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Relation of CYP2C19 loss-of-function polymorphism to the occurrence of stent thrombosis

Betti Giusti[†], Anna Maria Gori, Rossella Marcucci & Rosanna Abbate
[†]*University of Florence and SOD Atherothrombotic Diseases, Department of Medical and Surgical Critical Care, AOU Careggi, Viale Morgagni 85, 50134 Florence, Italy*

Importance of the field: Major adverse cardiovascular events including stent thrombosis associated with residual platelet reactivity on antiplatelet treatment in high risk vascular patients is a hot issue that needs a strong effort to be solved. Dual antiplatelet therapy with clopidogrel and aspirin prevents ischemic events and improves outcomes following acute coronary syndromes and percutaneous coronary intervention. However, adverse cardiovascular events occur in these patients, and several studies have shown that patients who suffer cardiovascular complications have high post-treatment platelet reactivity despite antiplatelet treatment. Clopidogrel requires conversion to active metabolite by CYP isoenzymes. Recently, CYP2C19*2 polymorphism (G681A nucleotide substitution) has been shown to be associated with decreased metabolisation of clopidogrel, poor antiaggregant effect and increased adverse cardiovascular events.

Areas covered in this review: This review summarises the principal studies contributing to establish the relationship between CYP2C19*2 polymorphism and adverse outcomes in high risk patients on clopidogrel treatment.

Take home message: Prospective studies are urgently needed to determine the clinical impact of a score that takes into account individual characteristics of patients – CYP2C19*2 genotypes, residual platelet reactivity, drug–drug interaction, as well as traditional and procedural risk factors – for the identification of the therapeutic strategy that provides the best benefit for the single subject.

Keywords: acute coronary syndromes, antiaggregant therapy, aspirin, clopidogrel, CYP2C19 loss-of-function polymorphism, drug eluting stent, major adverse cardiovascular events, percutaneous coronary interventions, stent thrombosis

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1. Introduction

Dual antiplatelet treatment with clopidogrel and acetyl salicylic acid (aspirin) is the standard treatment for the prevention of stent thrombosis (ST) [1-3]. Retrospective studies have shown that the discontinuation of clopidogrel, even after ≥ 6 months after stent implantation, is associated with an increased risk of thrombotic events in patients with drug-eluting stents (DESs) [4-7]. However, ST can also occur in patients taking clopidogrel and aspirin, and several retrospective and prospective studies [8-18] have shown that patients who suffer ST have a high post-treatment platelet reactivity despite the dual antiplatelet treatment, suggesting that platelet aggregation nonresponsiveness to clopidogrel is the main cause of the thrombotic event.

Multiple potential or well-documented mechanisms have been identified (**Figure 1**). Extrinsic or chronic mechanisms include inadequate drug compliance [19], drug–drug

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Article highlights.

- MACEs and in particular ST, a potentially lethal complication, are a critical clinical problem in the management of patients undergoing percutaneous coronary revascularisation with stent implantation. Dual antiplatelet treatment with aspirin and clopidogrel is mandatory in patients undergoing stent implantation. High on-treatment platelet reactivity has been associated with increased risk of thrombotic complications.
- The prodrug clopidogrel needs conversion to its active metabolite by hepatic CYP enzyme system. CYP2C19 is one of the most relevant CYP450 isoforms involved in the drug metabolism.
- Inadequate drug compliance, drug–drug interactions, age, diabetes mellitus, elevated body mass index and left ventricle ejection function, inflammation and accelerated platelet turnover are determinants of high on-clopidogrel platelet reactivity.
- CYP2C19*2 polymorphism is associated with decreased metabolisation of clopidogrel and poor antiplatelet effect both in healthy subjects and high risk vascular patients on dual antiplatelet therapy.
- CYP2C19*2 polymorphism is associated with increased adverse cardiovascular events and in particular the severe complication of ST.
- Future prospective studies, taking into account CYP2C19*2 genotypes, residual platelet reactivity, drug–drug interaction, as well as traditional and procedural risk factors, are needed for the identification of the therapeutic strategy that provides the best benefit for the single subject.

This box summarises key points contained in the article.

interactions [20-23], age, diabetes mellitus, elevated body mass index and left ventricle ejection function [14,19]. Transient mechanisms include inflammation [24], accelerated platelet turnover [25], reticulated platelets (RPs) [25,26], erythrocyte deformability [27,28] and ADAMTS-13 activity [29].

Among the possible mechanisms, common genetic variants could play a pivotal role in determining the individual susceptibility to antiplatelet drug response. Several polymorphisms in genes coding platelet components [glycoprotein (Gp)Ia, GpIIIa, GpIbalpha, GpVI, ADP receptor $P2Y_{12}$ (P2Y12), P-selectin, COX-1, COX-2,], eNOS or CYP enzyme isoforms (-3A4, -3A5) have been proposed and investigated [30-33]. As regards responsiveness to clopidogrel, studies in healthy subjects on clopidogrel treatment [34,35] and in acute coronary syndrome (ACS) patients undergoing percutaneous coronary interventions (PCIs) on dual antiplatelet treatment [36,37] showed that the CYP2C19*2 allelic variant is an independent predictor of antiplatelet treatment variability.

Recently, different groups have contemporarily demonstrated in different clinical settings and at different follow-up times that CYP2C19*2 is a determinant of the occurrence of major adverse cardiovascular events (MACEs) in patients on clopidogrel therapy [38-42] with an estimated risk factor ranging from 1.5 to 6.0.

In this article, we review the evidences on the relation of CYP2C19 loss-of-function polymorphism to the occurrence of coronary ST.

2. Clopidogrel metabolism

Diverse agonists are known to activate platelet aggregation and fibrinogen binding to the subsequently activated integrin GPIIb–IIIa complex. In this process, ADP is of particular importance because it is released by damaged cells and activated platelets, thus enhancing the action of many platelet activators [43]. ADP mediates platelet aggregation through its binding to two G protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$, acting together to achieve complete aggregation [44]. $P2Y_{12}$ also has an important role in amplifying the responses to other agonists. In fact, ADP is released from platelet dense granules regardless of the activating stimulus, be it thrombin, collagen, thromboxane A2 or other agonists. Therefore, $P2Y_{12}$ has a major role in arterial thrombosis and the concomitant inflammatory response and pharmacological targeting of this receptor has become an important strategy in cardiovascular disease. Clopidogrel, a $P2Y_{12}$ ADP-receptor antagonist, is a prodrug converted to its active thiol metabolite (SR26334) in two sequential oxidation steps, both occurring in the liver, by the hepatic CYP450 (**Figure 2**) [45,46]. Clopidogrel is absorbed in the duodenum: the P-glycoprotein coded by the ABCB1 gene is involved in the intestinal transport. Only ~ 15% of clopidogrel undergoes metabolism by CYP450. It is mostly hydrolysed by esterases to an inactive carboxylic acid derivative that accounts for 85% of clopidogrel related circulating compounds. The CYP450 mediated steps, whose good function is crucial for the adequate pharmacodynamic process, compete with the esterase pathway. Several CYP450 enzyme isoforms with different contributions are responsible for the oxidation of the thiophene ring of clopidogrel to 2-oxoclopidogrel (first oxidation step: CYP1A2, 35.8%; CYP2B6, 19.4%; and CYP2C19, 44.9%), which is further oxidised (second oxidation step: CYP2B6, 32.9%; CYP2C9, 6.76%; CYP2C19, 20.6%; and CYP3A4, 39.8%), resulting in the opening of the thiophene ring and the formation of both a carboxyl and a thiol group [45,47]. The latter forms a disulfide bridge with the two extracellular cysteine residues located on the ADP $P2Y_{12}$ receptor expressed on the platelet surface and causes an irreversible blockade of ADP binding for the platelet's life span [48]. Among the different CYP450 isoenzymes involved in clopidogrel metabolism, CYP2C19 contributes substantially to both oxidative steps of the clopidogrel conversion to its active metabolite explaining the pivotal role of genetic polymorphisms in the gene coding these enzyme isoforms. In particular, a single nucleotide polymorphism (SNP) (CYP2C19*2) has been associated with inactivation of the enzyme and impaired metabolism of drugs, including clopidogrel, via this pathway.

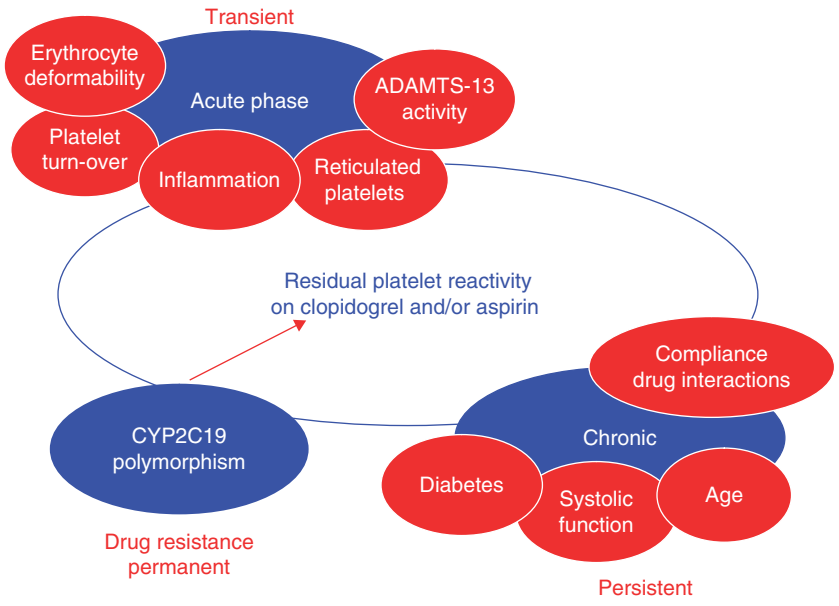

Figure 1. Mechanisms of clopidogrel response variability.

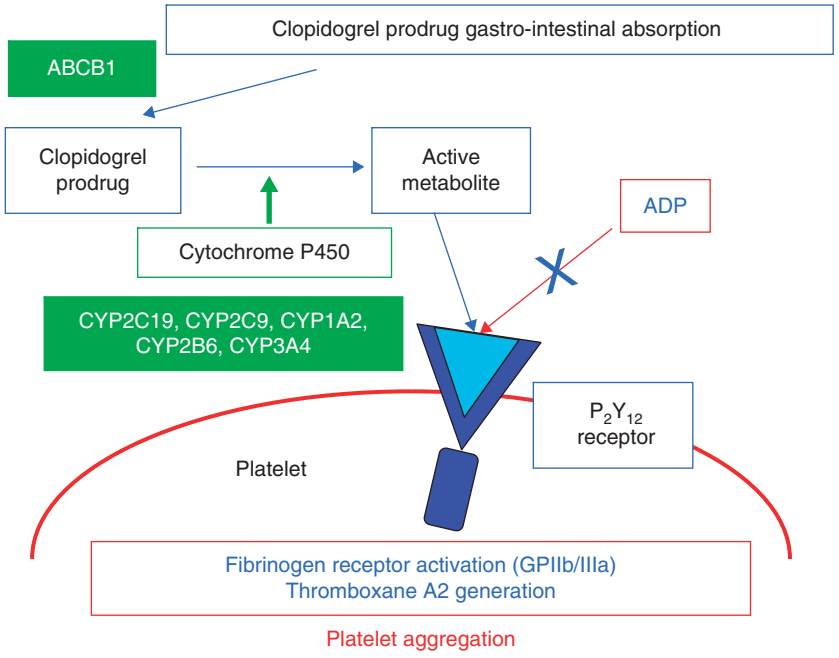

Figure 2. Mechanism of action of clopidogrel.

3. Non-pharmacogenetic mechanisms of high on-clopidogrel platelet reactivity

In this section, some of the numerous potential or well-documented non-pharmacogenetic mechanisms that could contribute to high on-clopidogrel platelet reactivity are reported (**Figure 1**). All these mechanisms could interact with the CYP2C19*2 polymorphism in determining the risk of occurrence of MACE, including ST, in high risk vascular patients.

3.1 Compliance

Clopidogrel non-compliance may represent a critical issue as cessation of clopidogrel therapy was observed in ~ 15% (within 30 days) of patients with coronary artery disease (CAD) [49] and in 18.4% (at 3 months) and ≤ 38.4%

(at 1 year) in a cohort of patients after stroke [50], and it was a well-known risk factor for ST [51]. With regard to compliance, it is critical to separate the evidence into acute (in-hospital) and chronic (outpatient) long-term setting.

3.2 Clopidogrel–proton pump inhibitor interaction

A growing body of evidences has recently been accumulated about the interaction between the proton pump inhibitors (PPIs) and clopidogrel in influencing platelet reactivity [22,52,53]. It is estimated that ~ 15% of an absorbed clopidogrel dose is converted to an active thiol metabolite, mainly by the hepatic CYP450 isoenzymes [46]. One of these isoenzymes, the CYP2C19 isoform, is involved in the metabolism of many of the PPIs, which are also inhibitors of the CYP2C19 isoenzyme in varying degrees [54]. The capability of PPIs to inhibit CYP2C19 activity would reduce the active metabolite of clopidogrel generation and cause a diminished platelet response to clopidogrel. In addition to this mechanism, PPIs may influence the antiplatelet clopidogrel response through the variability of intestinal absorption of clopidogrel [55,56]. PPIs are substrates and inhibitors of the intestinal efflux transporter P-glycoprotein, a key factor for intestinal absorption of clopidogrel [54]. Alterations in P-glycoprotein activity could potentially affect plasma concentrations of clopidogrel and hence the concentration of active metabolite. Gilard and coworkers documented for the first time that the contemporary administration of clopidogrel and omeprazol was associated with a reduced platelet inhibition related to $P2Y_{12}$ receptor in 105 CAD patients [57]. In the OCLA (Omeprazole CLopidogrel Aspirin study) trial [52] 140 CAD patients undergoing stent implantation on dual antiplatelet treatment were randomised to receive omeprazole 20 g/day or placebo for 7 days. This treatment significantly decreased the effect of clopidogrel on platelet reactivity as tested by platelet vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay [52]. The influence of pantoprazole and esomeprazole on the clopidogrel antiplatelet response was assessed in two different studies that evaluated 300 and 100 patients undergoing PCI, respectively [22,53]. In these *ex vivo* studies, the intake of pantoprazole or esomeprazole was not able to impair the platelet inhibition by clopidogrel. A recent *ex vivo* study has evaluated the effect of pantoprazole and omeprazole on inhibition by clopidogrel in 104 non-ST-segment elevation (NSTE) ACS patients and found, after 1 month of treatment, a significantly reduced platelet inhibition by omeprazole but not by pantoprazole [58]. Contrasting data about the capability of PPI administration to influence the risk of clinical events are available. In 13,636 patients with acute myocardial infarction (AMI) treated with clopidogrel, the use of PPIs was associated with an increased risk of AMI [20]. However, the use of pantoprazole did not significantly affect the risk of AMI, whereas the other PPIs were associated with an increased risk of ~ 40%. In the retrospective study of 8,205 patients with ACS, the use of clopidogrel plus PPIs (at discharge, during follow-up or both) was associated with an increased risk of death or rehospitalisation for ACS compared with the use of clopidogrel without PPIs. By analysing the different PPIs in relation to adverse outcomes, the earlier mentioned study failed to demonstrate a significant difference between omeprazole and raboprazole [23]. Recently, the authors of the PRINCIPLE-TIMI (Prasugrel in comparison to Clopidogrel for inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction) 44 and the TRITON-TIMI (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition-Thrombolysis in Myocardial Infarction) 38 trials retrospectively analysed data in order to verify the possible interactions between the use of PPIs, platelet function and adverse cardiovascular events in relation to both clopidogrel and prasugrel. In both trials, the use of PPIs was not significantly associated with an increased risk of ischemic events in patients treated with clopidogrel and with prasugrel and it was confirmed at the subgroup analysis with the single PPI (omeprazole, pantoprazole, esomeprazole and lansoprazole) in both sets of patients [21].

3.3 Age

The available evidence demonstrates that advanced age is associated with a significantly high percentage of patients with high on-clopidogrel platelet reactivity. One of the possible causes is related to the fact that the conversion of clopidogrel to its active metabolite may be impaired in older patients [59,60]. Recently, in a prospective study [61] that evaluated 191 patients who underwent PCI, ADP-inducible platelet reactivity increased linearly with age after adjustment for cardiovascular risk factors, type of intervention, medication, C-reactive protein and renal function. At variance, the study by Price and coworkers [62] evaluating various factors that may affect ADP-inducible platelet reactivity in patients on continuous clopidogrel therapy demonstrated no effect of age $\geq$ 75 years on ADP-inducible platelet reactivity [62]. This discrepancy may be attributable to the duration of clopidogrel therapy. Most of patients in the study by Gremmel *et al.* received a loading dose of clopidogrel prior to angioplasty, while the Price study assessed platelet aggregation mainly in patients on continuous clopidogrel therapy [61,62]. Therefore, the attenuation of clopidogrel-mediated platelet inhibition at an advanced age may be a phenomenon of the initial phase of antiplatelet therapy.

3.4 Diabetes mellitus

Diabetes mellitus is a clinical condition characterised by enhanced platelet reactivity that exposes diabetic patients to an increased risk of atherothrombotic events. In fact, platelets in diabetics respond more frequently even to subthreshold stimuli, are consumed more rapidly and thus contribute to accelerated thrombopoiesis compared to platelets in non-diabetic individuals [63]. It has been documented that high on-clopidogrel platelet reactivity is more prevalent in diabetic than in non-diabetic patients [64-66]. In particular, type 2

diabetic patients undergoing elective PCI have approximately a fourfold increase in the number of non-responders at 24 h following a standard 300 mg loading dose: moreover, platelet reactivity is persistently elevated in diabetic patients even in the maintenance phase of clopidogrel and is highest among those requiring insulin therapy [64].

3.5 Inflammation

Recent studies have documented a significant and independent association between platelet hyper-reactivity and both routinary inflammatory markers (such as erythrocyte sedimentation rate and leukocyte number) [67] and unbalanced pro-inflammatory/anti-inflammatory cytokine levels [24]. In ACS patients on dual antiplatelet treatment, during the acute phase of the disease, pro-inflammatory cytokine levels not sufficiently compensated by anti-inflammatory cytokines are able to modulate platelet hyper-reactivity [24]. Interestingly, Antonino and coworkers demonstrated that patients treated with long-term clopidogrel and aspirin therapy who underwent elective PCI had less inflammation, indicated by specific biomarkers (C reactive protein and other proinflammatory cytokines), compared with clopidogrel-naive patients, suggesting that long-term clopidogrel therapy is associated with an anti-inflammatory effect [68].

3.6 Platelet turnover and reticulated platelets

An elevated turnover of the circulating platelet pool yields a larger population of young platelets that are more reactive than an older population of platelets. The younger platelets had increased mean volume and a greater number of dense granules than older circulating ones [69]. In both healthy subjects and in patients with stable CAD, it has been demonstrated that an increased platelet turnover, as reported by the proportion of circulating RPs, exhibit significantly increased aggregation and activation responses even after dual antiplatelet therapy [26,69-71]. Moreover, in 372 ACS patients on dual antiplatelet therapy the number of patients with suboptimal or 'low response' to aspirin or clopidogrel or both was clustered among patients with a high proportion of circulating RPs [25].

3.7 von Willebrand factor and ADAMTS-13

The adhesion and activation of platelet at sites of vascular injury is mediated by von Willebrand factor (VWF). VWF, which is released from storage sites or secreted by endothelial cells as ultra-large VWF (ULVWF), is rapidly cleaved through intravascular proteolysis by ADAMTS-13 [72]. In the acute phase of CAD and after the vascular injury induced by a PCI, endothelial cells are activated and high amounts of ULVWF are released [73]. ULVWF forms are able to binds the glycoprotein 1b–IX complex and aggregate platelets [74]. In physiological conditions the large multimeric forms are rapidly cleaved by the ADAMTS-13 on the surface of the endothelium, resulting in the generation of the normal multimeric pattern of VWF [72,75]. When ADAMTS-13 activity is reduced, the ULVWFs accumulate and can induce platelet aggregation [72]. High on-clopidogrel platelet reactivity is associated with lower ADAMTS-13 activity accompanied by elevated VWF levels [29], suggesting a modulatory effect of the interplay between ADAMTS-13 and VWF on the response to antiplatelet therapy in patients with a marked endothelial activation, such as those with ACS who have undergone PCI.

3.8 Red blood cells and leukocytes

Red blood cells and leukocytes are significantly implicated into the pathogenesis of arterial thrombosis. Platelet–leukocyte interactions play an important role in both adhesion and activation of platelets, but red blood cells are also involved in the interactions between platelets and vessel wall. In patients with ACS the inhibition of platelets induced by aspirin is modulated not only by the direct action on platelets, but also by the erythrocyte deformability and the white blood cell count [27] suggesting that all blood components, including red blood cells, should be taken into account. Some hemorheologic variables are also associated with high on-treatment platelet reactivity in patients with ACS on dual antiplatelet therapy. In particular, decreased erythrocyte deformability is significantly associated with increased platelet aggregation induced by ADP [28]. A possible explanation for this association is that erythrocytes with decreased deformability tend to release a larger amount of ADP in the circulation compared to those without [76] and ADP released can compete against clopidogrel for purinergic receptors present on the platelet surface.

4. Association of CYP2C19 loss-of-function polymorphism with high on-clopidogrel platelet reactivity

Several polymorphisms in different genes coding isoforms of CYP450 have been investigated as possible determinants of the high on-clopidogrel platelet reactivity (genetic variants in CYP3A4, CYP3A5, CYP1A2, CYP2B6, CYP2C9, CYP2C19, ABCB1 genes). For the majority of the investigated polymorphisms results are negative or inconsistent. On the contrary, consistent data are available concerning a polymorphism in one isoform of the CYP450, the CYP2C19. 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 metaboliser phenotype [77].

4.1 Studies in healthy subjects

In 2006, Hulot and coworkers [34] demonstrated for the first time that the CYP2C19 loss-of-function allele is associated with a marked decrease in platelet responsiveness to clopidogrel in 28 young healthy male volunteers treated for 7 days with clopidogrel 75mg/day (**Table 1**). Fontana and coworkers [78] replicated the CYP2C19*2 allele influence on

Table 1. Association of CYP2C19*2 loss-of-function polymorphism with response variability to clopidogrel treatment in healthy subjects.

Study	No. of subjects	Treatment	Functional evaluation	CYP2C19*2 carriers (%)	Association
Hulot *et al*. [34]	28 Caucasian males	75mg/day for 7 days	10 μM ADP-induced PA	28.6	Yes
Fontana *et al*. [78]	94 Caucasians	300 mg the first day, then 75 mg/day for 7 days	20 μM ADP induced PA	27.7	Yes
Brandt *et al*. [35]	74 Caucasians (57 males/ 17 females)	300 mg	20 μM ADP-induced PA Active metabolite by LC + MS	25.8	Yes
Kim *et al*. [79]	24 Koreans	300 mg the first day, then 75 mg/day for 7 days	5 μM ADP-induced PA PCC by LC + MS	66.6 (selected)	Yes
Umemura *et al*. [80]	47 Japanese	300 mg	20 μM ADP-induced PA VASPph Active metabolite by LC + MS	61.7	Yes
Mega *et al*. [39]	162 (130 males/ 32 females)	300 – 600 mg ± 75 mg/day for 7 days	20 μM ADP-induced PA Active metabolite by LC + MS	34.0	Yes

LC + MS: Liquid chromatography with tandem mass spectrometry; PA: Platelet aggregation; PCC: Plasma concentrations of clopidogrel; VASPph: Vasodilator-stimulated phosphoprotein phosphorylation state of whole blood for which we performed a dual colour flow cytometric assay.

clopidogrel responsiveness in 94 healthy subjects by assessing the ADP-induced platelet aggregation after the 1-week course of clopidogrel (300 mg on day 1, then 75 mg/day) and showed that the CYP2C19*2 polymorphism explained 10% of the variability of clopidogrel responsiveness also when the analysis was adjusted for age, platelet count, hematocrit, collagen lag time and fibrinogen and von Willebrand levels. To determine the relationship between genetic variation in CYP450 isoenzymes and the pharmacokinetic/pharmacodynamic response to prasugrel and clopidogrel, Brandt and coworkers [35] genotyped healthy subjects participating in studies evaluating pharmacokinetic and pharmacodynamic response to prasugrel (60 mg, n = 71) or clopidogrel (300 mg, n = 74) for CYP1A2, CYP2B6, CYP2C19, CYP2C9, CYP3A4 and CYP3A5 genetic variants. In subjects receiving clopidogrel, the presence of the CYP2C19*2 loss-of-function variant was significantly associated with lower exposure to clopidogrel active metabolite, lower inhibition of platelet aggregation at 4 h and poor-responder status. Similarly, CYP2C9 loss-of-function variants, polymorphisms in another gene coding a different CYP450 isoenzyme, were significantly associated with lower exposure to clopidogrel active metabolite, lower inhibition of platelet aggregation at 4 h and poor-responder status. For prasugrel, there was no relationship observed between CYP2C19 or CYP2C9 loss-of-function polymorphisms and exposure to the active metabolite of prasugrel or pharmacodynamic response.

Recently, Mega and coworkers [39] have also confirmed that in 162 healthy subjects on a 300-mg dose of clopidogrel, carriers of at least one CYP2C19 reduced-function allele (~ 30% of the study population) had a relative reduction of 32.4% in plasma exposure to the active metabolite of clopidogrel, as compared with noncarriers. Carriers also had an absolute reduction in maximal platelet aggregation in response to clopidogrel that was nine percentage points less than that seen in noncarriers.

A total of 24 Korean subjects were divided into three groups on the basis of their CYP2C19 genotype by Kim and coworkers [79]: homozygous extensive metabolisers (homoEMs, n = 8), heterozygous EMs (heteroEMs, n = 8) and poor metabolisers (PMs, n = 8). After a single 300-mg loading dose of clopidogrel on day 1, followed by a 75-mg/day maintenance dose from days 2 to 7, they measured the plasma levels of clopidogrel and assessed its antiplatelet effect as inhibitory effect on ADP-induced platelet aggregation according to the CYP2C19 genotype. The mean clopidogrel AUC for PMs was 1.8- and 2.9-fold higher than that for heteroEMs and homoEMs, respectively, and their C_{max} values were 1.8- and 4.7-fold higher than that of heteroEMs and homoEMs, respectively. PMs also exhibited a significantly lower antiplatelet effect than heteroEMs or homoEMs.

Umemura and coworkers [80], in 47 healthy Japanese subjects, determined plasma concentrations of the active metabolite before and at 0.25, 0.5, 1, 2, 4 and 8 h after treatment with clopidogrel 300 mg. Moreover, before and at 1, 4, 6 and 24 h after the treatment, they measured inhibition of platelet aggregation to ADP and VASP. They confirmed that the *2 allele is a determinant for the formation of the active metabolite and demonstrated that the pharmacokinetic parameters, AUC and C_{max} of the active metabolite were well

correlated with platelet reactivity index by VASP and inhibition of platelet aggregation to ADP. Their findings suggest that low or no responsiveness to clopidogrel is mainly caused by the lower metabolic formation of the metabolite, but not because of the poor sensitivity of the platelet P2Y12 receptor. They also observed that CYP2C19 polymorphism showed a marked interethnic difference in the incidence of carriers of CYP2C19*2 polymorphism. The prevalence of carriers is much greater (18 – 23%) in Asian people than that (~ 3%) in Caucasian populations [81]. In a meta-analysis [82], 21% of patients treated with clopidogrel achieved low or no *ex vivo* inhibition of ADP-induced platelet aggregation in American or European populations. Based on the results in this study, it is estimated that > 30% of Asian patients show less responsiveness to clopidogrel.

Recently, in the Pharmacogenomics of Antiplatelet Intervention Study, Shuldiner and coworkers [83] administered clopidogrel 300 mg p.o. as a loading dose followed by 75 mg/day for 7 days to 429 healthy Amish persons and measured response by *ex vivo* platelet aggregometry (baseline platelet aggregation and at 1 h following the last dose of clopidogrel). In this population, a genome-wide association study was performed followed by genotyping the loss-of-function CYP2C19*2 variant. They observed that platelet response to clopidogrel was highly heritable ($h^2 = 0.73$; $p < 0.001$). A total of 13 SNPs on chromosome 10q24 within the CYP2C18–CYP2C19–CYP2C9–CYP2C8 cluster were associated with diminished clopidogrel response. These SNPs were in strong linkage disequilibrium with each other with rs12777823 being the most significantly associated SNP ($p = 1.5 \times 10^{-13}$ for the additive model). The rs12777823 polymorphism was in strong linkage disequilibrium with the CYP2C19*2 variant, and was associated with diminished clopidogrel response, accounting for 12% of the variation in platelet aggregation to ADP ($p = 4.3 \times 10^{-11}$). No other genomic region revealed association signals that displayed exceess genome-wide significance ($p < 1.0 \times 10^{-7}$). These data suggested that in this population of healthy subjects the common loss-of-function CYP2C19*2 variant could account for most of the association signal detected in the genome-wide association study.

4.2 Studies in high risk vascular patients

In 2007, Giusti and coworkers [36] studied two polymorphisms affecting clopidogrel metabolism (one in the +12 position of intron 10 of the CYP3A4 gene and one a G681A substitution determining a splicing alteration of the CYP2C19 gene) and one polymorphism of the P2Y12 ADP platelet receptor (T744C marker of the H1/H2 haplotype) (**Table 2**). The role of these three polymorphisms in modulating platelet function in ACS patients on dual antiplatelet treatment who has undergone PCI was evaluated. Three polymorphisms were found to be important in interfering with the mechanism of action of clopidogrel: the polymorphisms of the two isoforms of the CYP450, by interfering with the formation of the active metabolite that binds irreversibly to the platelet ADP receptor P2Y12, and the polymorphism of the receptor by interfering with its function. In that paper, for the first time, it was reported that in 1,419 high risk vascular patients on dual antiplatelet treatment the *2 allele of the CYP2C19 gene was associated with higher platelet aggregability and residual platelet reactivity. Carriers of the *2 allele of the polymorphism had significantly higher platelet aggregation values than noncarriers of the *2 allele after 2 and 10 μM ADP, and after arachidonic acid (AA) stimuli. In particular, homozygous subjects for the *2 allele had significantly higher platelet aggregation after ADP stimuli than heterozygous carriers. No differences in platelet aggregation values were observed after the three different stimuli in the overall studied population according to the other two polymorphisms investigated (P2Y12 T744C and CYP3A4 IVS10 + 12G/A). The genotype distribution of CYP2C19 polymorphism significantly differed between patients with and without residual platelet reactivity (RPR) evaluated by 10 μM ADP induced platelet aggregation. The prevalence of carriers of the *2 allele was higher in patients with ADP-RPR than in those without. Similar results were also observed in patients with AA-RPR as compared to those without. In the multivariate linear regression model with ADP 2 or 10 μM or log(AA) platelet aggregation as dependent variable and age, gender, hypertension, diabetes mellitus, dyslipidemia and smoking habit as independent variables, age, diabetes and CYP2C19*2 polymorphisms remained significant and independent risk factors for antiplatelet treatment variability.

Several other groups confirmed that the *2 allele of the CYP2C19 gene is associated with higher platelet aggregability and residual platelet reactivity in similar and different clinical settings.

Frere and coworkers [84] in 603 patients with non-ST elevation ACS demonstrated that the CYP2C19*2 allele influences post-treatment platelet reactivity and clopidogrel response assessed by ADP induced platelet aggregation, VASP phosphorylation index and ADP induced P-selectin expression. They also demonstrated no effect of CYP3A4*1B and CYP3A5*3 [84].

Trenk and coworkers [37] studied and followed-up 797 patients undergoing elective coronary stent implantation; they showed an association between CYP2C19*2 polymorphism and high (> 14%) ADP residual platelet aggregation on clopidogrel treatment.

In 237 symptomatic CAD patients (both stable angina or ACS patients), Geisler and coworkers [85] observed that CYP2C19*2 carriers showed significantly increased residual platelet aggregation compared with noncarriers.

In 60 patients undergoing elective PCI in the randomised PRINC (Plavix Response in Coronary Intervention) trial, Gladding and coworkers [86] measured platelet function using the VerifyNow P2Y12 analyser after a loading dose of 600 or split 1,200 mg and a maintenance dose of 75 or 150 mg/day. CYP2C19*1*1 carriers had greater platelet inhibition 2 h after

Table 2. Association of CYP2C19*2 loss-of-function polymorphism with response variability to clopidogrel treatment in high risk vascular patients.

Study	No. of subjects	Treatment	Functional evaluation	CYP2C19*2 carriers (%)	Association
Giusti *et al*. [36]	1,419 PCI + stent	600 mg LD + 75 mg/day maintaining	10 μM ADP-induced PA	31.4	Yes
Frere *et al*. [84]	603 NSTE	600 mg LD	10 μM ADP-induced PA VASPph ADP-induced P-selectin	27.6	Yes
Trenk *et al*. [37]	797 PCI + stent	600 mg LD + 75 mg/day maintaining	5 and 20 μM ADP-induced PA ADP-induced P-selectin, activated GPIIa/IIIb, CD63, CD40 L, CD41 (GPIIb)	30.7	Yes
Geisler *et al*. [85]	237 PCI	600 mg LD + 75 mg/day maintaining	20 μM ADP-induced PA	26.0	Yes
Gladding *et al*. [86]	60 PCI	600/1,200 mg LD + 75/150 mg/day	VerifyNow P2Y12		Yes
Jinnai *et al*. [87]	30 PCI (Japanese)	300 mg LD + 75 mg/day maintaining	5 μM ADP-induced PA	56.0	Yes

GPIIa/IIIb: Glycoprotein IIa/IIIb (PAC-1); GPIIb: Glycoprotein IIb; LD: Loading dose; NSTE: Non-ST elevation; PA: Platelet aggregation; PCI: Percutaneous coronary intervention; VASPph: Vasodilator-stimulated phosphoprotein phosphorylation state of whole blood for which we performed a dual colour flow cytometric assay.

a 600-mg dose, compared with platelet inhibition in CYP2C19*2 or *4 carriers and CYP2C19*17 carriers. CYP2C19*2 or *4 carriers had greater platelet inhibition with the higher loading dose than with the lower dose at 4 h and responded better with the higher maintenance dose regimen.

The association of the CYP2C19*2 polymorphism with a residual platelet reactivity was also observed in Japanese patients by Jinnai and coworkers [87], in whom a higher prevalence of the carriers of the *2 allele was found (56%) with respect to their Western counterparts (~ 30%). On the other hand, the same authors had previously reported that the effectiveness of clopidogrel in the Japanese is weaker than that in Western people [88], but the ratios of CYP2C19 poor metabolisers in the Western population are less (~ 1 – 7%) when compared to that in Asians (~ 12 – 23%) [89].

5. Association of CYP2C19 loss-of-function polymorphism with the occurrence of major adverse cardiovascular events

Recently, different groups contemporarily demonstrated in different clinical settings and at different follow-up times that CYP2C19*2 is a determinant of the occurrence of MACE and in particular of ST (**Table 3**) [38-42]. Only one paper failed to demonstrate the role of the CYP2C19*2 loss-of-function polymorphism in the occurrence of MACE or ST. In fact, the paper by Trenk and coworkers [37] did not demonstrate a relationship between genotype and increased occurrence of adverse 1-year clinical outcome (death from any cause and myocardial infarction) of elective PCI with drug-eluting or bare-metal stents (**Table 3**). They showed an association between CYP2C19*2 polymorphism and platelet function phenotype (see previous paragraph) as well as an association of high-on clopidogrel platelet reactivity with poor clinical outcome. On the other hand, in their patient population they had only 280 patients out of 797 with at least one DES. In the group of patients with at least one DES, the percentage of patients with an event was 3.3% (3 of 90) among carriers of the *2 allele versus 2.1% (4 of 190) among wild-type homozygotes ($p = 0.684$).

Simon and coworkers [38], in 2,208 patients with an AMI and on clopidogrel therapy, assessed the relation of allelic variants of genes modulating clopidogrel absorption [ATP-binding cassette, subfamily B (MDR/TAP), member 1 (ABCB1)], metabolic activation (CYP3A5 and CYP2C19) and biologic activity [purinergic receptor P2Y, G-protein coupled, 12 (P2RY12) and integrin, beta 3 (ITGB3)] to the risk of death from any cause, non-fatal stroke or myocardial infarction during 1 year of follow-up.

None of the selected polymorphisms in CYP3A5, P2RY12 or ITGB3 were associated with a risk of an adverse outcome. Patients with two variant alleles of ABCB1 (TT genotype) had a higher rate of cardiovascular events at 1 year than those with the ABCB1 wild-type genotype (CC) [15.5 versus 10.7%; adjusted hazard ratio (HR), 1.72; 95% confidence interval (CI), 1.20 – 2.47]. Moreover, they demonstrated that patients carrying CYP2C19 loss-of-function alleles (*2, *3, *4 or *5)

Table 3. Association of CYP2C19*2 loss-of-function polymorphism with occurrence of MACEs or intra-ST alone.

Study	No. of subjects	Treatment	Outcome (follow-up)	CYP2C19 alleles	Association
Trenk *et al*. [37]	797 PCI + stent	600 mg LD + 75 mg/day maintaining	Death + MI (1 year)	*2	No
Simon *et al*. [38]	2,208 AMI (1535 PCI)	300 mg mean LD + 75 mg/day maintaining	Death + non-fatal stroke + MI (1 year)	Any two*2,*3,*4,*5	Yes (HR = 1.98) Yes (HR = 3.58)
Mega *et al*. [39]	1,477 ACS	300 mg LD + 75 mg/day maintaining	Cardiovascular death + MI + stroke (15 months) ST (15 months)	*2	Yes (HR = 1.53) Yes (HR = 3.09)
Collet *et al*. [40]	259 MI (< 45 years)	600 mg LD + 75 mg/day maintaining	Death + MI + coronary revascularisation (6 months) ST (6 months)	*2	Yes (HR = 3.69) Yes (HR = 6.02)
Giusti *et al*. [41]	772 PCI + DES	600 mg LD + 75 mg/day maintaining	Cardiovascular death + ST (6 months) ST (6 months)	*2	Yes (OR = 2.70) Yes (OR = 3.43)
Sibbing *et al*. [42]	2,485 PCI + stent	600 mg LD + 75 mg/day maintaining	ST (30 days)	*2	Yes (HR = 3.81)

ACS: Acute coronary syndrome; AMI: Acute myocardial infarction; DES: Drug-eluting stent; HR: Hazard ratio; LD: Loading dose; MACEs: Major adverse cardiovascular events; MI: Myocardial infarction; OR: Odds ratio; PCI: Percutaneous coronary intervention; ST: Stent thrombosis.

had a higher rate of subsequent cardiovascular events during 1 year of follow-up (HR 1.98, 95%CI 1.10 – 3.58) that those who were not. The presence of two variant alleles of CYP2C19, but not ABCB1, was found to be associated with an increase by a factor of 3.58 (95%CI 1.71 – 7.51) in the rate of cardiovascular events among the 1,535 patients who underwent PCI during hospitalisation as compared with those who did not.

Mega and coworkers [39] tested the association between functional genetic variants in CYP genes [CYP2C19, CYP, family 2, subfamily C, polypeptide 9 (CYP2C9), CYP, family 2, subfamily B, polypeptide 6 (CYP2B6), CYP3A5, CYP, family 3, subfamily A, polypeptide 4 (CYP3A4), and CYP, family 1, subfamily A, polypeptide 2 (CYP1A2)], plasma concentrations of active drug metabolite and platelet inhibition in response to clopidogrel in 162 healthy subjects (see previous paragraph). Furthermore, they examined the association between these genetic variants and cardiovascular outcomes in a separate cohort of 1,477 subjects with ACS who were treated with clopidogrel in the TRITON-TIMI (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis in Myocardial Infarction) 38 trial. In the cohort of 1,477 ACS patients who were treated with clopidogrel in the TRITON-TIMI 38 trial, they demonstrated that carriers of the reduced-function CYP2C19*2 allele had significantly lower levels of the active metabolite of clopidogrel, diminished platelet inhibition and a higher rate of MACE at 15 months (death from cardiovascular causes, myocardial infarction or stroke; HR 1.53, 95%CI 1.07 – 2.19) and an increase by a factor of 3 in the risk of ST (HR 3.09, 95%CI 1.19 – 8.00).

In 259 young patients (< 45 years) who survived a first myocardial infarction and were exposed to clopidogrel treatment for ≥ 1 month, Collet and coworkers [40] assessed whether the CYP2C19*2 polymorphism affected long-term prognosis in those chronically treated with clopidogrel. They chose as primary end-point a composite of cardiovascular death, non-fatal myocardial infarction and urgent revascularisation occurring during exposure to clopidogrel and ST as secondary end-point. In this cohort of premature myocardial infarction patients, the primary end-point occurred more frequently in carriers than in noncarriers [15 versus 11 events; HR 3.69 (95%CI 1.69 – 8.05), $p = 0.0005$]. Similar results were obtained in relation to the secondary end-point for which they observed an increase in the risk of ST by a factor of 6 [8 versus 4 events; HR 6.02 (1.81 – 20.04), $p = 0.0009$]. After multivariable stepwise Cox regression analysis considering in the model all the baseline characteristics, especially those known to be associated with recurrent acute coronary thrombosis and poor response to clopidogrel (including body mass index, smoking status, diabetes status, stent implantation, initial ST-segment elevation myocardial infarction and use of PPIs) if they were significant at $\alpha = 0.20$ in the univariate model, the CYP2C19*2 genetic variant was the only independent

predictor of cardiovascular events [HR 4.04 (1.81 – 9.02), p = 0.0006].

In the framework of the Low Responsiveness to Clopidogrel and Sirolimus- or Paclitaxel-Eluting Stent Thrombosis (RECLOSE) trial, Giusti and coworkers [41] evaluated the role of the CYP2C19*2 polymorphism in the occurrence of drug-eluting ST (primary end-point) and the composite feature of cardiac mortality and drug-eluting ST (secondary end-point) in a 6-month follow-up of 772 patients undergoing PCI on dual-antiplatelet treatment.

Patients with ST or the composite of cardiac mortality and ST showed a higher prevalence of carriers of the *2 allele (54.1 versus 31.3%; p = 0.025 and 51.7 versus 31.2%; p = 0.020, respectively). In this clinical setting of high risk vascular patients, the authors demonstrated that the CYP2C19*2 polymorphism was an independent risk factor for ST when the effect of the polymorphism was evaluated in a model adjusted for high on-clopidogrel platelet reactivity evaluated by ADP induced platelet aggregation (ADP-RPR), traditional cardiovascular risk factors and clinical and procedural risk factors for ST (such as total chronic occlusion, multivessel disease, bifurcation lesion, AMI, previous myocardial infarction, total stent length and left ventricular ejection fraction) (OR 3.43, 95%CI 1.01 – 12.78). Moreover, subjects with the contemporary presence of the CYP2C19*2 allele and ADP-RPR showed a strong risk of ST or ST and cardiac mortality (OR 5.79, 95%CI 1.04 – 39.01 and 11.45, 95%CI 1.84 – 71.27, respectively).

Sibbing and coworkers [42] assessed the impact of the CYP2C19 loss-of-function polymorphism on ST following PCI performed after pre-treatment with clopidogrel. The primary end-point of the study was the incidence of definite ST within 30 days following PCI. A total of 2,485 patients with CAD undergoing PCI were enrolled in this study.

The cumulative 30-day incidence of ST was significantly higher in CYP2C19*2 allele carriers (*1/*2 heterozygotes or *2/*2 homozygotes) versus CYP2C19 wild-type homozygotes (*1/*1) (1.5% in CYP2C19*2 allele carriers versus 0.4% in CYP2C19 wild-type homozygotes, HR 3.81, 95% CI1.45 – 10.02, p = 0.006, after adjustment for confounding variables). The risk of ST was highest (2.1%) in patients with the CYP2C19 *2/*2 genotype (p = 0.002).

Recently, Sofi and coworkers [90] performed a meta-analysis on data available for a total of 7,950 patients from six cohort prospective studies who were followed for a time ranging from 6 months to 8.4 years [37-42] reinforcing the observation that the CYP2C19*2 polymorphism is associated with the increased risk of MACE and in particular ST. The summary risk ratios for included cohort prospective studies showed a significant association between the CYP2C19*2 polymorphism and an increased risk of MACE in the follow-up [RR: 1.80 (1.02 – 3.19); p = 0.04]. When studies evaluating ST (n = 4) for a total of 4,975 patients were considered, the presence of the variant allele was associated with an increased risk of ST [RR: 2.82 (1.43 – 5.56); p = 0.0001].

6. Expert opinion: towards personalised medicine

Due to the large body of evidences accumulated, the issue of whether, in subjects with ACS, the identification of CYPC19*2 carriers is appropriate to improve the risk stratification, the therapeutic management of patients and consequently to reduce the occurrence of MACEs and in particular ST could be raised.

Several strategies could be considered to solve the problem of the high on-treatment platelet reactivity despite the dual antiplatelet treatment in high risk vascular patients: a higher dosage of clopidogrel, a different antiplatelet drug among those at present under clinical evaluation (prasugrel, ticagrelor, cangrelor) or the addition of a third antiplatelet drug such as ilostal.

Bonello and coworkers [91] demonstrated that individually tailored loading dose of clopidogrel to obtain a VASP index < 50% after a first clopidogrel loading dose of 600 mg in patients with high on-treatment platelet reactivity translated into an improved outcome [91,92]. Recently, the same group observed that high on-treatment platelet reactivity in homozygotes for CYP2C19*2 loss-of-function polymorphism could be overcome by dose adjustment according to platelet reactivity monitoring [93]. Patients with a VASP < 50% after the first clopidogrel loading dose were considered good responders. Patients with a VASP ≥ 50% despite the 600-mg loading dose were considered to have high on-treatment platelet reactivity. For these patients clopidogrel was adjusted individually, before PCI, to obtain a VASP index < 50% using up-to three additional loading dose of 600 mg each prescribed at 24-h intervals. As regards new antiplatelet drugs, the TRITON-TIMI 38 trial demonstrated that, compared with clopidogrel, treatment with prasugrel resulted in a significantly lower rate of ischemic events and more bleeding among patients presenting with ACS with planned PCI [94]. Both clopidogrel and prasugrel are prodrugs that require biotransformation to active metabolites by CYP450 enzymes. Although the active metabolites of both drugs have similar affinity for the P2Y12 receptor *in vitro*, the *in vivo* differences in response appear to be mediated predominantly by differences in the metabolic pathways leading to the formation of the active metabolites [95]. Esterases shunt the majority of clopidogrel to a dead-end inactive pathway, with the remaining prodrug requiring two separate CYP-dependent oxidative steps [47]. In contrast, esterases are part of the activation pathway with prasugrel, and prasugrel is oxidised to its active metabolite in a single CYP-dependent step, without an apparent dead-end inactive pathway [96]. Among 238 healthy subjects, Mega and coworkers [97] did not observe significant attenuation of the pharmacokinetic or the pharmacodynamic response to prasugrel in carriers versus noncarriers of at least one reduced function allele for any of the CYP gene tested (CYP2C19, CYP2C9, CYP2B6, CYP3A5, CYP1A2). Consistent with these findings, in 1,466 patients with ACS

treated with prasugrel no significant associations were found between any of the tested CYP genotypes and risk of cardiovascular death, myocardial infarction or stroke [97]. On the other hand, to the best of our knowledge, we cannot exclude the fact that other polymorphisms, different from those involved in clopidogrel pharmacogenetics, in genes coding for different CYP450 isoforms or proteins involved in the functional pathway of new antiplatelet agents might determine a variability in their activity and therapeutic efficacy.

Several important issues should be considered in evaluating the possibility of a tailored therapy that takes in account the knowledge of CYP2C19*2 genotype. First of all the result of the genotyping test would be available at the time of starting clopidogrel and during the early high risk phase of the ACS together with the evaluation of the phenotype, such as ADP-induced platelet aggregation [14,15,98,99], VerifyNow System [16,17], the more specific flow cytometric assay of P2Y12-mediated effect on phosphorylation of VASP in PGE1-treated platelet [92,99] or a more global approach, such as Multiplate whole blood aggregometry [100]. The functional evaluation of the high on-clopidogrel platelet reactivity has the potential benefit of identifying those who are homozygous for the wild-type CYP2C19*1 allele but still have a poor pharmacodynamic response to clopidogrel. In fact, the findings by Giusti and coworkers [41], indicating that the presence of ADP-RPR and the CYP2C19*2 polymorphism was the strongest predictor of ST complication or the composite of ST and cardiac mortality with respect to the CYP2C19*2 polymorphism and ADP-RPR per se, provide the two insights that i) mining of the CYP2C19*2 polymorphism as a risk factor for ST is only partially linked to its role in determining the ADP-RPR phenotype observed in the acute periprocedural phase; and that ii) other genetic and acquired determinants of RPR in addition to the CYP2C19*2 polymorphism might have a role in determining the clinical outcome in these high risk vascular patients.

Several new drugs (prasugrel, ticagrelor, cangrelor) that target P2Y12 are being developed and will be available soon. These drugs achieve consistently high levels of receptor inhibition, and potentially provide solutions to the problem of variability in the response to clopidogrel. Nevertheless, the possibility of a higher incidence of bleeding or other drug–drug interaction or possible nonresponsiveness due to other genetic or acquired factors needs to be further explored for these new drugs.

In conclusion, a large body of evidence exists demonstrating that the CYP2C19*2 genetic variant is a major determinant of prognosis in high risk vascular patients who have undergone PCI and have received clopidogrel treatment. Available data underline the urgent need of prospective studies aimed at clarifying whether a more intense clopidogrel therapy or the use of a different drug (e.g., prasugrel) designed for correction of inadequate suppression of platelet reactivity could improve the outcome, incidence of MACE and in particular of ST, in high risk vascular patients without increasing the major bleeding complications. These studies, besides the traditional cardiovascular risk factors, and the clinical and procedural risk factors for MACE and ST, should take into account the genetic profile as well as the residual platelet reactivity phenotype and the possible drug interactions (especially with drugs metabolised by the same CYP450 isoforms) to identify the better pharmacological strategy to apply for the more efficacious management and safety of these high risk vascular patients.

Declaration of interest

The authors state no conflicts of interest and have received no payment in preparation of this manuscript.

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Affiliation

Betti Giusti[†1] PhD, Anna Maria Gori[2] BS, Rossella Marcucci[2] MD PhD & Rosanna Abbate[3] MD
[†]Author for correspondence
[1]Researcher in Clinical Pathology, University of Florence and SOD Atherothrombotic Diseases, Department of Medical and Surgical Critical Care, AOU Careggi, Viale Morgagni 85, 50134 Florence, Italy
Tel: +390557949420; Fax: +390557949418; E-mail: betti.giusti@unifi.it
[2]University of Florence - SOD Atherothrombotic Diseases, Department of Medical and Surgical Critical Care, AOU Careggi, Viale Morgagni 85, 50134 Florence, Italy
[3]Professor, University of Florence - SOD Atherothrombotic Diseases, Department of Medical and Surgical Critical Care, AOU Careggi, Viale Morgagni 85, 50134 Florence, Italy