a cerebrovascular event, death due to other cardiovascular causes (i.e. pulmonary embolism, aortic disease, cardiovascular intervention)

Myocardial infarction

Myocardial infarction was defined in accordance with the universal definition. Any one of the following criteria meets the diagnosis for myocardial infarction.

1. Detection of rise and/or fall of cardiac biomarkers (preferably troponin) with at least one value above the 99th percentile of the upper reference limit (URL) together with evidence of myocardial ischemia with at least one of the following:

    • Symptoms of ischemia;

    • ECG changes indicative of new ischemia (new ST-T changes or new left bundle branch block [LBBB]);

    • Development of pathological Q waves in the ECG;

    • Imaging evidence of a new loss of viable myocardium or a new regional wall motion abnormality.

2. Sudden, unexpected cardiac death, involving cardiac arrest, often with symptoms suggesting myocardial ischemia, and accompanied by presumably new ST elevation,

or new LBBB, and/or evidence of a fresh thrombus by coronary angiography and/or at autopsy, with death occurring before blood samples could be obtained or before the appearance of cardiac biomarkers in the blood.

3. For percutaneous coronary interventions (PCI) in patients with normal baseline troponin values, elevations of cardiac biomarkers above the 99th percentile URL were considered as being indicative of peri-procedural myocardial necrosis. By convention, increases in biomarkers of > 3 x 99^{th} percentile URL were designated as defining PCI-related myocardial infarction.

4. For coronary artery bypass grafting (CABG) in patients with normal baseline troponin values, elevations of cardiac biomarkers above the 99th percentile URL were considered as being indicative of peri-procedural myocardial necrosis. By convention, increases in biomarkers of > 5 x 99^{th} percentile URL plus new pathological Q waves or a new LBBB, or angiographically documented new graft or native coronary artery occlusion, or imaging evidence of a new loss of viable myocardium were designated as defining CABG-related myocardial infarction.

5. Pathological findings of an acute myocardial infarction.

Stroke

Stroke was defined as an acute episode of neurological dysfunction attributable to a central nervous system vascular cause. Stroke had to be documented by imaging (CT scan or magnetic resonance imaging [MRI] scan) or autoptic evidence. When possible stroke was classified as:

- Primary ischemic stroke, defined as an acute episode of focal brain, spinal, or retinal dysfunction caused by an infarction of central nervous system tissue and documented by imaging.

- Primary hemorrhagic stroke, defined as an acute episode of focal or global brain, spinal, or retinal dysfunction caused by non-traumatic intraparenchymal, intraventricular, or subarachnoid hemorrhage as documented by neuroimaging or autopsy. Microhemorrhages (<10 mm) evident only on MRI were not considered to be a hemorrhagic stroke.

BARC major bleeding

We defined major bleeding as BARC (Bleeding Academic Research Consortium) categories 3 to 5:

BARC 3:

- overt bleeding plus hemoglobin drop of 3 to <5 g/dL and/or any transfusion with overt bleeding (BARC 3a);

- or overt bleeding plus hemoglobin drop ≥ 5 g/dL and/or cardiac tamponade and/or bleeding requiring surgical intervention for control (excluding dental/nasal/skin/hemorrhoid) and/or bleeding requiring intravenous inotropes (BARC 3b);

- or intracranial hemorrhage (does not include microbleeds or hemorrhagic transformation; does include intraspinal) confirmed by autopsy or imaging or lumbar puncture and/or intra-ocular bleed compromising vision (BARC 3c).

BARC 4:

- CABG-related bleeding within 48 hours.

BARC 5:

- Fatal bleeding that directly causes death with no other explainable cause. BARC Fatal Bleeding is categorized as either *definite* or *probable* as follows:

- Type 5a

Definite fatal bleeding is bleeding that is directly observed or confirmed on autopsy or imaging.

- Type 5b

Probable fatal bleeding is bleeding that is clinically suspicious as the cause of death, but the bleeding is not directly observed and there is no autopsy or confirmatory imaging. The site of fatal bleeding is specified as intracranial, gastrointestinal, retroperitoneal, pulmonary, pericardial, genito-urinary, or other.

Stent thrombosis

Stent thrombosis was evaluated using the Academic Research Consortium criteria (4). Definite stent thrombosis was defined as the presence of angiographic or pathological confirmation of partial or total thrombotic occlusion within the peri-stent region and at least one of the following additional criteria: acute ischemic symptoms; ischemic ECG changes; elevated cardiac biomarkers. The definition of probable stent thrombosis required presence of unexplained death within 30 days after the procedure or acute myocardial infarction involving the target-vessel territory without angiographic confirmation.

STATISTICAL ANALYSIS

The sample size of the study was calculated on the assumption that the cumulative incidence of the primary end-point in the standard of care arm at 12 months was

25%. Given a relative risk reduction in the pharmacogenomic arm of 20%, 95% power and a type alpha error of 5%, the calculated sample size was 1,806 patients in each arm.

Descriptive statistics were used to compare the baseline characteristics of the two groups in order to test the randomisation process. The primary analysis was based on the intention-to-treat principle. Cox proportional hazard models were used to analyse the data relating to the primary and secondary end-points. The proportional hazards assumption for the Cox regression model was confirmed using the Schoenfeld residuals test. An Andersen–Gill intensity model analysis was not pre-specified, but was carried out in order to account for repeated occurrences of all of the components of the primary end-point during the study period, using a time-dependent model with separate hazard ratios before and after one month, 6 months and 12 months.

The cumulative incidence of the end-points during the 12 months of follow-up was graphically represented by means of Aalen-Johansen curves, and the significance of the difference between the sub-distribution of the hazards was tested using the Fine-Gray model. All of the tests were two sided at a significance level of 0.05. No interim analysis was planned and no multiplicity test correction was made. The statistical analyses were performed using R Statistical Software version 3.1.3.

RESULTS

A total of 888 patients were recruited between 12 June 2013 and 18 February 2015; 448 patients were randomised to the pharmacogenomic arm and 440 patients to the standard of care arm. This represents 24.6% of the pre-specified sample size because, on 18 February 2015, the Ethics Committee of Modena (Italy) required that the trial should be prematurely stopped and all of the patients followed up as planned, because of the lack of *in vitro* diagnosis (IVD) certification for the ST Q3 instrument.

The mean age of the population was 70.4±12 years (range 35-97), with 28.4% of the patients aged >80 years. Virtually all of the patients (96.4%) experienced the typical rise and fall of cardiac markers indicating an acute myocardial infarction and 96% underwent coronary angiography. Seventy three percent underwent revascularisation: 62.4% by means of a percutaneous coronary intervention and 11% by means of coronary artery by-pass grafting. The concomitant treatments were those currently used for patients with acute coronary syndromes. The two groups were well balanced in terms of the patients' baseline characteristics (Table 1).

Genotype

Table 2 shows the genotype distributions of the patients of the pharmacogenomic arm. Briefly, genotyping revealed that 47.1% had at least one copy of the ABCB1 3435 allele, and 26.4% were homozygous; 29.2% had at least one copy of the loss-of-function CYP2C19*2 allele, and 4.3% were homozygous; and 31.3% had at least one copy of the gain-of-function CYP2C19*17 allele, and 7.8% were homozygous.

The distribution of the P2Y12 receptor antagonists used during the acute phase and the 12 months of follow-up in the pharmacogenomic arm was clopidogrel in 43.3%,

prasugrel in 7.6% and ticagrelor in 42.6% of the patients. The corresponding figures in the standard of care arm were clopidogrel 50.7%, prasugrel 8.4% and ticagrelor 32.7%.. Respectively, 6.5% and 8.2% of the patients did not receive any P2Y12 receptor antagonists, mainly because of misdiagnosis, normal coronary angiograms or the concomitant use of oral anticoagulants. The difference in the distribution of the treatments with different P2Y12 receptor antagonists between the two arms was statistically significant (P=0.02).

Outcomes

One patient in the pharmacogenomic arm was lost to follow-up. The primary end-point occurred in 71 patients (15.9%) in the pharmacogenomic arm and in 114 patients (25.9%) in the standard of care arm (hazard ratio 0.58; 95% confidence interval, 0.43 to 0.78; P < 0.001) (Figure 2, Panel A). The vast majority of the primary end-points saved in the pharmacogenomic arm (31 out of 43; 72.1%) were observed in patients receiving clopidogrel. Among these patients, 48 (24.7%) in the pharmacogenomic arm and 79 (35.4%) in the standard of care arm experienced the primary end-point (hazard ratio 0.68; 95% confidence interval, 0.47 to 0.97; P=0.03) (Figure 2, Panel B).

Definite or probable stent thrombosis was observed in only eight patients, three in the pharmacogenomic arm and five in the standard of care arm, thus precluding an outcome analysis of this secondary end-point.

Ischemic end-points occured in 58 patients (13%) in the pharmacogenomic arm and 94 (21.4%) in the standard of care arm (hazard ratio 0.57; 95% confidence interval, 0.41 to 0.8; P < 0.001) (Figure 2, Panel C). Bleeding end-points occurred in 19 patients (4.2%) in the pharmacogenomic arm and 30 (6.8%) in the standard of care

arm (hazard ratio 0.62; 95% confidence interval, 0.35 to 1.1; P = 0.1) (Figure 2, Panel D).

Repeated ischemic and bleeding end-points were also less frequent in patients randomised to the pharmacogenomics arm: 85 *vs* 136 end-points (hazard ratio 0.61; 95% confidence interval, 0.47 to 0.8; P<0.001) .

DISCUSSION

Over last 10 years, there have been substantial advances in our understanding of the genetic variability associated with clopidogrel responses . However, although these genetic associations have been widely replicated and the effect sizes are sufficiently large to be predictive in the clinical setting, there are limited examples of the use of pharmacogenetic data concerning clopidogrel metabolism to guide clinical practice.

This prospective, randomized multicenter study provides evidence that the use of genomic medicine to select P2Y12 receptor antagonist can be successfully incorporated into the clinical care of patients with acute coronary syndromes. Acute coronary syndromes may be considered one of the most challenging clinical settings in which to use pharmacogenetic data to guide clinical practice because of the urgency of the situation and the need to start drug treatment promptly. In our case, this was made feasible by the development of a bedside instrument capable of providing genotype results within 70 minutes of blood sampling.

In patients with acute coronary syndromes, the selection of a P2Y12 receptor antagonist (clopidogrel, ticagrelor and prasugrel) is based on their individual clinical characteristics in order to obtain the best trade-off between ischemic events and bleeding complications. The main finding of the present study is that basing the

selection on the combination of the patients' clinical characteristics and genetic data related to clopidogrel metabolism led to a significantly lower rate of ischemic and bleeding events in comparison with usual practice. While it is relatively easy to explain why a knowledge of a patient's genetic data may lead to a better clinical outcome, it is more difficult to explain the magnitude of this effect. It is even possible that the effect size estimated in our sample size calculation could have been simply underestimated. A retrospective analysis of the Triton TIMI 38 trial by Mega et al. showed that the absolute difference in cardiovascular death, non-fatal myocardial infarction or non-fatal stroke was 7.3 percent between the most and least favourable ABCB1 and 2C19*2 genetic profile, with a relative difference of 50 percent. Another possible explanation of the magnitude of the effect size may be related to the percentage of patients receiving clopidogrel or ticagrelor in the two arms. As ticagrelor proved to be more effective than clopidogrel in the Plato trial(15), it is possible that some of the benefit may have been related to the more frequent use of ticagrelor in the pharmacogenomic arm. Finally, the ascertainment bias associated with the single-blind nature of the study may also partially account for the large magnitude of the effect size.

Given that the study was prematurely interrupted by the Ethical Committee and there are consequently large confidence intervals in the estimated relative risk reduction in the primary end-point, it would be unwise to evaluate the cost effectiveness of the pharmacogenomic approach to selecting P2Y12 receptor antagonists. Two larger randomised clinical trials with structured and standardise study protocols based on CYP2C19 genotyping are ongoing and will provide important results in the near future, including a cost effectiveness analysis (NCT 01761786; NCT 01742117)

Conclusions

In the present study, we demonstrate that the implementation of multiple genotyping to guide antiplatelet therapy in patients with acute coronary syndromes the implementation is feasible across multiple institutions. Our data also demonstrate that a more personalised approach to the selection of antiplatelet therapy leads to a clinically meaningful reduction in the combined end-point of ischemic and bleeding complications. Future studies of genotype-guided antiplatelet therapy may be of value to determine the cost-efficacy of the genotyping approach in the challenging setting of acute coronary syndromes before implementing it in everyday clinical practice.

Funding acknowledgements

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TABLE 1. Demographic, clinical, angiographic and treatment characteristics of the study population.

Characteristics	All patients (No=888)	Pharmacogenomic arm (No=448)	Standard of care arm (No=440)
Demographics			
Mean age ± SD, years	70.9 (± 12.2)	71.1 (± 12.3)	70.7 (± 12.1)
-<70 years	361/888 (40.6)	186/448 (41.5)	175/440 (39.8)
-70-80 years	275/888 (31.0)	130/448 (29.0)	145/440 (33.0)
->80 years	252/888 (28.4)	132/448 (29.5)	120/440 (27.2)
Female sex - No.(%)	283/888 (32.0)	153/448 (34.2)	130/440 (29.6)
Cardiovascular risk factors - No (%)			
-Family history	198/888 (22.3)	96/448 (21.4)	102/440 (23.2)
-Hypertension	660/888 (74.3)	331/448 (73.9)	329/440 (74.8)
-Dyslipidemia	483/888 (54.4)	251/448 (56.0)	232/440 (52.7)
-Diabetes mellitus	235/888 (26.5)	113/448 (25.2)	122/440 (27.7)
-Habitual smoker	200/888 (22.5)	92/448 (20.5)	108/440 (24.6)
-BMI (kg/m^2) - No			
<25	117/888 (13.2)	62/448 (13.8)	55/440 (12.5)
25-30	619/888 (69.7)	311/448 (69.4)	308/440 (70.0)
>30	152/888 (17.1)	75/448 (16.8)	77/440 (17.5)
History - No (%)			
-Previous MI	191/888 (21.5)	96/448 (21.4)	95/440 (21.6)
-Previous stable angina	50/888 (5.6)	25/448 (5.6)	25/440 (5.7)
-Previous PCI ¶	169/888 (19.0)	81/448 (18.1)	88/440 (20.0)
-Previous CABG	80/888 (9.0)	43/448 (9.6)	37/440 (8.4)
-Previous stroke	63/888 (7.1)	35/448 (7.8)	28/440 (6.4)
-Peripheral arterial disease	95/888 (10.7)	40/448 (8.9)	45/440 (10.2)
-Permanent atrial fibrillation	41/888 (4.7)	22/448 (4.9)	19/440 (4.3)
-Chronic kidney disease	76/888 (8.6)	35/448 (7.8)	41/440 (9.3)
-COPD	71/888 (8.0)	33/448 (7.4)	38/440 (8.6)
Acute coronary syndrome - No (%)			
-STEMI	248/888 (28.0)	117/448 (26.1)	131/440 (29.8)
-NSTEMI	608/888 (68.4)	315/448 (70.3)	293/440 (66.5)
-Unstable Angina	17/888 (1.9)	8/448 (1.8)	9/440 (2.1)
-No acute coronary syndrome †	15/888 (1.7)	8/448 (1.8)	7/440 (1.6)
Coronary angiography - No (%)			
-Angiography performed	855/888 (96.2)	433/448 (96.6)	422/440 (95.9)
-Single-vessel disease	265/855 (30.9)	124/433 (28.6)	141/422 (33.4)

-Two-vessel disease	234/855 (27.3)	125/433 (28.8)	109/422 (25.8)
-Multi-vessel disease	231/855 (27.0)	119/433 (27.4)	112/422 (26.5)
-Left main coronary artery	105/855 (12.2)	60/433 (13.8)	45/422 (10.6)
-Left anterior descending coronary artery	460/855 (53.8)	236/433 (54.5)	224/422 (50.6)
-Circumflex coronary artery	315/855 (36.8)	174/433 (40.1)	140/422 (33.1)
-Right coronary artery	406/855 (47.4)	203/433 (46.8)	203/422 (48.1)
-Other vessels	265/855 (30.9)	125/433 (28.8)	140/422 (33.1)
Revascularisation - No (%)			
PCI	532/855 (62.2)	268/433 (61.8)	264/422 (64.2)
CABG	92/855 (10.7)	49/433 (11.3)	43/422 (10.1)
Medical treatment - No (%) ‡			
Aspirin	860/888 (97.0)	437/448 (97.6)	423/440 (96.1)
Beta-blocker	751/888 (84.6)	382/448 (85.3)	369/440 (83.9)
ACEi and ARB	856/888 (74.1)	342/448 (76.0)	316/440 (71.8)
Lidip-lowering drug	761/888 (85.6)	386/448 (86.2)	375/440 (85.2)
Calcium-channel inhibitor	243/888 (27.4)	120/448 (26.8)	123/440 (28.0)
Warfarin	48/888 (5.4)	21/448 (4.7)	27/440 (6.1)

P>0.05 for all comparisons. BMI: body mass index; MI: myocardial infarction; PCI: percutaneous coronary intervention; CABG: coronary artery by-pass grafting; COPD: chronic obstructive pulmonary disease. STEMI: ST elevation myocardial infarction. NSTEMI: non-ST-elevation myocardial infarction. ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers.

¶ A total of 93% PCIs involved stenting.

† No acute coronary syndrome: this category includes patients with a misdiagnosis of acute coronary syndrome (i.e. myocarditis).

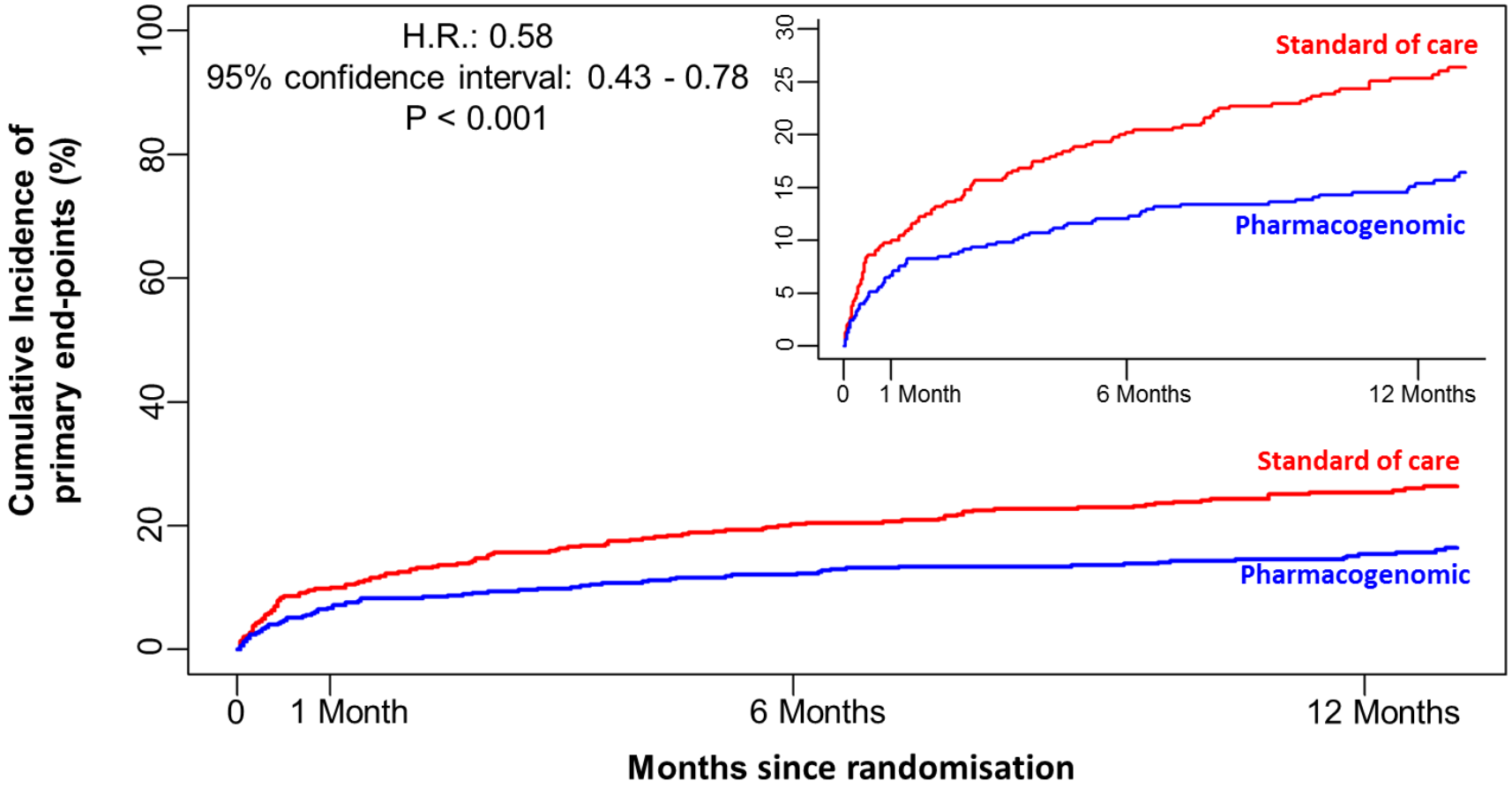
‡ All medical treatments were prescribed during hospitalisation and upon discharge.

TABLE 2. Frequency distribution of genetic variants and selected P2Y12 receptor antagonist

Characteristics	Pharmacogenomic arm (No=448)	Standard of care arm (No=440)
ABCB1 3435 genotype, No (%)		
Wild type (C/C)	119/448 (26.5)	NA
Heterozygous (C/T)	211/448 (47.1)	NA
Homozygous (T/T)	118/448 (26.4)	NA
CYP2C19*2 genotype, No (%)		
Wild type (*1/*1)	298/448 (66.5)	NA
Heterozygous (*1/*2)	131/448 (29.2)	NA
Homozygous (*2/*2)	19/448 (4.3)	NA
CYP2C19*17 genotype, No (%)		
Wild type (*1/*1)	273/448 (60.9)	NA
Heterozygous (*1/*17)	140/448 (31.3)	NA
Homozygous (*17/*17)	35/448 (7.8)	NA
P2Y12 receptor antagonist, No (%)*		
Clopidogrel	194/448 (43.3)	223/440 (50.7)
Prasugrel	34/448 (7.6)	37/440 (8.4)
Ticagrelor	191/448 (42.6)	144/440 (32.7)
No P2Y12 receptor antagonist §	29/448 (6.5)	36/440 (8.2)

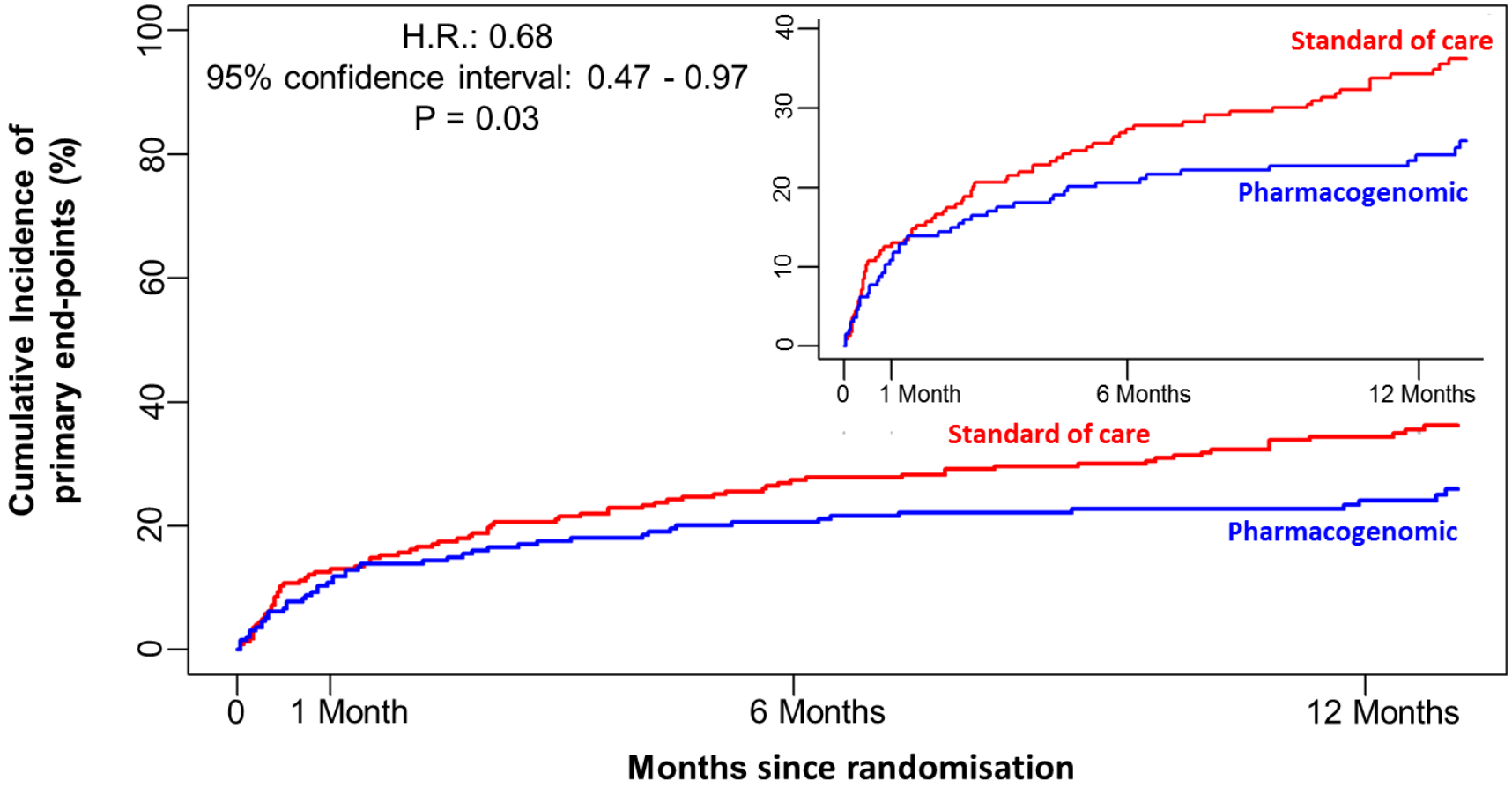
* P2Y12 receptor antagonist: P2Y12 receptor antagonist prescribed during hospitalisation and upon discharge. P value for global comparison = 0.02

§ No P2Y12 receptor antagonist: patients who did not receive any P2Y12 receptor antagonists during hospitalisation or upon discharge (because of misdiagnosis, a high bleeding risk, or concomitant oral anticoagulant therapy). ¶ P2Y12 receptor antagonist switch: any switch from one P2Y12 receptor antagonist to another during the 12-month follow-up.



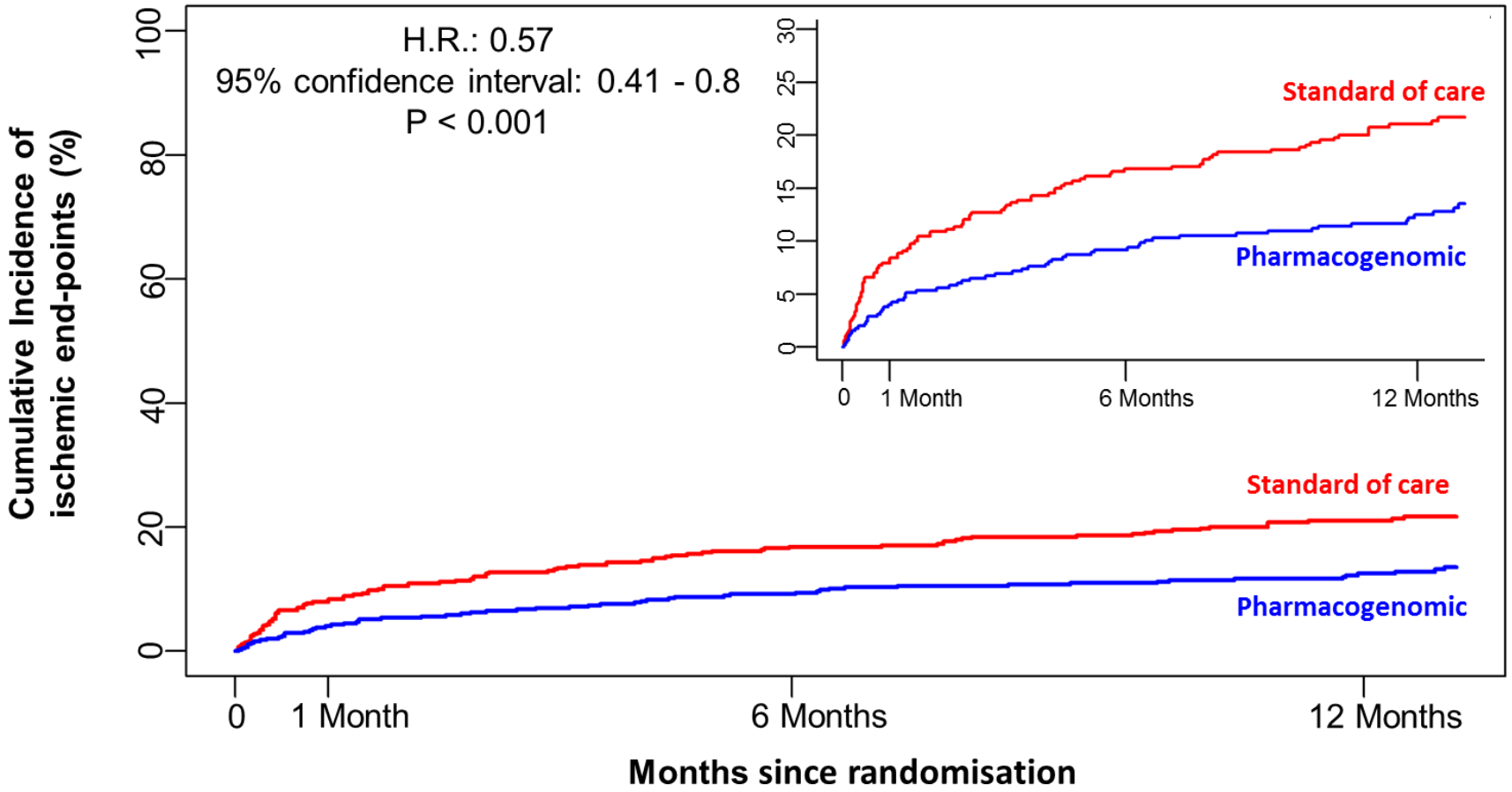
No. at risk

Pharmacogenomic arm	448	416	390	295
Standard of care arm	440	397	349	280



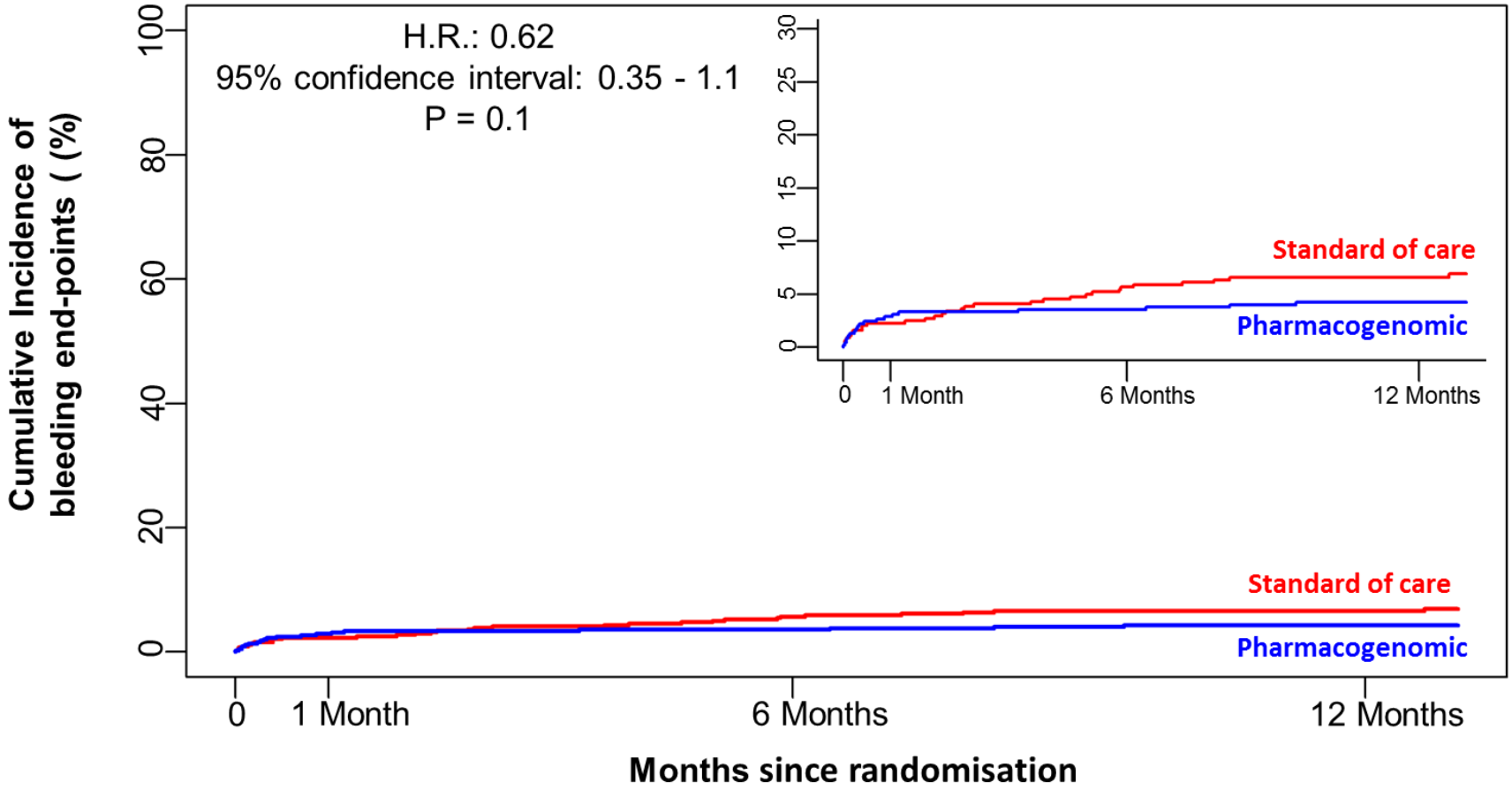
No. at risk

Pharmacogenomic arm	194	173	152	102
Standard of care arm	223	195	161	127



No. at risk

Pharmacogenomic arm	448	428	402	304
Standard of care arm	440	404	362	294



No. at risk

Pharmacogenomic arm	448	423	406	311
Standard of care arm	440	420	385	319

Supplementary Material – Figure 1

Subgroup	All patients	Standard of care arm (%)	Pharmacogenomic arm (%)	HR (95% CI)	Interaction P-value
All patients	888	25.9	15.9	0.58 [0.43-0.78]	<0.001
Age at randomiation (yrs)					0.07
<75 yrs	400	16.9	6.9	0.38 [0.22-0.67]	
≥75 yrs	488	36.9	26.7	0.71 [0.5-1]	
Sex					0.56
Male	605	24.2	13.6	0.53 [0.36-0.78]	
Female	283	30	20.3	0.64 [0.4-1.02]	
Qualifying MI					0.91
STEMI	244	20	11.4	0.56 [0.29-1.08]	
All the rest	644	28.4	17.4	0.57 [0.41-0.8]	
History of diabetes					0.32
Diabetes	235	35.2	25.7	0.72 [0.45-1.15]	
No diabetes	653	22.3	12.5	0.53 [0.36-0.78]	
Chronic kidney disease					0.87
Chronic kidney disease	76	51.2	31.4	0.56 [0.27-1.15]	
No chronic kidney disease	812	23.3	14.5	0.6 [0.43-0.82]	
Coronary artery disease					0.41
Multivessel disease	465	27.6	20.5	0.71 [0.49-1.03]	
Single vessel disease	387	21.5	7.5	0.33 [0.18-0.6]	
Previous revascularization					0.67
Previous revascularization	215	33.3	18.7	0.52 [0.3-0.9]	
No Previous revascularization	673	23.5	15	0.61 [0.43-0.87]	
Percutaneous revascularization					0.68
Percutaneous revascularization	532	22.7	12.7	0.54 [0.35-0.82]	
No percutaneous revascularization	356	30.7	20.6	0.62 [0.41-0.94]	

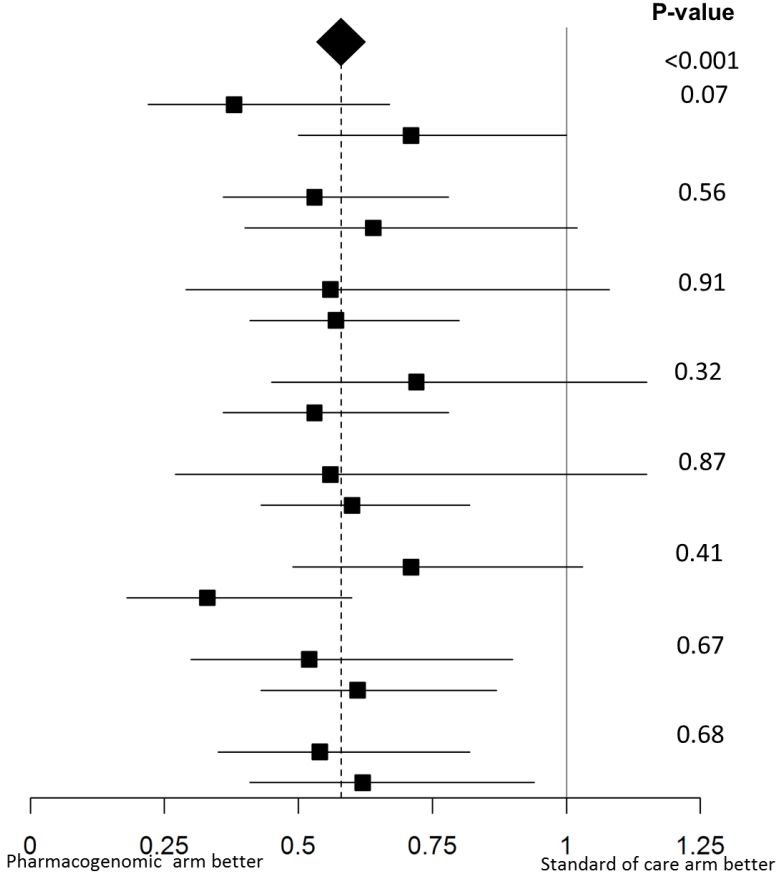


Figure legends

Figure 2. Cumulative incidence of ischemic and bleeding events at 12 months according to study arm.

Panel A shows the Aalen-Johansen curves of cumulative incidence of primary end-point (cardiovascular death, non-fatal MI, non-fatal stroke and BARC defined mayor bleeding 3 to 5). Data for the primary end-point are also shown in the subgroup of patients who received clopidogrel during the index hospitalization (Panel B). In panel C are displayed the Aalen-Johansen curves of cumulative incidence of ischemic endpoints and in panel D the Aalen-Johansen curves of cumulative incidence of bleeding endpoints.

The difference between sub-distribution hazard of the 2 study arms in all panels were tested using the Fine-Gray model.

The insets show the same data on an enlarged y axis.

Figure 1S. Hazard ratios and rates of the primary endpoint, according to selected subgroups of study patients.

The primary endpoint was defined as the composite of cardiovascular death, non-fatal MI, non-fatal stroke and BARC defined mayor bleeding 3 to 5. The percentages are estimates of the rate of the endpoint at 12±1 months of follow up. For each subgroup, the squares represent the point estimate of the effect of the strategy of selection of P2Y12 inhibitors. The horizontal lines indicates the 95% confidence intervals. The overall effect of strategy of selection of P2Y12 inhibitors in pharmacogenomics and standard of care arm is represented by the

diamond, and the dashed vertical line represents the corresponding overall point estimate.

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