The platelet P2Y12 receptor for adenosine diphosphate:
congenital and drug-induced defects

Marco Cattaneo

Unità di Medicina 3 – Ospedale San Paolo
Dipartimento di Medicina, Chirurgia e Odontoiatria. Università degli Studi di Milano
Milano, Italy

Correspondence:
Marco Cattaneo, M.D.
Unità di Medicina 3
Ospedale San Paolo – Università degli Studi di Milano
Via di Rudini 8
20142 Milano, ITALY
Phone +390250323095
Fax +39 0250323089
e-mail: marco.cattaneo@unimi.it
Abstract

P2Y$_{12}$, the G$_i$-coupled platelet receptor for adenosine diphosphate (ADP), plays a central role in platelet function. Patients with congenital P2Y$_{12}$ defects display a mild-to-moderate bleeding diathesis, characterized by mucocutaneous bleedings and excessive post-surgical and post-traumatic blood loss. Defects of P2Y$_{12}$ should be suspected when ADP, even at high concentrations (≥10 μM), is unable to induce full, irreversible platelet aggregation. Tests that evaluate the degree of inhibition of adenylyl cyclase by ADP should be used to confirm the diagnosis. Drugs that inhibit P2Y$_{12}$ are potent antithrombotic drugs, attesting the central role played by P2Y$_{12}$ in platelet thrombus formation. Clopidogrel, the most widely used drug that inhibits P2Y$_{12}$, is effective both in monotherapy and in combination with acetylsalicylic acid. The most important drawback of clopidogrel is its inability to inhibit adequately P2Y$_{12}$-dependent platelet function in about 1/3 of patients, who, therefore, are not protected from major cardiovascular events (MACE). New drugs, such as prasugrel and ticagrelor, which effectively inhibit P2Y$_{12}$ in the vast majority of patients, proved to be more efficacious than clopidogrel in preventing MACE. Despite the fact that they increase the incidence of major bleedings, the net clinical benefit is in favour of the new P2Y$_{12}$ inhibitors.
Adenosine diphosphate and the platelet P2Y Receptors

Adenosine diphosphate (ADP) plays a key role in platelet function. When it is secreted from the platelet dense granules where it is stored, it amplifies the platelet responses induced by other platelet agonists and stabilizes platelet aggregates. The ADP-induced signal is mediated by P2Y receptors, which are G-coupled 7-membrane-spanning proteins that are present in almost any kind of cell, whose ligands are purine and pyrimidine nucleotides. From a phylogenetic and structural point of view, two distinct P2Y receptor subgroups with a relatively high level of structural divergence have been identified: the first subgroup includes the Gq-coupled subtypes (P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11), while the second subgroup includes the Gi-coupled subtypes (P2Y12, P2Y13 and P2Y14). Human platelets express two distinct receptors for ADP: P2Y1 and P2Y12. The Gq-coupled P2Y1 receptor mediates a transient rise in cytoplasmic Ca\textsuperscript{2+}, platelet shape change and rapidly reversible aggregation, while the Gi-coupled P2Y12 receptor mediates inhibition of adenylyl cyclase and amplifies the platelet aggregation response. Concomitant activation of both the Gq and Gi pathways by ADP is necessary to elicit normal aggregation.

P2Y\textsubscript{12}

P2Y\textsubscript{12}, which maps to chromosome 3q21-q25, is present in platelets, endothelial cells, glial cells, and smooth muscle cells. It contains 342 aminoacid residues, including four extracellular Cys residues at positions 17, 97, 175 and 270: Cys 97 and Cys 175, which are linked by a disulphide bridge and are important for receptor expression; two potential N-linked glycosylation sites at its extra-cellular amino-terminus may modulate its activity (Figure 1). P2Y\textsubscript{12} receptors exist predominantly as homo-oligomers situated in lipid rafts. Upon treatment with the active metabolite of clopidogrel (which inhibits P2Y\textsubscript{12} function, see later), the homo-oligomers are disrupted into non-functional dimers and monomers that are sequestered outside the lipid rafts.

P2Y\textsubscript{12} signalling

ADP and some of its analogues, such as 2-methylthio-ADP and (N)-methanocarba-2-methylthio-ADP, stimulate P2Y\textsubscript{12}, while ATP and its triphosphate analogues act as antagonists.
The P2Y12 receptor is coupled to inhibition of adenylyl cyclase activity mostly through activation of Gαi2 and has a critical requirement for lipid rafts. It must be noted however that, although inhibition of adenylyl cyclase via Gαi2 is a key feature of platelet activation by ADP, it bears no causal relationship to platelet aggregation. Therefore, other signaling events downstream of Gαi2 are required for activation of integrin αIIbβ3 and subsequent platelet aggregation. Several studies demonstrated a crucial role for different isoforms of phosphoinositide 3-kinase (PI3-K) in ADP-dependent P2Y12 receptor-mediated amplification of platelet activation. In addition, studies of platelets from P2Y1 knockout mice and of normal platelets in the presence of specific P2Y1 antagonists showed that ADP, at higher concentrations than those commonly used to activate platelets, induces slow and sustained PI3-K-dependent platelet aggregation, which is not preceded by platelet shape change.

**Role of P2Y12 in platelet function**

Although ADP by itself is unable to cause the secretion of platelet dense granules, its interaction with P2Y12 greatly amplifies platelet secretion induced by agonists such as thromboxane A2 (TXA2) and thrombin receptor activating peptide (Figure 2). This effect, which is probably mediated by PI3-K, was observed both at physiological and micromolar concentrations of extracellular Ca2+, in the presence of acetylsalicylic acid (ASA), and independently of the formation of large platelet aggregates, demonstrating that it is a direct effect of P2Y12, rather than secondary to P2Y12-mediated amplification of aggregation.

P2Y12 plays an essential role in the stabilization of platelet aggregates induced by thrombin or TXA2 (Figure 2), which is mediated by PI3-K.

Studies of human platelets congenitally lacking P2Y12 and of P2Y12 knockout mice demonstrated that platelet aggregation and secretion induced by a range of platelet agonists were impaired. P2Y12 receptor stimulation by released ADP contributes to inhibition of adenylyl cyclase, activation of serine-threonine kinase Akt in platelets, tyrosine phosphorylation, extracellular signal-regulated kinase 2 (ERK2) activation, Rap1B activation, Rac activation and Ca2+ mobilization induced by other agonists.

Cattaneo M – P2Y12 receptor defects
Early studies demonstrated that P2Y12 is an important mediator of shear-induced platelet aggregation by using platelets from individuals treated with the anti-thrombotic drug ticlopidine or from a patient with congenital P2Y12 deficiency. This effect of P2Y12, which was later confirmed using specific, direct P2Y12 antagonists, is dependent upon PI3-K activation.

As already mentioned, inhibition of AC via G\(\alpha_i\)2 by ADP bears no causal relationship to platelet activation. However, it may substantially contribute to platelet thrombus formation in vivo by counteracting the antiplatelet effect of prostacyclin (Figure 2) or other substances that stimulate adenylyl cyclase.

P2Y12 shares with P2Y1 the ability to contribute to collagen-induced platelet microparticle formation in whole blood, and to contribute to the formation of platelet-leukocyte aggregates mediated by platelet surface P-selectin exposure, which results in tissue factor exposure at the surface of leukocytes. However, only the P2Y12 receptor was found to be involved in the exposure of phosphatidylserine by thrombin or other platelet agonists and in tissue factor-induced thrombin formation in platelet-rich plasma.

**Congenital defects of P2Y12**

**Congenital deficiency of P2Y12**

Congenital P2Y12 deficiency is an autosomal recessive disorder. The first patient with severe P2Y12 deficiency (VR) was described in 1992. He had a lifelong history of excessive bleeding, prolonged bleeding time (15-20 min), reversible aggregation in response to weak agonists and impaired aggregation in response to low concentrations of collagen or thrombin. However, the most typical feature was that ADP, even at very high concentrations (>10 \(\mu\)M), did not induce full and irreversible platelet aggregation. Other abnormalities of platelet function were: i) no inhibition by ADP of prostaglandin E\(_1\) (PGE\(_1\))-stimulated platelet adenylyl cyclase, but normal inhibition by epinephrine; ii) normal shape change and borderline-normal mobilization of cytoplasmic Ca\(^{2+}\) induced by ADP; iii) presence of approximately 30% of the normal number of binding sites for \[^{33}\text{P}]2\text{MeSADP} on fresh platelets or \[^3\text{H}]\text{ADP} on formalin-fixed platelets (which are associated with the ADP receptor P2Y\(_1\)). Five additional patients with severe P2Y12 deficiency, belonging to
4 kindreds, were subsequently described: one French man (ML), a26 two Italian sisters (IG and MG), a Japanese woman (OSP-1) a and a British woman of Asian descent (Table 1).

The study of the son of patient MG allowed the characterization of heterozygous P2Y12 deficiency: ADP-induced platelet aggregation was reversible for ADP concentrations ≤10 µM, but was full and irreversible for concentrations of ADP ≥10 µM; the inhibition of PGE1-induced increase in platelet cyclic AMP was impaired, albeit not completely absent; the number of platelet binding sites for [33P]2MeS-ADP was intermediate between his mother’s and normal subjects’; finally, the platelet secretion was impaired. Because the secretion defect in this patient’s platelets was not associated with impaired production of TXA2 or low concentrations of platelet granule contents, it is very similar to that described in patients with an ill-defined and probably heterogeneous group of congenital defect of platelet secretion, sometimes referred to with the general term “primary secretion defect”.

Molecular defects

The P2Y12 gene of patient VR and of the British patient with Asian descent displayed homozygous base pair deletions in the open reading frame, resulting in frameshifts and premature truncation of the protein. The P2Y12 gene of sisters IG and MG displayed an identical single base pair deletion (378delC), resulting in a frameshift and premature truncation of the protein. As only alleles encoding the mutated DNA sequence were found by PCR analysis, the patients were considered homozygous for the 378delC mutation. However, a subsequent study revealed that they suffer from P2Y12 deficiency owing to haploinsufficiency and to the 378delC mutation in their remaining allele. Patient OSP-1 is homozygous for a single nucleotide substitution in the transduction initiation codon (ATG to AGG). The molecular defect that is responsible for P2Y12 deficiency in patient ML is less well defined: one mutant allele contains a deletion of 2 base pairs, resulting in a frameshift and early truncation of the protein. Surprisingly, the other allele did not display any mutation: the findings that the patient’s platelets contained P2Y12 transcripts derived from the mutant allele only and that his daughter, who had a heterozygous phenotype, inherited the mutant allele from her father and a normal allele from her mother, suggest that patient ML has an additional, as yet unknown mutation that silences his normal allele.
Congenital dysfunction of P2Y12

One patient (AC) with congenital bleeding disorder associated with abnormal P2Y12-mediated platelet responses to ADP, whose platelets display normal number of dysfunctional P2Y12 receptors has been described. Platelets from this patient displayed reduced and reversible aggregation in response to 4 μM ADP, similar to normal platelets with a blocked P2Y12 receptor. However, the response to 20 μM ADP, albeit still decreased and reversible, was more pronounced and was further inhibited by a P2Y12 antagonist, indicating residual receptor function. ADP failed to lower adenylyl cyclase activity stimulated by PGE1 in the patient’s platelets. Analysis of the patient’s P2Y12 gene revealed, in one allele, a G to A transition changing the codon for Arg256 in TM6 to Gln and, in the other, a C to T transition changing the codon for Arg265 in EL3 to Trp (Table 1). Neither mutation interfered with receptor surface expression but both altered receptor function, since ADP inhibited the forskolin-induced increase of cyclic AMP markedly less in cells transfected with either mutant P2Y12 than in wild type cells. These observations, in accordance with previous studies of the P2Y1 receptor,46,47 helped to identify regions in TM6 and EL3, whose structural integrity is necessary for normal receptor function (Figure 1). A heterozygous point mutation in the same region of the molecule, which changed codon 258 coding for proline (CCT) to threonine (ACT) (Pro258Thr), was described in a patient with mild bleeding disorder and severely impaired ADP-induced platelet aggregation.48 Since the proline to threonine substitution alters the protein hydrophobicity, size and rotational mobility, it is likely to affect the function of P2Y12.

Finally, a heterozygous mutation, predicting a lysine to glutamate (Lys174Glu) substitution in P2Y12, was identified in one patient with mild type 1 von Willebrand disease (VWD).49 Platelets from this patient showed reduced and reversible aggregation in response to ADP, up to 10 μM. The reduced response was associated with an approximate 50% reduction in binding of [3H]2MeS-ADP. Considering that Lys174 is situated in the second extracellular loop of P2Y12, adjacent to Cys175, which may be important for the expression of the ADP binding site receptor, and that a hemagglutinin-tagged Lys174Glu P2Y12 variant showed surface expression in Chinese hamster ovary cells, it is likely that the Lys174Glu mutation is responsible for disruption of the ADP binding site of the receptor.
It is interesting to note that, for reasons that are presently unclear, two patients with heterozygous dysfunctional P2Y12 (Pro258Thr and Lys174Glu) display a much more severe impairment of ADP-induced platelet aggregation compared to the two patients who are heterozygous for P2Y12 deficiency\cite{26,34} and to the two children of patient AC, who are heterozygous for the Arg265Gln mutation\cite{32} (Table 1).

**Bleeding diathesis**

Patients with defects of P2Y12 experience mucocutaneous bleedings and excessive post-surgical or post-traumatic blood loss. The severity of their bleeding diathesis is variable. The bleeding scores of patient VR and of the two sisters MG and IG, which was calculated using a standardized questionnaire that was developed to investigate patients with type-1 von Willebrand Disease\cite{50}, were 8, 7 and 13, respectively, (normal values ≤3) (unpublished data). After extensive investigation of hemostasis parameters, which included measurement of the activity of clotting and fibrinolytic factors and the search for known polymorphisms of hemostasis proteins, we found no explanation for the discrepancy in the severity of bleeding manifestations in the two sisters MG and IG.

The bleeding score of GL, the son of patient MG, was normal; however, it must be noted that this young boy had not yet experienced situations that could challenge the hemostatic system at the time of our investigation. His bleeding time, despite the mild defect of P2Y12, was prolonged (13 minutes).

**Diagnosis and treatment**

The diagnosis of P2Y12 defects is rather simple: they should be suspected when ADP, even at relatively high concentrations (≥10 μM), is unable to induce full, irreversible platelet aggregation, while inducing normal shape change. Tests that evaluate the degree of inhibition of adenylyl cyclase by ADP, by measuring either the platelet levels of cyclic AMP or the phosphorylation of vasodilator-stimulated phosphoprotein (VASP)\cite{51} after the exposure of platelets to PGE1, should be used to confirm the diagnosis (Table 2).

The intravenous infusion of the vasopressin analogue desmopressin (0.3 μg/kg) shortened the prolonged bleeding time of patient VR from 20 minutes to 8.5 minutes.\cite{36} After the infusion of
desmopressin, which was repeated twice at 24 hour intervals, the patient underwent a surgical intervention for disc hernia repair, which was not complicated by excessive bleeding. Although the efficacy of desmopressin in reducing bleeding complications of patients with defects of primary hemostasis is anecdotal,\textsuperscript{52} its administration is generally without serious side effects.

**Drugs targeting P2Y\textsubscript{12}**

Drugs that target P2Y\textsubscript{12} reduce the incidence of arterial thrombosis, as documented by the results of several randomized clinical trials (Table 3). Due to space limitations, this review will not analyze the results of these randomized clinical trials in detail: the interested reader is referred to recently published reviews.\textsuperscript{53,54}

**Thienopyridines**

First and second generation thienopyridines: ticlopidine and clopidogrel

Ticlopidine and clopidogrel (Figure 3) are pro-drugs that need to be converted \textit{in vivo} by the hepatic cytochrome P-450 (CYP) enzymatic pathway to active metabolites, which covalently bind to P2Y\textsubscript{12} by forming a disulfide bond with cysteine residues, thereby irreversibly inhibiting the receptor.\textsuperscript{53}

Several randomized clinical trials documented the clinical efficacy of these drugs in the prevention of major adverse cardiovascular events (MACE)\textsuperscript{55-64} (Table 3). Their efficacy was essentially similar to that of acetylsalicylic acid (ASA) in patients with stable disease of cerebral or coronary arteries. For patients with acute coronary syndromes, undergoing medical treatment only or in combination with percutaneous coronary intervention (PCI), the addition of thienopiridines to ASA proved highly efficacious. In contrast, the combination of clopidogrel and ASA was not more effective than monotherapy in low-moderate risk patients with stable disease, but increased the incidence of bleeding.

Due to its toxicity,\textsuperscript{53} ticlopidine has been almost completely replaced by clopidogrel in the clinical practice.

Despite its proven antithrombotic efficacy, clopidogrel has some important drawbacks:\textsuperscript{54} 1) its antiplatelet effects are delayed, due to the need for metabolism of the pro-drug; 2) there is substantial inter-individual variability in platelet inhibition; 3) its ability to irreversibly inhibit P2Y\textsubscript{12}
may represent a problem for patients who need to undergo coronary bypass (CABG) surgery, because the incidence of post-operative bleeding complications is higher than in patients not treated with clopidogrel. While the onset of action of clopidogrel can be accelerated by giving patients a loading dose of 300-600 mg, the solution of the other two problems appears more difficult.54

The high inter-individual variability of the response to clopidogrel is a clinically relevant issue, as it has been demonstrated that poor responders are not adequately protected from MACE.65 About 1/3 of treated patients do not display adequate inhibition of P2Y12-dependent platelet function; this condition is associated with loss of function mutations of CYP,66-68 with the homozygous 3435C→T mutation of ABCB1, a gene encoding for the efflux pump P-glycoprotein, a key protein involved in thienopyridine absorption,69,70 and may be exacerbated by negative interference with common adjunctive medications, such as proton pump inhibitors.68

Tailored treatment of patients, based on the results of platelet function tests or of CYP genotyping, has been proposed to solve the problem of clopidogrel resistance.54 This approach cannot be recommended in daily clinical practice yet, because the best laboratory method to monitor the effects of clopidogrel on platelet function still needs to be identified, standardized (for pre-analytical and analytical variables) and validated in the clinical setting. Several recent studies demonstrated that the agreement among different laboratory tests to identify poor responders is rather low and that assessment of platelet response to clopidogrel is highly test-specific.71-76 Recent studies showed that the search for loss of function mutations of CYP is not very accurate in predicting the response to clopidogrel.77,78 In addition, preliminary experiments that evaluated the effects of increasing the dose of clopidogrel in resistant patients gave results that are incompletely satisfactory, because many patients remained “resistant” to clopidogrel, even after repeated administrations of high doses of the drug.79,80

Mostly based on the aforementioned consideration, a recent consensus paper concluded that until the results of large-scale trials of personalized antiplatelet therapy are available, the routine use of platelet function measurements in the care of patients with cardiovascular disease cannot
be recommended. Therefore, the use of new P2Y₁₂ antagonists that are able to induce predictable and adequate inhibition of platelet function in all patients is desirable.

**Prasugrel, a new thienopyridine**

Prasugrel is a new thienopyridine, with much more rapid and consistent inhibitory effects on platelet aggregation than clopidogrel. It has a distinct chemical structure, which permits conversion to its active metabolite with less dependence on CYP enzymes than clopidogrel (Figure 3).

Consequences of the different metabolism of prasugrel, compared to that of clopidogrel, are:

1) faster appearance and higher concentration of its active metabolite in circulating blood;
2) faster and greater mean inhibition of P2Y₁₂-dependent platelet function;
3) no influence of the CYP genotype on its pharmacokinetics, pharmacodynamics and antithrombotic activity;
4) much lower inter-individual variability in inhibition of P2Y₁₂-dependent platelet responses and very low prevalence of subjects who display “resistance” to the drug.

The aforementioned more favourable characteristics of prasugrel compared to clopidogrel result in greater clinical benefit, as shown by the results of TRITON TIMI-38, which evaluated 13,608 high-risk patients with acute coronary syndromes who required PCI. Patients were randomized to receive prasugrel 60-mg loading dose followed by 10 mg/day or clopidogrel 300 mg followed by 75 mg/day for 6-15 months. Prasugrel was associated with fewer ischemic events but more non CABG- and CABG-related major and fatal bleedings (Table 4).

Based on the results of the TRITON TIMI-38 trial, prasugrel is generally considered a more potent antiplatelet agent than clopidogrel, to be used only in high-risk patients or for a short period, while treatment with clopidogrel should be preferred in the remaining situations. However, it is incorrect to say that prasugrel is more potent than clopidogrel, as both ex vivo and in vitro studies demonstrated that the active metabolites of the two compounds have the same potency. The different clinical efficacy and safety of prasugrel compared to clopidogrel is mostly explained by the fact that very few treated patients are “resistant” to prasugrel. Because protection from thrombotic events and exposure to bleeding risk are a function of the degree of inhibition of P2Y₁₂-dependent platelet function, the higher efficacy and the lower safety of prasugrel compared to clopidogrel are simply explained by the fact that prasugrel protects from MACE and exposes to the risk of bleeding
more patients than clopidogrel. Based on the results of published studies, it can be predicted that, if tailored treatment with clopidogrel were successful in all patients displaying hyporesponsiveness to the drug, incidences of MACE and bleedings in patients given tailored clopidogrel treatment would be similar to those observed in patients given prasugrel.54

Therefore, prasugrel appears an attractive solution to some of the problems that are associated with the use of clopidogrel. However, prasugrel does not solve the problem associated with the slow offset of action, because, like clopidogrel, it causes irreversible inhibition of P2Y12. Some direct P2Y12 antagonists with such characteristics are currently under development.

**Direct P2Y12 inhibitors**

**Ticagrelor**

Ticagrelor belongs to the new chemical class cyclopentyl-triazolo-pyrimidines (Figure 4): it does not require conversion to an active metabolite and has a half life of 7-8.5 h.85,86 After oral administration, it rapidly and reversibly inhibits P2Y12 via a mechanism that is noncompetitive with ADP, suggesting the existence of an independent receptor binding site.87 In phase II trials, ticagrelor more rapidly and effectively inhibited platelet aggregation and with less variability than clopidogrel.88,89 A study that compared the onset and offset of action of clopidogrel and ticagrelor showed that, despite the greater mean antiplatelet effect of ticagrelor, inhibition of platelet aggregation at 24 hours after the last dose was equivalent in ticagrelor- and clopidogrel-treated patients, which is indicative of a faster offset of effect.90 Considering that, due to its short half life, ticagrelor needs to be administered every 12 hours, which might negatively affect the compliance of patients under chronic treatment, these data have relevant practical implications, because they suggest that patients who miss 1 dose of ticagrelor will have a level of platelet inhibition at 24 hours after the last dose that is not inferior to that of patients undergoing chronic clopidogrel therapy. Dyspnea was reported in 10-20% of patients treated with ticagrelor in phase II trials, although none of the incidents was considered to be serious.89,90 The pathogenesis of dyspnea during ticagrelor treatment is unclear, although it has been hypothesized that it may be mediated by adenosine.54
The results of PLATO trial, in which ticagrelor (180 mg LD, 90 mg b.i.d, MD) was compared to clopidogrel (300-600 mg LD, 75 mg daily MD) for prevention of MACE in patients with non-ST or ST elevation acute coronary syndromes (2/3 of them underwent PCI) showed that ticagrelor decreases the incidence of MACE, compared to clopidogrel (Table 4). Very importantly, ticagrelor also decreased the incidence of cardiovascular and total mortality. There was a higher incidence of TIMI major non-CABG-related bleedings in patients who received ticagrelor, compared with those treated with clopidogrel. The incidence of major CABG-related bleedings was similar in the two groups. Therefore, similarly to the TRITON-TIMI 38 trial, the PLATO trial showed that a more consistent, adequate inhibition of P2Y₁₂-dependent platelet function than that achieved with standard doses of clopidogrel is associated with greater antithrombotic efficacy and higher risk of non-CABG-related major bleedings. The higher incidence of clinically irrelevant dyspnea in ticagrelor-treated patients was confirmed in the PLATO study.

Cangrelor

Cangrelor (Figure 4) belongs to a family of analogues of ATP that are relatively resistant to breakdown by ectonucleotidases and display high affinity for the P2Y₁₂ receptor, which is reversibly inhibited by the drug. Cangrelor does not require conversion to an active metabolite and is immediately active after intravenous infusion, with a half-life of 3-6 minutes.

Two trials, which compared cangrelor to clopidogrel in patients requiring PCI, were prematurely terminated due to insufficient evidence of superiority of cangrelor. Cangrelor is still being studied as a bridge for patients who need to suspend thienopyridines before surgery.

Bleeding events associated with treatment with P2Y₁₂ inhibitors

As already mentioned, patients with congenital P2Y₁₂ defects have a bleeding diathesis of variable severity: it was therefore not unexpected that drugs targeting P2Y₁₂ increase the incidence of bleedings. Although the value of laboratory tests of hemostasis for the prediction of bleeding events in patients with acute coronary syndromes under antithrombotic treatment is extensively being evaluated, a recent study showed that a simple bleeding score, based on 6 readily available clinical and laboratory variables (female sex, advanced age, elevated serum creatinine and white
blood cell count, anemia, type of acute coronary syndrome), plus the anticoagulation regimen used, may provide a rapid tool to predict the rate of major bleeding in these patients.\textsuperscript{94}

**Major bleedings and the risk of mortality**

Severe bleeding complications during antithrombotic therapy have negative consequences, not only because they may be fatal, disabling, and expose the patients to the risks that are associated with blood transfusion: they are also associated with poor prognosis of the patients, whose risk of death is increased during a follow-up of up to 1 year.\textsuperscript{94-96} The nature of the relationship between major bleeding complications and long-term mortality is unclear. Despite the fact that this association remained statistically significant after adjustment for confounders, it is still possible that bleeding may simply be a marker of an underlying severe disease state, which exposes the patient to increased risk of mortality. A direct causal effect of major bleeding on the long-term risk of death is unlikely and, as a matter of fact, it is ruled out by the observation that major bleeding after CABG surgery is not associated with increased mortality.\textsuperscript{94,96} Yet, the possibility that non-CABG-related major bleeding may be indirectly causally associated with increased mortality of patients under treatment with antithrombotic drugs is biologically plausible. Antithrombotic drugs are usually withheld in patients who experience major bleeding, and this exposes them to high risk of MACE. In keeping with this hypothesis is the observation that major bleeding was also associated with increased risk of ischemic events, such as myocardial infarction and stroke.\textsuperscript{97}

**Old thienopyridines vs ASA**

Randomized clinical trials that compared old thienopyridines (ticlopidine or clopidogrel) to ASA showed that the risk of bleeding was not different between the two treatment arms (Table 3). This observation is somewhat surprising, considering that \textit{in vitro} and \textit{in vivo} experiments demonstrated that ADP plays a more important role in platelet thrombus formation than thromboxane A\(_2\). Although there are many plausible explanations for these unexpected results, the high prevalence of non-responders to old thienopyridines, who are not exposed to the risk of bleeding, is the most plausible.

**Combined treatment with clopidogrel and ASA**
Combined treatment with clopidogrel and ASA is associated with increased bleeding compared to monotherapy. If this is a fair price to pay when treating patients with ACS, in consideration of the net clinical benefit associated with combined therapy, it is unacceptable for secondary prophylaxis of patients with stable disease or for primary prophylaxis of patients at risk, because the higher bleeding risk is not counterbalanced by antithrombotic efficacy in these settings (Table 3).

New P2Y<sub>12</sub> inhibitors vs clopidogrel

The higher incidence of bleeding complications that was observed in patients treated with the new P2Y<sub>12</sub> antagonists prasugrel and ticagrelor, compared to clopidogrel (Table 4), is mostly explained by the fact that the new drugs effectively inhibit P2Y<sub>12</sub>-dependent platelet function in the great majority of treated patients, who will not only be protected from MACE, but will also be exposed to higher risk of bleeding, compared to patients treated with clopidogel who do not respond adequately to the drug. This hypothesis is biologically plausible and is supported by the observation that patients who respond adequately to clopidogrel not only are better protected from MACE, but also experience an increased incidence of bleedings, compared to poor responders.

P2Y<sub>12</sub> inhibitors and CABG-related bleeding

A variable percentage of patients with acute coronary syndromes (<10%) need to undergo CABG surgery. It has been demonstrated that clopidogrel treatment within about 4 days of the procedure is associated with increased blood loss, reoperation for bleeding, increased transfusion requirements and prolonged intensive care unit and hospital stays.<sup>54</sup> For this reason, when the clinical conditions of the patients allow it, clopidogrel is usually withheld for 5 days before CABG, in order to restore the hemostatic competency of the patient. This procedure was followed for all patients undergoing CABG in randomized clinical trials that compared the new P2Y<sub>12</sub> antagonists to clopidogrel. For this reason, the incidence of CABG-related bleeding complications should not be considered when evaluating the risk of bleeding associated with the new anti-P2Y<sub>12</sub> drug, for the simple reason that patients were off-treatment when they underwent CABG. Differences among P2Y<sub>12</sub> antagonists in this setting should be evaluated on the basis of the time needed to withhold treatment before surgery to restore hemostatic competency. Considering that withholding
antiplatelet treatment exposes patients to high risk of MACE, it is obvious that drugs with reversible mechanism of action and short half life, such as ticagrelor, may be preferable to drugs that irreversibly inhibit the receptor.
Authorship

I searched, read the literature, and wrote the paper.

Conflict of interest

Marco Cattaneo participated to advisory board meetings and received lecture honoraria by

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Table 1. Characteristics of the described patients with congenital P2Y$_{12}$ defects.

<table>
<thead>
<tr>
<th>Patient identification</th>
<th>References</th>
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<td>VR</td>
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<td>p.[Gln98fs]+[Gln98fs]</td>
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<td>ML</td>
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<td>p.[Phe240fs]+[?]*</td>
<td>Severely reduced</td>
<td>Reduced and reversible</td>
</tr>
<tr>
<td>IG and MG</td>
<td>26,44</td>
<td>p.[0]+p.Thr126fs*</td>
<td>Severely reduced</td>
<td>Reduced and reversible</td>
</tr>
<tr>
<td>OSP-1</td>
<td>40</td>
<td>p.[0]+[0]$\dagger$</td>
<td>Not tested</td>
<td>Reduced and reversible</td>
</tr>
<tr>
<td>??</td>
<td>41</td>
<td>p.[Gly12fs]+[Gly12fs]</td>
<td>Not tested</td>
<td>Reduced and reversible</td>
</tr>
<tr>
<td>CL</td>
<td>34,45</td>
<td>p.[Phe240fs]+[=]</td>
<td>Intermediate</td>
<td>Full and irreversible</td>
</tr>
<tr>
<td>GL</td>
<td>26,44</td>
<td>p.[0]+[=]$\dagger$</td>
<td>Intermediate</td>
<td>Full and irreversible</td>
</tr>
<tr>
<td>AC</td>
<td>32</td>
<td>p.[Arg256Gln]+[Arg265Trp]</td>
<td>Normal</td>
<td>Reduced and reversible</td>
</tr>
<tr>
<td>MC and FC</td>
<td>32</td>
<td>p.[Arg265Trp]+[=]</td>
<td>Normal</td>
<td>Full and irreversible</td>
</tr>
<tr>
<td>GS</td>
<td>48</td>
<td>p.[Pro258Thr]+[=]</td>
<td>Not tested</td>
<td>Reduced and reversible</td>
</tr>
<tr>
<td>PII.1</td>
<td>49</td>
<td>p.[Lys174Glu]+[=]</td>
<td>Intermediate</td>
<td>Reduced and reversible</td>
</tr>
</tbody>
</table>

Patient CL is the daughter of patient ML; patient GL is the son of patient MG; MC and FC are the son and the daughter of patient AC.

*No mutations were found in one allele of patient ML; however, the findings that the patient’s platelets contained P2Y$_{12}$ transcripts derived from the mutant allele only and that his daughter (CL) inherited the mutant allele from her father and a normal allele from her mother, suggest that patient ML has an additional, as yet unknown mutation that silences his normal allele (see text for details).

$\dagger$ Failure of expression of the P2Y$_{12}$ protein (p.[0]) in patient OSP-1 was associated with homozygous single nucleotide substitution in the transduction initiation codon (ATG to AGG).

$\dagger$ p[0] was associated with partial or complete P2Y$_{12}$ gene deletion in patients IG, MG and GL.
Table 2. Diagnosis of severe congenital P2Y\textsubscript{12} defects.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <strong>Clinical hallmarks</strong></td>
<td></td>
</tr>
<tr>
<td>Lifelong history of mucocutaneous bleedings; excessive</td>
<td></td>
</tr>
<tr>
<td>post-surgical or post-traumatic blood loss</td>
<td></td>
</tr>
<tr>
<td>2. <strong>Laboratory hallmarks</strong></td>
<td></td>
</tr>
<tr>
<td>• <strong>Screening test</strong>: inability of high concentrations of ADP</td>
<td></td>
</tr>
<tr>
<td>((\geq 10 \mu\text{M})) to induce full and reversible</td>
<td></td>
</tr>
<tr>
<td>platelet aggregation (light transmission aggregometry)</td>
<td></td>
</tr>
<tr>
<td>• <strong>Confirmatory test</strong>: inability of ADP to inhibit PGE\textsubscript{1}-stimulated adenyl cyclase</td>
<td></td>
</tr>
<tr>
<td>(measurement of platelet cyclic AMP or phosphorylation of</td>
<td></td>
</tr>
<tr>
<td>vasodilator-stimulated phosphoprotein)</td>
<td></td>
</tr>
</tbody>
</table>


Table 3. Main results of major double-blind, randomized, controlled clinical trials with ticlopidine or clopidogrel

<table>
<thead>
<tr>
<th>Clinical trial</th>
<th>Ref.</th>
<th>Patients</th>
<th>Treatments</th>
<th>Cardiovascular end-points</th>
<th>Fup</th>
<th>Efficacy*</th>
<th>Bleeding events**</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATS 55</td>
<td></td>
<td>Recent thromboembolic stroke</td>
<td>1. Ticlopidine 2. Placebo</td>
<td>Stroke, MI, or vascular death</td>
<td>24 mo</td>
<td>23.3% (1.0-40.5)</td>
<td>6.5%</td>
</tr>
<tr>
<td>TASS 56</td>
<td></td>
<td>Recent transient or mild persistent focal cerebral or retinal ischemia</td>
<td>1. Ticlopidine 2. ASA</td>
<td>Non-fatal stroke or death from any cause</td>
<td>3 y</td>
<td>12% (-2%-26%)</td>
<td>9.0%</td>
</tr>
<tr>
<td>ISAR 57</td>
<td></td>
<td>CAD patients: PCI and stent implantation</td>
<td>1. Ticlopidine+ASA 2. Heparin/VKA+ASA</td>
<td>Cardiac death or AMI, CABG, or repeated PCI</td>
<td>4 wk</td>
<td>0.25 (0.06-0.77)</td>
<td>0.00-0.019</td>
</tr>
<tr>
<td>SAR 58</td>
<td></td>
<td>CAD patients: PCI and stent implantation</td>
<td>1. Ticlopidine+ASA 2. Heparin/VKA+ASA 3. ASA</td>
<td>AMI, death, repeat PCI, stent thrombosis at angiography</td>
<td>30 d</td>
<td>1 vs 2: 0.88 (0.55–1.43) 1 vs 3: 3.06 (1.57-5.97)</td>
<td></td>
</tr>
<tr>
<td>CAPRIE 59</td>
<td></td>
<td>Atherosclerotic vascular disease</td>
<td>1. Clopidogrel 2. ASA</td>
<td>Ischemic stroke, AMI, or vascular death</td>
<td>1.9 y</td>
<td>8.7% (0.3-16.5)</td>
<td>9.27% vs 9.28%</td>
</tr>
<tr>
<td>CHARISMA 60</td>
<td></td>
<td>clinically evident CV disease or multiple risk factors</td>
<td>1. Clopidogrel + ASA 2. Placebo + ASA</td>
<td>MI, stroke, or CV death</td>
<td>28 mo</td>
<td>0.93 (0.83-1.05)</td>
<td>F: 1.53 (0.83-2.82) M: 1.25 (0.97-1.61) Mod: 1.62 (1.27-2.08)</td>
</tr>
<tr>
<td>MATCH 61</td>
<td></td>
<td>recent ischemic stroke or TIA and ≥ 1 risk factor</td>
<td>1. Clopidogrel+ASA 2. Clopidogrel+placebo</td>
<td>Ischemic stroke, MI, vascular death, or rehospitalisation for acute ischemia</td>
<td>18 mo</td>
<td>6.4% (-4-6-16.3)</td>
<td>LT: 1.26 (0.62 - 1.88) M: 1.36 (0.86 - 1.86)</td>
</tr>
<tr>
<td>CURE 62</td>
<td></td>
<td>Acute coronary syndromes</td>
<td>1. Clopidogrel+ASA 2. Placebo+ASA</td>
<td>CV death, nonfatal AMI, or stroke</td>
<td>12 mo</td>
<td>0.80 (0.72-0.90)</td>
<td>LT: 1.21 (0.95–1.56) M: 1.38 (1.13–1.67) T: 1.69 (1.48–1.94)</td>
</tr>
<tr>
<td>COMMIT 63</td>
<td></td>
<td>Suspected AMI</td>
<td>1. Clopidogrel+ASA 2. Placebo+ASA</td>
<td>(1) Death, reinfarction, or stroke; (2) death from any cause</td>
<td>Up to 28d</td>
<td>(1) 0-91 (0.96-0.97) (2) 0.93 (0.87-0.99)</td>
<td>F: -0.1 (SE-0.5) M: 0.4 (0.7) m: 4.7 (1.7)</td>
</tr>
<tr>
<td>PCI CURE 64</td>
<td></td>
<td>NSTE ACS undergoing PCI in the CURE study</td>
<td>1. Clopidogrel+ASA 2. Placebo+ASA</td>
<td>CV death, AMI, or urgent target-vessel revascularisation</td>
<td>30 d</td>
<td>0.70 (0.50-0.97)</td>
<td>LT: 0.92 (0.38–2.26) M: 1.13 (0.61-2.10) m: 1.33 (0.59-3.03)</td>
</tr>
</tbody>
</table>

*Results are reported as: relative risk reduction,55,56,59,60 relative risk,57,58,60,62,63,64 (95% C.I.).

**When available, data on major (M), life-threatening (LT), fatal (F), moderate (Mod.), minor (m) and total (T) bleeding events are reported as: total incidence,55,56,59 relative risk,57,58,60,61,62,64 excess per 1,000 patients,63 (95% C.I.).

Doses of the antiplatelet agents:

Ticlopidine: 250 mg b.i.d.55-58
ASA: 650 mg b.i.d.,55 325 mg q.d.,58,59 100 mg b.i.d.,57 160 mg q.d.;63 75-325 mg q.d.,62,64 75-160 mg q.d.50 75 mg q.d.61
Clopidogrel: 75 mg q.d. in all randomized clinical trials, plus 300 mg loading dose in the CURE trial.62,64

Abbreviations: CAD coronary artery disease; PCI percutaneous coronary intervention; CABG, coronary artery bypass surgery; VKA, vitamin K antagonist; AMI, acute myocardial infarction; CV, cardiovascular; ASA, acetyl salicylic acid.
Table 4. Main results of two double-blind, randomized clinical trials that compared prasugrel and ticagrelor to clopidogrel for the treatment of patients with ACS

<table>
<thead>
<tr>
<th>Clinical trial</th>
<th>Ref</th>
<th>Patients</th>
<th>Treatments</th>
<th>Primary end-points</th>
<th>Follow up</th>
<th>Efficacy HR (95%CI)</th>
<th>TIMI major Bleedings* HR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRITON TIMI38</td>
<td>82</td>
<td>ACS with scheduled PCI</td>
<td>1. Prasugrel + ASA 2. Clopidogrel + ASA</td>
<td>CV death, nonfatal stroke, nonfatal AMI</td>
<td>6-15 mo</td>
<td>0.81 (0.73-0.90)</td>
<td>1.32 (1.03-1.68)</td>
</tr>
<tr>
<td>PLATO</td>
<td>91</td>
<td>ACS with or without ST elevation</td>
<td>1. Ticagrelor + ASA 2. Clopidogrel + ASA</td>
<td>CV death**, AMI, stroke</td>
<td>12 mo</td>
<td>0.84 (0.77-0.92)</td>
<td>1.25 (1.03-1.53)</td>
</tr>
</tbody>
</table>

Although the investigators of PLATO trial elaborated original criteria to classify the severity of bleeding episodes, they also calculated the incidence of bleedings based on TIMI criteria, which are reported in this Table for easier comparison with the results of the TRITON TIMI 38 trial.

* the non CABG-related bleedings only are reported (see text for more details).

** the incidence of death from any cause was significantly decreased by ticagrelor, compared to clopidogrel

Doses of the antiplatelet agents:
- Prasugrel: 60 mg loading dose + 10 mg q.d.
- Ticagrelor: 180 mg loading dose + 90 mg b.i.d
- Clopidogrel: 300 mg (TRITON TIMI 38) and 300-600 mg (PLATO) LD + 75 mg q.d.
- Aspirin: 100 mg q.d. (TRITON TIMI 38) and 75-325 mg q.d. (PLATO)

Abbreviations: ACS, acute coronary syndrome; PCI percutaneous coronary intervention; AMI, acute myocardial infarction; CV, cardiovascular; ASA, acetyl salicylic acid; HR, hazard ratio.
Figure legends

Figure 1. Predicted secondary structure of the human P2Y<sub>12</sub> receptor. Black circles highlight the sites of aminoacid substitution in patients with dysfunctional P2Y<sub>12</sub> (Table 1). TM, transmembrane region; EL, extracellular loop; IL, intracellular loop.

Figure 2. Central role of P2Y<sub>12</sub> in platelet function. See text for details. Legend of symbols: green arrow, activation; truncated red line, inhibition; blue line ending with a (+), amplification; dotted black line, secretion.

Figure 3. Metabolic pathways for the transformation of thienopyridines to their active metabolites. CYP, cytochrome P450.

Figure 4. Chemical structures of the direct P2Y<sub>12</sub> inhibitors ticagrelor and cangrelor.
Figure 1

[Diagram of P2Y12 receptor structure with labeled domains and sites for glycosylation and phosphorylation]
Figure 3

- Ticlopidine
  - CYP2B6
  - CYP2C19
  - other
  - Ticlopidine thiolactone (2-Oxo-ticlopidine)
  - Ticlopidine active metabolite

- Clopidogrel
  - CYP2C19
  - CYP1A2
  - CYP2B6
  - Clopidogrel thiolactone (2-Oxo-clopidogrel)
  - Clopidogrel active metabolite

- Prasugrel
  - Estersases (hCE2)
  - Prasugrel thiolactone (R-99139)
  - Prasugrel active metabolite
The platelet P2Y<sub>12</sub> receptor for adenosine diphosphate: congenital and drug-induced defects

Marco Cattaneo