
# UNIVERSITÀ DEGLI STUDI DI MILANO

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# Does the inhibition of $P2Y_{12}$ inhibit the production of thromboxane $A_2$ by platelets?

Mariangela Scavone

Matr.n. R09136

Tutor:

Chiar.mo Prof. Marco Cattaneo

Coordinatore del Dottorato:

Chiar.mo Prof. Marco Cattaneo

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# SUMMARY

Patients with acute coronary syndromes (ACS) are treated with dual antiplatelet therapy (DAPT), which includes aspirin, an inhibitor of thromboxane A2 ($TxA_2$) production, and an antagonist of the $P2Y_{12}$ receptor for ADP. Based on the recent observation that $P2Y_{12}$ antagonists also inhibit the platelet production of $TxA_2$, it has been suggested that patients with ACS might be safely treated with $P2Y_{12}$ antagonists only. However, the observation that platelets congenitally deficient of $P2Y_{12}$ synthesize normal amounts of $TxA_2$ contrasts the results obtained with $P2Y_{12}$ antagonists. To test whether the reported inhibitory effect of $P2Y_{12}$ antagonists on $TxA_2$ production is due to off-target effects, or secondary to inhibition of platelet aggregation (PA). Serum $TxB_2$ was measured in 2 patients with inherited $P2Y_{12}$ deficiency and 7 healthy subjects in presence/absence of increasing concentrations of $P2Y_{12}$ antagonists added *in vitro*, and in 20 patients treated with 10 mg/d prasugrel ($P2Y_{12}$ antagonist) or placebo for 14 days in a randomized, double-blind, cross-over study. $TxB_2$ levels were also measured after stimulation of citrate-PRP by collagen (0,5µg/mL) or arachidonic acid (1mM) in an aggregometer in presence/absence of $P2Y_{12}$ antagonists, under stirring and non-stirring conditions (PA does not occur without stirring). $P2Y_{12}$ antagonists did not decrease serum $TxB_2$ levels both *in vitro* and *ex vivo* (prasugrel-treated patients). They partially inhibited $TxB_2$ production under stirring, but not under non-stirring conditions.

In conclusion, $P2Y_{12}$ antagonists do not inhibit the platelet $TxA_2$ production; therefore, there is no pharmacological evidence that aspirin should be withheld in patients with ACS.

# 1. INTRODUCTION

Patients with acute coronary syndromes (ACS) are treated with dual antiplatelet therapy, which includes aspirin, an inhibitor of thromboxane A2 ($TxA_2$) production, and an antagonist of the $P2Y_{12}$ receptor for ADP. Based on the recent observation that $P2Y_{12}$ antagonists also inhibit the platelet production of $TxA_2$, it has been suggested that patients with ACS might be safely treated with $P2Y_{12}$ antagonists only. However, the observation that platelets congenitally deficient of $P2Y_{12}$ synthesize normal amounts of $TxA_2$ contrasts the results obtained with $P2Y_{12}$ antagonists.

The aim of this study will be to better understand the effect of $P2Y_{12}$ antagonists on platelet $TxA_2$ production.

## 1.1 Platelets

### 1.1.1 Platelet biology

Platelets are small anuclear blood cells, with a discoid shape ranging between 1 to 3 μm in diameter. These cell fragments originate from the cytoplasm of megakaryocytes (MKs) in the bone marrow and circulate in the human bloodstream for about 10 days. Platelets lack genomic DNA [1] but contain megakaryocyte-derived messenger RNA (mRNA) and the translational machinery needed for protein synthesis including ribosomes, and initiation and termination factors [2]. Furthermore, platelets contain three types of secretory organelles known as α-granules, δ-granules (dense) and lysosomes, which are generated by the budding of small vesicles containing granule cargo from the *trans*-Golgi zone of the Golgi complex in MKs [3]. The number of α-granules per platelet depends on cell size but and may range between 40 and 80. They contain many proteins, such as coagulation factor V, thrombospondin, P-selectin, von Willebrand Factor (vWF) and fibrinogen. The δ-granules, compared with α-granules, are smaller, fewer, and have high morphological variability. They are rich in ATP and ADP, serotonin, pyrophosphate, calcium, and magnesium. Human platelets also contain few lysosomes (no more than 3), which contain at least 13 acid hydrolases. Other organelles present in the platelet cytoplasm include a small number of simple mitochondria involved in energy metabolism, glycosomes [4], electron dense chains and clusters [5], and tubular inclusions [6].

### 1.1.2 Platelet in primary hemostasis

The main role of blood platelets is to ensure primary haemostasis, which means the rapid cessation of bleeding after tissue trauma and the maintenance of the integrity of the endothelium, in part through the release of proangiogenic cytokines and growth factors. The balance between blood fluidity and rapid thrombus formation in response to injury is regulated by endothelial cells, which synthesize either inhibitors or activators of platelet aggregation and blood clotting [7,8]. Under normal physiological conditions, platelets circulate close to the endothelium without establishing/ forming stable adhesion contacts. The anti-adhesive phenotype of vascular endothelium cells towards platelet is maintained by at least 4 intrinsic pathways. The arachidonic acid-prostacyclin (PGI2) and the L-arginine-nitric oxide (NO) pathways inhibit platelet activation by the stimulation of cAMP and cGMP production respectively, whereas endothelial ecto-adenosine diphosphatase (ecto-ADPase/CD39) is involved in ADP metabolism, which is necessary to prevent premature platelet activation at the vessel wall. Furthermore, thrombomodulin rapidly inhibits the prothrombotic effect of α thrombin, reducing platelet activation and fibrin generation (Figure 1).

At sites of vascular injury, platelets interact with the damaged vessel, to form a platelet aggregate. The initial platelet tethering at the surface and subsequent platelet-platelet cohesion are typically differentiated into the following steps: adhesion, activation, secretion and aggregation of platelets [9].

- *Platelet Adhesion*

  After vascular injury, such as rupture or erosion of the vessel wall, subendothelial matrix proteins such as collagen, von Willebrand factor (vWF), fibronectin and laminin become exposed to the circulating blood. These proteins support platelet adhesion via the engagement of specific receptors, thus rapidly recruiting individual platelets at the site of subendothelial damage. The initial tethering of platelets occurs via the interaction between glycoprotein Ib (GPIb), a component of GPIb-V-IX platelet complex, and exposed collagen-bound vWF. This bond has a rapid dissociation rate and is therefore unable to support stable adhesion, resulting in platelet translocation along the vessel wall. Translocating platelets engage with collagen in the vessel wall through their adhesion receptors glycoprotein VI (GPVI) and GPIa. GPVI is the major collagen receptor, whose stimulation induces the intracellular calcium flux necessary for stable platelet adhesion, cytoskeletal reorganization, integrin glycoprotein IIb/IIIa (αIIbβ3) activation and the release of soluble agonists.

- *Platelet Activation and Secretion*

After the initial adhesion, platelets undergo the repair process that requires a quick response to autocrine and paracrine mediators. Platelets experience a complex series of morphological and biochemical changes that lead to the release of platelet granular content such as ADP and serotonin (5-HT), as well as to the synthesis of $TxA_2$. These endogenous agonists act to enhance platelet activation by interacting with specific G-protein coupled receptors expressed on the platelet membrane. Briefly, ADP and 5-HT are released from platelet dense granules and bind their specific receptors. Activation of the $5\text{-}HT_{2A}$ receptor by 5-HT and the P2Y1 receptor by ADP (both coupled to a Gq protein) induces an increase in intracellular $Ca^{2+}$ levels, whereas activation of $P2Y_{12}$ (couple to Gi protein) by ADP activates PI3kinase and inhibits adenylate cyclase. $TxA_2$ is synthesized in activated platelets starting from arachidonic acid (AA) by cyclooxygenase (COX). Once formed, $TxA_2$ diffuses across the platelet membrane and activates other platelets through the interaction with two surface membrane $TxA_2$ receptors, TPα and TPβ, coupled to the proteins $G_q$ and $G_{12}$ or $G_{13}$, which activate phospholipase C (PLC). This enzyme degrades membrane phospholipids, thus releasing secondary messengers inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates intracellular protein kinase C (PKC), which causes protein phosphorylation, whereas IP3 increases cytosolic $Ca^{2+}$ levels from the endoplasmic reticulum. In addition platelets provide a catalytic surface necessary for local production of thrombin thus enhancing platelet activation. Indeed, at the site of injury prothrombin is proteolytically cleaved to form thrombin, a serine protease that converts soluble fibrinogen into insoluble strands of fibrin. Subsequently, thrombin mediates cleavage of the N-terminal extradomain of protease-activated receptors (PAR)-1 and (PAR)-4, that increases intracellular calcium ($Ca^{2+}$). The generation of thrombin is contingent upon the expression of tissue factor (TF) on the surface of fibroblasts, smooth muscle cells, endothelial cells and leukocytes. Thrombin is among the most potent stimulators of platelets.

- *Platelet Aggregation*

Aggregation is the amplification step that involves accumulation of platelets into the hemostatic thrombus through release of soluble agonists that enhance recruitment of further platelets. The stimulation of Gq and Gi signaling pathways leads to activation of the glycoprotein complex GPIIb/IIIa. Activated GPIIb/IIIa binds multiple ligands, including vWF [10] [11], fibrinogen [12], fibrin and fibronectin [13], able to form stable platelet aggregates [14]. The primary hemostatic plug is

consolidated by fibrin generation at the site of injury. Platelet activation is under tight negative control to limit and contain thrombus formation within the boundaries of the lesion in the vessel wall.

### 1.1.3 Role of platelets in arterial thrombosis and atherosclerosis

Arterial thrombosis and atherosclerosis have been considered separate entities with different pathogenic mechanisms, natural histories and therapies. Arterial thrombosis is primarily mediated by platelets and fibrin [15], whereas pathogenesis of atherosclerosis is multifactorial but is mainly promoted by altered function of endothelial and smooth muscle cells, deposition of lipids metabolized abnormally and oxidized in the vascular wall, and the local infiltration of leukocytes [16] [17]. However, it is becoming clear that the cellular and biochemical interactions underlying thrombosis are also directly relevant to atherosclerosis [18] .

*Arterial thrombosis*

Arterial thrombosis is an acute complication that occurs after rupturing or erosion of unstable atherosclerotic plaque in the blood vessels. In the case of coronary heart disease it causes heart attack, whereas in the case of cerebrovascular disease it leads to stroke. The most abundant components of the occlusive arterial thrombi formed are platelets with fibrin. On the other hand, platelets seem to be less relevant in the pathogenesis of venous thromboembolism.

Platelet thrombus formation is thought to occur in successive stages. After vascular injury, under conditions of rapid blood flow that occur in stenotic diseased arteries, platelets adhere to the exposed subendothelium (platelet adhesion), are activated (platelet activation) and secrete their granule contents (platelet secretion), including some platelet agonists (ADP and serotonin) which contribute to the recruitment of additional platelets to form aggregates (platelet aggregation) by interacting with specific platelet receptors. In this pathological condition the mechanism supporting platelet adhesion and aggregation at the site of vascular injury follows the physiological processes previously described in the paragraph 1.1.2.

*Atherosclerosis*

Atherosclerosis is in part an inflammatory disease, affecting medium and large size arteries through the accumulation of fatty substances, cholesterol, cellular waste product, calcium and fibrin within the arterial intima resulting in atherosclerotic plaques. Atherosclerosis starts when these deposits (plaque) lead to the inner surface of the blood vessel becoming damaged and the narrowing of the

lumen, making it harder for blood to flow through. Plaques may induce partial or total obstruction of blood flow through the artery (stenosis) and in the case that a piece of the plaque breaks off, trigger the formation of a blood clot (thrombus), causing heart attack or stroke.

Several factors promote the process of atherosclerosis, they are known as risk factors and include [19]:

- Cigarette smoke
- Decreased physical activity
- Consumption of unhealthful foods (rich in salt, fat and calories)
- Harmful use of alcohol
- High blood pressure (hypertension)
- Diabetes
- High levels of cholesterol and triglycerides
- Overweight and obesity
- Male gender
- Genetic disposition
- Psychological factors (e.g. stress, depression)
- Other risk factors (e.g. excess homocysteine)

Platelets play a central role in arterial thrombosis but may also participate in the development and progression of atherosclerotic plaque. Platelets promote the progression of plaque formation by forming platelet/leukocyte aggregates and via adhesion to the endothelium. Specific pro-inflammatory signals make endothelial cells more adhesive toward platelets, stimulating the production of various platelet-derived inflammatory molecules that provide a positive feedback loop for the activation of further endothelial cells. Indeed, under inflammatory conditions platelets can adhere to the intact but activated endothelial cells. [20] [21] [22]. Additionally an increase of circulating activated platelets promotes atherosclerosis. Endothelial-bound platelets are highly effective at recruiting leukocytes from flowing blood and also enhance leukocyte adhesion and transmigration to the site of the proinflammatory stimulus.

Activated-platelets induce shorter plaque formation by releasing and exposing P-selectin on plasma membrane, which binds to the P-selectin glycoprotein ligand 1 (PSGL-1) receptor on monocyte cell surface, thus forming platelet-monocyte aggregates. Once activated, platelets are capable of time-dependent synthesis of protein mediators, such as interleukin-1β, which cause an increase in the release of chemokines and up-regulate molecules that promote adhesion of neutrophils and monocytes to the endothelium [23]. Another important mediator released from platelets is the CD40 ligand which triggers an inflammatory response of the endothelial cells [24]. This ligand is stored in α-

granules of resting platelets and becomes rapidly exposed on cell surface following platelet activation [25]. CD40 ligand undergoes cleavage over a period of minutes to hours, generating a functional soluble fragment. The soluble CD40 ligand is released into the extracellular environment inducing endothelial cells to produce reactive oxygen species, [26] adhesion molecules, chemokines [24], and tissue factor [27] leading to an inflammatory response. The interaction between platelets, endothelial cells and leukocytes thus establishes a localised inflammatory response that can accelerate the early formation of atherosclerosis lesions. In addition platelets release platelet-derived growth factor (PDGF), which stimulates smooth muscle proliferation and angiogenesis in the plaque [28].

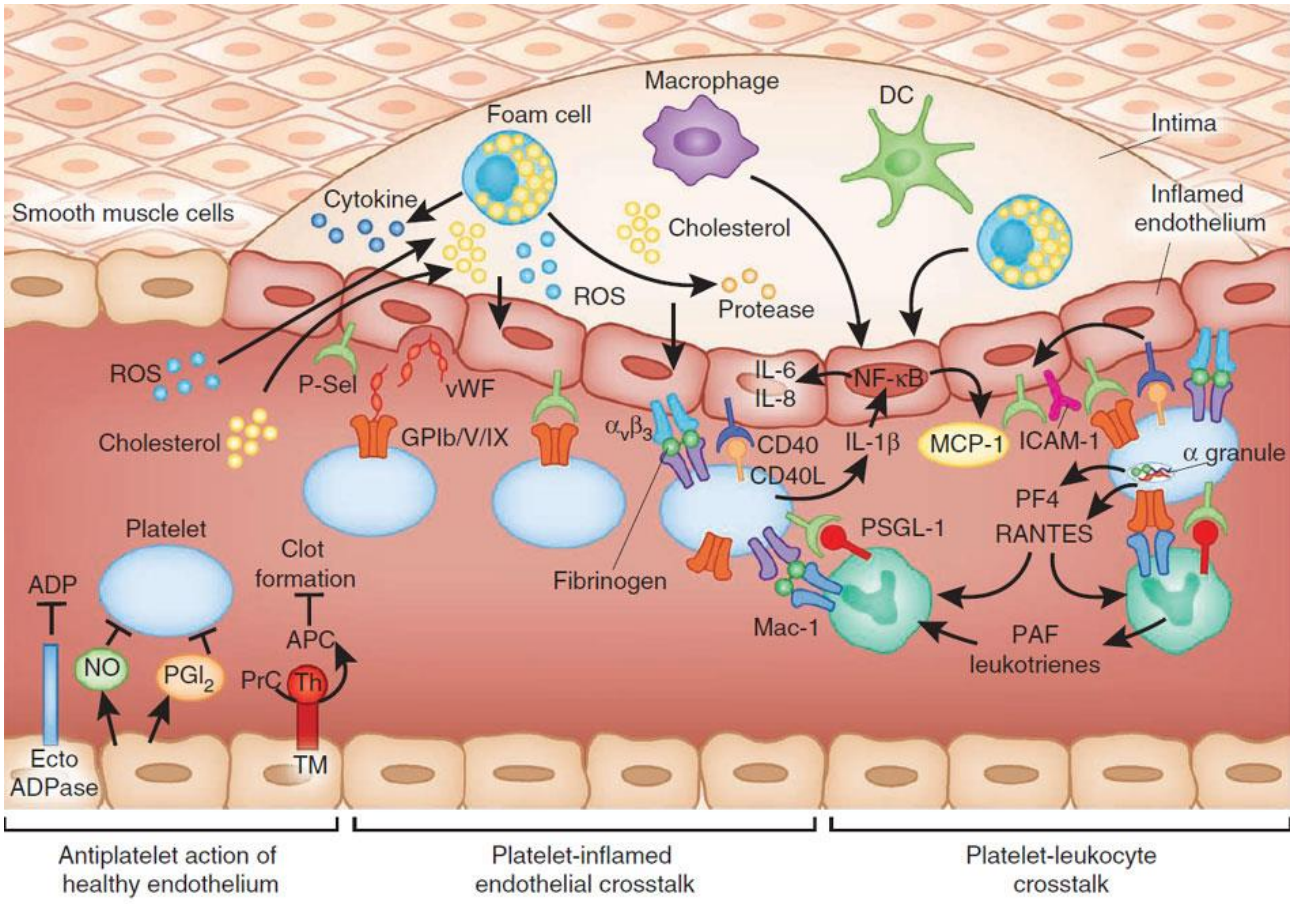



**Figure 1.** Picture modified from [29] The antiadhesive phenotype of endothelial cells is maintained through four intrinsic pathways: ecto-ADPase, prostaglandin I2 (PGI2), nitric oxide (NO) and the thrombomodulin (TM)-activated protein C (APC) pathways.

## 1.2 **Cardiovascular disease**

Cardiovascular disease (CVD) refers to a class of diseases that affect the cardiovascular system, which involves the heart, brain and blood vessels (arteries and vein). According to the World Health Organization classification 2008, CVD, caused mostly by atherosclerosis and/or hypertension, is the major cause of morbidity and mortality worldwide. This is confirmed by the Framingham Heart Study, which found that atherothrombosis significantly reduces life expectancy.

CVD of coronary arteries, known as coronary artery disease (CAD) is associated with chronic stenosis and is characterized by unchanged or slowly progressing symptoms over time.

When the CAD condition is documented, patients are treated with daily dose of aspirin in order to prevent potential adverse cardiovascular events, in fact in these patients there is a relevant net-clinical benefit of long-term administration of aspirin. [30] (table 1)

Stable CAD become unstable when dynamic stenosis or occlusion occur due to plaque rupture, with subsequent exposure of subendothelial vessel structures to blood flow, leading to thrombus formation in the artery. The ruptured atherosclerotic plaque, fissure, erosion, or their combination, may lead to lack of oxygen with a perfusion imbalance that lead to a myocardial ischemia. Prolonged ischemia can lead to myocardial infarction [31]. This spectrum of clinical conditions is referred to as acute coronary syndrome and is classified on the basis of the electrocardiogram (ECG) test:

1. ST-segment elevation myocardial infarction (STEMI);
2. non-ST-segment elevation ACS (NSTE-ACS), which may be divided, on the basis of cardiac troponin measurements, into:
   - non-ST-segment elevation myocardial infarction (NSTEMI), if there is an increased value for troponin;
   - ustable angina.

These criteria for diagnosis reflect the pathophysiological conditions: NSTE-ACS occurs when the thrombus is not completely occlusive or is so only transiently, while STEMI is caused by the intra-coronary thrombus completely occluding the vessel. The management of patients with ischemic coronary disease consists of 2 approaches: revascularization (percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG)) and systemic theray (aspirin, $P2Y_{12}$ antagonists, lipid-lowering therapy, angiotensin-converting enzyme inhibitors, and β-blockers) ( table 1).

**Table 1.** Table modified from [32]**.** Guidelines for Antithrombotic Therapy in the prevention and treatment of thrombosis among patients with CAD (according to European Society of Cardiology, ESC[33] [34])

| Treatment | Preclinical CAD | Stable CAD | Elective PCI | NSTE-ACS | STEMI |
|---|---|---|---|---|---|
| **Aspirin** | II-A | I-A | I-A | I-A | I-A |
| **Clopidogrel** | - | I-A | I-A | I-B[a] | I-C |
| **Prasugrel** | - | - | - | IIa-B<br>I-B[b] | I-B |
| **Ticagrelor** | - | - | - | I-B | I-B |
| **GPIIb-IIIa antagonists** | - | - | IIa-C (bailout situation only) | I-B only in high risk PCI[c]<br>Not for routine use (IIIa) | IIa-A[d] IIa-B[e] IIb-B[f]<br>No benefit of upstream therapy (III-B) |

[a] Only if prasugrel or ticagrelor are not an option

[b] In case of known coronary anatomy, intent to PCI and no pretreatment with $P2Y_{12}$-inhibitors

[c] At the time of PCI

[d] Abciximab

[e] Eptifibatide

[f] Tirofiban

**Classes of recommendation:**

Class I: Benefits >>> Risk: Procedure /treatment should be performed/administered.

Class IIa: Benefits >> Risk: Additional studies with focused objective needed; it is reasonable to perform procedure/administer treatment.

Class IIb: Benefits ≥ Risk: Additional studies with broad objective needed; additional registry data would be helpful; procedure/treatment may be considered.

Class III: Risk > Benefits: Procedure /treatment should not be performed/administered since it is not helpful and may be harmful.

**Levels of evidence:**

**Level A:** Multiple populations evaluated; data derived from multiple randomized clinical trials or meta-analysis

**Level B:** Limited populations evaluated; data derived from a single randomized trial or nonrandomized studies

**Level C:** Very limited populations evaluated; only consensus opinion of experts, case studies, or standard of care.

### 1.3 Pharmacological treatment of CAD: antiplatelet drugs

ADP and $TxA_2$ are released when platelets adhere to the vessel wall and therefore act as mediators, with an autocrine and paracrine positive feedback loop, that amplify platelet response to stimulation. Both ADP and $TxA_2$ act selectively on single pathways of platelet aggregation. The discovery of the importance of platelets in cardiovascular disease led to the common use of aspirin and $P2Y_{12}$ receptor antagonists in clinical practice.

#### 1.3.1 Aspirin

##### 1.3.1.1 Thromboxane A2 pathway

$TxA_2$ belongs to the family of eicosanoids, which includes prostaglandins, prostacyclin, leukotrienes, and epoxygenases. In humans eicosanoids derive from arachidonic acid, a 20-carbon fatty acid containing four double bonds, which is liberated from membrane phospholipids following an intracellular increase in calcium and the action of phospholipase A2 or C. [35] Once liberated arachidonic acid is converted by COX-1 in two independent enzymatic reaction steps, producing first prostaglandin (PG) $G_2$, via a cyclooxygenase function, and subsequently to $PGH_2$, via a peroxidase function. $PGH_2$ is the substrate for several synthases that generate a range of bioactive prostanoids such as PGD2, PGE2, PGI2 and $TxA_2$. While almost all human tissue is capable of generating PGH2, its metabolite is tissue-specific and depends on the presence of tissue-specific enzyme. In platelets, thromboxane synthase (TXS) is responsible for the conversion of $PGH_2$ into $TxA_2$, a known strong platelet agonist (Figure 2). Once produced, $TxA_2$ has a short half-life (30 seconds) and acts on neighboring cells via autocrine or paracrine systems. $TxA_2$ undergoes rapid non-enzymatic hydrolysis to the inactive TxB2 [36], further metabolized to 2,3-dinor-TxB2 and 11-dehydro-TxB2, eventually excreted in urine. Endothelial cells, on the other hand, contain PGI2 synthase, which produces prostacyclin. [35] There are three isoforms of the COX enzyme. COX-1 is constitutively expressed mostly in platelets. While the gene for COX-2 is present in nucleated cells, it is only expressed when induced in presence of inflammatory stimuli [37]. COX-3 is the third isoform which derives from the same gene as COX-1; in humans COX-3 mRNA is most abundant in the cerebral cortex and heart [38].

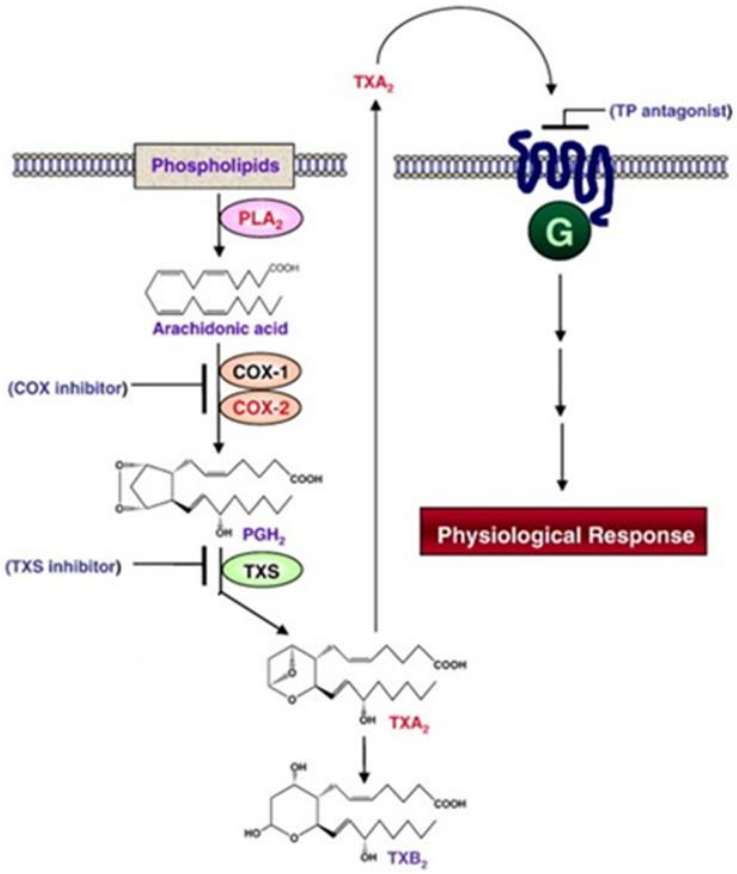



**Figure 2.** Modified from [39] **Thromboxane pathway**: PLA2, phospholipase A2; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; TXS, thromboxane synthase; PGH2, prostaglandin H2; $TxA_2$, thromboxane A2; TXB2, thromboxane B2;

*Thromboxane A2 receptor*

$TxA_2$ exerts its action through the specific G protein-coupled $TxA_2$ receptor (TP). The TP receptor is a member of the G-protein-coupled receptor superfamily, which in turn regulates several effectors, including phospholipase C, guanine nucleotide exchange factor of the small G protein Rho (RhoGEF) and adenylyl cyclase. TP is expressed in various tissues such as platelets, endothelial cells, smooth muscle cells, monocytes, macrophages, kidney, heart and spleen cells. [40] In human platelets two TP isoforms have been described, TPα and TPβ, which occur by alternative splicing of their common mRNA. [41] [42] Although mRNA for both isoforms have been isolated from platelets [43], TPα seems to be the predominant isoform expressed on the platelet surface [44]. The wide distribution of TP suggests that $TxA_2$ is involved in a wide range of physiological and pathophysiological conditions. Genetic variations affecting TP receptors are associated with a mild bleeding phenotype. To date, two quantitative defects causing reduced TP receptor expression have

been identified, (c167dupG) [45] and (W29C) [46] as well as one qualitative defect caused by a point mutation (R60L) [47] in the first cytoplasmic loop of TP. Another mutation (D304N) [48] associated with reduced ligand binding to TP, has been described . TP defects may be suspected in patients with defective response to the $TxA_2$ analog U46619, arachidonic acid, and collagen.

#### 1.3.1.3 Aspirin Pharmacology

Aspirin exerts its cardioprotective effect by targeting one of the major activators of platelet $TxA_2$ synthesis, so consequently treatment with aspirin is fundamental to the management of patients with cardiovascular disease at different levels (Table 1).

Aspirin irreversibly inhibits COX-1 [49] [50] by acetylating Ser529 residue. Aspirin inhibits not only $TxA_2$ production by COX-1 but also the synthesis of other prostanoids via inhibition of COX2.

Aspirin is rapidly absorbed in the stomach and upper small intestine, primarily by passive diffusion of non-dissociated acetylsalicylic acid (ASA) across gastrointestinal membranes. Generally, peak plasma level occurs 30 to 40 minutes after the ingestion of aspirin and the reduction of $TxA_2$ is evident after 1 hour. However, other formulations of ASA, such as the enteric-coated form, takes up to 3-4 hours to reach peak plasma levels. Aspirin is an effective anti-thrombotic agent when used long term at doses between 50 and 100 mg/day [51] [52]. In contrast, randomized trials have shown no evidence for increase of efficacy of aspirin (>300mg/day) as antithrombotic agent at higher doses, there is however a dose-dependent effect on gastroenterological complications [51] [52] [53]

### 1.3.2 ADP/ $P2Y_{12}$ antagonists

#### 1.3.2.1 ADP pathway

Adenosine diphosphate (ADP), the first known low molecular weight platelet aggregating agent, plays an important role in platelet function despite being a weak platelet agonist. As such, it only induces platelet shape change and reversible aggregation in humans. Platelet secretion and secondary aggregation observed after stimulation with ADP of normal, human citrated platelet-rich plasma are due to the aggregation-dependent formation of $TxA_2$. ADP is released in high concentration from platelet dense granules where it is stored and amplifies platelet responses induced by other agonists [54] [55] and stabilizes platelet aggregate [56] [57]. ADP stimulates two specific G protein coupled P2 receptors on the platelet surface. The transduction of its signal involves both a transient rise in free cytoplasmic calcium, mediated by the Gq-linked P2Y1 receptor, and inhibition of adenylyl cyclase, which is mediated by the Gi-linked $P2Y_{12}$ receptor [58]. The activation of P2Y1 receptor by ADP mediates platelet shape change and initiates platelet aggregation, whereas $P2Y_{12}$ amplifies the platelet aggregation response. [59] Concomitant activation of both G protein-coupled receptors is essential to elicit normal platelet aggregation [60] [58] (Figure 3)

$P2Y_{12}$ *receptor*

The P2 receptors, which interact with purine and pyrimidine nucleotides, are divided into two groups: G protein-linked or metabotropic, termed P2Y, and ligand-gated ion channels or ionotropic, termed P2X [61]. The P2Y receptors are seven-membrane-spanning proteins with a molecular mass of 41 to 53 kD after glycolysation [61]. The carboxyl terminal domain is on the cytoplasmatic side, whereas the amino terminal domain is exposed to extracellular environment. The mechanisms of signal transduction are shared by most seven-membrane-spanning receptors, and include activation of phospholipase C and regulation of adenylyl cyclase activity. The Gq coupled receptor P2Y1 leads to activation of β-isoforms of phospholipase C (PLC) and triggers the mobilization of Ca2+ into the cytoplasm. The Gi coupled receptor $P2Y_{12}$ leads to inhibition of adenyl cyclase (AC) with an increase of platelet cyclic adenosine monophosphate (cAMP). Co-interaction of the P2Y1 and $P2Y_{12}$ is necessary for normal ADP-induced platelet aggregation, in fact separate inhibition of either of them with selective antagonists results in a dramatic decrease in aggregation [62] [63] [60]. The stimulation of the ADP receptors, predominately the $P2Y_{12}$ receptor, assists to activation of integrin GP IIb/IIIa (fibrinogen receptor) [64] [65]. $P2Y_{12}$ is important for both normal hemostasis and

pathologic thrombosis result this receptor is relevant target for antiplatelet drug and research is very interested in this field.

*Congenital defect of* $P2Y_{12}$*receptor*

Patients with defects of the platelet $P2Y_{12}$ receptors have been described. Congenital $P2Y_{12}$ deficiency is an autosomal recessive disorder, characterized by life-long history of excessive bleeding, prolonged bleeding time, abnormalities of platelet aggregation similar to those observed in patients with defects of platelet secretion (reversible aggregation in response to weak agonists and impaired aggregation in response to low concentrations of collagen or thrombin), except that the aggregation response to ADP was severely impaired.

The diagnosis of $P2Y_{12}$ defects should be suspected when ADP, even at high concentrations (>10 µM), fails to induce full and irreversible platelet aggregation, but induces normal platelet shape change and borderline-normal mobilization of cytoplasmatic $Ca^{2+}$ induced by ADP. Furthermore, platelets show no inhibition by ADP of prostaglandin $E_1$-stimulated platelet adenylyl cyclase, but normal inhibition by epinephrine. In addition, the presence of approximately 30% of the normal number of binding sites for [33P]2MeSADP on fresh platelets or [3H]ADP on formalin-fixed platelets (which are associated with the ADP receptor P2Y1).

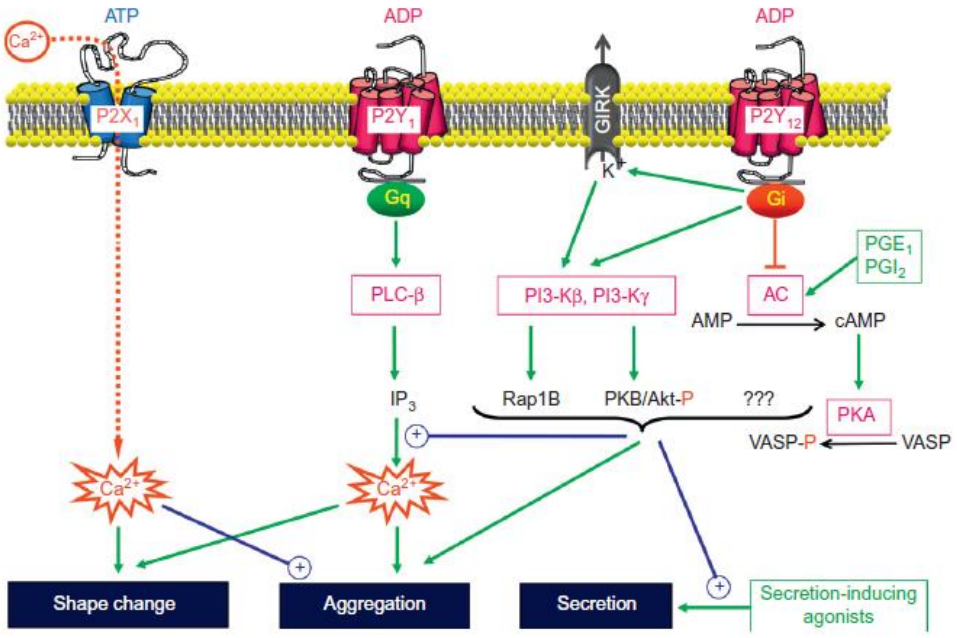



**Figure 3.** Figure modified from [32]. Role of $P2Y_{12}$ in platelet aggregation**.** ADP interact with $P2Y_{12}$, a seven-transmembrane receptor that is coupled to Gi protein. This bond induces platelet aggregation and amplifies the aggregation response that is induced by other agonists or by ADP itself, by interacting with its other platelet receptor,

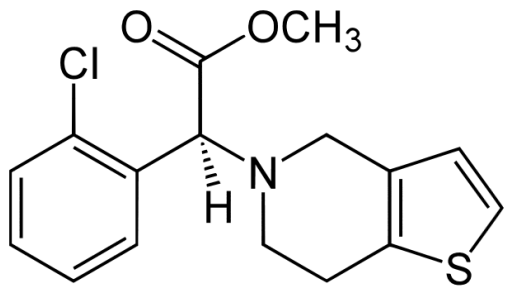
P2Y1. $P2Y_{12}$ stabilizes platelet aggregates and amplifies the secretion of platelet dense granules stimulated by secretion-inducing agonists (coupled to Gq). $P2Y_{12}$ is coupled to inhibition of adenylyl cyclase (AC) through Gi, this function does not appear to be directly related to $P2Y_{12}$ -mediated platelet activation. However, it could have important implications in vivo, where platelets are exposed to the inhibitory prostaglandin PGI2 (prostacyclin), which inhibits platelet aggregation by increasing platelet cyclic adenosine monophosphate (cAMP) through activation of AC mediated by Gs: inhibition of AC by $P2Y_{12}$counteracts the inhibitory effect of prostacyclin, thereby favoring the formation of platelet aggregates in vivo.

### 1.3.3.2 $P2Y_{12}$ antagonists pharmacology

Thienopyridines (such as ticlopidine, clopidogrel and prasugrel) irreversibly inhibit $P2Y_{12}$, while ticagrelor and cangrelor are reversibly-binding inhibitors. [66] [67]

**Clopidogrel**

*Pharmacology*




*Molecular mass: 321.82 g/mol*

**Figure 4.** Chemical structure of clopidogrel

Ticlopidine and Clopidogrel are structurally related compounds, but ticlopidine has been almost completely replaced by clopidogrel in clinical practice, due to the its citotoxicity (neutropenia, thrombotic thrombocytopenic purpura) [32]. In fact, clopidogrel resulted a better tolerated and safer drug than ticlopidine. Clopidogrel is absorbed as a pro-drug in the intestine and then modified via two competing pathways in the liver. In one, clopidogrel is rapidly metabolized by human carboxylesterase 1 (hCE1) to an inactive acid metabolite [68], about 75% of clopidogrel administrated is converted through this step [69]. In the second pathway, clopidogrel is metabolized in a two-step process, first to 2-oxo-clopidogrel by CYP2C19, CYP1A2 and CYP2B6, subsequently into the active metabolite by CYP2B6, CYP3A2, CYP2C9 and CYP2C19 and in inactive acid metabolite of 2-Oxo-Clopidogrel by esterase [68]. The active metabolite irreversible binds to $P2Y_{12}$ by forming a covalent disulfide bond with cysteine residues. The consequence of an irreversible inhibition is that

stays on for entire life span of a circulating platelets [70]. Gastrointestinal side effects are also described during treatment with clopidogrel, even if clinically less severe than in aspirin-treated patients [71].

Despite its proven antithrombotic efficacy clopidogrel has some important limitations:

1. The need for metabolism to active metabolite causes a delayed antiplatelet effect, with a maximum plateau of inhibition of ADP-induced platelet aggregation from 4 to 5 days after daily dose of 75 mg of clopidogrel. However, the delayed of action of clopidogrel can be reduced to approximately 2-5 hours by a loading dose of 300-600 mg [72] [73].
2. There is interindividual variability in the response to clopidogrel, which is due mostly to interindividual differences in the extent of metabolism of the prodrug in the liver (mutation of CYP2C19 and other CYP isoform) [74] [75] [32]. Furthermore, the variables that can affect the pharmacodynamics response to clopidogrel are several as lack of compliance, reduced absorption, interaction with other drugs, age, high body mass index, diabetes mellitus, renal insufficiency in diabetes mellitus, pre-existed variability in platelet response to ADP, increased platelet turnover, tobacco smoking.

*Clinical Trials*

Long-term administration of clopidogrel was associated with a modest but statistically significant advantage over aspirin in reducing adverse cardiovascular outcomes in patients with established cardiovascular disease in the CAPRIE trial. In other large well designed multicenter trials the addition of clopidogrel to aspirin therapy improved outcomes in patients with acute coronary syndromes (Table 2). Results of several large randomized trial, therefore, have established clopidogrel as an effective and well tolerated antiplatelet agent for the secondary prevention of ischaemic events in patients with various cardiovascular conditions, including those with ischaemic stroke or acute coronary syndromes. In addition, treatment guidelines from the US and Europe acknowledge the importance of clopidogrel in contemporary cardiovascular medicine.

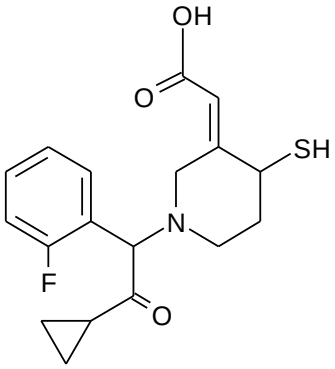
**Table 2.** Clinical Trial of Clopidogrel

| Trial | Treatment | N | Patients | Primary endpoint | Follow-up | Primary endpoint data (%) | Safety (%) | REF. |
|---|---|---|---|---|---|---|---|---|
| ***Clopidogrel vs ASA*** | | | | | | | | |
| **CAPRIE** | CLO 75 mg/d vs ASA 325 mg/d | 19.185 | Previous AMI, Ischemic stroke, or PAD | Composite of outcome of ischemic stroke, MI o vascular death | 1.9 years | 5.3 vs 5.8 (p=0.043) | No difference | [71] |
| ***Clopidogrel + ASA vs ASA*** | | | | | | | | |
| **CHARISMA** | CLO 75 mg/d plus low dose ASA 75-162 mg/d vs PL plus low dose ASA | 15.603 | CVD or multiple risk factor | Composite of MI, stroke or death from CV causes | 28 months | 6.8 vs 7.3 (p=0.22) | Severe bleeding 1.7 vs 1.3 (p=0.09) Moderate bleeding 2.1 vs 1.3 (p<0.001) | [76] [77] |
| ***Clopidogrel + ASA vs ASA*** | | | | | | | | |
| **CURE** | CLO 300 mg LD, then 75 mg/d plus ASA 75-325 mg/d vs ASA | 12.562 | NSTE ACS, unstable angina | Composite of death from CV causes, nonfatal MI and stroke | 3-12 months | 9.3 vs 11.4 (p<0.001) | Major bleeding 3.7 vs 2.7 (p=0.001) | [78] |
| **COMMIT** | CLO 75 mg/d plus ASA 162 mg/d vs PL plus ASA | 45.852 | STEMI | Composite of CV death, re-infarction, or stroke | 28 days | 9.2 vs 10.1 (p=0.002) | Fatal bleeding 0.32 vs 0.32 (p=0.92) | [79] |
| **CREDO** | CLO 300 mg LD plus ASA 325 mg or PL 3-24 h before PCI, then CLO 75 mg/d plus ASA 325 mg/d throught day 28 then CLO 75mg/d in the LD group or PL | 2.116 | CAD undergoing PCI | 28 days: Composite of death, MI or urgent TVR 1 year: Composite of death, MI or stroke | 1 year | 28 d: 6.8 vs 8.3 (p=0.23) 1 y: 8.5 vs 11.5 (p=0.02) | Major bleeding 8.8 vs 6.7 (p=0.07) | [80] |
| **(Double-dose Clopidogrel vs standard dose of Clopidogrel )+ (high dose ASA vs low dose ASA)** | | | | | | | | |
| **CURRENT-OASIS 7** | CLO 300 mg LD then 75 mg/d, or 600 mg LD then 150 mg/d on days 2-7, then 75 mg/d on day 8-30 plus ASA ≥ 300 mg LD then 75-100 mg/d or 300 mg/d | 25.086 | ACS undergoing PCI | Composite of CV death, MI or stroke | 30 days | 4.2 vs 4.4 (p=0.3) | Major bleeding 2.5 vs 2.0 (p=0.01) | [81] |
| ***Clopidogrel + ASA vs ASA*** | | | | | | | | |
| **CLARITY- TIMI 28** | CLO 300 mg LD then 75 mg/d plus ASA 150-325 mg LD then 75-162 mg/d vs ASA | 3.491 | Acute STEMI, received a fibrinolytic agent | Composite of occluded infart related artery on angiography, death or MI before angiografy | 30 days | 15.0 vs 21.7 (p<0.001) | Major bleeding 1.9 vs 1.7 (p=0.8) | [82] |

**CLO**= Clopidogrel; **ASA**= Aspirin; **AMI**= acute myocardial infarction **MI**= myocardial infarction; **PAD**= peripheral artery disease; **PL**= placebo; **CAD**= cardiovascular disease; **CV**= cardiovascular; **LD**= loading dose; **ACS**= Acute coronary syndrome; **STEMI=** ST-segment elevation MI; **NSTE=** non-ST-segment elevation MI**; PCI=**percutaneous coronary interventio

**Prasugrel**

*Pharmacology*




*2-acetoxy-5-(α-cyclopropylcarbonyl-2-fluorobenzyl)-4,5,6,7-tetra-hydrothieno[3,2-c]pyridine;*
*Molecular mass: 349.42;*

**Figure 5.** Chemical structure of prasugrel and the metabolic pathway of prasugrel leading to its active metabolite [83]

Prasugrel is a thienopyridyl pro-drug that is rapidly metabolized to its active metabolite R-138727 (Figure 5 ) and irreversibly inhibits platelet $P2Y_{12}$ receptors. Oral administration of prasugrel is 10 and 100 times more effective on an equal-dose on inhibition of platelet aggregation than clopidogrel and ticlopidine, respectively [84], although the active metabolites for prasugrel and clopidogrel have equipotency at the $P2Y_{12}$ receptor in vitro [85]. In addition, prasugrel has a more rapid onset of action than clopidogrel and is less dependent to CYP enzyme than clopidogrel, as has a distinct chemical structure that allow a more efficient conversion to its active metabolite. The biotrasformation of prasugrel to its active metabolite, 2-[1-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene] acetic acid, requires ester bond hydrolysis by carboxylesterase (hCE)-2 in the intestine [86] [83] forming the thiolactone, and then an oxidation by intestinal and epatic cytochrome P450–mediated, mainly CYP3A and CYP2B6 [87], with smaller contributions by CYP2C9 and CYP2C19 [87].

The different pharmacokinetics and pharmacodynamics of prasugrel compared with clopidogrel can be summarized as follows [88]:

1. fast appearance of its active metabolite in circulating blood within 15 minutes of dosing, which reaches maximal plasma concentration at ≈30 minutes;
2. higher mean area under the concentration-time curve of the active metabolite of prasugrel 60 mg than that of clopidogrel 600 mg;

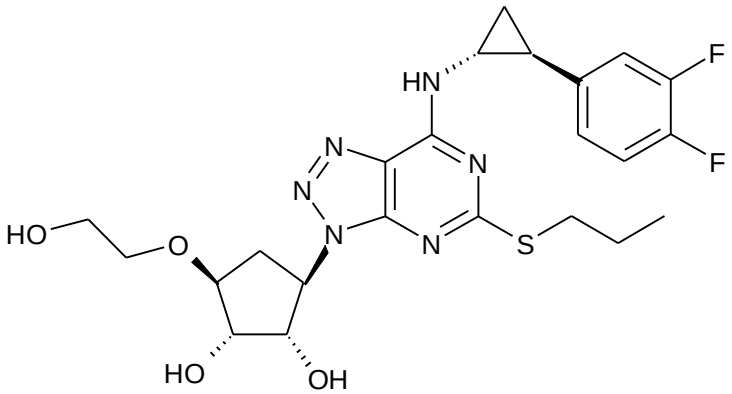
3. faster and greater mean inhibition of $P2Y_{12}$-dependent platelet function after a 60-mg loading dose and 10-mg maintenance dose than after a 300- or 600-mg loading dose and 75- or 150-mg maintenance dose of clopidogrel;
4. no influence of the CYP genotype on its pharmacokinetics and pharmacodynamics;
5. much lower interindividual variability in the inhibition of P2Y12-dependent platelet responses and extremely low prevalence of subjects who display resistance to prasugrel.

***Clinical trials***

The more favorable pharmacokinetics and pharmacodynamics of prasugrel compared to clopidogrel result in greater clinical benefit as showed by data of the phase III TRITON TIMI-38 trial, in which patients were randomized to receive prasugrel 60 mg loading dose followed by 10 mg/ day or clopidogrel 300 mg loading dose followed by 75 mg / day for 6-15 months. The study was a randomized, double-blind, parallel group, multinational trial, which evaluated 13,608 high risk patients with ACS who require PCI [89]. Prasugrel was associated with fewer ischemic events but higher incidence of bleeding complications.

**Ticagrelor**

*Pharmacology*




*(1S,2S,3R,5S)-3-[7-{[(1R,2S)-2-(3,4-Difluorophenyl)cyclopropyl]amino}-5-(propylthio)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy)cyclopentane-1,2-diol.*

*Molecular mass = 522.57;*

**Figure 6.** Chemical structure of ticagrelor.

Ticagrelor (previously known as AZD6140) belongs to a new chemical class of compounds, the cyclopentyl-triazolo-pyrimidines, which target the $P2Y_{12}$ receptor (figure 6) [90,91]. It is the first reversibly binding oral $P2Y_{12}$ receptor antagonist with a pIC50 value of 7.9 for inhibition of 30 µM ADP-induced aggregation of human-washed platelets and no significant affinity for other P2 receptor at concentrations >3 µM [90].

In contrast to the other antiplatelet drugs, ticagrelor appears to inhibit $P2Y_{12}$ receptor in a noncompetitive manner suggesting the existence of an independent receptor binding site, making it an antagonist [67]. The drug does not need activation whereby the pharmacological effect of ticagrelor is not influenced by CYP genotypes. It gives a more complete and consistent platelet inhibition compared to clopidogrel [92]. Ticagrelor has a rapid beginning of action, reaching maximal inhibition of platelet function in about 2 hours [93] [94]. Although the plasma half-life of ticagrelor is 6-8 hours [93] [95], the duration of its inhibitory effect is much longer, because platelet function returns to near normal levels after about 5 days following cessation of treatment [96]. Ticagrelor has been shown to inhibit erythrocyte adenosine uptake likely via the equilibrative nucleoside transporter 1 (ENT1) [97] and to augment adenosine-induced coronary blood flow, possibly contributing to reduced myocardial infarction size [98].

Briefly, ticagrelor has the potential to address many of the limitations of thienopyridine therapy:

1. is not a prodrug and therefore does not require metabolic activation, has a rapid and reversible concentration dependent inhibitory effect on the $P2Y_{12}$ receptor;
2. provides greater and more consistent inhibition of ADP-induced platelet aggregation than clopidogrel;
3. offers the potential for greater flexibility in the management of patients at risk for thrombotic events due to rapid onset and offset of antiplatelet effect;

***Clinical trials***

The clinical relevance of ticagrelor was evaluate in the phase III clinical trial PLATO, in which ticagrelor (180 mg loading dose, 90 mg bid maintenance dose) was compared to clopidogrel (300-600 mg loading dose, 75 mg daily maintenance dose) for the prevention of major adverse cardiac events (MACE) among patients with non-ST or ST elevation acute coronary syndrome (ACS). The main results showed that ticagrelor reduced the rate of a composite of death from vascular causes,

myocardial infarction (MI), or stroke (9.8% versus 11.7%)[99]. The incidence of bleeding complications was higher in ticagrelor-treated patients, compared to clopidogrel-treated patients.

### 1.4 Role of $P2Y_{12}$ in thromboxane A2 production

Recent studies showed that inhibition of $P2Y_{12}$ is associated with markedly decreased platelet production of $TxA_2$ [100] [101] [102] [103]. Based on these findings, it has been suggested that patients with ACS might be safely treated with $P2Y_{12}$ antagonists only. However, the observation that platelets congenitally deficient of $P2Y_{12}$ synthesize normal amounts of $TxA_2$ contrasts the results obtained with $P2Y_{12}$ antagonists. Therefore, the role of $P2Y_{12}$ in stimulation of the platelet production of $TxA_2$ is still unclear.

# 2. AIMS OF THE STUDY

The aim of this study was to evaluate the effect of $P2Y_{12}$ antagonists on platelet $TxA_2$ production.

In particular, specific objectives of the study were:

1. to evaluate whether or not $P2Y_{12}$ inhibitors have **off-target effects** that affect $TxA_2$ platelet production.
2. to evaluate whether or not the reduction of TxB2 production due to $P2Y_{12}$ inhibitors recently reported by some authors is **secondary to the inhibition of platelet aggregation**.
3. to assess whether inhibition of $P2Y_{12}$ synergizes with aspirin in inhibiting the platelet production of $TxA_2$.

# 3. DESIGN OF THE STUDY

**1.** Serum TxB2 levels were measured in patients treated with 10 mg prasugrel q.i.d. or placebo for 14 days (double blind, randomized study).

Serum TxB2 levels were measured after *in vitro* addition of $P2Y_{12}$ inhibitors at high concentration to blood samples from:

- healthy subjects
- patients with severe inherited $P2Y_{12}$ defects

**2.** TxB2 levels were measured in the supernatant platelet-poor plasma after stimulation of platelet-rich plasma (PRP) with platelet agonists in:

- healthy subjects
- patients with severe inherited $P2Y_{12}$ defects

under the following experimental conditions:

- By stirring the samples of PRP at a constant speed: constant stirring of PRP samples is essential to allow platelet aggregation to occur
- Under no-stirring conditions, which prevent the formation of detectable platelet aggregates

**3.** A. Platelet aggregation and TxB2 production after stimulation of PRP with platelet agonist in the presence of aspirin and cangrelor, alone and in combination

B. Serum TxB2 levels were measured in CAD patients on chronic treatment with aspirin or aspirin plus clopidogrel

# 4. MATERIALS AND METHODS

## 4.1 Study population

*Healthy subjects*

Blood samples from 24 apparently healthy male (n=14) and female (n=10) volunteers (range: 20-63 years of age) were studied. They were recruited among the laboratory personnel and medical students of our institution. All subjects abstained from any drug known to affect platelet function for at least 10 days before blood sampling were enrolled.

*Patients with $P2Y_{12}$ deficiency:*

Three patients with bleeding diathesis and defects of $P2Y_{12}$ were studied: two patients with inherited, severe $P2Y_{12}$ deficiency (one man, aged 77 y and one woman, aged 65 y) [104] [105] and one patient with heterozygous $P2Y_{12}$ deficiency [105] [106] (Table 3)

**Table 3.** Clinical baseline characteristics of patients with $P2Y_{12}$ defects

| **Patient** | **1** [104] | **2** [105] | **3** [105] [106] |
|---|---|---|---|
| **Congenital defect** | Severe$P2Y_{12}$ deficiency | Severe$P2Y_{12}$ deficiency | Haploinsufficiency of $P2Y_{12}$ |
| **Clinical characteristics** | Moderate/severe bleeding diathesis | Moderate/severe bleeding diathesis | No overt bleeding diathesis |
| **Platelet function** | Severely impaired responses to ADP | Severely impaired responses to ADP | Mild abnormalities of platelet function |

PRINA study patients:

Twenty consecutive patients, aged 23-69 years, were recruited and participated in the PRasugrel INAsthma (PRINA) study after providing written informed consent. Patients with allergic asthma (>1 year) with mild and stable asthma without chronic medication, except for the use of inhaled low dose of steroids or the use of inhaled beta2-agonist on demand disease, non-smoking, were recruited at the Pneumology Unit of Ospedale San Paolo. All patients abstained from any drug known to affect platelet function for at least 10 days before blood sampling, and throughout the duration of the study.

Patients were randomized in a cross-over study, in double-blind treatment, placebo-controlled. Patients were treated with 10 mg prasugrel q.i.d. or placebo for 14 days with a washout ≥ 15 days (Figure 7). Compliance was assessed by interview. The study was approved by the ethics committee of Ospedale San Paolo, Milan, Italy and all subjects provided informed consent (ClinicalTrials.gov Identifier: NCT01305369).

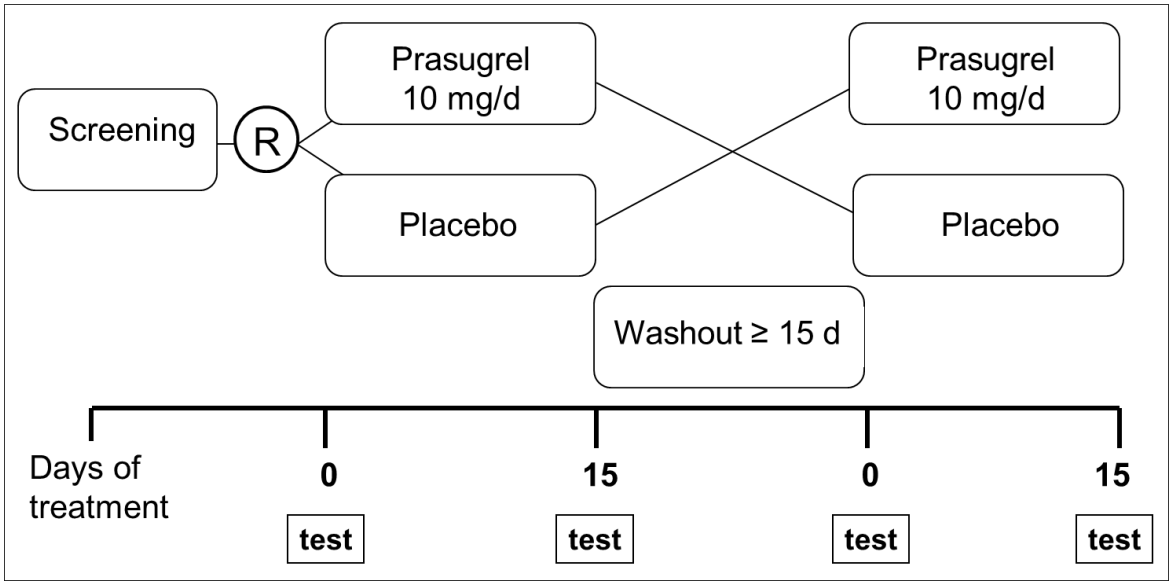



**Figure 7**. PRINA study design

*CAD study patients*

In this study 49 stable CAD patients were enrolled from the Cardiology Unit, at Ospedale San Paolo, Milan (IT) after providing written informed consent. All patients were on chronic treatment (≥4 weeks) with daily dose (100 mg) of enteric coated acetylsalicylic acid (Cardioaspirin, Bayer, DE), 25 patients were also on treatment with a daily dose (75 mg) of clopidogrel (Plavix, Sanofi Aventis, FR). Patients were eligible for the study if they met all of the following inclusion criteria: 1) over than 18 years of age, 2) free of warfarin or drugs known to affect platelet function (e.g. non-steroidal anti-inflammatory drugs, ticlopidine, dipyridamol) in the two weeks before the blood sampling, 4) any ischemic event or revascularization procedure not less than 3 months before 5) any drug or alcohol abuse.

To optimize compliance and uniform pharmacokinetics, all subjects were asked to ingest ASA or ASA plus clopidogrel at 9:00 AM of the day preceding blood sampling (day -1) and the blood samples were taken exactly 24 h after the last dose.

The study was conducted in agreement with the Helsinki-II declaration and was approved by the Ethics Committees of the Ospedale San Paolo (N° 958), Milano, IT.

### 4.2 Reagents

Arachidonic acid (AA), epinephrine, ADP and indomethacin were supplied by Sigma Aldrich (Milano, IT). Horm collagen from Mascia Brunelli (Milano, IT). Ticagrelor (TIC), Active metabolite of Clopidogrel (CAM) and Active metabolite of Prasugrel (PAM) were kindly provided by AstraZeneca R&D (Mölndal Sweden). Cangrelor (Cang) was from The Medicines Company.
All concentrations of reagents reported in the text have been expressed as final concentrations.

### 4.3 Blood sampling

Patients had to refrain from smoking for at least 2h before blood sampling; a light breakfast was allowed in the morning of the study. Blood samples were collected from an antecubital vein, using a 21 gauge butterfly needle and a tourniquet, released soon after needle insertion. The first 3 mL of blood were collected into K-EDTA and analyzed by coulter hematology analyzer (Beckman Coulter, Milano, IT), the following blood was collected in into plastic tubes (PP) containing trisodium citrate (109 mM, 1:9, v/v) or Lepirudin 25 ug/mL, 16.000 ATU/mg (Verum Diagnostica, Munich, DE), gently mixed, allowed " to rest" at room temperature for 15 min, and

used for platelet aggregation studies. For phosphorylated-VASP assay.3 mL of blood were collected into a commercial tube containing trisodium citrate (109 mM, 1:9, v/v).

### 4.4 Platelet aggregation experiments

To assess the platelet aggregation light transmission aggregometry (LTA) was used according to manufacturer's instructions by Chrono-Log 560 (Havertown, PA, USA) or by the Platelet Aggregation Profiler (model PAP-8E, Biodata, Horsham, PA, USA). Platelet rich plasma (PRP) was obtained by centrifugation of citrate or hirudin whole blood samples at 200 x g for 10 min at room temperature as recommended [107] Autologous platelet-poor plasma (PPP) was obtained by further centrifugation at 1.400 x g for 15 min at room temperature. Autologous PPP was used to set the instrument's 100% light transmission, while the un-stimulated PRP was used to set 0% light transmission. The individual platelet count of the PRPs was not adjusted to a pre-determined range, because this procedure may induce artefacts [108]. All aggregation tests were performed within 3 hours after blood collection. PRP was placed into a test tube containing a stir bar, $P2Y_{12}$ antagonists or vehicle were added and pre-incubated at 37°C for 10 min without stirring followed by 2 min under stirring (1.000 r.p.m). Subsequently, platelet aggregation was induced by arachidonic acid (1 mM), collagen (0,5 or 10 µg/mL), or ADP (10µM) then recorded for 3 min. Maximal aggregation response to each agonist was considered and expressed as percentage.

In the set experiments without stirring, after addition of platelet agonist, the test tubes were placed at 37°C under soft stirring, just 100 r.p.m. for 10 seconds to well mix the agonist, then stopped the stirring, and recorded for 3 min.

In some sets of experiments, only cangrelor (10 uM) was used, epinephrine (1µM) was added at the same time with the agonists (collagen 0,5 µg/mL or 10 µg/mL) and platelet aggregation was performed both in citrate-PRP and hirudin-PRP.

### 4.5 TXB2 measurements

*Sample preparation*

Serum was prepared from non-anticoagulated venous blood, allowed to clot at 37°C exactly for 1 hour, centrifuged at 1,400 x g for 15 min at RT, and stored at -80°C until the analysis.

For the in vitro studies: 10 mL of non-anticoagulated venous blood were collected and divided in aliquots of 1 mL. Immediately after, 10 µL of DMSO or $P2Y_{12}$ inhibitors at different concentrations (10 µM and 100 µM) were added to the aliquots and allowed to clot in the conditions previously described.

Supernatants were obtained by centrifuging PRP at the end of the observation period of aggregation induced by AA or collagen. Cyclooxygenase activity was halted by the addition of indomethacin (20 µM) at 13.000×g for 1 min at RT and stored at -80°C until the analysis.
All data are expressed as pmoles/$10^8$ plts considering the appropriated platelet count.

*Quantification of TXB2 by EIA*

$TxA_2$ has a short half-life under physiological conditions, $TxA_2$ levels are typically monitored by measurement of TXB2 produced by non-enzymatic hydration of $TxA_2$. TxB2 levels, were measured by using a selective, competitive enzyme immunoassay (Thromboxane B2 EIA kit, Cayman Chemical Company, Ann Arbor,MI, USA) in serum and in the supernatants of citrated-hirudin PRP, according to the manufacturer's instructions. Frozen samples were thawed at 37°C and diluted between 1:2 and 1:3000 with buffer and tested in duplicated. The plate was read at 405 nm wavelength by a standard 96-well plate reader (TecanTM Sunrise). Samples were assayed parallel to known TxB2 standards, prepared as outlined in the manufacturer's instructions, and a maximum binding control. The percentage binding of known standards was calculated in reference to the maximum binding control wells, plotted against the logarithm of concentration and analyzed by non-linear regression. Unknown samples were expressed in a similar fashion, interpolated from this standard curve and corrected for dilution. Samples with results outside the standard curve were re-assayed with appropriate dilution. The detection limit of the assay is 11 pg/mL. Data are expressed as pmoles/108 plts based on the platelet count in K-EDTA samples.

**4.6 VASP phosphorylation assay**

In order to evaluate the degree on $P2Y_{12}$ inhibition, in patients of PRINA and CAD study commercial VASP assay (Diagnostica Stago, Asnières, Stago, FR) was performed and the degree of $P2Y_{12}$ receptor inhibition was measured as percentage of platelet reactivity index (PRI).
The phosphorylation of vasodilator-stimulated phosphoprotein (VASP), an intraplatelet actin regulatory protein, is dependent on the level of activation of the platelet $P2Y_{12}$ receptor, which is targeted by $P2Y_{12}$ antagonist. The VASP phosphorilation analysis was performed according to the manufacturer's instructions within 48 hours after blood collection using PLT VASP/P2Y12 kit (Diagnostica Stago, Asnières, FR). Briefly, the anticoagulated blood samples were incubated with PGE1 alone or in combination with ADP for 10 min at room temperature and fixed with paraformaldehyde. The platelets were then permeabilized with non-ionic detergent and labelled using a primary monoclonal antibody against 239-phosphorylated VASP (16C2), followed by a

secondary fluorescein isothiocynate-conjugated polyclonal goat anti-mouse antibody. The samples were analysed by dual colour flow cytometer (FC500, Beckman-Coulter) and the platelet population was identified from its forward and side scatter distribution, 10.000 platelets were gated. The analysis allowed to compare the two tested conditions and to evaluate for both samples the capacity of ADP to inhibit VASP phosphorilation. The results were expressed as the platelet reactivity index (PRI) calculated using corrected mean fluorescence intensities (MFIc), reflecting VASP phosphorilation, of samples incubated with PGE1 alone or PGE1 plus ADP according to the following calculation:

PRI = [(MFIc PGE1- MFIc (PGE1+ADP)) / MFIc PGE1] X 100

### 4.7 Statistical Analysis

All statistical analyses were performed using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Normal distribution was evaluated by D'Agostino-Pearson test. Parametric or non-parametric tests were used as appropriated. Paired t-test or Wilcoxon test were employed for comparison between two groups. For comparison among multiple groups one-way ANOVA or Friedman test were used followed by Dunn' post hoc test.

Differences were considered statistically significant with p-value $<0.05$. Results were expressed as median with range.

# 5. RESULTS

## 5.1 Population characteristics

Clinical and demographic characteristics of the subjects studied are summarized in table 4. Only patients enrolled in CAD study had personal history of one or more of the following: coronary stenosis (18%), myocardial infarction (61%), angina (8%), percutaneous coronary intervention (59%), coronary artery by-pass grafting (20%), without differences between the group treated with aspirin and that treated with aspirin plus clopidogrel.

## 5.2 Serum TXB2 experiments

### 5.2.1. Ex vivo experiments

*Levels of serum TxB2 in healthy subjects and in patients with $P2Y_{12}$ deficiency*

The levels of serum $TxB_2$ in two patients with congenital severe $P2Y_{12}$ deficiency and in one patient with haploinsufficiency of the $P2Y_{12}$ gene were comparable to those measured in a group of healthy subjects (n=56) (Table 5).

*Levels of serum TxB2 in patients on daily treatment with prasugrel (PRINA study)*

Serum TxB2 levels, measured before and after treatment with prasugrel or placebo in the PRINA study were not significantly different (figure 8), despite the fact that prasugrel was highly effective in inhibiting $P2Y_{12}$ , as shown by the VASP phosphorylation assay (figure 9).

### 5.2.2. In vitro experiments

*Levels of serum TxB2 in the presence and absence of* $P2Y_{12}$ *inhibitors added in vitro*

As the *ex vivo* studies showed that the $P2Y_{12}$ antagonism or deficiency does not affect the serum TXB2 production, in order to test whether $P2Y_{12}$ antagonists have off-target effects on the platelet production of $TxA_2$, serum TxB2 levels were determined after the in vitro addition of cangrelor, ticagrelor, CAM, and PAM or vehicle at high concentrations. The levels of serum $TxB_2$ in presence of $P2Y_{12}$ antagonists at all tested concentrations were not decreased (Table 5).

**Table 4.**: Population characteristics

| | ***Healthy Subjects (n=24)*** | ***P1*** | ***P2*** | ***P3*** | ***PRINA patients (n=20)*** | ***CAD patients (ASA) (n=24)*** | ***CAD patients (ASA + CLO) (n=25)*** |
|---|---|---|---|---|---|---|---|
| **Sex (M/F)** | 14/10 | M | F | M | 14/6 | 20/4 | 23/2 |
| **Age (Y)** | 27 (20-63) | 77 | 65 | 29 | 44 (23-69) | 73 (51-83) | 65(45-8) |
| **WBC (x $10^9$/L)** | 6.8 (4.0-8.5) | 6.9 | 2.9 | 4.4 | 7.2 (4.4-8.7) | 6.9 (3.5-10.2) | 7.1 (3.1-11.4) |
| **RBC (x $10^{12}$/L)** | 4.5 (3.7-6.0) | 4.4 | 4.1 | 5.0 | 4.8 (3.9-7.0) | 4.5 (3.8-5.7) | 4.6 (3.0-5.5) |
| **Hb (g/dL)** | 13.1(9.8-16.3) | 13.13 | 12.4 | 15.2 | 14.5 (11.6-17.4) | 13.9 (11.8-16.3) | 14.0 (9.8 - 16.6) |
| **Hematocrit (%)** | 39.7(31.5-50.3) | 39.5 | 37.7 | 45.8 | 42.9 (34.8-50.2) | 43.0 (37.7-49.7) | 42.8 (28.8-50.4) |
| **Platelets (x $10^9$/L)** | 207 (143-329) | 170 | 118 | 139 | 243 (163-381) | 169 (102-314) | 190 (136-308) |
| **MPV, (fL)** | ND | 9.8 | 10.6 | ND | 8.3 (6.8-12.1) | 9.6 (7.7-12.0) | 10.0 (7.0-11.6) |
| ***Medications*** | | | | | | | |
| **Oral hypoglycemic agents** | - | - | - | - | - | 2/24 | 10/25 |
| **Statins** | - | - | - | - | - | 14/24 | 25/25 |
| **Diuretics** | - | - | - | - | - | 8/24 | 3/25 |
| **Antiulcer** | - | - | - | - | - | 11/24 | 17/25 |
| **Antihypertensive** | - | - | - | - | - | 18/24 | 23/25 |

**Table 5.** SerumTxB2 levels in patients with congenital $P2Y_{12}$ defects and in healthy subjects (n=7), in presence/absence of in vitro $P2Y_{12}$ inhibitors

| ***$TxB_2$: pmoles/$10^8$ plts** | | **Patient 1: homozygous $P2Y_{12}$ deficiency** | **Patient 2: homozygous $P2Y_{12}$ deficiency** | **Patient 3: heterozygous $P2Y_{12}$ deficiency** | **Healthy subjects (n=7)** | **Normal range (n=56)** |
|---|---|---|---|---|---|---|
| **Vehicle** | | 262,7 | 158,2 | 112,1 | 181,6 (96,6 - 488,7) | 64,9 - 482,3 |
| **Cangrelor** | **10 µM** | 325,5 | 190,6 | ND | 189,9 (112,3 -388,7) | - |
| | **100 µM** | 275,8 | 229,5 | ND | 256,4 (75,5 – 439,0) | - |
| **Ticagrelor** | **10 µM** | 252,8 | 192,4 | 125,7 | 320,5 (94,4 – 415,7) | - |
| | **100 µM** | 200,2 | 166,4 | 77,4 | 221,0 (84,1 – 292,7) | - |
| **Clopidogrel active metabolite** | **10 µM** | 277,2 | 196,2 | 76,3 | 222,2 (103,1 -484,8) | - |
| | **100 µM** | 288,3 | 181,8 | 75,5 | 238,1 (107,6 -606,9) | - |
| **Prasugrel active metabolite** | **10 µM** | 266,6 | 171,9 | 98,2 | 180,8 (89,1 - 362,5) | - |
| | **100 µM** | 248,0 | 154,6 | 70,6 | 177,0 (146,9-591,1) | - |

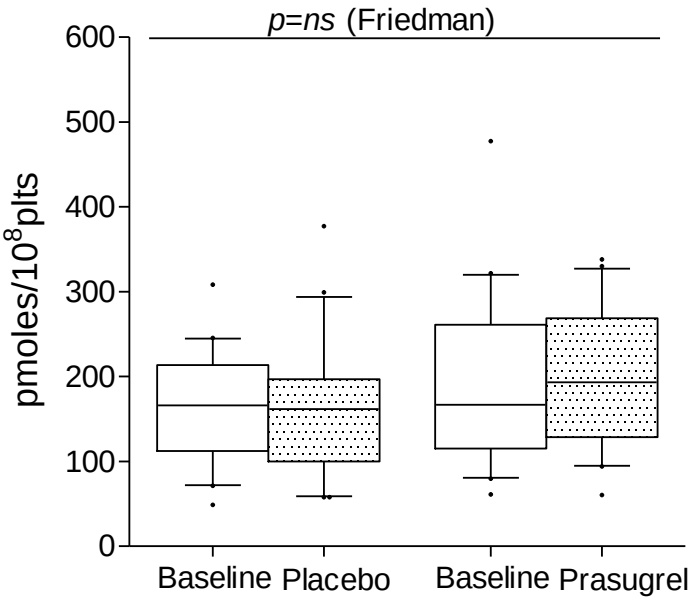



**Figure 8.** Serum $TxB_2$ levels in 20 patients with bronchial asthma before and after 14 day treatment with prasugrel (10 mg q.i.d.) or placebo. The $TxB_2$ levels were determined in duplicate by a commercial EIA. Data are shown as median (line in box), 25–75% percentile (box), and 10–90% percentile (whiskers) and were analysed by repeated measures and non-parametric statistical test ( Friedman test, $p = 0.42$ ).

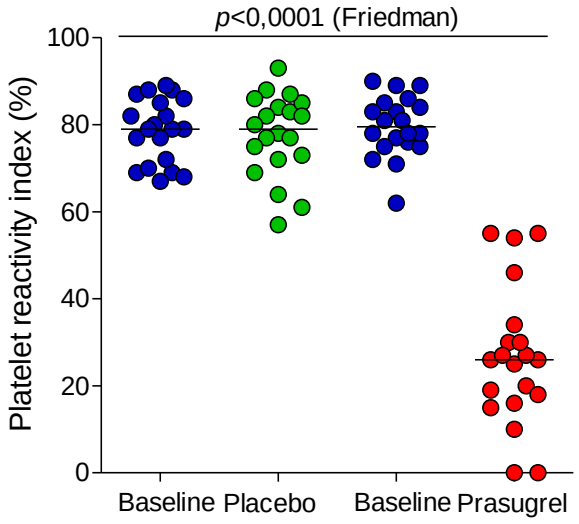



**Figure 9.** Platelet VASP phosphorylation, expressed as Platelet Reactivity Index (PRI), in patients (n=20) with asthma 15 days after treatment with placebo or prasugrel (10 mg/day). Data are shown as median and were analysed by repeated measures and non-parametric statistical test plus Dunn's multiple comparison post-test.

### 5.3 Effects of $P2Y_{12}$ antagonists on TXB2 production by platelet-rich plasma stimulated by agonists in an aggregometer

*Experiments with citrate-anticoagulated platelet-rich plasma from healthy subjects.*

Two different platelet agonists were chosen to test the effect of $P2Y_{12}$ inhibitors on TXB2 production and aggregation of platelet-rich plasma in citrate anticoagulant: exogenous arachidonic acid, and collagen, which induces TXB2 production from endogenous arachidonic acid. When experiments were performed under stirring conditions (which allow platelet aggregation to occur), each $P2Y_{12}$ antagonist (Cangrelor, Ticagrelor, CAM or PAM) caused a statistically significant and similar reduction of platelet aggregation and of TxB2 production by platelets that had been stimulated by arachidonic acid or collagen (Figure 10). In contrast, when experiments were performed under non-stirring conditions (which prevent the formation of detectable platelet aggregates), no platelet aggregation was detected and no differences in TXB2 production were observed in presence/absence of $P2Y_{12}$ antagonists (Figure 10). All tested $P2Y_{12}$ inhibitors caused severe inhibition of platelet aggregation induced by ADP (10 μM) (data not shown).

*Experiments with citrate-anticoagulated platelet-rich plasma from patients with inherited severe* $P2Y_{12}$ *deficiency.*

As expected, the aggregation of $P2Y_{12}$ deficient platelets in response to arachidonic acid or collagen was lower than that of healthy subjects. The in vitro addition of $P2Y_{12}$ antagonists did not further inhibit platelet aggregation (Figure 10). TxB2 production by $P2Y_{12}$-deficient platelets was comparable to that observed by normal platelets in the presence of $P2Y_{12}$ antagonists, and was not further reduced by $P2Y_{12}$ antagonists added in vitro, both under stirring and non-stirring conditions (Figure 10).

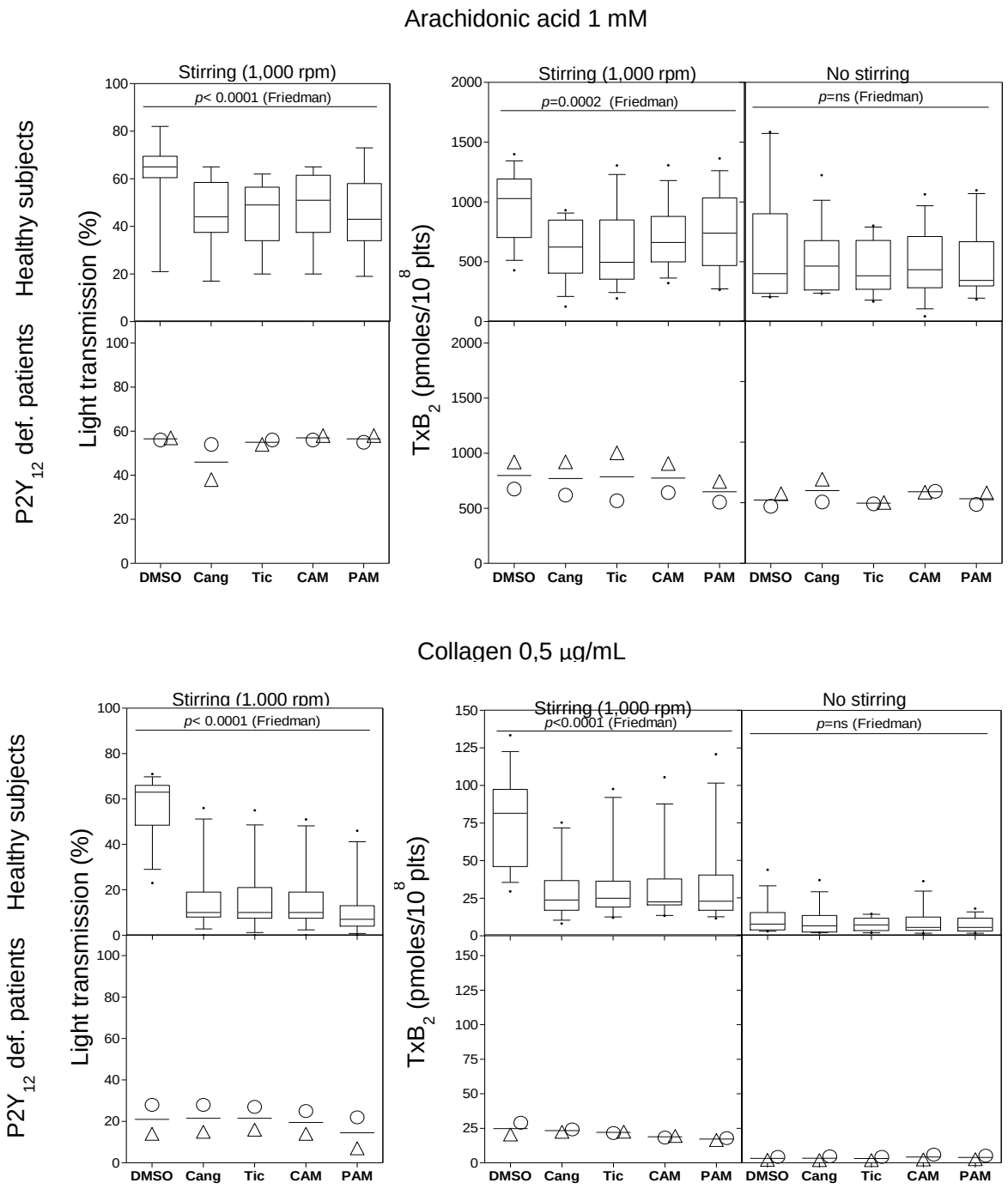



**Figure 10.** Platelet aggregation (measured by LTA) and $TxB_2$ production in citrate PRP of healthy subjects (n=13) (upper graphs) and two patients with inherited, severe $P2Y_{12}$ deficiency (lower graphs), after stimulation with arachidonic acid (1 mM) or collagen (0,5 µg/mL). Each antagonist (10µM) or vehicle was added to PRP and pre-incubated at 37°C without stirring for 10 min, followed by 2 min pre-incubation with stirring. Once mixed, PRP was stimulated with agonist under two different conditions: with stirring and with no stirring. Platelet aggregation was monitored for 3 min, after which samples were centrifuged and the supernatant platelet-poor plasma and the supernatant platelet-poor plasma was separated and stored at -80 °C until assay of TxB2 levels by EIA (see Materials and Methods for details). The data are shown as medians (line in box), 25–75% percentiles (box), and 10–90% percentiles (whiskers). Data were analysed by Friedman for repeated measures using a non-parametric statistical test plus Dunn's multiple comparison post-test.

### 5.4 Further evidence that the inhibitory effect of $P2Y_{12}$ antagonists on TxB2 production by platelets is mediated by the inhibition of platelet aggregation.

In order to test further the hypothesis that the described inhibitory effect of $P2Y_{12}$ antagonists on TxB2 production by platelets is secondary to the inhibition of platelet aggregation, we performed additional studies, under different experimental conditions.

#### 5.4.1 Effects of hirudin anticoagulant and of epinephrine

As the aggregation-dependent platelet production of TxB2 is greatly enhanced under conditions of low plasma [Ca2+], we compared the effects of cangrelor on collagen-induced platelet aggregation in citrate-PRP (low [Ca2+]) and hirudin-PRP (physiological [Ca2+]). The extent of both platelet aggregation and TxB2 production induced by collagen was greater in citrate-PRP than in hirudin-PRP (Table 6) Cangrelor dramatically inhibited platelet aggregation and TxB2 production both in citrate-PRP and hirudin-PRP. The addition of epinephrine, which, like ADP/ $P2Y_{12}$ stimulates an inhibitory G-protein through its interaction with its receptor, restored platelet aggregation and TxB2 production in the presence of cangrelor both in citrate-PRP and hirudin-PRP.

**Table 6.** Effect of epinephrine on platelet aggregation and TxB2 production induced by collagen (0.5 μg/ml) in citrate-PRP and hirudin-PRP from healthy subjects

| **Collagen 0,5 ( μg/mL )** | **Vehicle (n=6)** | **Cangrelor (n=6)** | **Cangrelor + epinephrine (n=6)** |
|---|---|---|---|
| **Citrate-PRP** | | | |
| Platelet aggregation (percent) | **63** (8-70) | **11** (0-37) | **59** (19-70) |
| TxB2 (pmoles/$10^8$ plts) | **33.6** (12.3-75.3) | **11.8** (7.91-22.8) | **20.4** (8.8-51.1) |
| **Hirudin-PRP** | | | |
| Platelet aggregation (percent) | **45** (1-99) | **1** (0-3) | **44** (7-100) |
| TxB2 (pmoles/$10^8$ plts) | **6.9** (4.1-19.1) | **3.9** (2.1-8.8) | **6.6** (2.8-15.1) |

### 5.4.2 Effects of MRS2500, an inhibitor of the platelet P2Y1 receptor for ADP

The addition of MRS2500 (an inhibitor of P2Y1, the other ADP receptor on platelets) to citrate-PRP had effects on collagen-induced platelet responses that were similar to those observed after the addition of $P2Y_{12}$ antagonists: platelet aggregation and TxB2 production were significantly inhibited under stirring conditions, while no inhibitory effects on TxB2 production were observed under non-stirring conditions (Figure 11).

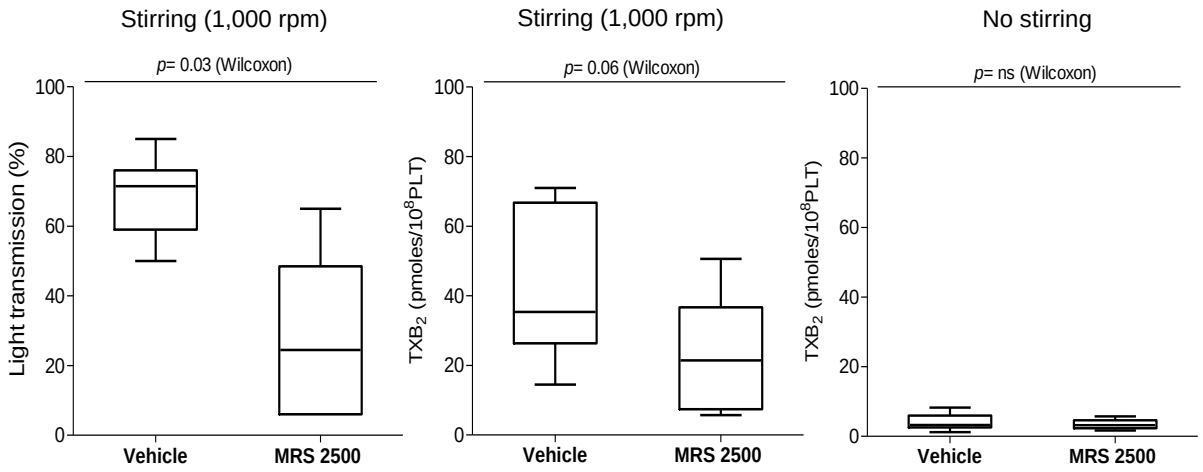



**Figure 11.** In vitro effects of MRS2500, an inhibitor of the P2Y1 platelet receptor for ADP, on platelet aggregation and TxB2 production induced by collagen 0.5 µg/mL in citrate PRP of healthy subjects (n= 6). MRS2500 or vehicle was added to PRP and pre-incubated at 37°C without stirring for 10 min, followed by 2 min pre-incubation with stirring. Once mixed, PRP was stimulated with agonist under two different conditions: with stirring and with no stirring. Platelet aggregation was monitored for 3 min, after which samples were centrifuged and the supernatant platelet-poor plasma and the supernatant platelet-poor plasma was separated and stored at -80 °C until assay of TxB2 levels by EIA (see Materials and Methods for details). The data are shown as medians (line in box), 25–75% percentiles (box), and 10–90% percentiles (whiskers). Data were analyzed by Friedman for repeated measures using a non-parametric test plus Dunn's multiple comparison post-test.

## 5.5 Comparison of the inhibitory effects of aspirin and cangrelor, alone and in combination, on platelet aggregation and TxB2 production

Experiments were performed using citrate-PRP and hirudin-PRP, both low (0.5 ug/ml) and high (10 ug/ml) concentrations of collagen

### 5.5.1 Collagen 0.5 ug/ml

Aspirin and cangrelor inhibited platelet aggregation and TxB2 production induced by collagen 0.5 ug/ml (Figure 12). With the exception of platelet aggregation in hirudin-PRP, in which the 2 inhibitors had a similar effect, aspirin proved to be a better inhibitor than cangrelor. The combination of aspirin and cangrelor did not significantly inhibit collagen-induced platelet aggregation and TxB2 production further, compared to aspirin alone (figure 12).

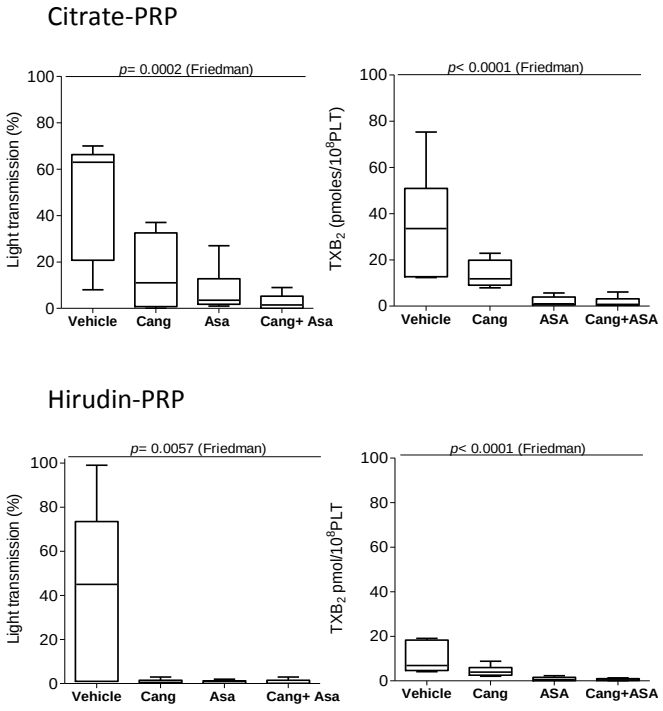



**Figure 12.** Effects of cangrelor and aspirin, alone and in combination, on platelet aggregation and TxB2 production induced by collagen 0.5 µg/mL in citrate or hirudin PRP. Each compound or vehicle was added to PRP and pre-incubated at 37°C without stirring for 10 min, followed by 2 min pre-incubation with stirring. Once mixed, PRP was stimulated with agonist under two different conditions: with stirring and with no stirring. Platelet aggregation was monitored for 3 min, after which samples were centrifuged and the supernatant platelet-poor plasma was separated and stored at -80 °C until assay of TxB2 levels by EIA (see Materials and Methods for details). The data are shown as medians (line in box), 25–75% percentiles (box), and 10–90% percentiles (whiskers). Data were analyzed by Friedman for repeated measures using a non-parametric test plus Dunn's multiple comparison post-test.

### 4.5.2. Collagen 10 ug/ml

Aspirin and cangrelor only partially inhibited platelet aggregation induced by collagen 10 ug/ml both in citrate-PRP and hirudin-PRP: no statistically significant differences were observed in the degree of inhibition by the 2 drugs, when they were added alone. The combination of aspirin and cangrelor dramatically reduced the extent of platelet aggregation. Both cangrelor and aspirin significantly inhibited TxB2 production both in citrate-PRP and hirudin-PRP, but aspirin proved to be more effective. The combination of aspirin and cangrelor completely abolished TxB2 production (figure 13).

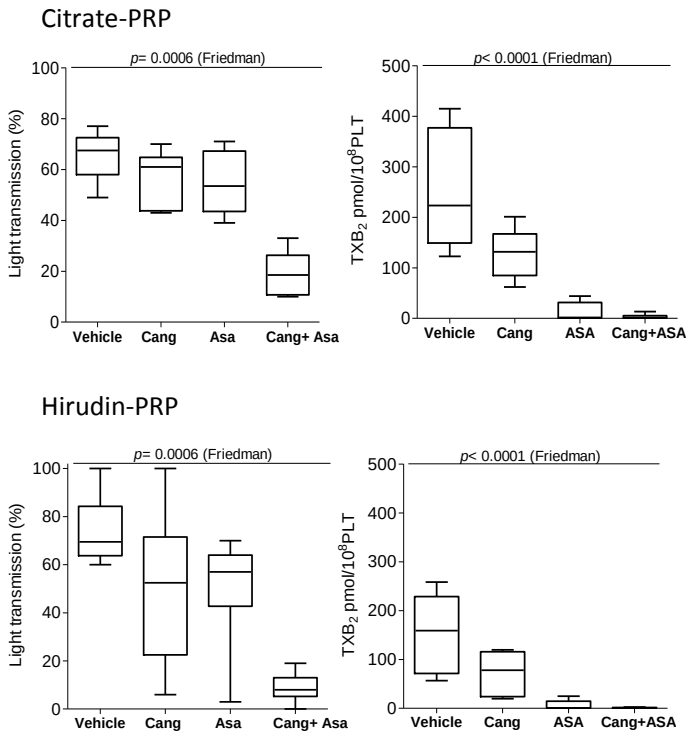



**Figure 13.** Effects of cangrelor and aspirin, alone and in combination, on platelet aggregation and TxB2 production induced by collagen 10 µg/mL in citrate or hirudin PRP. Each compound or vehicle was added to PRP and pre-incubated at 37°C without stirring for 10 min, followed by 2 min pre-incubation with stirring. Once mixed, PRP was stimulated with agonist under two different conditions: with stirring and with no stirring. Platelet aggregation was monitored for 3 min, after which samples were centrifuged and the supernatant platelet-poor plasma was separated and stored at -80 °C until assay of TxB2 levels by EIA (see Materials and Methods for details). The data are shown as medians (line in box), 25–75% percentiles (box), and 10–90% percentiles (whiskers). Data were analyzed by Friedman for repeated measures using a non-parametric test plus Dunn's multiple comparison post-test.

## 5.6. Serum TXB2 levels in CAD patients on chronic treatment with aspirin or aspirin plus clopidogrel

Levels of serum TXB2 were very low in CAD patients treated with aspirin or aspirin plus clopidogrel both 3h and 24h after the last drug intake. There was no statistically significant difference in the serum TxB2 levels in patients treated with aspirin alone compare to patients treated with aspirin plus clopidogrel both at 3h and 24h after the last drug intake (Figure 14).

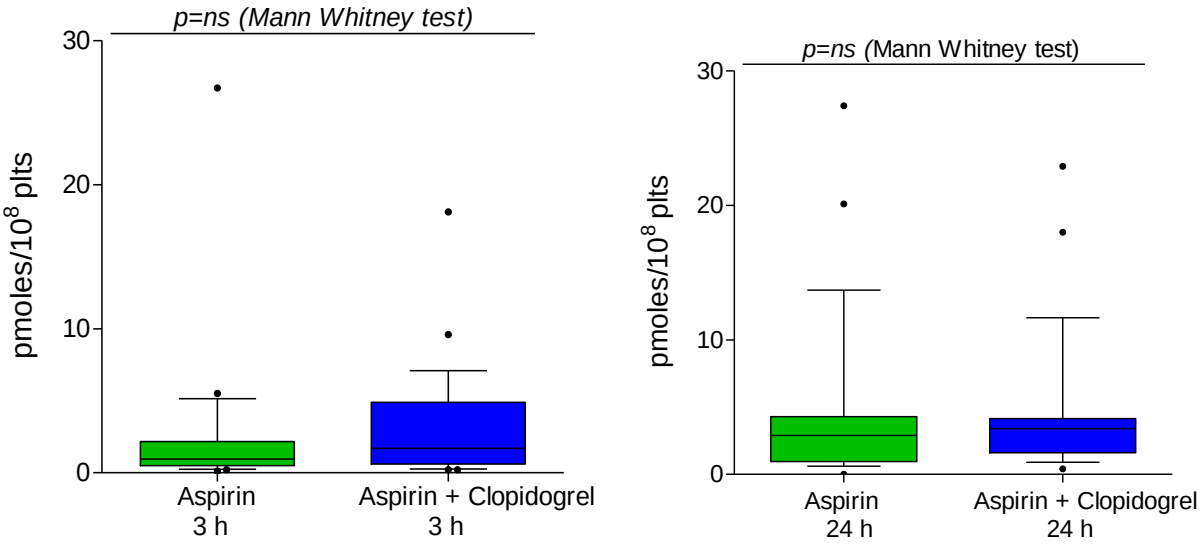



**Figure 14**. Serum $TxB_2$ levels of 24 patients on chronic daily treatment with aspirin (100 mg) and 25 patients on chronic daily treatment with aspirin (100 mg) plus clopidogrel (75 mg). $TxB_2$ levels were assayed in duplicate using a commercial EIA (see Materials and Methodsa for details). Data are shown as medians (line in box), 25–75% percentiles (box), and 10–90% percentiles (whiskers), and were analyzed the Mann Whitney test (*p*= ns).

# 6. DISCUSSION AND CONCLUSIONS

Current management of ACS patients targets primary drivers of platelet aggregation: $TxA_2$ and ADP by a combination therapy of aspirin and a $P2Y_{12}$ antagonist. Based on the recent observation [101] [102] [109] that $P2Y_{12}$ antagonists also inhibit the platelet production of $TxA_2$, it has been suggested that patients with ACS might be safely treated with $P2Y_{12}$ antagonists only [110]. In contrast, our previous observations demonstrated that $P2Y_{12}$ deficiency did not lead to a reduction of serum TxB2 [104] [105].

Given the need to provide novel insight into the controversial issue about the effect of $P2Y_{12}$ receptor antagonism on $TxA_2$ generation, in this study we tested the hypotheses that the reported inhibitory effect of $P2Y_{12}$ antagonists on TxA2 production may be: 1) due to off-target effects, (addressed by the *in vitro, ex vivo* effect of $P2Y_{12}$ antagonists) 2) secondary to the inhibition of platelet aggregation (addressed by the *in vitro* addition of P2Y12 antagonists in presence or absence of aggregation).

The major conclusion of the present study is that the reported reduction of $TxA_2$ production after the addition of $P2Y_{12}$ antagonist is not due to unspecific effects of the compounds as they do not affect serum $TxA_2$ levels both *in vitro* and ex vivo, but only secondary to the inhibition of platelet aggregation.

In addition, we also demonstrated that $P2Y_{12}$ antagonist is not able to reduce TXB2 in a comparable extent as aspirin, and even the combination of both does not add any benefit in terms of reduction of TxB2 production neither in *in vitro* nor in *ex vivo* experiments.

Firstly, we confirmed that serum $TxA_2$ of 3 patients with congenital $P2Y_{12}$ defects, either haploinsufficiency or severe deficiency, were in the normal range.

However, these data did not exclude that administration of $P2Y_{12}$ antagonists may affect TxB2 production as suggested by Bhavaraju et al [101]. Our hypothesis is based on recent findings that support the idea that irreversible $P2Y_{12}$ antagonists (tienopyridines) have off-target activity affecting the bleeding, not entirely $P2Y_{12}$ dependent [111].

Focusing on the off-target activity of $P2Y_{12}$ antagonists (cangrelor, ticagrelor, PAM and CAM) we observed that their *in vitro* addition during the serum preparation did not modify the TxB2 levels in healthy subjects compared to the vehicle. All compounds were added at two different concentrations both in excess respect to the effective peak plasma concentration to secured complete pharmacological inhibition of $P2Y_{12}$ receptor. These results were similar to those found in patients with congenital $P2Y_{12}$ deficiency and were quite consistent across them.

Consistent with these *in vitro* data, results of PRINA trial reported that subjects receiving prasugrel had normal serum TxB2 levels and not significantly reduced compared to the placebo. Prasugrel was chosen because it is a third-generation thienopyridine, with faster onset of action and a more uniform inhibition of platelet function compared to clopidogrel. Indeed, in our study population we found a very low residual platelet reactivity (median PRI 26%, 0-55). Hence, this controlled, randomized study provides proof-of-concept that pharmacological inhibition of the platelet $P2Y_{12}$ receptors does not affect serum TxB2 production neither directly nor by unspecific effects due to in vivo formed metabolites.

Taken together, this findings are not in agreement with Bhavaraju et al and Kidson-Gerber et al. studies [101] [109]. We do not have an explanation for this discrepancy, but in our experience, the assessment of TxB2 production has high inter-intra individual variability over time as the tables 2 and figure 1 show.

With this in mind, we controlled the variability, indeed the design of the pilot study virtually excluded the possibility that any difference was due to the variability between individuals and the in vitro experiments were performed starting from the same blood sample.

In addition, it is important to note that serum TxB2 levels found here are quite different from those reported by Bhavaraju et al and Kidson-Gerber et al., although our reference range, obtained from a large number of healthy subjects, was consistent with those reported by others [112] table 2

Hence, our study provides multiple consistent lines of evidence that neither deficiency nor blockade of $P2Y_{12}$ receptor do lead to the reduction of TxB2 generation under thrombin stimulus.

However, the results reported here are in contrast with some studies which showed that ADP-directly induced TxB2 generation in human platelets [100] and that $P2Y_{12}$ inhibitors are able to reduce in vitro platelet aggregation and $TxA_2$ production induced by several agonists [103] [113] [114] [102].

Thus, we conducted in vitro experiments on citrated-PRP to provide novel insight in the understanding if the reduction of TxB2 due to $P2Y_{12}$ antagonists is only secondary to the inhibition of platelet aggregation.

As expected, we found that $P2Y_{12}$ antagonists caused a reduction of platelet aggregation and a consequent reduction of $TxA_2$ synthesis when agonists such as AA and collagen, able to directly stimulate $TxA_2$ synthesis, were employed.

However, these experiments simultaneously repeated under non-stirring conditions, when platelet aggregation does not occur, showed no reduction of TxB2 production in presence of $P2Y_{12}$ antagonists.

The same pattern of results was found in both groups, healthy subjects and in 2 patients with severe, congenital $P2Y_{12}$ deficiency. In both cases, $P2Y_{12}$ receptor resulted strongly inhibited as demonstrated by ADP-induced platelet aggregation.

Our data suggest that synthesis of endogenous $TxA_2$ is triggered by the close platelet-to-platelet contact that occurs during platelet aggregation, in agreement with the well-established knowledge that the ADP via $P2Y_{12}$ receptor can induce directly platelet aggregation, but not $TxA_2$ production [115] [116].

Indeed platelet-to platelet contacts caused by weak agonists, such as ADP, causes the formation of trace amounts of $TxA_2$ and consequent secretion of ADP. When platelets aggregate and reach a threshold level trigger $TxA_2$ generation, which causes platelet secretion and, in collaboration with secreted ADP, a second and irreversible wave of aggregation by interacting with its receptor $P2Y_{12}$.

In agreement with this, the stimulation of Giα family member Gzα by a weak agonist as epinephrine is able to completely reverse the cangrelor inhibition of collagen induced platelet, but is not able to reverse TxB2 production completely, confirming that weak agonist is not able to directly induce TxB2 production.

The results of this study provide novel insight in the understanding the effect of ADP/ $P2Y_{12}$ receptor pathway on the $TxA_2$ production that may have a clinical relevance because is still argue the better medication strategy to prevent blood from clotting and, at the same time, to minimize the complications due to the therapy after a stent procedure.

The main contribution in this field will come from the results of GLOBAL LEADER trial (NCT 01813435) currently ongoing. This study will evaluate "Comparative Effectiveness of 1 Month of Ticagrelor Plus Aspirin Followed by Ticagrelor Monotherapy Versus a Current-day Intensive Dual Antiplatelet Therapy in All-comers Patients Undergoing Percutaneous Coronary Intervention With Drug-eluting Stent Use". In agreement with some recent studies [113] [103], the rationale of this trial is based on the hypothesis that aspirin may be not required for long-term secondary prevention of recurrent ischemic events.

However, this hypothesis does not fit with the findings presented here, therefore we also investigated whether strong $P2Y_{12}$ receptor antagonism leads to additional antiaggregatory effect to aspirin by comparing, in vitro and ex vivo, individual and combined effects of them. Our data

suggest that aspirin provides an additional antiaggregatory effect to a $P2Y_{12}$ inhibitor (cangrelor) owing to a reduction in the aggregation driven- $TxA_2$ formation (collagen 0.5 µg/mL). This effect is also more appreciable when the aggregation is not $TxA_2$ dependent (collagen 10 µg/mL). These observations indicate, just in vitro, that the combination of aspirin and a $P2Y_{12}$antagonist leads to a greater benefit in terms of inhibition of platelet activation compared to that produced by strong $P2Y_{12}$ blockade alone.

However, we were aware that the effect found here may be affected by the anticoagulant used. As it is known, the low extracellular calcium is able to overemphasize the in vitro role and production of TxB2 as it happens with citrate anticoagulant. However, it was not our case, because our results show the same pattern when the experiments were conducted either in citrated PRP and at physiological concentration of calcium by using hirudin.

Consistent with all results reported here, we also observed in a pilot study, that the chronic administration of clopidogrel has no additional effect on serum TxB2 production in CAD patients receiving aspirin compared to patients in treatment with aspirin only. That was consistent in patients either responders or non-responders to clopidogrel (as indicated by their PRI VASP assay, no correlation was found between the PRI and serum TXB2, data not shown). In conclusion, our study demonstrates that neither the antagonism nor the antagonists of $P2Y_{12}$ receptor are able to directly reduce $TxA_2$ generation in vitro as well as ex vivo and that $P2Y_{12}$ receptor is crucial for $TxA_2$ generation only as a secondary player. Consistent with these findings, to date, there is no clinical, nor pharmacological/ physiological evidence that aspirin should be withheld in patients with acute coronary syndromes, who are generally treated with aspirin plus $P2Y_{12}$ antagonists.

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