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Effect of prasugrel in patients with asthma: results of PRINA, a randomized, double-blind, placebo-controlled, cross-over study

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Acknowledgments

I thank my wife, my children and my parents who in different ways positively contribute to my life and my work. Marco Cattaneo fascinated me with scientific research and I am particularly fortunate to have his scientific support; I would like to dedicate this thesis to him.

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List of abbreviations

LTs	Cysteinyl-leukotrienes
PD15	Provocative dose of mannitol causing ≥15% drop in FEV1
FEV1	Forced Expiratory Volume in 1 Second
FeNO	Fractional exhaled nitric oxide
LSD	Least Significant Difference
ADP	adenosine diphosphate
VASP	vasodilator-stimulated phosphoprotein
PRI	platelet reactivity index

Summary

Introduction: Although experimental studies demonstrated that platelets are pro-inflammatory cells, no randomized studies tested the anti-inflammatory effect of antiplatelet agents in humans. The platelet $P2Y_{12}$ receptors mediated bronchial inflammation in a mouse model of asthma, suggesting that $P2Y_{12}$ represents a pharmacological target for asthma. ***Objectives:*** In this proof-of concept, placebo-controlled, randomized, cross-over study we tested the effects of the $P2Y_{12}$ antagonist prasugrel on bronchial hyper-reactivity of asthmatic patients. *Patients/Methods:* Twenty-six asthmatic patients were randomly and blindly allocated to prasugrel (10mg o.d.) or placebo for 15 days. After ≥15-day wash-out, patients were crossed-over to the alternative treatment. Before and after each treatment, patients underwent bronchial provocation test with mannitol and measurement of fractional exhaled nitric oxide (FeNO). Inhibition of $P2Y_{12}$-dependent platelet reactivity (PRI) was measured by the VASP phosphorylation assay. ***Results:*** The provocative dose of mannitol causing a 15%-drop in FEV1 tended to increase from 142 mg (95%CI 82-202) to 187 mg (113-262) after prasugrel (p=0.09) and did not change after placebo (136 mg; 76-196 and 144 mg; 84-204 , p=0.65). FeNO did not change after either treatment. PRI decreased from 80%(77-83) to 23%(7-29) after prasugrel (p<0.001) and remained unchanged after placebo. ***Conclusions:*** Our proof-of-concept, randomized, controlled study is the first one to test in vivo the anti-inflammatory effects of platelet inhibition in human patients. Its results suggest that pharmacological inhibition of $P2Y_{12}$ receptors may slightly reduce the bronchial inflammatory burden and lays the groundwork for further studies, with clinical end-points.

1. INTRODUCTION

1.1 Asthma

1.1.2 Definition of asthma

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheezing, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation.

1.1.2 Description of asthma

Asthma affects people of all ages, but it most often starts during childhood. Asthma is characterized by variable symptoms of wheezing, shortness of breath, chest tightness and/or cough and variable expiratory airflow limitation. Symptoms and airflow limitation vary over time and in intensity. These variations are often triggered by factors such as exercise, allergen, or irritant exposure, change in weather, or respiratory infections. Symptoms and airflow limitation may resolve spontaneously, or in response to medication and may sometimes be absent for weeks or months at a time. Patients can experience a minor inconvenience, while for others asthma can be a major problem that interferes with daily activities and may lead to life-threatening exacerbations. Asthma is usually associated with airway hyperresponsiveness to direct or indirect stimuli, and with chronic airway inflammation.

- *Asthma phenotype:*

Asthma symptoms range from minor to severe and vary from person to person. Some patients have infrequent asthma attacks, have symptoms only at certain times, such as when exercising, or have symptoms all the time (1-3).

For some people, asthma symptoms flare up in certain situations:

- Exercise-induced asthma, which may be worse when the air is cold and dry
- Occupational asthma, triggered by workplace irritants, such as chemical fumes, gases or dust
- Allergy-induced asthma, triggered by particular allergens, such as pet, dander, cockroaches or pollen

Asthma signs and symptoms include:

- Shortness of breath
- Chest tightness or pain
- Trouble sleeping caused by shortness of breath, coughing or wheezing
- A whistling or wheezing sound when exhaling
- Coughing or wheezing attacks that are worsened by a respiratory virus, such as a cold or the flu

1.1.3 Asthma diagnosis

The first step in diagnosing asthma (4) is to identify patients at risk of, or with significant likelihood of having chronic airways disease and to rule out other possible conditions, such as a respiratory infection or chronic obstructive pulmonary disease

(COPD). This first step is based on a detailed medical history and physical examination. A diagnosis of asthma should be suspected if there is a history of: recurrent wheezing, coughing or difficulty breathing and these symptoms occur or worsen due to exercise, viral infections, allergens or air pollution. The diagnosis of allergic asthma is more likely when the person also has an allergy and a family history of asthma.

Tests to measure lung function

- Spirometry (5). This test estimates the narrowing of bronchial tubes by checking how much air can be exhaled after a deep breath and how fast it can be breathed out.

- Methacholine challenge (6, 7). Methacholine is a known asthma trigger that, when inhaled, will cause mild constriction of airways. The methacholine challenge involves the inhalation of increasing concentrations of this substance that causes airway narrowing in those predisposed. If negative it means that a person does not have asthma; if positive, however, it is not specific for the disease

- Nitric oxide test (8). This test measures the amount of the gas, nitric oxide, that patients have in their breath. When airways are inflamed, a sign of asthma, patients may have higher than normal nitric oxide levels.

Other tests include:

- Allergy testing. This can be performed by skin test or blood test. Allergy tests can identify allergy to pets, dust, mold and pollen. If important allergy triggers are identified, this can lead to a recommendation for allergen immunotherapy.

- Sputum eosinophils. This test looks for certain white blood cells (eosinophils) in the mixture of saliva and mucus (sputum) which patients discharge during coughing. Eosinophils are present when symptoms develop and become visible when stained with a rose-colored dye (eosin).

- Imaging tests. A chest X-ray and high-resolution computerized tomography (CT) scan of lungs and nose cavities (sinuses) can identify any structural abnormalities or diseases (such as infection) that can cause or aggravate breathing problems.

1.2 Platelets

1.2.1 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 (9) but contain megakaryocyte-derived messenger RNA (mRNA) and the translational machinery needed for protein synthesis including ribosomes, and initiation and termination factors (10). 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 (11). The number of α-granules per platelet depends on cell size 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 (12), electron dense chains and clusters (13), and tubular inclusions (14).

1.2.2 Platelets in primary hemostasis

The main role of blood platelets is to ensure primary hemostasis, 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 (15, 16). 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 (17).

- *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 TxA2. 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-HT2A receptor by 5-HT and the $P2Y_1$ receptor by ADP (both coupled to a Gq

protein) induces an increase in intracellular Ca2+ levels, whereas activation of $P2Y_{12}$ (couple to Gi protein) by ADP activates PI3kinase and inhibits adenylate cyclase. TxA2 is synthesized in activated platelets starting from arachidonic acid (AA) by cyclooxygenase (COX). Once formed, TxA2 diffuses across the platelet membrane and activates other platelets through the interaction with two surface membrane TxA2 receptors, TPα and TPβ, coupled to the proteins Gq and G12 or G13, 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 Ca2+ 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 (Ca2+). 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 (18, 19), fibrinogen (20), fibrin and fibronectin (21), able to form stable platelet aggregates (22). 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.

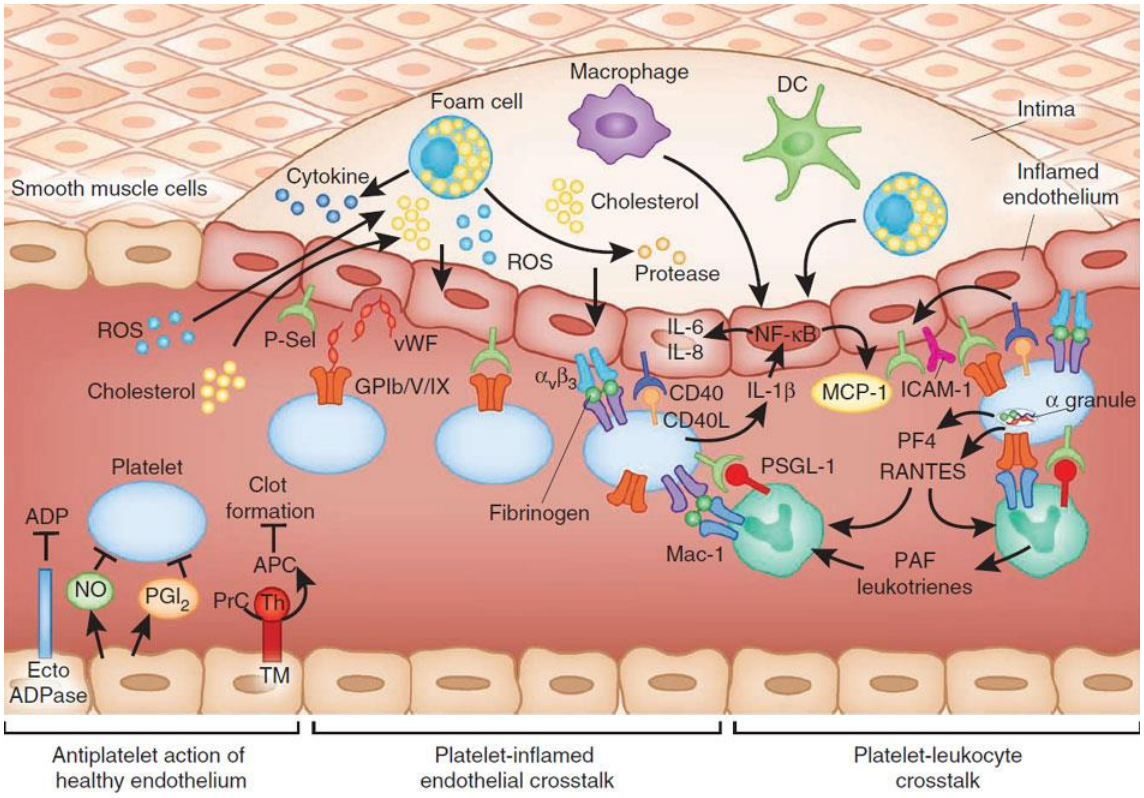


Figure 1. Modified from (23) The antiadhesive phenotype of endothelial cells is maintained through four intrinsic pathways: ecto-ADPase, prostaglandin I2 (PGI2), nitric oxide (NO) and the thrombomodulin (TM)-activated protein C (APC) pathways.

1.2.3 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 (24), 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 (25, 26). However, it is becoming clear that the cellular and biochemical interactions underlying thrombosis are also directly relevant to atherosclerosis (27).

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 1.2.2.

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 (28):

- 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 (29-31). 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 (32). Another important mediator released from platelets is the CD40

ligand which triggers an inflammatory response of the endothelial cells (33). This ligand is stored in α-granules of resting platelets and becomes rapidly exposed on cell surface following platelet activation (34). 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 (35), adhesion molecules, chemokines(33), and tissue factor (36) leading to an inflammatory response. The interaction between platelets, endothelial cells and leukocytes thus establishes a localized 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 (37).

1.3 The platelet $P2Y_{12}$ receptor for adenosine diphosphate

1.3.1 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 (38, 39) and stabilizes platelet aggregate (40, 41). 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 $P2Y_1$ receptor, and inhibition of adenylyl cyclase, which is mediated by the Gi-linked $P2Y_{12}$ receptor (42). The activation of $P2Y_1$ receptor by ADP mediates platelet shape change and initiates platelet aggregation, whereas $P2Y_{12}$ amplifies the platelet aggregation response (43). Concomitant activation of both G protein-coupled receptors is essential to elicit normal platelet aggregation (42, 44) (Figure 2)

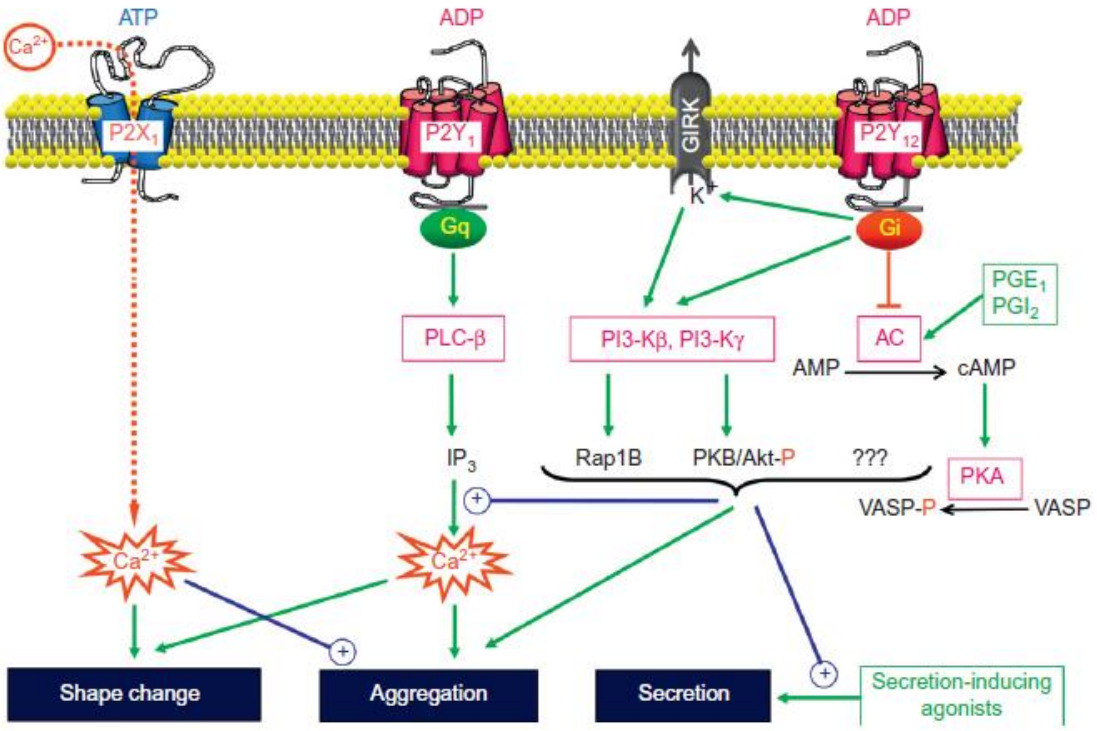


Figure 2. Figure modified from (45). Role of $P2Y_{12}$ in platelet aggregation**.** ADP interact with $P2Y_{12}$, a seven-transmembrane receptor that is coupled to Gi protein. This bond induces platelet aggregation and amplifies the aggregation response that is induced by other agonists or by ADP itself, by interacting with its other platelet receptor, $P2Y_1$. $P2Y_{12}$ 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.

1.3.2 $P2Y_{12}$ receptor

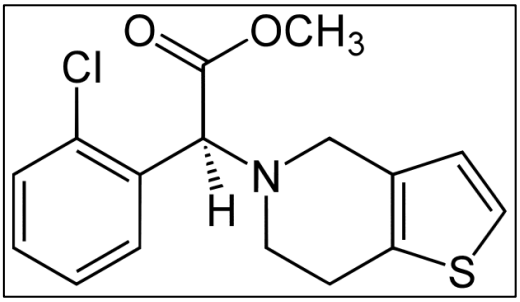
The P2 receptors, which interact with purine and pyrimidine nucleotides, are divided into two groups: G protein-linked or metabotropic, termed P2Y, and ligand-gated ion channels or ionotropic, termed P2X (46). The P2Y receptors are seven-membrane-spanning proteins with a molecular mass of 41 to 53 kD after glycolysation (46). 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 $P2Y_1$ leads to activation of β-isoforms of phospholipase C (PLC) and triggers the mobilization of Ca^{2+} 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 $P2Y_1$ 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 (44, 47, 48). The stimulation of the ADP receptors, predominately the $P2Y_{12}$ receptor, assists to activation of integrin GP IIb/IIIa (fibrinogen receptor)(49, 50). $P2Y_{12}$ 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.

1.4 Antiplatelet drugs: ADP/ $P2Y_{12}$ antagonists

Thienopyridines (ticlopidine, clopidogrel and prasugrel) irreversibly inhibit $P2Y_{12}$, while ticagrelor and cangrelor are reversibly-binding inhibitors (51, 52).

1.4.1 Clopidogrel

Pharmacology



(+)-(S)-methyl 2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate

Molecular mass: 321.82 g/mol

Figure 3. 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 cytotoxicity (neutropenia, thrombotic thrombocytopenic purpura). 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 (53), about 75% of clopidogrel administrated is converted through this step (54). 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

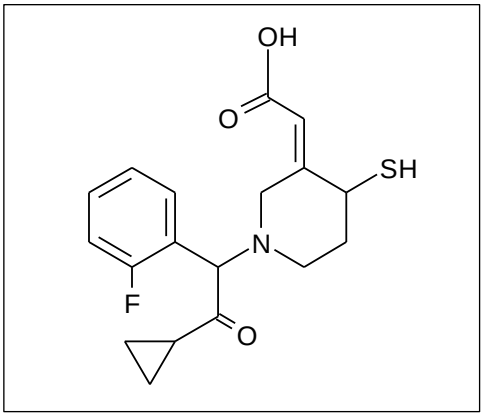
inactive acid metabolite of 2-Oxo-Clopidogrel by esterase (53). The active metabolite irreversible binds to $P2Y_{12}$ by forming a covalent disulfide bond with cysteine residues. The consequence of an irreversible inhibition is that stays on for entire life span of circulating platelets (55). Gastrointestinal side effects are also described during treatment with clopidogrel, even if clinically less severe than in aspirin-treated patients (56).

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 (57, 58).
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)(59, 60). 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.

1.4.2 Prasugrel

Pharmacology



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

Figure 4. Chemical structure of prasugrel (61)

Prasugrel is a thienopyridyl pro-drug that is rapidly metabolized to its active metabolite R-138727 (Figure 4) 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 (62), although the active metabolites for prasugrel and clopidogrel have equipotency at the $P2Y_{12}$ receptor in vitro (63). 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 allows 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 (61, 64) forming the thiolactone, and then an oxidation by intestinal and hepatic cytochrome P450–mediated,

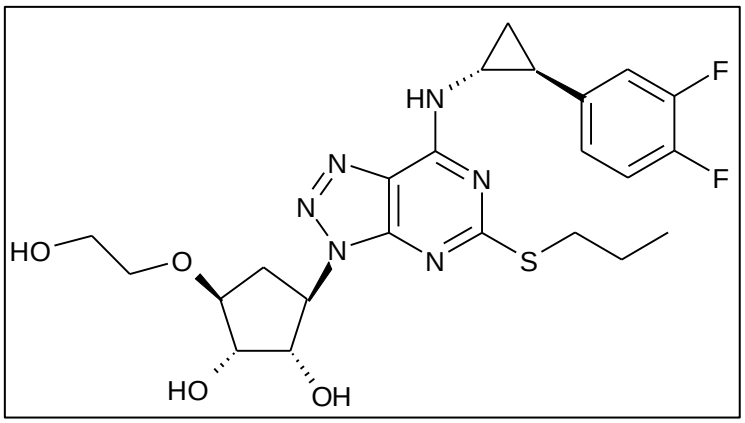
mainly CYP3A and CYP2B6 (65), with smaller contributions by CYP2C9 and CYP2C19 (65).

The different pharmacokinetics and pharmacodynamics of prasugrel compared with clopidogrel can be summarized as follows (66):

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 $P2Y_{12}$-dependent platelet responses and extremely low prevalence of subjects who display resistance to prasugrel.

1.4.3 Ticagrelor

Pharmacology



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

Molecular mass = 522.57;

Figure 5. 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 5) (67, 68). 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 (67).

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 (52). 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 (69). Ticagrelor has a rapid beginning of action, reaching maximal inhibition of platelet function in about 2 hours (70, 71). Although the plasma half-life of ticagrelor is 6-8 hours (70, 72), 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 (73). Ticagrelor has been shown to inhibit erythrocyte adenosine uptake likely via the equilibrative nucleoside transporter 1 (ENT1) (74) and to augment adenosine-induced coronary blood flow, possibly contributing to reduced myocardial infarction size (75).

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;

1.5 The role of platelets $P2Y_{12}$ receptor in inflammation and asthma

In addition to their well characterized and established role in hemostasis and thrombosis, platelets also have inflammatory functions and influence innate and adaptive immune responses (76-78). They express toll-like receptors, interact with activated endothelium, undergo chemotaxis, "prime" leukocytes for efficient tissue recruitment, activate other inflammatory cells, exert phagocytosis, release biologically active substances, including adhesive proteins, vasoactive and pro-inflammatory mediators(76-78).

Allergic bronchial asthma is a chronic inflammatory disease that impairs the quality of life, is associated with significant mortality rate and negatively impacts on health care and social costs. In1981, it was shown that platelets are activated during antigen-induced bronchoconstriction (79). More recently, it was shown that pplatelets accumulate in lungs of asthmatic patients, are required for airway wall remodelling and recruitment of inflammatory cells in murine allergic lung inflammation (80, 81) and migrate into the lungs of ovalbumin-sensitized and -challenged mice by an IgE-dependent mechanism(82).

Cysteinyl-leukotrienes (LTs) LTC_4, LTD_4 and LTE_4 are biologically active lipids that play a role in allergic asthma (83). They are formed in eosinophils, basophils and mast cells by 5-lipoxygenase, which catalyses the conversion of arachidonic acid into LTA_4, an unstable intermediate that is enzymatically converted into either LTB_4 or LTC_4, which is transported into the extracellular space where a gamma-glutamyl-

transpeptidase forms LTD_4, which is finally converted into the stable metabolite LTE_4 (84. Cysteinyl-LTs interact with G protein-coupled receptors, CysLT1R, CysLT2R and GPR99 {Hui, 2001 #10, 85, 86). Platelets adhere to leukocytes and amplify the production of Cysteinyl-LTs (83), express $CysLT_1R$ and $CysLT_2R$ and, when exposed to LTD_4 or LTE_4, release RANTES, a powerful eosinophil chemoattractant (87).

In the last few years, the pro-inflammatory role of adenine nucleotides interacting with their platelet P2 receptors has emerged. In particular, it was shown that the platelet $P2Y_{12}$ receptor for ADP significantly contributed to the pro-inflammatory effects of cysteinyl leukotrienes (CysLT) in experimental models of asthma in mice. LTE_4 enhances inflammatory cell recruitment in lungs of sensitized mice, which is abrogated by platelet depletion, by treatment with the anti-$P2Y_{12}$ thienopyridine drug clopidogrel, and in mice lacking $P2Y_{12}$, but not in mice lacking $CysLT_1R$ and $CysLT_2R$ (88). Moreover, intranasal administration of LTC_4 in sensitized mice before ovalbumin challenges potentiated the recruitment of eosinophils in the bronchoalveolar lavage, which was dependent on $CysLT_2R$, on $P2Y_{12}$ and platelets (89). Therefore, the platelet $P2Y_{12}$ may represent an ideal pharmacological target for the treatment of allergic asthma.

Despite the profusion of experimental studies demonstrating the role of platelets in inflammatory diseases in general and in allergic asthma in particular, no randomized, controlled study tested whether inhibition of platelet function has beneficial effects in patients with inflammatory diseases.

2. AIM OF THE STUDY

The aim of the study was to test whether or not inhibition of the platelet $P2Y_{12}$ receptor by the $P2Y_{12}$ antagonist prasugrel (66) reduces bronchial hyper-reactivity in patients with asthma: "PRasugrel IN Asthma" (PRINA), a proof-of-concept randomized, double-blind, cross-over, placebo-controlled study.

3. MATERIALS AND METHODS

3.1 Study design

Randomized, double blind (Subject, Caregiver, Investigator, Outcomes Assessor), cross-over, placebo-controlled, prospective study. The study was approved by the Ethical Committee of Ospedale San Paolo, Milan, Italy and was conducted in agreement with the principles of the Helsinki Declaration. All patients gave their written informed consent to the study. Randomization was performed in sequential blocks. Patients were blindly and randomly allocated to treatment "A" (placebo) or "B" (prasugrel 10 mg o.d., Efient, Eli Lilly and Daiichi-Sankyo) for 15 days. After ≥15 days wash-out, patients who had been allocated to treatment "A" were allocated to treatment "B", and viceversa (Figure 6). Measurements were done at baseline and on day 15 after each treatment, at the same time (+/-1 h) of the day. Primary end-point was variation in airway hyper-responsiveness, recorded as reduction of FEV1 with the mannitol test; secondary end-points were variations in nitric oxide expiration and in inflammatory markers in sputum.

The study is registered with ClinicalTrials.gov, number NCT01305369; EudraCT number: 2010-023945-31.

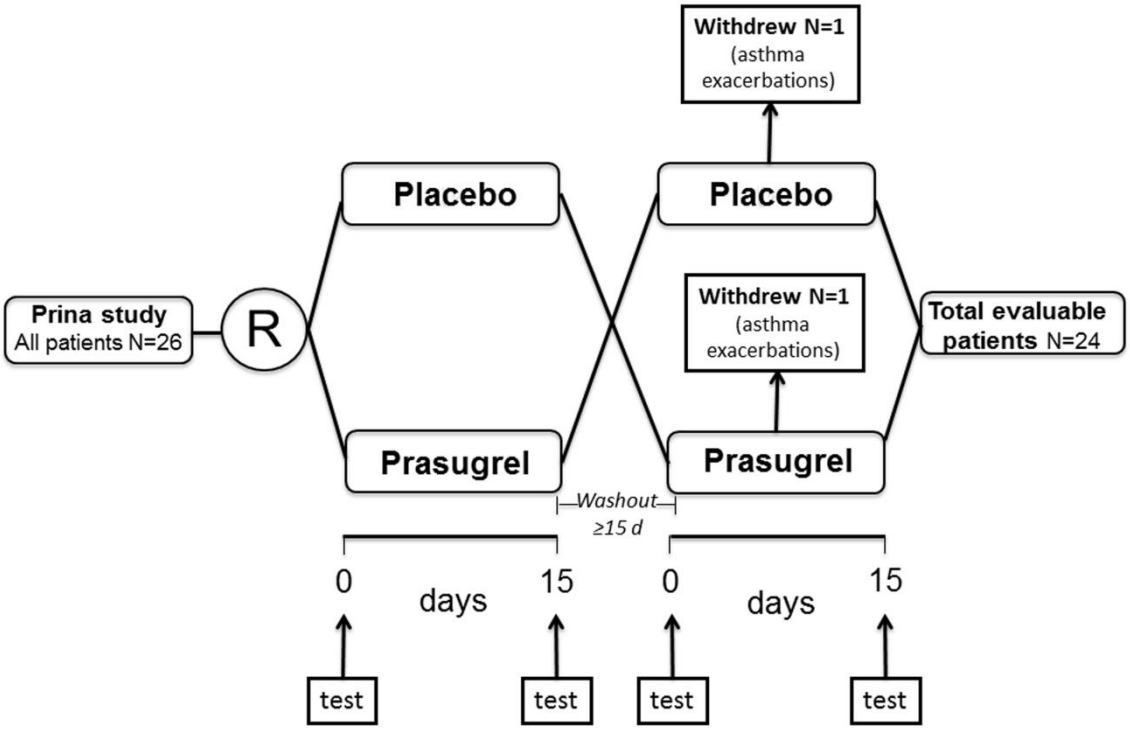


Figure 6. Schematic representation of the study design and diagram of enrolled patients. Prasugrel was given at 10 mg o.d.

3.2 Study Patients

Patients (18-74 years) with mild and stable asthma of >1 year duration, without chronic medication, except for the use of inhaled beta2-agonists on demand, or low-dose inhaled steroids, with positive mannitol bronchial challenge test were eligible. All patients were followed at the Pneumology Unit of Ospedale San Paolo, Milan, Italy. Asthma was diagnosed based on the occurrence of episodic wheezing, chest tightness and/or dyspnea and was objectively confirmed according to methacholine airway hyper-

responsiveness (PC20 FEV1 <16mg/ml) and positive skin test to common allergens, as suggested by international guidelines (www.ginasthma.com).

Exclusion criteria included pregnancy/lactation, active bleeding or conditions contraindicating treatment with antithrombotic drugs, previous TIA or stroke, age ≥75 years, body weight <60 Kg, other indications for anti-thrombotic therapy, systolic blood pressure >180 mmHg or diastolic blood pressure >110 mmHg, use of non-steroid antinflammatory drugs (NSAIDs) in the previous 7 days.

3.3 Measurements

3.3.1 Mannitol bronchial challenge test

In the mannitol bronchial challenge test, airway responsiveness was measured by bronchial provocation with dry powder mannitol (Osmohale™) using doses of mannitol up to 160 mg. The test was terminated when FEV1 decreased by at least 15% from baseline or after a cumulative mannitol dose of 635 mg. The provocative dose of mannitol causing ≥15% drop in FEV1 (PD15) was calculated by linear interpolation (6).

3.3.2 Mediators of airway inflammation in sputum

The mannitol challenge test was also used to induce sputum for airways inflammation assessment (7). Cell counts were performed in the induced sputum samples, to ensure adequacy for analysis. Thereafter, samples were centrifuged and supernatants stored for analysis of inflammatory markers (90).

3.3.3 Fractional exhaled nitric oxide (FeNO)

Fractional exhaled NO (FeNO) was measured using the portable multi-gas analyzer (NIOX MINOH, Aerocrine, Sweden), in accordance with published guidelines (8). The portable analyzer ensures a constant expiratory flow of 50±5 ml/s, with an accuracy of ±10% (91). Measurement was performed with the patient in a sitting position, after resting ventilation, without nose clip, before performing the examination of pulmonary function and airway hyper-responsiveness. Mean values of 3 independent measurements were calculated for each patient at each time point.

3.3.4 VASP phosphorylation assay

The inter-individual variability of pharmacological response to drugs inhibiting the platelet $P2Y_{12}$ receptor is high, particularly with clopidogrel (92, 93). For this reason, we elected to treat our patients with prasugrel, a third generation thienopyridine, which causes less variable inhibition of platelet $P2Y_{12}$ than clopidogrel (66). Inhibition of platelet $P2Y_{12}$ was evaluated by the Platelet Reactivity Index (PRI), using the vasodilator stimulated phosphoprotein (VASP) phosphorylation assay (Platelet VASP; Biocytex, Marseille, France) (92). The phosphorylation of vasodilator-stimulated phosphoprotein (VASP), an intraplatelet actin regulatory protein, by PGE1 is inhibited by the interaction of ADP with its $P2Y_{12}$ receptor, which is targeted by $P2Y_{12}$ antagonist. Briefly, the anticoagulated blood samples were incubated with PGE1 alone or in combination with ADP at room temperature for 10 min and fixed with paraformaldehyde. 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 the ability 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

3.4 Calculated Sample Size and Statistical Testing

In the absence of data regarding the effectiveness of prasugrel in reducing bronchial hyper-responsiveness, a sample size of 26 patients was calculated as needed to detect a decrease of 0.5 standard deviations in bronchial hyperresponsiveness with a power of 80%. All the examined variables followed a normal distribution according to the Kolmogorov-Smirnov test, thus allowing the use of parametric tests to analyse the data. Data are reported as means and 95% confidence intervals. Categorical data are reported as counts and percentages. Differences before and after each treatment were calculated by analysis of variance (ANOVA) for repeated measures. Post-hoc pairwise comparisons were performed with Least Significant Difference (LSD) test. All tests for statistical significance were two-tailed and $P<0.05$ was chosen as cut-off for statistical

significance. Analyses were carried out using SPSS for Windows 19.0 (SPSS Inc., Chicago, IL,USA).

4. RESULTS

4.1 Study patients' characteristics

We enrolled 26 patients (10 women, aged 43 years; 95%CI 39-48) in 10 months. Two women withdrew from the study due to asthma exacerbations during the second treatment (1 with placebo, 1 with prasugrel). Therefore, a complete set of data was available for 24 patients (Figure 6), whose characteristics are summarized in Table 1.

Table 1. Characteristics of the patients enrolled in the study

Characteristics	N=24
Sex, female (%)	8 (33)
Age, years; mean (95%CI)	43 (38-48)
Body Mass Index (BMI), mean (95%CI)	25.4 (24-27)
Basal FEV1 (%), mean (95%CI)	90 (82-98)
Duration of asthma in years, mean (95%CI)	18 (13-24)

CI= confidence interval

4.2 Primary Efficacy Measure

The effects of prasugrel and placebo on the changes in airway hyper-responsiveness, recorded as reduction of FEV1 (forced expiratory volume in 1-s) of at least 15% with the mannitol test induction, were compared by calculation of the difference between the increase in the average mannitol dosage from baseline to treatment. Differences in the provocative dose of mannitol causing a 15% drop in FEV1

(PD15) before and after placebo or prasugrel were statistically significant ($p<0.001$, ANOVA for repeated measures). PD15 tended to increase from 142 mg (82-202) to 187 mg (112-262) after prasugrel ($p=0.09$), while it remained virtually unchanged after placebo (136 mg; 76-196 and 144 mg; 84-204, $p=0.65$) (Figure 7). Differences between PD15 values after prasugrel and those before and after placebo were borderline statistically significant ($p=0.050$ and $p=0.052$). Delta-PD15 ($PD15_{after\ treatment} - PD15_{baseline}$) was 45.2 mg (-5-95) after prasugrel and 7.3 mg (-27-41) after placebo ($p=0.16$).

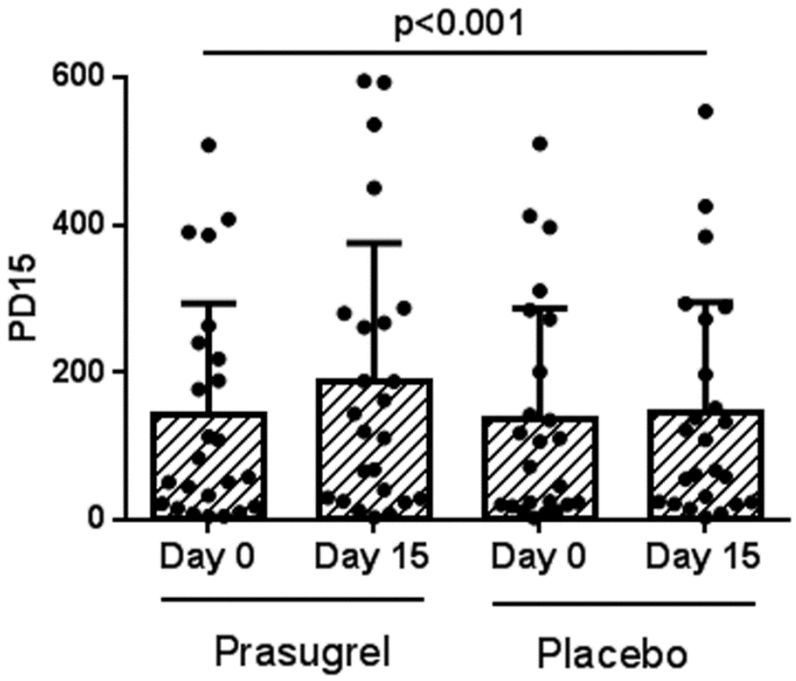


Figure 7. Bar graph and individual dots of the PD15 (dose of mannitol causing a decrease in FEV1 of 15%) in patients with allergic asthma before and 15 days after treatment with placebo or prasugrel (10 mg o.d.). P value: ANOVA for repeated measures. Post-hoc analysis by Least Significant Difference test: before prasugrel v*s*. after prasugrel, p= *0.09*; before placebo *vs*. after placebo, *p= 0.65*; after prasugrel *vs*.

before placebo, *p= 0.050*; after prasugrel *vs*. after placebo, *p=0.052*. The delta-PD15 ($PD15_{after\ treatment} - PD15_{baseline}$) values tended to be higher after prasugrel (45.2 mg; 95%CI -5-95) than after placebo (7.3 mg; 95%CI -27-41) (p=0.16).

4.3 Secondary Efficacy Measure

4.3.1 Changes in measurement of nitric oxide expiration, as a surrogate marker of airway lung inflammation

Fractional exhaled nitric oxide FeNO, a surrogate marker of eosinophilic airway lung inflammation, did not change significantly after treatment with prasugrel (from 23;17-29 to 27;19-35 ppb; P=0.160) or placebo (from 25;18-32 to 28; 21-35 ppb; P=0.33).

4.3.2 Changes in measurement of mediators of airway inflammation in sputum

We could not perform the programmed measurement of markers of inflammation in induced sputum, because a complete set of 4 samples was obtained in one patient only, due to the inadequacy of either one or two samples of the remaining patients, based on the presence of insufficient numbers of cells at optical microscopy.

4.3.3 Platelet VASP phosphorylation

The Platelet Reactivity Index (PRI), measured by the VASP phosphorylation assay, decreased from a mean baseline value of 80% (77-83) to 23% (17-29) following prasugrel treatment (p<0.001), while it did not change following placebo treatment (78%; 74-82 vs 78%; 75-81 , P=0.88) (Figure 8). Three patients displayed PRI>50%

post-prasugrel, which is the cut-off value identifying poor responders to $P2Y_{12}$-antagonists (92).

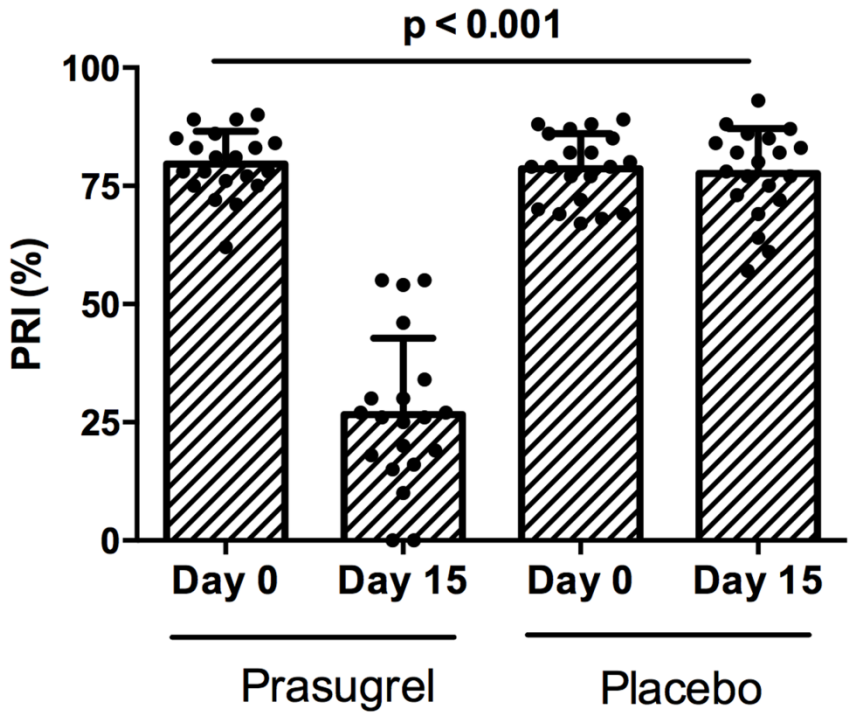


Figure 8. Bar graphs and individual dots of the effects of treatment with placebo or prasugrel (10 mg o.d.) of patients with chronic bronchial asthma for 15 days on platelet $P2Y_{12}$-dependent reactivity (platelet reactivity index, PRI), measured by the Vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay. P value: ANOVA for repeated measures. Post-hoc analysis by Least Significant Difference test: before prasugrel v*s*. after prasugrel, p< *0.001*; before placebo *vs*. after placebo, *p= 0.88*; after prasugrel *vs*. before placebo, *p<0.001*; after prasugrel *vs*. after placebo, *p<0.001*

5. DISCUSSION AND CONCLUSIONS

The main finding of this study is that in patients with mild chronic asthma, treatment with prasugrel tended to attenuate airway hyper-responsiveness, measured by the mannitol challenge test. The provocative dose of mannitol causing a 15% drop (PD15) in FEV1 tended to increase following treatment with prasugrel, although the difference with baseline values did not quite reach statistical significance (p=0.09). The design of this study virtually excludes the possibility that this difference is caused by variability among individuals. An increase in PD15 reflects a reduction in airway inflammation, because the mannitol test, like other indirect airway challenges, more closely reflects active airway inflammation than direct challenges, such as the metacholine test (94). The greater specificity of the mannitol test for detecting changes in airway hyper-responsiveness in asthma patients is likely explained by the fact that it mimics the normal pathophysiology of bronchial asthma, causing the release of mediators of bronchoconstriction (6). Although the increased PD15 observed in our patients after prasugrel administration did not reach statistical significance, likely due to the small number of evaluable subjects and the wide inter-individual variability of baseline values, it does suggest that prasugrel may attenuate airway hyper-responsiveness in patients with allergic bronchial asthma, likely reducing active airway inflammation.

Although $P2Y_{12}$ is less promiscuous than $P2Y_1$, which is the second platelet receptor for ADP, it is also expressed by cells other than the platelets (95). As a consequence, it remains to be established whether prasugrel affects bronchial hyper-responsiveness through inhibition of $P2Y_{12}$ on platelets or on other cells. The former hypothesis is supported by the results of experimental studies that demonstrated the important role of platelet $P2Y_{12}$ in the recruitment of inflammatory cells in lungs of sensitized mice challenged with cysteinyl-LT (88, 89).

The mechanism by which the platelet $P2Y_{12}$ contributes to the effects of cysteinyl-LT is uncertain. Because LTE4 shows negligible activity at CysLT1R and CysLT2R, its biological effects are likely mediated by a third, elusive receptor, which was tentatively identified with $P2Y_{12}$, based on computer modelling and the demonstration that LTE4 signals through $P2Y_{12}$ in transfected cells

(96). However, more recently GPR99 was potentially identified as the elusive receptor for LTE4 (86). Moreover, studies that demonstrated the important role played by platelet P2Y12 in LTE4- or LTC4-induced enhanced recruitment of inflammatory cells in the lungs of sensitized mice failed to show that CysLT interact directly with the platelet $P2Y_{12}$, which might therefore play an indirect, albeit important role in the process (83, 88, 89).

None of the patients enrolled in our study experienced pathological bleeding of any severity, in line with the evidence that the incidence of bleeding complications in patients treated with $P2Y_{12}$ inhibitors in monotherapy is low (97).

In our study, FeNO, a surrogate marker of eosinophilic airway lung inflammation, did not change significantly after treatment with prasugrel or placebo. Therefore, FeNO may be a less sensitive marker than the mannitol inhalation test, which is considered a very sensitive means to detect bronchial hyper-responsiveness associated with airway lung inflammation.

Unfortunately, we could not perform the programmed measurement of markers of inflammation in induced sputum, due to the difficulty to obtain adequate samples with a sufficient numbers of cells at optical microscopy.

Due to the nature of our proof-of-concept study, we enrolled a small number of patients with allergic asthma and did not consider clinical end-points. Patients with severe, chronic allergic asthma on treatment with systemic steroids have higher levels of LTE4, compared with patients with mild/moderate asthma (98). Although patients with severe asthma might be more sensitive to therapeutic effect of $P2Y_{12}$ antagonists, they were excluded from our study because concomitant treatment with steroids might influence the study end points.

Evidence of the involvement of platelets in inflammatory processes in general and of platelet $P2Y_{12}$ in the pathogenesis of allergic asthma stemmed from studies in animal models.The demonstration that $P2Y_{12}$ variants are associated with lung function in a large family-based asthma cohort provided the first human evidence supporting a role for $P2Y_{12}$ in this disorder (99). To the

best of our knowledge, our randomized, double-blind, placebo-controlled, cross-over study is the first to provide proof-of-concept that pharmacological inhibition of the platelet $P2Y_{12}$ receptors may be useful in the treatment of patients with asthma. This hypothesis should be tested in randomized trials with clinical end-points.

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