Coagulation Disorders Unit, Division of Hematology
Department of Medicine, Helsinki University Central Hospital, Finland

Wihuri Research Institute, Helsinki, Finland

and

Department of Medicine, Institute of Clinical Medicine
Faculty of Medicine, University of Helsinki, Finland

INDIVIDUAL VARIATION IN *IN VITRO* EFFICACY OF ANTIPLATELET MEDICATION
—
ASPIRIN AND CLOPIDOGREL

Aino Lepäntalo

Academic dissertation

To be presented with the permission of the Faculty of Medicine,
University of Helsinki, for public examination in the auditorium 3, Meilahti Hospital,
Haartmaninkatu 4, on January 19th, 2007, at 12 noon.

Helsinki 2007

SUPERVISOR

Docent Riitta Lassila
Coagulation Disorders Unit
Division of Hematology
Department of Medicine
Helsinki University Central Hospital
Helsinki, Finland

REVIEWERS

Professor Heikki Vapaatalo
Department of Pharmacology
Institute of Biomedicine
University of Helsinki
Helsinki, Finland

Professor Christian Gachet
Institut National de la Sante et de la Recherche Medicale
Etablissement Francais du Sang-Alsace
Strasbourg, France

OFFICIAL OPPONENT

Professor Stefan Lethagen
Copenhagen Hemophilia Center
Rigshospitalet
Copenhagen, Denmark

ISBN 978-952-92-1505-8 (paperback)
ISBN 978-952-10-3657-6 (PDF)
http://ethesis.helsinki.fi
Tampereen Yliopistopaino Oy
Tampere 2007

To My Father

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals. The original publications are reproduced with the permission of copyright holders.

I Lepäntalo A, Beer JH, Siljander P, Syrjälä M, Lassila R. Variability in platelet responses to collagen – comparison between whole blood perfusions, traditional platelet function tests and PFA-100. *Thromb Res* 2001;103:123-133

II Lepäntalo A, Mikkelsson J, Reséndiz JC, Viiri L, Backman JT, Kankuri E, Karhunen PJ, Lassila R. Polymorphisms of COX-1 and GP VI associate with the antiplatelet effect of aspirin in coronary artery disease patients. *Thromb Haemost* 2006;95:253-259

III Lepäntalo A, Virtanen KS, Heikkilä J, Wartiovaara U, Lassila R. Limited early antiplatelet effect of 300 mg clopidogrel in patients with aspirin therapy undergoing percutaneous coronary interventions. *Eur Heart J* 2004;25:476-483

IV Lepäntalo A, Virtanen KS, Reséndiz JC, Mikkelsson J, Viiri L, Karhunen P, Lassila R. Limited antiplatelet effect of clopidogrel in patients with aspirin therapy undergoing percutaneous coronary interventions – Inhibition of $P2Y_{12}$ receptor *in vivo* and *in vitro*. Submitted.

MAJOR ABBREVIATIONS

AA	arachidonic acid
ACS	acute coronary syndromes
ADP	adenosine-diphosphate,
ARMX	$P2Y_{12}$-receptor antagonist AR-C69931MX
ATC	Antithrombotic Trialists´Collaboration
ATP	adenosine-triphosphate
A3P5P	$P2Y_1$-receptor antagonist adenosine 3,5 diphosphate
BMI	body mass index
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CADP	PFA-100® cartridge with collagen and ADP
cAMP	cyclic adenosine- 3´,5´-monophosphate
CEPI	PFA-100® cartridge with collagen and epinephrine
COX	cyclo-oxygense
CYP 450	cytochrome P450
GI	gastrointestinal
GP	glycoprotein
MI	myocardial infarction
NSAID	non-steroidal anti-inflammatory drug
NTPDase-1	nucleoside triphosphate diphosphohydrolase/endothelial ecto-adenosine phosphatase (ADPase)/CD39
PAD	peripheral arterial disease
PAR	protease-activated receptor
PCI	percutaneous coronary intervention
PG	prostaglandin
PFA-100®	Platelet Function Analyzer-100®
PPACK	D-phenylalanyl-1-prolyl-1 arginine chloromethyl ketone
PRP	platelet rich plasma
SNP	single nucleotide polymorphism
TIA	transient ischemic attack
Tx	thromboxane
VASP	vasodilator stimulated phosphoprotein
vWf	von Willebrand factor

The term aspirin is used in this thesis instead of acetylsalicylic acid (ASA).
The term epinephrine is used in this thesis instead of adrenaline.

ABSTRACT

Antiplatelet medication is known to decrease adverse effects in patients with atherothrombotic disease. However, despite ongoing antiplatelet medication considerable number of patients suffer from atherothrombotic events.

The aims of the study were 1) to evaluate the individual variability in platelet functions and compare the usability of different methods in detecting it, 2) to assess variability in efficacy of antiplatelet medication with aspirin (acetylsalicylic acid) or the combination of aspirin and clopidogrel and 3) to investigate the main genetic and clinical variables as well as potential underlying mechanisms of variability in efficacy of antiplatelet medication.

In comparisons of different platelet function tests in 19 healthy individuals PFA-100® correlated with traditional methods of measuring platelet function and was thus considered appropriate for testing individual variability in platelet activity.

Efficacy of ongoing 100mg aspirin daily was studied in 101 patients with coronary artery disease (CAD). Aspirin response was measured with arachidonic acid (AA)-induced platelet aggregation, which reflects cyclo-oxygenase (COX)-1 dependent thromboxane (Tx) A_2 formation, and PFA-100®, which evaluates platelet activation under high shear stress in the presence of collagen and epinephrine. Five percent of patients failed to show inhibition of AA-aggregation and 21% of patients had normal PFA-100® results despite aspirin and were thus considered non-responders to aspirin.

Interestingly, the two methods of assessing aspirin efficacy, platelet aggregation and PFA-100®, detected different populations as being aspirin non-responders. It could be postulated that PFA-100® actually measures enhanced platelet function, which is not directly associated with TxA_2 inhibition exerted by aspirin.

Clopidogrel efficacy was assessed in 50 patients who received a 300mg loading dose of clopidogrel 2.5 h prior to percutaneous coronary intervention (PCI) and in 51 patients who were given a loading dose of 300mg combined with a five day treatment of 75mg clopidogrel daily mimicking ongoing treatment. Clopidogrel response was assessed with ADP-induced aggregations, due to its mechanism of action as an inhibitor of ADP-induced activation. When patients received only a loading dose of clopidogrel prior to PCI, 40% did not gain measurable inhibition of their ADP-induced platelet activity (inhibition of 10% or less). Prolongation of treatment so that all patients had reached a plateau of inhibition exerted by clopidogrel, decreased the incidence of non-responders to 20%.

Polymorphisms of COX-1 and GP VI, as well as diabetes and female gender, were associated with decreased *in vitro* aspirin efficacy. Diabetes also impaired the *in vitro* efficacy of short-term clopidogrel. Decreased response to clopidogrel was associated with limited inhibition by ARMX, an antagonist of $P2Y_{12}$-receptor, suggesting the reason for clopidogrel resistance to be receptor-dependent.

Conclusions: Considerable numbers of CAD patients were non-responders either to aspirin, clopidogrel or both. In the future, platelet function tests may be helpful to individually select effective and safe antiplatelet medication for these patients.

INTRODUCTION

Atherothrombotic disease is the most common cause of mortality in developed countries. Atherosclerosis in connected with obliterative lesions ranging from minor stenosis to total occlusions. These lesions cause a variety of rheological changes, leading to increased shear forces and thus enhanced risk of thrombus formation. Acute thrombosis occurs when plaque rupture causes platelet activation and formation of a platelet plug at the injury site. Atherosclerotic changes in arteries are universal, but the major clinical manifestations of the disease are symptoms originating either from occluded coronary arteries, cerebral or carotid arteries, peripheral arteries or combination of these depending on where they form. Thus, acute formation of thrombi can respectively cause heart infarction, stroke or critical limb ischemia. Platelets play a critical role in thrombus formation especially in the arterial environment. Occurrences of acute events are also influenced by lipids, inflammation and vasoactivity, which have all been linked to platelet activation. Platelets have also been shown to take part in the development of atherosclerosis and postulated to regenerate stenosis of arteries which have been treated with angioplasty or stenting (see Le Breton et al 1996, see Ruggeri 2002).

Several antiplatelet drugs have been developed to inhibit platelet activity in acute thrombotic situations as well as to prevent adverse events. Antiplatelet therapy is one of the most effective therapies for treatment of atherothrombotic disease (see Patrono et al 2004). Considering the recently proved role of platelets in atherosclerosis the long-term use of antiplatelet drugs could also be postulated to slow down progression of the disease.

Aspirin was discovered over 100 years ago for analgesia. Later on it was shown to inhibit platelet activity and is the still most commonly used antiplatelet drug. It has been shown to decrease the risk of atherothrombotic events by 25% (see Antithrombotic Trialists´ Collaboration (ATC) (2002). Recently, an ADP-receptor inhibitor, clopidogrel, was developed. It is at least as effective as aspirin and their combination has proven beneficial in acute thrombotic states (CAPRIE Steering Committee 1996, CURE investigators 2001, Steinhubl et al 2002). In addition, clopidogrel has been shown to have a better efficacy and safety profile than previous alternatives to aspirin (CAPRIE 1996).

Despite antiplatelet medication considerable numbers of patients suffer from atherothrombotic events (ATC 2002). Of the patients who have undergone coronary artery angioplasty 1% develop stent thrombosis (Cutlip et al 2001). Due to this sustained incidence of thrombosis the phenomenon of individually variable responses to antiplatelet medication has become of interest lately. As knowledge of platelet function and of factors affecting it has increased, as well as the availability and user-friendliness of platelet function tests, the subject has become salient. Different environmental and genetic factors are known to affect an individual's platelet activity and are beginning to be better understood, at the same time new antiplatelet drugs have been developed, thus offering actual alternatives to aspirin.

In conclusion, there are patients who do not seem to achieve adequate protection against atherothrombotic events by the present antiplatelet medication and increased knowledge of this phenomenon would help us to detect patients at increased risk. If these patients could be detected by platelet function tests or genetic testing, enhanced or alternative antiplatelet medication could be prescribed. At the same time a population of patients suffer from bleeding events due to the efficacy of antiplatelet medication, which is excessive for their individual needs. Thus, tailored antiplatelet medication would benefit individuals at both ends of the range of variation when it comes to the efficacy of antiplatelet medication.

REVIEW OF THE LITERATURE

1 Platelet structure and functions

1.1 Platelet structure

Platelets are small, approximately 2µm in diameter, anucleated cells that derive from megacaryocyte cytoplasm. Their development is controlled mainly by thrombopoietin, but also many cytokines and hormones (see Kauhansky 1995). Platelets circulate for approximately 10 days, before removal by macrophages (see George 2000). Numbers of platelets in blood vary between 150-350 10^9/L. The main functions of platelets include normal hemostasis as well as vessel constriction and repair. Platelets also participate in pathophysiological processes such as thrombosis, bleeding, inflammation, tumor growth and promotion of atherosclerosis (see Ruggeri 2002) (Fig 1).

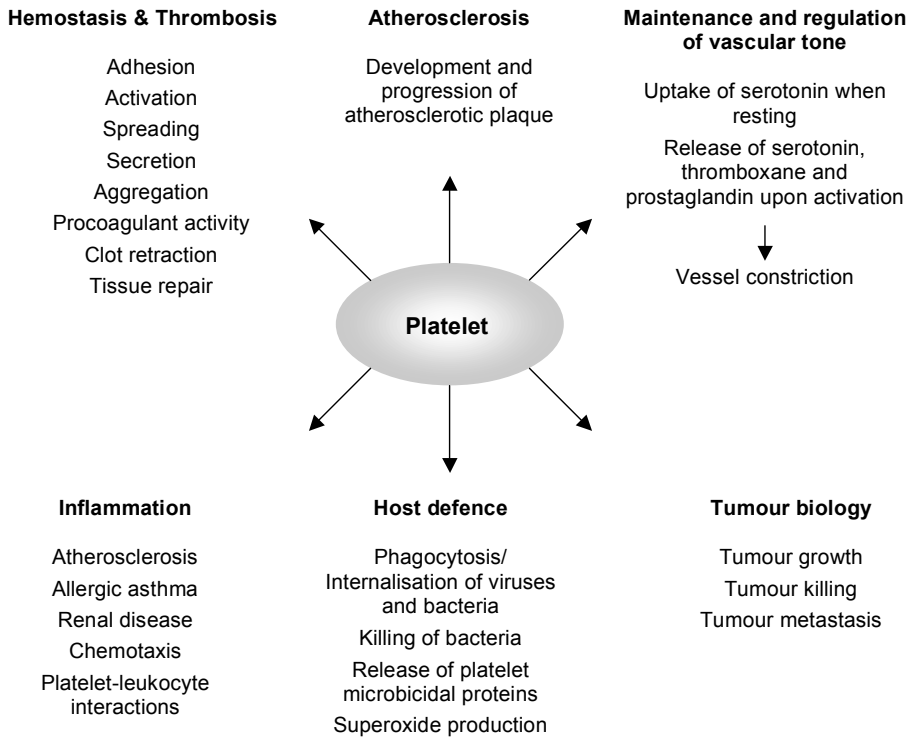


Figure 1. *Different functions of platelets. Platelets are involved in many pathophysiological processes in addition to hemostasis and thrombosis. Modified from Harrison (2005).*

1.2 Platelet activation

As platelets are activated they change from their normal disc shape to a sphere with long dendritic extensions (Fig 2). The shape change is brought about by actin and myosin in platelet cytoplasm (see George 2000). During the shape change secretory granules are organized into the center of the platelet. Platelets have three types of secretory granules; dense granules, α-granules and lysosomes (Table 1). The contents of secretory granules are either produced by platelets and megakaryocytes or acquired from plasma via endocytosis and pinocytosis facilitated by the canalicular system. Secretory granules release their contents into plasma during platelet activation, which enhances further activation. The granule contents also have procoagulant activities (see Rendy & Brohard-Bohn 2001).

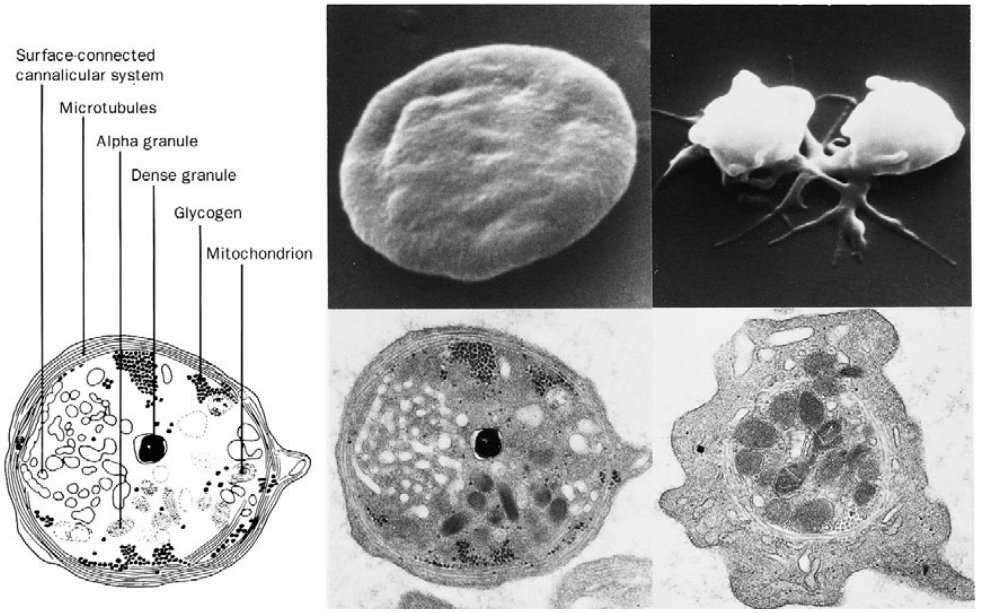


Figure 2. *Top photographs are scanning electron micrographs demonstrating the disc shape of normal circulating platelets and the spherical form of activated platelets with pseudopodia (x 10 000-20 000). Lower transmission electron micrographs of resting and activated platelets, respectively (x 21 000-30 000). In activated platelets the normal structures presented in the drawing of a normal platelet change as pseudopodia form and a microtubular ring constricts centralizing secretory granules. Electron micrographs are courtesy of JG White and M Krumwiede, University of Minnesota. Reproduced from George (1998) with permission.*

Table 1. *The major granule contents and membrane components of platelets.*

Alpha-granules – Adhesion and repair

Major granule contents

- Platelet-specific proteins
  -platelet factor 4,
  β-thromboglobulin
- Adhesive glycoproteins
  - fibrinogen, vWf, thrombospondin, fibronectin, vitronectin
- Hemostasis factors, co-factors and inhibitors
  - fibrinogen, factors V, VII, XI, XIII, protein S, plasminogen, plasminogen activator inhibitor 1
- Mitogenic and angiogenic factors
  - platelet derived GF, vascular endothelial GF, transforming GF-β
- Other proteins
  - albumin, immunoglobulins

Granule membrane components

- P-selectin
- Receptors*: GP IIb/IIIa, GP Ib/IX, GP VI, PECAM

Dense granules – Pro-aggregation factors

Major granule contents

- ADP, ATP
- serotonin (5-HT), histamine
- Ca^{2+}, Mg^{2+}

Granule membrane components

- P-selectin
- receptors*: GP IIb/IIIa, GP Ib

Lysosomes – Clearing factors

- acid proteases, acid hydrolases

Cytoplasm

- factor XIII, tissue factor pathway inhibitor

Modified from Rendu & Brohard-Bohn (2001). GF=growth factor, * Receptors are also expressed at the platelet plasma membrane

1.3 Platelet adhesion and aggregation

Platelet activation is induced at the site of vessel wall injury, which is represented by rupture of an atherosclerotic plaque. As the vessel wall is injured subendothelial collagen and other platelet activating factors such as von Willebrand factor (vWf) are exposed (Fig 3). Initially plasma vWf binds to exposed collagen. The first contact between platelet receptors and matrix components depends to a large extent on the shear stress at the site of injury. In conditions of low shear stress matrix proteins cause activation at the injury site. In conditions of high shear stress, as in arteries, activation and adhesion depend largely on glycoprotein (GP) Ib-V-IX complex, which interacts with immobilized vWf. This interaction causes initial tethering of circulating platelets to the vessel wall. Thus, platelets slow down and roll over a vWf-coated surface. The rolling ends with firm attachment through GP Ia/IIa, which has become available via activation of rolling platelets. Firm attachment mediated by GP Ia/IIa also allows low-affinity GP VI to interact with collagen.

Interactions of platelet GP VI with collagen induce further collagen-dependent activation of platelets. However, recent studies have suggested that the differential roles of GP Ia/IIa and GP VI are not this simple, but are in fact modulated by changes at the extracellular matrix of the vessel wall induced by specific metalloproteinases, and that both of these receptors participate in adhesion and aggregation alike (see Ruggeri 2002, see Farndale et al 2004) (Fig 4).

The initial adhesion is followed by recruitment of additional platelets into the growing platelet plug. Platelets are activated by factors at the injury site, but more importantly further activation is mediated by agonists released from the secretory granules of previously activated platelets. Adenosine-diphosphate (ADP) and thromboxane (Tx) A_2 are crucial secondary mediators of platelet activation. As platelets are activated GP IIb/IIIa-receptors undergo conformational changes to become active. Activated GP IIb/IIIa mediates platelet-platelet interaction, aggregation, by several ligands of which fibrinogen is most abundant (Bennett & Vilaire 1979, see Michelson 2003).

After a platelet plug has been formed, it is then stabilized to prevent premature disaggregation. It has been suggested that outside-in signalling through cell surface integrins and tyrosine kinases of receptors have central roles in this phase of thrombus formation. Platelets also participate in localization, amplification and maintenance of the coagulant response at the injury site (Ilveskero et al 2001, see Michelson 2003).

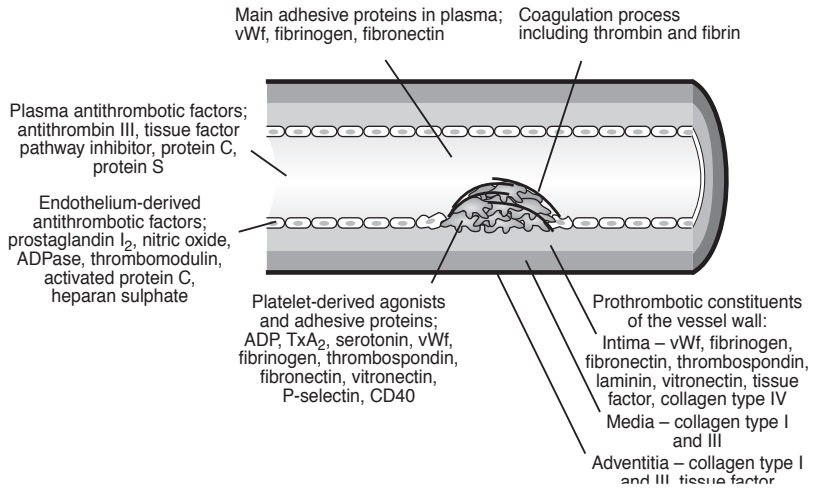


Figure 3. *Antithrombotic and prothrombotic molecules synthesized by endothelial cells and platelets. Antithrombotic factors circulate in plasma and are excreted from endothelium and platelets to limit the thrombus to the site of injury. Prothrombotic factors are excreted from endothelial cells and activated platelets, platelet activation mediates also coagulation process and prothrombotic constituents revealed at the injured vessel wall initiate thrombus formation. Modified from Ruggeri (2002).*

Platelets also regulate their own activation at the site of a platelet plug to prevent uncontrolled expansion of the thrombi. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) is an inhibitory receptor which mediates the inhibitory pathway. In addition, antithrombotic factors circulate in plasma and are secreted by platelets (see Ruggeri 2002) (Fig 3). Nitric oxide, prostacyclin and endothelial ecto-nucleotidase (NTPDase) are believed to be the most important endothelial regulators of the platelet activity. Nitric oxide and prostacyclin cause platelet inhibition and vasodilatation and NTPDase neutralizes the prothrombotic releseate of platelets by metabolism of ADP (see Tan et al 2004, Marcus et al 2005.

1.4 Coagulation

Coagulation of blood has classically been described as a cascade dependent on adequate levels of coagulation proteins. However, recent advances in research have suggested a new model of coagulation as a cell-regulated overlapping process (Fig 5). Platelets support procoagulant reactions and vascular endothelial cells maintain anticoagulant properties of the vasculature. In healthy vessels the tissue factor pathway inhibitor (TFPI) inhibits coagulation factors. The complex formed by thrombin binding to thrombomodulin and protein C/protein S as well as an another complex of endothelial surface heparinoids and antithrombin act as anticoagulants at the site of injury, preventing excessive formation of thrombi (see Monroe & Hoffman 2005).

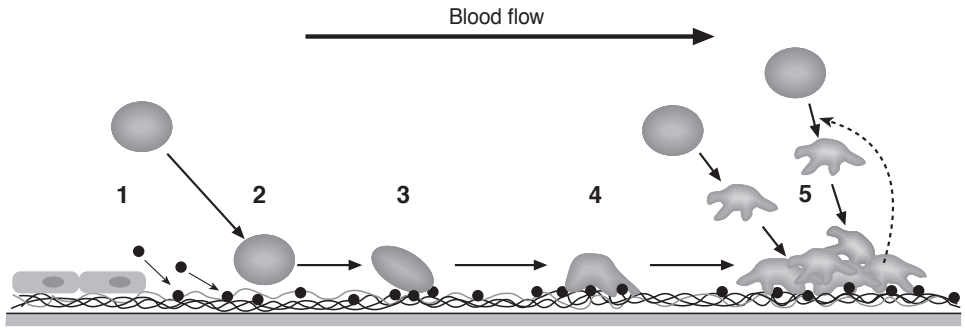


Figure 4. *A traditional model of platelet activation and adhesion to immobilized collagen.*
1) Binding of vWf molecules onto the collagen surface
2) Platelets moving on the surface – GPIb-V-IX complex and vWf interact
3) Tight binding of platelets on collagen by GP Ia/IIa
4) Collagen-induced activation of platelets by GP VI
5) Formation of a platelet aggregate – mediated by activated GP IIb/IIIa and its ligands vWf and fibrinogen. Activated platelets release prothrombotic agents which further activate other platelets. Reaction steps 3 and 4 occur almost simultaneously and recent studies suggest that both GP Ia/IIa and GP VI participate in adhesion and collagen-induced activation alike. Modified from Jung & Moroi (2000).

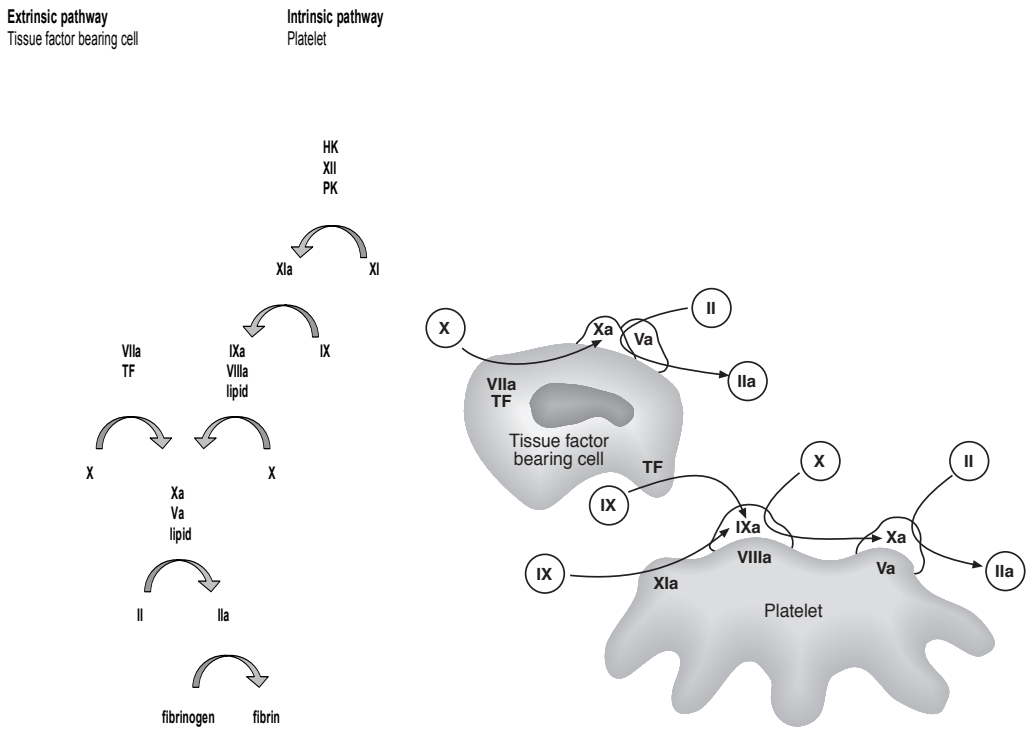


Figure 5A) *Classical view of the extrinsic and intrinsic pathways of coagulation. HK=high-molecular weight kininogen, PK=prekallikrein, II=prothrombin, IIa= thrombin.*
B) *Coagulation as a process controlled by cells. On the surface of tissue factor-bearing cells factor VIIa bound to tissue factor (TF) activates factor X. Activated factor Xa binds to factor Va and this complex activates factor II to IIa. Tissue factor on the cell surface also activates IX. On the surface of the platelet factor IXa is activated by factor XIa. Factor is IXa activated either at the surface of platelet or tissue factor bearing cell binds to surface VIIIa. Complex of IXa and VIIIa activates factor X, which binds to factor Va forming a complex. This complex activates factor II to IIa. Modified from Monroe & Hoffman (2005).*

2 Platelet receptors

2.1 Collagen receptors

Several glycoprotein receptors are present at the platelet surface. A number of these have been proposed as collagen receptors, but at present GP Ia/IIa and GP VI are believed to be the main ones responsible for collagen induced platelet activation. Fibrillar collagen of types I and III are the major constituents of the blood vessel wall, although type IV is also present in basement membranes (see Kehler 1995). These collagen types are also the most able to activate platelets and to cause adhesion and aggregation (see Sixma et al 1997).

The GP Ia/IIa receptor, integrin $\alpha_2\beta_1$, causes initial platelet adhesion to the subendothelial matrix. It requires activation by thrombin-, collagen- or ADP- pathways to become operational. It has been suggested that activation via thrombin- and ADP-pathway may result in two different conformations of activated GP Ia/IIa with different ligand activity (Jung & Moroi 2000). While the activation with ADP has been suggested independent of Ca^{2+} the activation by collagen, especially collagen monomers, is dependent on physiological concentrations of Mg^{2+} and Ca^{2+} (Siljander & Lassila 1999, Jung & Moroi 2001). The role of GP Ia/IIa in platelet adhesion on collagen is emphasized in environments of high shear (Alberio & Dale 1999). Monomeric collagen provides an important tool for studying the role of GP Ia/IIa as at physiological cation concentrations it is responsible for platelet adhesion (Siljander & Lassila 1999). In comparison, the second collagen receptor GP VI is reported to adhere only to fibrillar collagen (see Jung & Moroi 1998). Indeed, modulation of the collagens revealed at an injury site is believed to mediate variable roles of GP Ia/IIa and GP VI in adhesion to collagen. Variation in GP Ia/IIa activity has been shown to have clinical importance, and has been associated with the C807T polymorphism of the GP Ia/IIa gene (Kunicki et al 1993, Kritzik et al 1998). In addition, the C807T polymorphism has been associated with increased risk for atherothrombotic events (Carlsson et al 1999, Moshfegh et al 1999). However, regarding the role of these polymorphisms as predictors of atherothrombotic disease the multifactorial nature of the disease needs to be taken into account.

GP VI participates in initial platelet adhesion and activation in circulating blood in addition to the GP Ib-IX-V complex (see Andrews & Berndt 2004). In fact, it seems to be topographically associated with GP Ib-IX-V (see Farndale et al 2004). Patients deficient in GP VI lack the ability to form thrombi on a collagen surface under flow conditions, but clinically present with only mild bleeding tendencies (see Moroi & Jung 2004). However, the T13254C polymorphism of GP VI has been associated with myocardial infarction (Croft et al 2001). The differential roles of GP Ia/IIa and GP VI in initial platelet adhesion and aggregation related to collagen have not yet been indisputably determined.

2.2 Adhesion receptors

In addition to collagen receptors, there are other adhesion receptors such as GP Ib-V-IX complex, GP IIb/IIIa and receptors for fibronectin, vitronectin and thrombospondin. The GP Ib-V-IX complex causes tethering of platelets to perivascular vWf. This enables platelets to slow down at the point of vascular injury and to interact with collagen. GP Ib-V-IX is a complex of glycoproteins and the N-terminal globular domain of GP Ibα is the major ligand-binding region. Patients lacking GP Ib or GP IX have Bernard-Soulier syndrome which causes bleeding diasthesis (see Andrews & Berndt 2004).

GP IIb/IIIa, integrin-$\alpha_{IIb}\beta_3$, is the most abundant adhesion receptor. It requires a conformational change caused by platelet activation through adhesion receptors (GP Ib, GP VI, GP Ia/IIa), thrombin (protease activated receptors 1 and 4) or ADP ($P2Y_{12}$, $P2Y_1$, $P2X_1$) receptors to become active. GP IIb/IIIa binds mainly fibrinogen, but also vWf, fibronectin, vitronectin and thrombospondin. As GP IIb/IIIa binds to vWf, especially in flow conditions, it offers a second mechanism of platelet adhesion to the vessel wall in addition to GP Ib-V-IX. The receptor mediates aggregation by binding fibrin and vWf. GP IIb/IIIa function is Ca^{2+}-dependent. Patients with hereditary Glanzmann´s thrombastenia have decreased or functionally abnormal GP IIb/IIIa which causes bleeding symptoms (see Andrews & Berndt 2004).

2.3 Purinergic receptors

Purinergic receptors play an important role in thrombus formation under conditions of high shear stress. These receptors mediate activation by platelet derived ADP excreted from secretory granules of activated platelets. They constitute an autocrine mechanism for promotion of platelet aggregation (see Gachet 2005).

The purinergic-receptor family consists of P2X ligand gated channels ($P2X_{1-7}$) and G-protein-coupled P2Y receptors ($P2Y_{1,2,3,4,11,12,13,14}$) of which 7 and 8 subtypes, respectively, have been described to date (see Khakh et al 2003, see Gachet 2005). Platelets express mainly $P2Y_1$, $P2X_1$ and $P2Y_{12}$. In addition, some subtypes ($P2Y_{2,4,11,13}$, $P2X_{4,7}$) have been detected in platelets at very low levels and do not seem to have clinical significance (Wang et al 2003).

G_q-coupled $P2Y_1$ regulates Ca^{2+}-dependent signalling events, initiating platelet shape change and activating GP IIb/IIIa dependent platelet aggregation (Fig 6). These receptors initiate aggregation, reinforce the actions of the $P2Y_{12}$-receptor in response to ADP and participate in platelet aggregation induced by collagen and other platelet agonists (see Gachet 2005).
The $P2Y_{12}$ receptor is activated by ADP causing completion of aggregation of platelets in synergistic action with $P2Y_1$, ADP-dependent amplification of aggregation induced by other agonists (TxA_2, thrombin, collagen chemokines or immune complexes), potentiation of the release reaction and stabilization of thrombi (see Gachet 2005). $P2Y_{12}$-receptor is coupled to G_i causing inhibition of adenylate cyclase and thus also cyclic adenosine 3´,5´-monophosphate (cAMP) formation (see Gachet 2001) (Fig 6). Activation of the $P2Y_{12}$-receptor causes inhibition of phosphorylation of the vasodilator stimulated phosphoprotein (VASP), mediated by cAMP-dependent protein kinase (PKA). Thus $P2Y_{12}$-receptor activation causes a decrease in the negative regulatory effect of GP IIb/IIIa normally caused by dephosphorylation of the VASP. Activation of $P2Y_{12}$ also causes stimulation of phosphatidyl inositol-3 kinase (PI-3K), which activates small GTPase RapIb and PKB/Akt, all three of which participate in platelet aggregation (see Gachet 2005).

Activation of both $P2Y_{12}$ and $P2Y_1$ receptors at the same time is a requirement for normal ADP-induced aggregation, and antagonism of either one causes a dramatic inhibition of platelet aggregation (Jin & Kunalipuli 1998).

The $P2X_1$-receptor is a ligand gated cation channel activated by adenosine triphosphate (ATP) (Fig 6). It causes rapid Ca^{2+} entry associated with platelet shape change and centralization of secretory granules. $P2X_1$ is unable to cause platelet aggregation by itself, but enhances collagen- and shear-induced aggregation. Indeed, flow conditions have been found to be critical for the full efficacy of the receptor (see Gachet 2005).

2.4 Thrombin receptors

Thrombin is a potent platelet activator which catalyzes cleavage of amino-terminal domains of G-protein coupled protease-activated receptors (PARs). Proteolysis of PAR-receptors causes tethered ligands to be exposed at the N-terminus of the receptor, which triggers signalling (Vu et al 1991 a & b, see Coughlin 1999). Of the three thrombin-activated PAR-receptors PAR-1 and PAR-4 are expressed in human platelets (Kahn et al 1999).

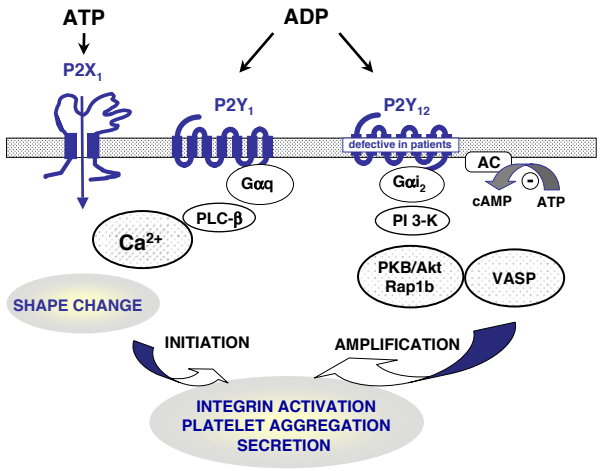


Figure 6. *The current model of platelet activation by ADP and ATP. Two ADP receptors are $P2Y_1$ and $P2Y_{12}$, while the $P2X_1$ receptor is activated by ATP. $P2Y_1$ receptor is coupled to G_q, causes Ca^{2+}, shape change and initiation of aggregation. G_i-coupled $P2Y_{12}$ receptor causes completion and potentiation of the aggregation. $P2Y_{12}$ receptor-mediated adenylate cyclase inhibition is not directly responsible for the activation of the GP IIb/IIIa and subsequent aggregation. Dephosphorylation of the vasodilator-stimulated phosphoprotein (VASP), which negatively regulates GP IIb/IIIa, and a PI3K-dependent activation of the small GTPase Rap1B and PKB/Akt are involved in $P2Y_{12}$ receptor-mediated aggregation. Reproduced from Gachet (2005), with permission.*

PAR-1 is a high affinity receptor, activated by low concentrations of thrombin (<2nM) (Kahn et al 1999). It uses GP Ibα as a cofactor for cleavage and also acts synergistically with ADP to amplify its responses (see Gachet et al 1997, De Candia 2001, see Adam et al 2003). ADP is released from dense granules when a platelet is activated by a primary agonist. Thus ADP-receptors, including $P2Y_{12}$, become functional and part take in thrombin-induced aggregation. ADP has been shown to be responsible for stabilization of thrombin-induced aggregates (Cattaneo et al 1990). PAR-4 is a low affinity receptor activated by higher concentrations of thrombin independently of ADP (see Adam et al 2003).

2.5 TxA_2-receptor

Thromboxane A_2 (TxA_2) is a potent platelet activator, produced as COX enzymes convert arachidonic acid (AA) to prostaglandin $(PG)H_2$, which can be further converted by thromboxane synthase to TxA_2. Optionally PGH_2 can also be converted to other prostaglandins PGD_2, PGE_2, $PGF_2\alpha$ or PGI_2. TxA_2 synthesis is caused by platelet activation by other agonists and the product acts rapidly *in situ* as it has a short half-life of 30 seconds. TxA_2 itself causes further platelet activation (see Dogné et al 2004).

The TxA_2- receptor, officially called TP-receptor, belongs to the G-protein coupled receptor family. Classically they are characterized by signalling via G_q, but also via other proteins.

Two different isoforms of the receptor exist, both coded by the same gene, but generated by alternative splicing (Hirata et al 1991, see Dogné et al 2004).

2.6 Adrenergic receptors

Both adrenergic and dopaminergic receptors are expressed in human platelets. Actions of catecholamines are mediated by α_2-adrenergic receptors, potentiating the effects of other agonists and in high concentrations inducing platelet aggregation and secretion (see Anfossi & Trovati 1996). In physiological conditions epinephrine mainly acts as by potentiating platelet activation induced by other agonists (Lanza et al 1988). Interestingly, epinephrine significantly potentiates shear-induced platelet activation dependable on vWf and GP Ib (Goto et al 1992 & 1996, Mustonen & Lassila 1996). Adrenergic receptors are linked, via G proteins, to the same intracellular pathways as $P2Y_{12}$ (Jin & Kunapuli 1998). Epinephrine is therefore postulated to synergize with ADP-induced responses mediated by $P2Y_1$-receptors (see Conley & Delaney 2003). The α_{2A}-receptor has the highest affinity of adrenergic receptors and epinephrine is the most potent catecholamine affecting platelet function (see Anfossi & Trovati 1996). The number of α_{2A}-receptors has been suggested to change with different conditions (see Kerry & Scurton 1985). Epinephrine has also been shown to attenuate the aspirin efficacy treatment (Mustonen et al 2001).

Dopaminergic receptors are classified to postsynaptic D_1-like receptors, interacts with Gs protein and influence the activation of adenylate cyclase, and presynaptic D_2-like receptors, which inhibit cAMP synthesis via Gi proteins as well as cause hydrolysis of phospatidyl inositol, activate K^+ channels and modulate intracellular Ca^{2+} levels (see Anfossi & Trovati 1996).

2.7 $5HT_2$-receptor

Platelets have a serotoninergic receptor $5HT_2$, activation of which causes rise in cytoplasmic Ca^{2+} and a shape change. Serotonin is a weak agonist of platelets and act synergistically with other platelet agonists (Baumgartner & Born 1968, Pletsher A 1987).

3 Assessment of platelet function

3.1 Platelet function tests

Several techniques for measuring platelet function have been developed. Traditionally they have been used to assess platelet function defects and bleeding tendency prior to surgery. Recently, platelet function tests have been used in atherothrombotic disease to predict clinical outcomes and to monitor antiplatelet drugs (see Michelson 2004). Platelet function tests in atherothrombotic disease have been established in research use and not as much in clinical practice, as the evidence to support their use on a regular basis is still inadequate.

All techniques of measuring platelet activation and aggregation are sensitive to several variables. Normal platelet function consists of activation, adhesion, spreading, release reactions, aggregation, exposure of a procoagulant surface, microparticle formation and clot retraction (Fig 7). Different test reflect all these variables alternatively and their sensitivity and specificity in doing so vary. The large number and variety of either pathological or drug mediated platelet defects challenge the platelet function tests. Phlebotomy, anticoagulation, sample transit and

handling in the laboratory are all sources of potential artefacts as platelets are easily activated, but also desensitized (see Harrison 2005).

Several challenges for measuring platelet activation exist (Table 2). However, the interest in platelet function tests has recently increased with new, plausible objectives like monitoring of efficacy of antiplatelet medication.

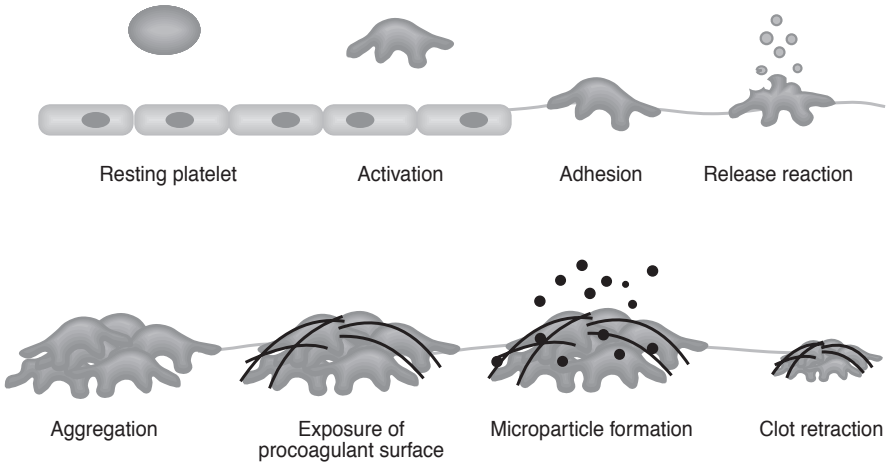


Figure 7. *Platelet activation and functions. Resting platelets circulate in blood and their activation is inhibited by local antithrombotic factors such as an intact endothelial surface. At activation platelets change shape. Adhesion to prothrombotic matrix revealed under injured endothelium follows. Activation of platelets causes release reactions, where platelets release the mostly prothrombotic contents of their secretory granules. Platelet activation enables aggregation, which causes platelets to attach to already adhered platelets, thus forming a platelet clot and localizing the formation of a thrombus. Platelets also have procoagulant properties, which enhance coagulation forming fibrin clot which stabilizes the thrombus. Strong platelet activation causes microparticle formation and shedding. Microparticles contain prothrombotic and antithrombotic proteins. Platelets participate in clot retraction as their actin and myosin cause contraction of the platelets. Clot retraction stabilizes the clot and participates in the initiation of wound healing.*

Table 2. *Platelet function tests.*

Basis of test	Name of the test	Advantages	Disadvantages	Clin	ASA	Clopi
In vivo cessation of blood flow by platelet plug	Bleeding time	In vivo; physiological	Non-specific; insensitive; high interoperator CV; invasive, can leave scar	No	No	No
Platelet-to-platelet aggregation	Aggregometry (turbidometric)	Historical gold standard, diagnostic	Poor reproducibility; non-physiologic; high sample volume; sample preparation; time consuming; expensive; no standardization; not specific; uncertain sensitivity	Yes	Yes with AA	Yes with ADP
	Aggregometry (impedance)	Whole blood assay	Insensitive; uncertain sensitivity; high sample volume; sample preparation; time consuming; expensive	Yes	Yes with AA	Yes with ADP
	Aggregometry (luminescence)	Combined aggregations and ADP release	Semi-quantitative	No	No	Yes?
	VerifyNow (Ultegra RPFA®)	Simple, rapid; point-of-care, low sample volume; no sample preparation; whole blood assay	No instrument adjustment	Yes	Yes with AA	Yes with ADP
	Plateletworks® (Helena Laboratories)	Minimal sample preparation; whole blood assay	Not well studied	No	No	Yes
Shear-induced platelet adhesion	Platelet perfusion studies	High shear; whole blood; versatile, sensitive	High sample volume; sample preparation; time consuming; requires experienced technician; poor reproducibility; no standardization	No	No	No
	IMPACT (cone and plate (let) analyzed)®, DiaMed	Simple, rapid; point-of-care, low sample volume; high shear; whole blood assay	Instrument not yet widely available	No	U. D	U. D
In vitro cessation of hight shear blood flow by platelet plug	PFA-100®	Simple, rapid; low sample volume; high shear; no sample preparation; whole blood assay	Dependent on vWf, hematocrit; not specific; no instrument adjustment	Yes	Yes	No

Basis of test	Name of the test	Advantages	Disadvantages	Clin	ASA	Clopi
Activation dependent changes in platelet surface	Platelet surface P-selectin or activated GP IIb/IIIa, leucocyte-platelet aggragtes (flow cytometry)	Low sample volume; whole blood assay	Sample preparation; expensive; requires flow cytometer and experienced operator	Yes	Yes with AA	Yes with ADP
Activation dependent signalling	VASP phosphorylation state (flow cytometry)	Directly dependent on clopidogrel´s target $P2Y_{12}$-receptor; low sample volume; whole blood assay	Sample preparation; expensive; requires flow cytometer and experienced operator	No	No	Yes
Activation dependent release from platelets	Platelet derived microparticles (flow cytometry)	Low sample volume; whole blood assay	Sample preparation; expensive; requires flow cytometer and experienced operator	No	No	No
	Serum thromboxane B_2	Directly dependent on aspirin target, COX-1	Indirect measure; not platelet specific	No	Yes	No
	Urinary 11-dehydro-thromboxane B_2	Directly dependent on aspirin target, COX-1	Indirect measure; not platelet specific; dependent on renal function; uncertain sensitivity; uncertain reproducibility; not widely evaluated	Yes	Yes	No
	Plasma sCD40L	Majority of plasma sCD40L is platelet-derived	Separation of plasma can result in artefactual platelet activation	Yes	No	No
	Plasma GP V	Platelet specific	Separation of plasma can result in artefactual platelet activation; reflect only thrombin-mediated platelet activation	No	No	No
	α-granule constituents in plasma: platelet factor 4, β-thromboglobulin, soluble P-selectin	Reflects platelet secretion	Separation of plasma can result in artefactual platelet activation; plasma soluble P-selectin also originates from endothelial cells	No	No	No
Gene analyses	Measurement of specific genotypic differences	Specific to tested polymorphisms	Platelet genome yet to be fully defined; Usability depends on extensive data on relevance of genotypic differences to platelet function and clinical endpoints	Yes	?	?

Modified from Michelson (2004), Harrison (2005) and Hankey & Eikelbloom (2006).
Clin= reported to predict clinical outcomes, ASA= monitoring of aspirin, clopi= monitoring of clopidogrel, U.D= under development, VASP= vasodilator-stimulated phosphoprotein, CV= coefficient of variation, RPFA= rapid platelet function analyzer, AA=arachidonic acid, vWf=von Willebrand factor.

3.2 Blood sample anticoagulation

Blood obtained from patients for platelet function tests needs to be anticoagulated to prevent it from clotting within minutes of blood collection. Citrate, PPACK (D-phenylalanyl, L-prolyl-L-arginine cholоromethyl ketone), hirudin and heparin are most commonly used to anticoagulate whole blood or platelet rich plasma (PRP). The role of Ca^{2+} in normal platelet function and Ca^{2+} chelation by common anticoagulants is one of the specific challenges of platelet function measurement (Phillips et al 1997, Andre et al 2003). Citrate is often used as an anticoagulant due to its availability in clinical practice. However, citrate causes the chelation of Ca^{2+} and Mg^{2+}, which are necessary for normal platelet function. For example, monomeric collagen is unable to activate platelets in the absence of Mg^{2+} (Siljander & Lassila 1999). Platelet activation induced by ADP or epinephrine are known to be enhanced in environment of low Ca^{2+}, when compared with physiological conditions (Lages & Weiss 1981, Lalau Keraly et al 1988, Packham et al 1989). In citrate anticoagulated blood ADP causes two phases of platelet aggregation. The secondary aggregation is caused by ADP-induced Tx formation and platelet release reaction, neither of which occurs under physiological concentrations of divalent cations (Pacham et al 1987 & 1989). In addition, primary phase of platelet aggregation is diminished in conditions of low Ca^{2+} concentrations when compared with physiological ones (Packham et al 1989). Due to the Tx formation under low Ca^{2+} concentrations, ADP-induced platelet aggregation has been used to detect Tx inhibition by aspirin.

Other anticoagulants can also be used, but they all have their specific problems for testing platelet function. PPACK, hirudin and heparin affect the physiological role of thrombin as they act as thrombin-inhibitors. Heparin is known to activate platelets under certain conditions (May & Heptinstall 2004). Choice of anticoagulant is also known to affect measured inhibition exerted by antiplatelet agents such as GP IIb/IIIa-inhibitors and $P2Y_{12}$-receptor antagonists (Phillips et al 1997, May & Heptinstall 2004).

3.3 Platelet aggregation studies

Platelet aggregometry, despite its development already in the 1960´s, is still considered as the gold standard of assessing platelet activation (Born 1962, Born & Cross 1963, see Born & Patrono 2006). Traditionally, turbidometric measurements of platelet aggregation are performed in platelet rich plasma separated from erythrocytes and white blood cells by centrifugation. Platelets are then activated by different agonists (eg. collagen, ADP, epinephrine). Platelet activation and aggregation are measured by increased light transmittance through the PRP solution as platelets, which in the beginning were spread out evenly in plasma, clump together in the cuvettes. In whole blood aggregations the sample is analysed with electrical impedance or light scattering (see Harrison 2005). Choice of anticoagulant influences the results obtained in platelet aggregation studies (see section 3.2).

The limitations of aggregation studies are that age, gender, race, medication and hematocrit levels are known to affect platelet aggregation responses. In addition, physical and mental stress as well as diet have been found to influence platelet activity, thus at least partly explaining the day-to-day variability presented in aggregation studies (see George & Shattil 1991). Even when the many pre-analytical and analytical variables affecting the results are controlled, the accuracy and reproducibility of the aggregations have limitations. Reproducibility is also limited by the fact that there is no standardisation when it comes to aggregation studies, thus agonists, scales and geometry of the optical system can vary from one laboratory to another (see Michelson 2004, see Harrison 2005).

Platelet aggregation tests have been found to be insensitive to small aggregate formation. When traditional aggregations in platelet-rich-plasma are studied the removal of the homeostatic properties of erythrocytes leads to deterioration in platelet function over time and the process of centrifugation may cause platelet activation or desentization (see Storey & Heptinstall 1999).

3.4 PFA-100® platelet function analyser

PFA-100® is a platelet function analyser (Dade-Behring AG, Düdingen, Switzerland) which was released in 1995 and has become one of the most studied new automated tests for measuring platelet function (see Favaloro 2002). PFA-100® measures platelet-related primary hemostasis in whole blood under high shear-rate conditions (4000 1/s) (Kundu et al 1995, Mammen et al 1998). Platelets interact with a collagen mesh spiked with either ADP (CADP) or epinephrine (CEPI), resulting in occlusion of an aperture in the respective cartridge. The results are expressed as closure times. The PFA-100® method has been suggested to be useful in detection of vWD and assessment of the antiplatelet effects of aspirin (see Cattaneo et al 1999, Feuring et al 1999, Favaloro et al 1999, see Favaloro 2002, Homoncik et al 2000).

PFA-100® measurements are usually performed in citrate anticoagulated blood. The concentration of 3.2% citrate has been recommended with the exception of measuring the antiplatelet effect of aspirin, where results obtained using 3.8% citrate anticoagulation have presented greater stability (see Jilma 2001, see Favaloro 2002).

PFA-100® has been called "*in vitro* bleeding time", but is actually more sensitive and has better reproducibility than bleeding time (Marshall et al 1997, Fressinaud et al 1998). VWf plays a major role in hemostasis occurring in the PFA-100® system (Chakroun et al 2004). In addition, PFA-100® is sensitive to many variables including the number of platelets, hematocrit, drug and dietary effects (see Jilma 2001). Low hematocrit or platelet count (under 0.1 or 10 x 10^9/L, respectively) will cause non-closure in the PFA-100® and thus preclude its normal use (see Harrison 2000, see Jilma 2001). The hematocrit levels correlate directly with closure times in PFA-100® (Escolar et al 1999).

PFA-100® is sensitive to some, but not all, blood-based deficiencies or defects. It lacks sensitivity to disorders of coagulation and fibrinogen defects, including hemophilia. Its sensitivity to defective platelet function depends on the severity of the disturbance. However, as noted earlier, PFA-100® has high sensitivity to disturbances in levels or properties of vWf (see Favaloro 2002) (Fig 8).

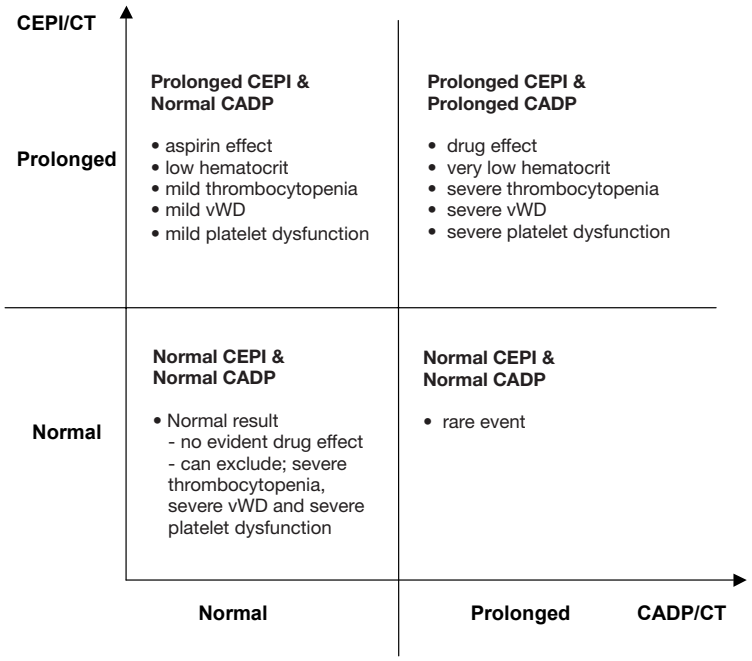


Figure 8. *Schematic illustration of the interpretation of PFA-100®. In PFA-100® blood flows at high shear rate through an aperture coated with collagen and either epinephrine (CEPI) or ADP (CADP). The time for the aperture to occlude is monitored and called the closure time (CT). vWf=von Willebrand factor. Modified from Favaloro (2002).*

3.5 Perfusion studies

Platelet perfusion studies assess platelet activation by collagen and shear stress followed by consequent adhesion and following aggregation *in vitro*. Studies are performed with whole blood and thus provide a close to physiological environment for studying platelet function in a high shear environment comparable with arteries (1600 s^{-1}) (Baumgartner 1973 and 1976, Turitto 1975, Turitto & Baumgartner 1975, Hall et al 1998). Immobilized collagen as the adhesive surface models the site of vascular damage (Baumgartner 1976). As perfusion studies are extremely time-consuming and require the expertise of the performing person they are only used in research laboratories and not in clinical practice. In comparison with aggregation studies perfusions provide interaction with other cells in whole blood as well as the high shear rate. Perfusion studies are versatile as the adhesive surface of collagen can be replaced by other adhesive materials and platelet function in response to added factors can also be studied. Hence perfusion studies are sensitive, but have variable reproducibility.

Automatized analyzers such as PFA-100® and IMPACT cone and plate(let) analyzer® have been developed to provide tests which have similar physiological shear forces as perfusion studies, but are easier to execute and have better reproducibility. However, with these tests some of the sensitivity and most of the versatility of perfusion studies are lost (see Harrison 2005).

3.6 Assessment of aspirin efficacy

The term aspirin resistance has been introduced to describe sustained platelet activity despite aspirin medication. The sustained activity can be divided into TxA_2-induced platelet activity and increased platelet function which is independent of TxA_2 formation. As aspirin inhibits TxA_2 formation and thus abolishes activity dependent on it, the former type has been suggested to be labelled *pharmacologic aspirin resistance* (see Wong et al 2004). The latter type has been proposed to be labelled *functional aspirin resistance*, as this phenomenon is characterized by increased platelet function mediated by activating pathways other than TxA_2 (see Wong et al 2004).

Recent studies assessing the prevalence and relevance of aspirin resistance have used different methods to determine aspirin resistance, which have included methods specific to TxA_2 formation and those measuring general platelet activity. Thus, the definition of aspirin resistance needs to be defined more clearly, as suggested by Wong et al (see 2004) and discussed further in section 5.1.

Several factors, such as the method chosen to measure platelet aggregation or activation, time allowed to measure platelet aggregation and different, often arbitrary, cut-off values to define resistance, influence measurement of platelet function when non-specific methods are used (see Cattaneo 2004). In addition, it has been suggested that when measuring efficacy of antiplatelet medication the tests should be performed both before and after drug intake, otherwise baseline platelet activity influences the results obtained (see Steinhubl et al 2005). However, termination of antiplatelet medication from patients with atherothrombotic disease is unethical and thus in practice this study design is often impossible to execute.

Due to the relatively large role of poor compliance to antiplatelet therapy in aspirin resistance, it has also been suggested that the role of non-compliance should be controlled by measurement of plasma concentrations of aspirin and salicylate (Poulsen et al 2005). This could be performed by high performance liquid chromatography to detect aspirin and salicylate in plasma within the first 10 h of ingestion (Cerletti et al 2003). However, measurement of plasma aspirin concentrations is severely limited by its short half life of only 20min.

AA-induced aggregation

Arachidonic acid (AA)-induced aggregation could be considered as the gold standard for assessing aspirin response, since it directly evaluates the capability of COX-1 to produce TxA_2 subsequent to platelet aggregation (Gum et al 2003). Therefore this method, although time-consuming, is valuable for detecting aspirin efficacy. Sustained AA-induced aggregation has also been associated with clinical endpoints in aspirin-treated patients (Gum et al 2003).

The use of AA-induced aggregation in determining aspirin resistance in previous studies has been criticized due to the use of high concentrations of AA, suspected to cause platelet lysis (detectable in an aggregometer by enhanced light transmission), and the fact that overall AA-induced aggregation is the result of TxA_2 and other agonists secreted by platelet granules (see Cattaneo 2004).

Platelet aggregation induced by several other agonists such as TxA_2-mimetics, collagen and ADP have also been used to determine aspirin resistance. These agonists, however, are

not specific to aspirin's mechanism of action and are only partially TxA_2-dependent. Thus, they measure general platelet activity instead of aspirin efficacy (see Hankey & Eikelboom 2006).

PFA-100® platelet function analyser

As PFA-100® has been reported to detect aspirin use it has been suggested as a method for monitoring the antiplatelet effect of aspirin. The closure time of the epinephrine-stimulated cartridge should be prolonged in aspirin-treated patients. The manufacturer of the PFA-100® refers to 170 s as the limit of detecting aspirin efficacy with a sensitivity of 95%.The closure time is followed for maximally 300 s (Feuring et al 1999, Homoncik et al 2000).

However, in the case of aspirin response, other mechanisms compensate and surpass the need for TxA_2 production when platelets are challenged in the PFA-100® system. It is well accepted that aspirin does not affect shear stress-induced platelet aggregation, one of the important features in the PFA-100® system (see Cattaneo 2004). In addition, the role of vWF is pivotal and high plasma levels associate with decreased closure times (Chakroun et al 2004). Then again, patients with cardiovascular disease and diabetes are known to have increased plasma levels of vWf (see Manucci 1998). The roles of GP Ib and GP IIb/IIIa are emphasized in PFA-100® due to high shear forces (Watala et al 2003). Thus, PFA-100® offers a means to measure platelet function which, however, is not specific to aspirin and can be overcome by several factors such as increased levels of vWF detected in cardiovascular disease and diabetes.

In studies comparing PFA-100® and AA-induced aggregation in detecting aspirin resistance, different populations of patients have been found as aspirin resistant (Hezard 2002, Gum et al 2003). It seems that PFA-100® is not specific for aspirin resistance. In a study by Andersen et al (2002) aspirin resistance measured with PFA-100® was not associated with Tx levels, but instead aspirin response was associated with P-selectin levels and the general secretory function of platelets (Andersen et al 2002). This refers to the fact that aspirin resistance measured with PFA-100® describes general platelet activity and not TxA_2-dependent activity. Thus according to Wong et al (see 2004), aspirin resistance measured with PFA-100® could be termed as *functional aspirin resistance*.

Blood or urine TxB_2 levels

As most platelet function tests measure general platelet function in which TxA_2 production is of marginal importance, Tx levels have been suggested as a measurement of aspirin effect (see Cattaneo 2004). TxB_2, the stable metabolite of TxA_2, can be measured from plasma, serum or urine. However, urinary TxB_2 levels, concentration of 11-dehydro-thromboxane B_2, is not platelet specific and depends also on renal function (Riutta et al 1992). As serum TxB_2 levels reflect both platelet and extra-platelet sources of Tx generation, they have been speculated not to be specific for aspirin (see Hankey & Eikelboom 2006).

3.7 Assessment of clopidogrel efficacy

ADP-induced aggregation

ADP-induced aggregation has been the most used tool for assessing the clopidogrel efficacy, due to clopidogrel´s mechanism of action as an antagonist of ADP-receptor $P2Y_{12}$ (see Cattaneo 2004). However, it has been criticized because it is not a specific measurement of $P2Y_{12}$-inhibition.

The inhibitory effect of clopidogrel can be overcome by platelet activation via other pathways, including platelet activation via the other ADP-receptor $P2Y_1$ (see Cattaneo & Gachet 1999, Cattaneo et al 2004). In fact, $P2Y_1$-receptor has been shown to activate platelets synergistically with α-adrenergic receptors, thus bypassing the need for simultaneous signalling of $P2Y_{12}$ and $P2Y_1$ for significant platelet activation (see Conley & Delaney 2003). In addition, the activity of $P2Y_1$ has been shown to vary widely in patients whose $P2Y_{12}$-receptors are blocked or in otherwise healthy individuals with a genetic $P2Y_{12}$-receptor deficiency (see Cattaneo 2004).

Other methods of studying platelet activation

Flowcytometric measurement of GP IIb/IIIa activation has been found to associate with response to thienopyridines (Hezard et al 2002). However, this test is not specific to ADP-induced activation and especially to inhibition of the $P2Y_{12}$-receptor. ADP-evoked fibrinogen binding has also been suggested as a test for clopidogrel efficacy, however, it is not specific to $P2Y_{12}$-activation, but also depends on GP IIb/IIIa activation (Järemo et al 2002, Schumann et al 2005).

Several studies have failed to measure clopidogrel efficacy with PFA-100®, despite the fact that in the CADP-cartridge platelet activation is induced by ADP in addition to collagen (Hezard et al 2002, Mueller et al 2003).

ADP-induced inhibition of adenylate cyclase, which is selectively mediated by $P2Y_{12}$, has been suggested for reliable monitoring of clopidogrel efficacy (see Cattaneo 2004, Almsherqi et al 2005). Normally activation of the $P2Y_{12}$-receptor would inhibit cAMP production and thus cAMP-dependent protein kinase (PKA)-mediated phosphorylation of vasodilator stimulated phosphoprotein (VASP) (Fig 6). As clopidogrel acts via $P2Y_{12}$-receptor its effect could be assessed by cAMP levels or VASP phosphorylation (Schwarz et al 1999). However, changes of these determinants are also to some extent dependent on activation of the epinephrine receptor α2A, which could produce some features of $P2Y_{12}$ signalling by repressing cAMP levels (see Conley & Delaney 2003).

4 Antiplatelet Medication

4.1 Aspirin

Mechanism of action

Aspirin was first synthesized in 1897 by German chemist Felix Hoffman and today it has an important position as an antiplatelet agent, among other indications. The antiplatelet effect of aspirin was first described by Morris in 1967. Aspirin exerts its effect by inhibiting the activity of prostaglandin (PG) H-synthase-1 and -2, also known as cyclooxygenase (COX)-1 and 2. COX-1 is known to predominate in platelets. Thus, in activated platelets COX-1 utilizes arachidonic acid (AA) to produce PGH_2, which is further converted to TxA_2, a potent platelet activator, as well as other prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2. The anti-platelet effect of aspirin is exerted by the irreversible inhibition of the COX-1 enzyme, inhibiting the production of TxA_2 (Roth et al 1975). The irreversibility is caused by acetylation of strategic serine residues (Ser529 in COX-1 and Ser516 in COX-2) of COX-channel causing prevention of substrate access to the catalytic site of the enzyme. Anucleated platelets are unable to resynthesize the enzyme and thus depend on platelet turnover for its expression (see Patrono et al 2004).

Aspirin inhibits COX-1 in 1/100 to1/50 concentrations compared to COX-2 (Cipollone et al 1997). Thus, smaller doses of aspirin are sufficient to inhibit COX-1, while inhibition of COX-2 is left incomplete. COX-2, which is inducibly expressed mostly in leukocytes and connective tissue cells and constitutively in certain organs, is not markedly affected by small aspirin doses (50-325mg) administered once daily.

Aspirin has been postulated to also have effects unrelated to TxA_2 inhibition. Platelet inhibition not related to COX-1 inhibition, enhancement of fibrinolysis, suppression of plasma coagulation and anti-inflammatory effects, have been reported (see Patrono et al 2004). Enhancement of fibrinolysis is caused by N-acetylation of lysyl residues of fibrinogen by aspirin (dose >650mg twice daily) (Björnsson et al 1989). Suppression of coagulation could be caused by vitamin K- counter effect of large doses of aspirin (>1500mg/d), inhibition of thrombin generation (dose >500mg) or acetylation of one or more clotting factors (Quick & Cleasceri 1960, Szczeklik et al 1996, see Patrono et al 2004). Anti-inflammatory effects of aspirin are not only due to inhibition of COX-2 activity but aspirin also modifies the interaction between platelets and either neutrophils or erythrocytes, protects endothelial cells from oxidative stress and improves endothelial dysfunction (López-Farré et al 1995, Podhaisky et al 1997, Santos et al 1997, Husain et al 1998, see Patrono et al 2004).

Pharmacokinetics

Aspirin is absorbed in the stomach and upper intestine. Normally peak plasma levels are reached within 30-40 min of aspirin uptake. However, when enterocoated aspirin is used the peak levels are reached between 3-4 h. Aspirin is rapidly cleared from the circulation; half-life of acetylsalicylic acid is 15-20 min. As the effects on Tx synthesis are irreversible they last for the life-span of platelets, gradually decaying if dosage is not repeated. As 10% of platelets are replaced every day, it has been estimated that after 5-6 days 50% of the platelets function normally.

Bleeding and other adverse effects

Bleeding complications are common in patients receiving aspirin therapy. The risk of major hemorrhage is approximately 1-2%. Aspirin-induced gastro-intestinal (GI) toxicity depends on the dose. GI toxicity is postulated to be caused by inhibition of PG synthesis and direct damage to gastric and intestinal mucosa, not by bleeding directly associated with TxA_2 inhibition (see Roth & Caverly 1994). Thus, low-doses (100-300mg/d) used for inhibiting platelet activity are associated with a 2% risk of GI bleeding, which is similar to other antiplatelet agents.

In contrast to the widely held belief, enteric-coated aspirin has not been proven safer compared to plain or buffered aspirin in relation to GI toxicity. Kelly et al (1996) showed 2.6, 2.7 and 3.1 times increased risks of upper GI bleeding with plain, enteric-coated and buffered aspirin, respectively, in a case-control study of 550 patients using daily aspirin doses lower than 325mg/d. Similar results have been reported by others (see Patrono et al 2004).

Primary prevention

Aspirin has been evaluated in six primary prevention trials (US Physicians, Primary prevention Project, Hypertension Optimal Treatment, UK Doctors, Thrombosis Prevention Trial and Swedish Angina Pectoris Aspirin Trial) including healthy, hypertensive, high-risk and stable angina patients. These trials showed that the level of cardiovascular risk is the major determinant of the absolute benefit of aspirin therapy, and aspirin use can be recommended only if a patient's risk for coronary events is above 1.5% per year (Sanmuganathan et al 2001, see Patrono et al 2004).

Secondary prevention

Risk reduction of 20-25% in adverse events has been shown with long-term aspirin use in patients with previous atherothrombotic events or in other high-risk categories (ATC 2002). In a large meta-analysis antiplatelet medication was associated with decreases in reinfarction, death and stroke in 18 7888 patients with a history of myocardial infarction, 18 270 patients with a history of cerebrovascular events and 9214 patients with peripheral arterial disease (Fig 9) (ATC 2002). These results were obtained from a number of different antiplatelet agents combined, but aspirin was the drug most commonly studied and there was no clear evidence of differences between aspirin and other antiplatelet drugs (ATC 2002) (Fig 10).

Aspirin in acute atherothrombotic syndromes

The antithrombotic effect of aspirin has been well established. The ISIS-2 (International Study of Infarct Survival) study proved the efficacy of a single 162.5mg dose at the time of acute coronary symptoms in preventing further atherothrombotic events and at the same time the treatment was shown to be safe (ISIS-2 Collaborative group 1988). Proportional risk reduction of adverse events was 30 %.

The aspirin efficacy in acute stroke has also been established with a risk reduction of approximately 11% (ATC 2002). In meta-analysis of altogether 40 821 patients with acute stroke there was a significant reduction of new strokes as well as cardiovascular deaths. Hemorrhagic and ischemic stroke were also separately assessed in almost all of these patients and antiplatelet medication seemed to increase hemorrhagic strokes by 1.9 per 1000 patients, but this was counter balanced with a reduction of ischemic strokes to 6.9 per 1000 patients (ATC 2002).

Aspirin in atrial fibrillation

Oral anticoagulation with warfarin is beneficial in reducing the risk of stroke in patients with atrial fibrillation (The Boston Area Anticoagulation Trial for Atrial Fibrillation Investigators 1990). In comparison with warfarin, aspirin (75-325mg/d) was found to be significantly less effective in preventing stroke. However, aspirin caused a risk reduction of 25% and thus its use in patients unable to receive anticoagulation therapy is recommended (see Patrono et al 2004).

Summary

Aspirin is beneficial in both acute atherothrombotic events and in the prevention of thrombosis in patients with stable atherothrombotic disease. The benefits of antithrombotic medication in these patients at the population level overcome the risks of adverse-effects. However, subgroups of patients who suffer from either thrombotic or bleeding complications exist.

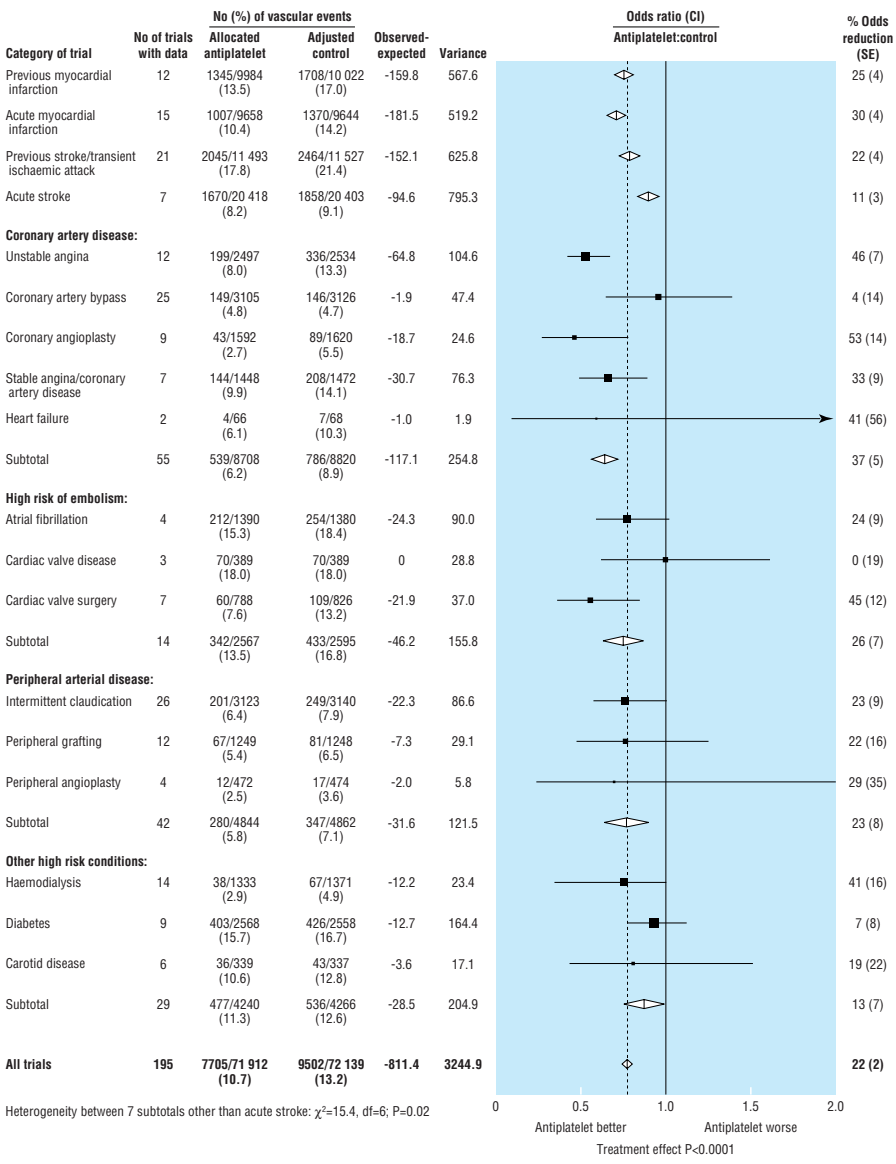


Figure 9. *Proportional effects of antiplatelet therapy on vascular event in 195 trials in high risk patients subdivided by disease category. Stratified ratio of odds of an event in treatment groups to that in control groups is plotted for each group of trials (black square) along with its 99% confidence interval (horizontal line). Meta-analysis of results for each main category and for all trials (and 95% confidence interval) is represented as an open diamond. Adjusted control totals have been calculated after converting any unevenly randomised trials to even ones by counting control groups more than once, but other statistical calculations are based on actual numbers from individual trials. Reproduced from the Antithrombotic Trialists´ Collaboration (2002), with permission.*

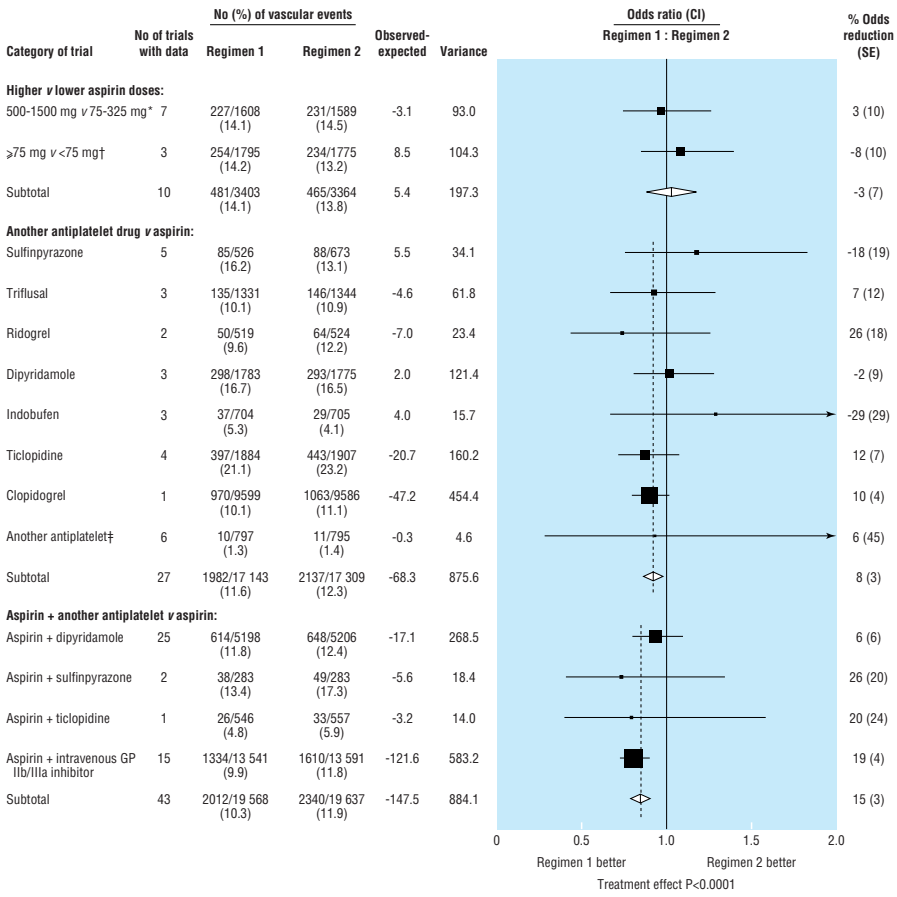


Figure 10. *Direct comparisons of proportional effects of different antiplatelet regimens on vascular events in high-risk patients. Only meta-analyses involving a total of 500 or more high risk patients are shown. *Includes one trial comparing 1400mg/day v 350mg/day, and another comparing 1000mg/day v 300mg/day among patients with who were also given dipyridamole. †Includes two trials comparing 75-325mg aspirin daily v <75mg aspirin daily and one trial of 500-1500mg aspirin daily v <75mg aspirin daily. ‡Includes cilostazol, sulotroban, trapidil, E5510, eptifibatide and GR32191B. Stratified ratio of odds of an event in regimen 1group to that in regimen 2 group is plotted for each group of trials (black square) along with its 99% confidence interval (horizontal line). Meta-analysis of results for all trials for particular comparison (and 95% confidence interval) is represented by an open diamond. Reproduced from the Antithrombotic trialists' Collaboration (2002), with permission.*

4.2 Clopidogrel

Mechanism of action

Clopidogrel is a thienopyridine derivative which irreversibly inhibits platelet $P2Y_{12}$-receptor (Hollopeter et al 2001). It is a prodrug which requires metabolism by liver cytochrome P450

to its active form before achieving its antiplatelet effect (Savi et al 1994b and 2000). Inhibition of $P2Y_{12}$-receptors is caused by formation of a disulfide bridge between the reactive thiol group of clopidogrel´s active metabolite and one or more cysteine residues of the platelet $P2Y_{12}$-receptor (Ding et al 2003, Savi et al 2006).

Clopidogrel selectively inhibits ADP-induced platelet activation but has also been shown to have an inhibitory effect on platelet activation by other agonists such as epinephrine. This is most likely due to blocking of the effect of secondarily released ADP (Gawaz et al 1996). ADP has an important role in thrombus formation as the escalating process of secondary activation of platelets is caused by agonists such as ADP released from the primarily activated platelets. However, it has been postulated that increased concentrations of platelet agonists other than ADP, such as Tx and thrombin, will cause platelet activation despite $P2Y_{12}$-receptor inhibition (see Patrono et al 2004).

Pharmacokinetics

Clopidogrel is absorbed and metabolised relatively rapidly. The plasma elimination half-life of the main systemic metabolite of clopidogrel, SR26334, is 8 h, but because of its irreversible mechanism of action a once daily administration of clopidogrel is sufficient (Savi et al 2000). In healthy volunteers platelet function increased gradually after discontinuation of clopidogrel treatment and was restored in 7 days (Weber et al 2001). Elimination of clopidogrel is 50% renal and 46% gastrointestinal.

In small studies with healthy individuals a 75mg daily dose was found to cause a plateau of platelet inhibition compared with doses ranging between 25 and 150mg, and at the same time be safer than 100 or 150mg doses when assessed with bleeding time (Thebault et al 1999b). In the same study the 75mg daily dose seemed to cause a steady state of platelet inhibition in 3 to 7 days after initiation of clopidogrel treatment (Thebault et al 1999b). Because of the lag in achieving the full platelet inhibitory effect loading doses have been introduced. In a similar setting of healthy individuals 300 to 400mg loading doses were found to be more effective compared to smaller loading doses (Savcic et al 1999). The effect of the loading dose seemed to be achieved within 2 h (Savcic et al 1999). However, in later studies including patients with coronary artery disease (CAD) the adequacy of these dosages and their timing has been questioned.

Bleeding and other adverse effects

Bleeding complications are obviously the most common adverse effects of clopidogrel treatment, however, they did not significantly differ from those of aspirin when clopidogrel was used instead of or in combination with low-dose aspirin (CAPRIE 1996, CURE 2001, Creager 1998). The overall incidence of bleeding complications was 9.3% in the CAPRIE study (1996) and the incidence of severe bleeding complications was 1.4% in patients on clopidogrel and 1.6% in patients on aspirin. In the CURE study (2001) non-life threatening, severe bleeding complications occurred more frequently with the combination of aspirin and clopidogrel than with aspirin alone. The incidence of life threatening bleeding complications did not differ between groups.

In addition, bloating, cramping, diarrhoea and rash were the most common adverse effects. Severe adverse effects, such as neutropenia (0.04%), thrombotic thrombocytopenic purpura

(1/200 000), and severe thrombosytopenia (0.2%), were rare and significantly less common than with ticlopidine (CAPRIE 1996, CURE 2001).

Primary prevention

Use of clopidogrel in the primary prevention of atherothrombotic diseases has not been specifically studied. The CHARISMA study comprised two patient populations: those with documented atherothrombotic disease and those at high risk due to multiple risk factors. The latter group received antiplatelet medication for primary prevention of atherothrombotic events. Use of antiplatelet agents such as aspirin is beneficial in primary prevention in patients with multiple risk factors (ATC 2002). Thus, the combination of clopidogrel and aspirin was expected to have increased beneficial effects in comparison with aspirin alone. However, in the CHARISMA study in patients with risk factors the combination treatment compared to aspirin alone did not decrease the risk of adverse vascular events. In addition, in the primary prevention group the overall risk for death was increased significantly with the combination treatment compared to aspirin (5.4% vs. 3.8%) (Bhatt et al 2006).

Secondary prevention

The CAPRIE study (1996) was the first large phase III trial, to test the efficacy and safety of clopidogrel in comparison with aspirin. Modestly better clopidogrel efficacy in comparison with aspirin was found, but the difference between these antiplatelet drugs was not large (P=0.04). After a 1.9 year follow-up the relative risk reduction with clopidogrel instead of aspirin treatment was 8.7% (5.32% vs. 5.83%). In a subanalysis it was shown, however, that patients with peripheral arterial disease (PAD) gained a superior benefit from clopidogrel when compared with patients with either primary cardio- or cerebrovascular disease (RRR 23.8%). In concordance, the benefit of clopidogrel over aspirin seemed to be increased in high-risk patients (Bhatt et al 2001). Clopidogrel was found to have a similar safety profile to aspirin. While the efficacy in secondary prevention with clopidogrel and aspirin was similar, the financial aspects of treatment in unselected populations become of interest as the cost of clopidogrel treatment was approximately 7 times that of aspirin (Gorelick et al 1999).

In the CHARISMA study (2006) some of the patients had documented atherothrombotic disease with either cardiovascular, cerebrovascular manifestations or symptomatic PAD (Bhatt et al 2006). In these patients the combination of aspirin and clopidogrel had similar efficacy and safety as aspirin treatment alone.

The clopidogrel efficacy versus the combination of clopidogrel and aspirin in patients with cerebrovascular complications was assessed in the MATCH study (Diener et al 2004). No differences in efficacy of the study regimens were observed. In addition, the combination treatment was associated with increased risk of life-threatening complications with an absolute risk increase of 1.3% (Diener et al 2004).

Also in patients with atrial fibrillation and one or more risk factors for stroke the traditional treatment with oral anticoagulation was found to be superior to the combination of aspirin and clopidogrel, in fact the study was stopped because of the clear evidence (ACTIVE investigators 2006). In patients with contraindications to oral anticoagulation the ongoing ACTIVE A trial has set out to determine the efficacy of combination treatment in comparison with aspirin alone (The ACTIVE Steering Committee 2006).

Clopidogrel as adjuvant treatment in PCI

The CLASSICS study compared combinations of either ticlopidine or clopidogrel with aspirin after either elective or unplanned coronary stenting in patients with CAD (Bertrand et al 2000). The two study regimens were found to be equally effective, but clopidogrel was safer than ticlopidine. Later Bhatt et al (2002a) conducted meta analysis of trials comparing ticlopidine and clopidogrel after percutaneous coronary intervention (PCI) and found the latter to be significantly better with decreased incidence of cardiac adverse events (4.0% vs. 2.1%) and mortality (1.1% vs. 0.5%) (Bhatt et al 2002a).

In the PCI-CURE trial of patients with acute coronary syndrome 10-day pre-treatment with clopidogrel prior to PCI was associated with 30% risk reduction of the combined endpoint of cardiovascular death, myocardial infarction (MI) and urgent revascularization (4.5% vs. 6.4 % in clopidogrel vs. placebo in addition to aspirin groups) (Mehta et al 2001). In addition, the combination of clopidogrel and aspirin was found to be superior in reducing cardiovascular morbidity and mortality during an 8 month treatment when compared to short-term treatment (RR 17%).

In elective patients the CREDO study proved that prolonged 1 year clopidogrel treatment after PCI reduced adverse events when compared with 1 month short-term treatment (27% RRR) (Steinhubl et al 2002). It also showed that a 300mg loading dose of clopidogrel administered more than 6 h prior to PCI caused 39% risk reduction of adverse events compared to no pre-treatment.

Clopidogrel has established a firm position in the treatment of CAD patients after PCI. Its efficacy and safety in addition to aspirin were shown in the CREDO (Steinhubl et al 2002) and CURE (2001) studies. The use of clopidogrel is currently recommended both prior to and after PCI (Bhatt et al 2002a, Steinhubl et al 2002).

Combination treatment with aspirin and clopidogrel was proven to be beneficial in patients with acute coronary syndromes also when treated conservatively (CURE 2001). Beneficial effects of aggressive antiplatelet therapy in acute situations with GP IIb/IIIa inhibitors and clopidogrel have been shown. In addition, no significant excess bleeding was found in patients who underwent coronary artery bypass grafting (CABG) if dual antiplatelet medication with aspirin was stopped more than 5 days prior to the procedure (CURE 2001).

Clopidogrel in acute atherothrombotic syndromes

The CURE trial (2001) compared the efficacy and safety of the combination of clopidogrel and aspirin to aspirin alone in patients with acute coronary syndromes without ST-segment elevation. The risk of atherothrombotic events in patients with dual antiplatelet medication was reduced 20% when compared with aspirin alone (9.3% vs. 11.4 %). The risk reduction rate for MI was most evident. In subgroup analyses it was found that patients with previous PCI had the most significant (40%) risk reduction.

The efficacy of combination treatment in acute cardiovascular syndromes has been shown when used in adjunction with standard treatments such as fibrinolytic therapy (COMMIT collaborative group 2005). Clopidogrel in addition to aspirin caused a 9% reduction in atherothrombotic events compared with aspirin alone in patients with acute MI (COMMIT

2005). Patients undergoing primary PCI were excluded, however, after the randomization to the study groups the patients received a variety of treatments (procedures, fibrinolysis, conservative treatment). Similar results were obtained in the CLARITY-TIMI trial which assessed the efficacy of the combination of aspirin and clopidogrel compared to aspirin in patients with acute MI, who received fibrinolytic therapy. In these 3491 patients who were also treated with fibrinolytics the absolute reduction of atherothrombotic complications in the group with combination treatment was 6.7% (Sabatine et al 2005).

The CARESS study evaluated the efficacy of the combination aspirin and clopidogrel instead of aspirin alone and found it to be more effective in reducing cerebral microemboli detected by Doppler ultrasound in patients with >50% carotid artery stenosis with recent transient ischemic attack (TIA) or stroke. Because of the small size of the study (N=110) the result was unable to reach clinical significance (Markus et al 2005).

Summary

Clopidogrel is beneficial in reducing atherothrombotic events in patients with acute thrombotic syndromes and elective PCI (Table 3). However, in primary and secondary prevention of patients with increased risk for thrombosis, it is not superior to aspirin (Table 3). The differences between these patient groups could result from enhanced platelet activity, and thus a need for more potent antiplatelet medication, in patients with acute plaque rupture or artificial vessel wall damage caused by PCI. Therefore, it could be postulated that in stable disease compensatory mechanisms overcome the need for and benefit of enhanced antiplatelet medication and in an unstable set up the excessive platelet activity is more successfully inhibited by the combination of aspirin and clopidogrel, thus causing clinical benefit. As the risk of bleeding with antiplatelet medication is obvious, use of extensive antiplatelet medication without significant benefit is not justified. Thus, based on recent studies, it seems that at the moment the use of clopidogrel can be recommended only in acute situations. However, as in the case of aspirin, subgroups of patients who would potentially receive increased benefit from clopidogrel and patients who are at unacceptable risk for bleeding if treated with clopidogrel, exist.

Table 3. *Summary of large, prospective, randomized studies on clopidogrel.*

	Title	**Patients, design**	**Setting**	**Results**
CAPRIE	Clopidogrel versus Aspirin in Patients at Risk of Ischeamic Events *CAPRIE steering committee* *Lancet 1996*	19 185 patients with recent stroke, recent MI or symptomatic PAD	1) clopi 75mg/d + placebo 2) ASA 325mg/d + placebo For 1-3 years	- Clopi is more effective than ASA in reducing risk of atherothrombotic events - Clopi is at least as safe as ASA

	Title	Patients, design	Setting	Results
CLASSICS	The Clopidogrel Aspirin Stent International Cooperative Study *Bertrand et al Circulation 2000*	1 020 patients after stent placement (either elective or acute procedures)	In addition to ASA 375mg/d 1) clopi LD 300mg + 75mg/d 2) clopi 75mg/d 3) ticlopidine 250mg twice a day For 28 days	-Clopi and ticlopidine combined with ASA are equally effective -Clopi is safer than ticlopidine -Loading dose of clopi is well tolerated
CURE	Clopidogrel in Unstable angina to prevent Recurrent Events Study *CURE Investigators N Eng J Med 2001*	12 562 patients with ACS, without ST-segment elevation	In addition to ASA 75mg-375mg 1) clopi LD 300 mg + 75mg/d 2) placebo For 3-12 months	-Addition of clopi is beneficial regardless of ASA dose in all patients with ACS. - Bleeding risks increase with increasing ASA dose
PCI-CURE	CURE - subgroup *Mehta et al Lancet 2001*	2 658 patients with ACS who underwent PCI	In addition to ASA 75-325mg/d 1) 10 days prior to PCI clopi 75mg/d + 28 days of open label thienopyridine + 8 months of clopi 75mg/d 2) placebo + 28 d open-label thinopyridine Altogether for 9 months	- Prolonged treatment with clopi is superior to no 1 month treatment -Pre-treatment with clopidogrel is beneficial
CREDO	The Clopidogrel for the Reduction of Events During Observation *Steinhubl et al JAMA 2002*	2 116 patients to undergo elective PCI	In addition to ASA 81-325mg/d 1) clopi LD 300mg + 75mg/d (12 months) 2) no loading dose + clopi 75mg/d (28 days) +placebo (ad 12 months) Altogether for 12 months	-Long term (1-year) clopi reduces adverse effects after PCI -Loading dose administered 3h prior to PCI does not have an effect -Loading dose >6h prior to PCI is associated with decreased risk of adverse events

	Title	Patients, design	Setting	Results
MATCH	Management of atherothrombosis with clopidogrel in high-risk patients with recent transient ischemic attack or ischemic stroke *Diener et al Lancet 2004*	7 599 patients with recent TIA or stroke	In addition to clopi 75mg/d 1) ASA 75mg/d 2) placebo For 18 months	-Combination of ASA and clopi is not superior to clopi -Combination is associated with increased risk for life-threatening bleeding
COMMIT	Clopidogrel and metoprolol myocardial infarction trial *COMMIT collaborative group Lancet 2005*	45 852 patients with recent (<24h) acute MI Patients undergoing primary PCI excluded	In addition to 162mg ASA 1) clopi 75mg/d 2) placebo For mean of 15 days	-Dual antiplatelet treatment caused reduction in atherothrombotic events
CLARITY - TIMI	Clopidogrel as Adjunctive Reperfusion Therapy-Thrombolysis in Myocardial Infarction *Sabatine et al N Eng J Med 2005*	3 491 patients with ST-elevation MI	In addition to fibrinolytic therapy and ASA 1) clopi LD 300mg + 75mg/d 2) placebo For 48-192 hours	-Clopi treatment in addition to ASA was associated with reduced risk or reinfarction and other atherothrombotic complications
ACTIVE W	Atrial fibrillation clopidogrel trial with irbesartan for prevention of vascular effects *Active investigators Lancet 2006*	6 706 patients with atrial fibrillation + one or more risk factors for stroke	1) oral anticoagulation (target INR 2.0-3.0) 2) clopi 75mg/d + ASA 75-100mg/d For 18 months	-Oral anticoagulation is superior to combination treatment -Study was stopped early, because of clear evidence
CHARISMA	Clopidogrel for high atherothrombotic risk and ischemic stabilization, management and avoidance trial *Bhatt et al N Eng J Med 2006*	15 603 patients with atherothrombotic disease or at high risk	In addition to low-dose ASA 75-162mg/d 1) clopi 75mg/d 2) placebo For 28 months	-In whole study population the efficacy and safety of clopi+ASA vs. ASA did not differ -In patients with no documented atherothrombotic disease clopi+ASA was found inferior and less safe to compared to ASA alone

LD=loading dose, ASA= aspirin, clopi= clopidogrel.

4.3 Other ADP-antagonists

The three purinergic-receptors, $P2Y_{12}$, $P2Y_{1}$ and $P2X_{1}$, are potential targets for antiplatelet medication although $P2Y_{12}$-receptors antagonists have been the most studied (Table 4). Prodrugs ticlopidine and clopidogrel were the first ADP-receptor inhibitors in clinical use. Later, ARC compounds, group of ADP-receptor inhibitors, which are also active *in vitro*, were developed. Cyclopentyl-triazolo-pyrimidine AZD6140 was further developed from ARC compounds and is now being launched as an oral antiplatelet agent (see van Giezen & Humphries 2005).

$P2Y_{1}$-receptor antagonism has a beneficial effect in reducing thrombus formation and promising preliminary studies on animals with antagonising MRS compounds have been performed and further studies are in progress (Lenain et al 2003, Hechler et al 2006). $P2X_{1}$ has also been suggested as a potential target for antiplatelet medication, and a newly identified antagonist NF449 has an inhibitory effect on platelet activation *in vitro* (Kassack et al 2004, Hechler et al 2005).

Ticlopidine and prasugrel

Ticlopidine and clopidogrel are structurally related thienopyridines. Ticlopidine was the first $P2Y_{12}$-receptor antagonist used clinically. Plasma levels of ticlopidine increase on repeated twice-daily dosing over 2 to 3 weeks. As ticlopidine has lag in full onset of action is not useful when rapid antiplatelet effect is needed. Its efficacy has been shown in clinical studies as a single antiplatelet drug or in combination with aspirin in patients with different manifestations of atherothrombotic disease. However, ticlopidine therapy has been associated with several adverse effects, some severe (hypercholesterolemia, neutropenia, thrombocytopenia, aplastic anemia and thrombotic thrombocytopenic purpura). In comparison with ticlopidine clopidogrel has a favourable adverse effect profile and accelerated onset of action (see Patrono et al 2004).

Prasugrel, also known as CS-747 or LY640315, is a new thienopyridine compound. Prasugrel is orally administered and irreversible in action by its active metabolite R-138727 (Niitsu et al 2005). At present there are ongoing phase 3 studies comparing prasugrel with clopidogrel in patients undergoing PCI or with MI (Wiviott et al 2006). Previously it has been reported to be more potent and to have more rapid onset of action than clopidogrel (Wiviott et al 2005).

AR-C69931MX

AR-C69931MX (ARMX) is an ATP analogue, ARC compound and specific antagonist of the $P2Y_{12}$-receptor and unlike clopidogrel is active *in vitro* (see Storey 2001). *In vitro* the maximal effect of ARMX is achieved at a 100nM concentration (Goto et al 2002, Storey et al 2002). ARMX inhibits aggregation, granule secretion, P-selectin expression and procoagulant activity of platelets (see Storey 2001). After development into an intravenous antiplatelet agent it has been renamed cangrelor. It has been proposed in clinical use with acceptable efficacy and safety profile as well as reversible action and short half life (see Storey 2001, Jacobsson et al 2002, Greenbaum et al 2006). In previous studies ARMX has also been found to be more effective in inhibiting platelet activation than clopidogrel and its onset of action is rapid (Storey et al 2002). ARMX has been found also to inhibit the $P2Y_{13}$-receptor (Marteau et al 2003, see van Giezen & Humphries 2005).

Table 4. *Compounds targeted for inhibiting ADP-induced activation of platelets.*

Compounds	$P2Y_1$	$P2Y_{12}$	Reference
Antagonists			
A2P5P	+		Boyer et al 1996
A3P5P	+		Boyer et al 1996
A3P5PS	+		Boyer et al 1996
MRS-2179	+		Boyer et al 1998, Brown et al 2000
MRS-2279	+		Nandanan et al 2000
MRS-2500	+		Kim et al 2003, Cattaneo et al 2004
AR-C66096MX		+	Ingall et al 1999, Humphries et al 2000
AR-C67085MX		+	Ingall et al 1999, Humphries et al 2000
AR-C69931MX		+	Ingall et al 1999, Humphries et al 2000
C1330-7		+	Hollopeter et al 2001
INS compounds		+	
AZD6140		+	Peters & Robbie 2004, van Giezen & Humphries 2005
NF449		+	Kassack et al 2004
Inhibitors			
Ticlopidine		+	Savi & Herbert 2000
Clopidogrel		+	Savi & Herbert 2000, Savi et al 2000
CS-747		+	Sugidachi et al 2000, Niitsu et al 2005

Modified from Gachet (2001 and 2005).

4.4 Dipyridamole

Dipyridamole is a pyrimidopyrimide derivative. It has antiplatelet properties, but its mechanism of action is not exactly known. It inhibits cyclic nucleotide phosphodiesterase, which results in accumulation of its substrate, platelet-inhibiting cyclic adenosine 3′,5′- monophosphate (cAMP) (Smith & Mills 1970). Another suggestion is that dipyridamole blocks the uptake of adenosine, which would cause stimulation of adenylate cyclase and thus increase cAMP. In addition, direct stimulation of PGI_2 synthesis and inhibition of its degradation by dipyridamole have been shown (Moncada & Korbut 1978). Dipyridamole also has vasodilatory effects.

Conventional formulations of dipyridamole had variable bioavailability and thus a new modified-release formulation has been developed (see Patrono et al 2004). Clinical trials with the conventional form together with aspirin or dipyridamole alone have achieved questionable efficacy. However, trials using the reformulated drug have shown improved efficacy in patients with cerebrovascular manifestations of atherothrombotic disease. In both the ESPS2-study (1996) and recent ESPRIT study the combination of aspirin and dipyridamole was found to be superior to aspirin alone in preventing vascular events after ischemic stroke (Diener et al 1996, The ESPIRIT study group 2006). In ESPIRIT study the incidence of adverse events was 13% in combination group and 16% in patients with aspirin treatment (ESPIRIT 2006).

4.5 GP IIb/IIIa antagonists

As their name implies the GP IIb/IIIa antagonists inhibit the platelet GP IIb/IIIa receptor responsible for aggregation. GP IIb/IIIa antagonists inhibit platelet aggregation regardless of the platelet activating agent (Coller et al 1989). The GP IIb/IIIa antagonists include monoclonal antibodies against the receptor, naturally occurring and synthetic peptides, as well as peptidomimetic and nonpeptide mimetics that compete with receptor ligands (eg. fibrinogen, vWf). The safety and efficacy of different parenteral GP IIb/IIIa antagonists, abciximab, tirofiban and eptifibatide, have been proven in several clinical trials in patients with acute coronary syndromes undergoing PCI (see Casserly & Topol 2002). This group of antiplatelet medication has been shown to improve both long- and short- term outcome after PCI performed in both acute and stable settings. Benefit in patients with acute coronary syndrome who are not scheduled for early revascularization has not been shown, although in subgroup analyses in some studies diabetic patients seemed to benefit from parenteral GP IIb/IIa antagonists (Roffi et al 2001, see Patrono et al 2004).

Oral GP IIb/IIIa antagonists have also been developed (xemilofiban, orbofiban, sibrafiban and lotrafiban) but in trials of over 40 000 patients they were not more effective than aspirin nor caused additional benefit on top of aspirin and may in fact increase mortality (see Patrono et al 2004).

5 Variability in aspirin efficacy

5.1 Definition, prevalence and relevance of non-response to aspirin

Definition

Although aspirin has been shown to decrease atherothrombotic events in high risk patients a considerable number still suffer from cardiovascular events while on aspirin (ATC 2002). The response to aspirin treatment varies among individuals as measured by different platelet function tests and bleeding time (Buchanan et al 2000, Gum et al 2001 and 2003, Zimmermann et al 2003), and the term "aspirin resistance" has been used to describe lack of measurable antiplatelet effect in laboratory tests (see Patrono 2003). Recently, however, there have been efforts to define the term aspirin resistance more specifically as failure of aspirin to inhibit thromboxane A_2 (TxA_2) production or TxA_2 dependent platelet activation (see Cattaneo 2004, see Hankey & Eikelboom 2006), hence the terms *pharmacological aspirin resistance* to describe TxA_2 dependent failure of aspirin and *functional aspirin resistance* to describe increased platelet activity in platelet function tests despite aspirin (see Wong et al 2004). Previously the term *clinical aspirin resistance* has also been used for situations where atherothrombotic complications occur despite aspirin medication. However, the term *treatment failure* is more precise in these cases and that phenomenon can also be observed with other drugs. A feature of aspirin resistance highlighted in recent reviews is the presumable, continuous nature of the phenomenon instead of a dichotomized on-off nature (see Hankey & Eikelboom 2006). This is a natural assumption considering the variability of all natural phenomena as well as different drug responses.

The definition of aspirin resistance as failure of aspirin to inhibit TxA_2 production derives from aspirin´s mechanism of action. As aspirin is supposed to inhibit COX-1, which normally converts AA to the precursors of TxA_2 (prostaglandin G_2/H_2), it should prevent or significantly

decrease the formation of TxA_2. Thus, TxA_2 production has been measured from serum, plasma or urine with the help of its stable metabolites TxB_2 and 11-dehydro TxB_2 to diagnose aspirin resistance. In these cases, subjects have been defined as aspirin resistant if they have increased levels of plasma, serum or urine TxB_2 while on aspirin (see Patrono 2003). However, in patients with measurable Tx levels non-compliance to aspirin treatment needs to be ruled out. This type of aspirin resistance could be labeled *pharmacological aspirin resistance* according to Wong et al (see 2004).

The second, popular method of defining aspirin resistance, which is equivalent to *functional aspirin resistance according* to Wong et al (see 2004), has been the use of platelet function tests. Different platelet function tests, agonists and cut-off levels of defining aspirin resistance have been used. In previous studies platelet activation by ADP, thrombin and collagen have among other methods been used to determine aspirin resistance. Platelet aggregometry induced by AA has historically been proposed as the gold standard for defining aspirin resistance and is still the most widely used method (see Hankey & Eikelboom 2006). Due to the fact that AA induces aggregation only if it can be converted to Tx by COX-1, the aspirin resistance measured with AA-induced aggregation could in fact be labeled pharmacological resistance. In general, the definition of aspirin resistance is problematic due to the fact that a standardized, reproducible valid, specific, easy to perform and affordable method does not exist. The problems associated with the previously mentioned methods were discussed in section 3.

Three different types of aspirin resistance have also been proposed by Weber et al (2002). In patients with *pharmacokinetic resistance* oral aspirin medication does not inhibit platelet function or TxA_2 synthesis, but addition of *in vitro* aspirin results in inhibition of TxA_2 synthesis and platelet activation. This type could be caused or mimicked by inadequate intake or dose of aspirin, reduced absorption of the drug or increased platelet turnover. *Pharmacodynamic resistance* causes neither *in vivo* nor *in vitro* response to aspirin and would be caused at the cellular level by eg. extra-platelet sources of TxA_2 production, altered binding to COX-1 or genetic polymorphisms affecting COX enzymes. The third type, *pseudo-resistance*, manifests itself as lack of inhibition of platelet activity despite complete inhibition of TxA_2 formation, thus representing activation of platelets mediated by pathways other than TxA_2 (Weber et al 2002). Thus, pharmacokinetic and pseudo resistance would fall into the category of *functional aspirin resistance* according to Wong et al (see 2004).

Prevalence

Based on different studies the prevalence of aspirin resistance has been estimated to be 5.5-61% (Table 5) (see Hankey & Eikelboom 2006). Response to aspirin has been measured in patients with different manifestations of atherotrombotic disease and is poor in approximately 20%. The prevalence depends on the method used to determine aspirin resistance as well as the group of patients studied (see also section 3.5). In patients studied at the postoperative stage or with more severe or unstable disease the prevalence is increased compared to the stable patients (Zimmermann et al 2003, Payne et al 2004).

Table 5. *Prevalence of aspirin resistance reported in selected studies.*

Author	Location of symptomatic atherosclerosis	Sample size	Aspirin dose mg/day	Platelet analysis technique	Frequency %
Buchanan et al 2000	CAD, CABG	289	325	Bleeding time	55
Peters et al 2001	CAD, stable	19	100	PFA-100®	63
Macchi et al 2002	CAD, stable	72	160	PFA-100®	29
Andersen et al 2002	CAD, stable	129	75-160	PFA-100®	37
Christiaens et al 2002	CAD, stable	50	>75	PFA-100®	20
Hezard et al 2002	CAD, PCI	50	75-300	PFA-100®, optical aggr.(ADP)	52 19-38
Macchi et al 2003	CAD, stable	72	160	PFA-100®	29
Gum et al 2003	CAD, stable	326	325	PFA-100®, optical aggr.(AA, ADP)	9.5 5.5
Wang et al 2003	CAD, stable	422	81-325	Ultegra RPFA®	23
Chen et al 2004	CAD, elective PCI	151	80-325	Ultegra RPFA®	19
Cotter et al 2004	CAD, 6months post MI	82	100	TxB_2	12
Mueller et al 1997	PAD	100	100	Whole blood aggr. (ADP, collagen)	60
Ziegler et al 2002	PAD	52	100	PFA-100®	10
Grotemeyer et al 1993	CVD	180	1500	Platelet reactivity index	33
Helgason et al 1994	CVD	306	325	Optical aggr (AA, ADP, collagen, epinephrine)	25
Grundmann et al 2003	CVD	53	100	PFA-100®	34
Alberts et al 2004	CVD	129	< 162 162-325	PFA-100®	56 28
Sane et al 2002	CHF	88	325	Flow cytometry, aggr.(collagen, ADP)	57

Modified from Poulsen et al (2005).
aggr.=platelet aggregation, Ultegra RPFA®=Ultegra Rapid Platelet Function Assay®, PFA-100®=Platelet Function Analyser-100®, CAD=coronary artery disease, CVD=cerebrovascular disease, PAD=peripheral arterial disease, CHF=chronic heart failure.

Relevance

The relevance of poor aspirin response in platelet function tests and studies assessing aspirin resistant TxA_2 generation will become important only if these findings can be reliably proved to associate with increased risk of atherothrombotic events. Interestingly, poor response to aspirin has recently been associated with clinical end points of cardiovascular, cerebrovascular and peripheral arterial disease in small studies, which could predict existence of such phenomenon.

Grotemeyer et al (1993) studied 181 patients with previous stroke. The authors state that in 90% of acute stroke patients platelet activation is known to be increased, but can be inhibited by 500mg of aspirin. Platelet activity was studied 12 h after stroke and oral dose of 500mg aspirin. Patients who at that time had increased activity assessed with the platelet reactivity index had increased risk of atherothrombotic events during the 24-month follow-up period. The severe limitations of this study were that platelet function was assessed only at one time point and the method used was unspecific, thus it is unknown if the increased platelet activity was a consequence of limited response to aspirin or simply reflects significantly increased platelet activity that was perhaps decreased by aspirin to some degree. In addition, platelet activity is known to be increased in acute atherothrombotic settings and thus these settings represent an extreme challenge for antiplatelet medication. It is unknown whether the increased platelet activity at the time of measurement was the reason or consequence of acute thrombotic event (Grotemeyer et al 1993).

Mueller et al (1997) studied 100 patients with intermittent claudication who were on 100mg/d aspirin treatment. Patients who developed re-stenosis of the percutaneous balloon angioplasty site during the 18-month follow-up had increased platelet activation in function measurements by ADP- and collagen-induced platelet aggregation. In all patients AA-induced aggregation was inhibited by aspirin. As platelet aggregation induced by AA, rather than by other agonists, can be considered more specific to aspirin, the results reflect association of re-stenosis with generally increased platelet activity and not specifically with aspirin resistance.

In the study by Eikelboom et al (2002) the aspirin efficacy was assessed by measurement of urine TxB_2 in patients with atherothrombotic disease. In this study 488 patients who suffered from thrombotic complications during the 5-year follow-up period were compared with 488 matched controls. TxB_2 levels associated with the incidence of clinical events. However, the dose of aspirin was not specified and patient compliance was not controlled. In addition, possible drug interactions were not assessed. Despite the previously mentioned short comings of the study, this relatively large assessment was significant as one of the first studies proving a trend of association between aspirin resistance and clinical treatment failure.

Gum et al (2003) reported that aspirin resistance measured with AA-induced aggregation, but not with PFA-100®, was associated with increased risk of atherothrombotic events in patients with CAD during follow-up of 2 years. Aspirin resistant patients had a 4.1 risk ratio for death, MI or cerebrovascular accident compared to aspirin responders. This study has been criticized for the lack of a comparison group and thus for only descriptive study design. However, in this study the AA-induced aggregation, a quite specific measurement of aspirin function, was found to be associated with aspirin efficacy to prevent clinical endpoints.

Grundmann et al (2003) assessed the aspirin resistance of 53 patients with either coronary or cerebrovascular manifestations of atherothrombotic disease. Thirty-five of them had had acute cerebrovascular symptoms within 3 days and 18 had been asymptomatic for the previous 24 months. Aspirin resistance was defined as failure of aspirin to prolong closure time in PFA-100®. Symptomatic patients had increased prevalence of aspirin resistance compared to asymptomatic patients. The authors concluded that aspirin resistance defined by PFA-100® could contribute to clinical treatment failure. The study by Grundmann et al (2003) was confronted with similar limitations as the study by Grotemeyer et al (1993). These are also the same problems that affect most studies on aspirin resistance, including those described later in this section.

Andersen et al (2002) studied the antiplatelet efficacy of 160mg/d aspirin (N=73) and the combination of 74mg/d aspirin and warfarin (INR 2.8-4.2) (N=58) in patients with previous acute MI. Aspirin efficacy in both treatment groups was detected by PFA-100® with a cut off point of 196s. The patients were followed up for 4 years and no difference in occurrence of atherothrombotic events in non-responders compared to responders to aspirin was found (36% vs. 24%) (Andersen et al 2002).

Non-response to aspirin has also been assessed with Ultegra-RPFA® and found to be associated with elevated incidence of myonecrosis after coronary intervention, despite the additional inhibition by a loading dose of clopidogrel (Chen et al 2004).
Ajzenberg et al (2005) studied the platelet activity of patients with recent stent thrombosis right after PCI. They found that these patients had increased platelet activity compared to controls and healthy subjects, despite dual antiplatelet medication with aspirin and clopidogrel. The limitation of the study was that as the platelet activity in the patients was measured within days (mean 4.5 d) of the incident the platelet activity could be at least partly associated with the recent thrombotic event. Thus no definite conclusions can be drawn on the role of increased platelet activity as a factor contributing to the incidence of stent thrombosis based on this study.

Wenaweser et al (2005) studied 23 patients with stent thrombosis after PCI and compared them with 50 matched patients who had undergone PCI but not suffered from stent thrombosis as well as 9 healthy volunteers. In this small study aspirin resistance measured with AA-induced aggregation seemed more common but was not significantly so in patients who had suffered from stent thrombosis than in patients who had not (48% vs. 32%). However, when patients with stent thrombosis were compared with healthy volunteers the difference was evident (48% vs. 0%, P=0.01). Clopidogrel resistance measured with ADP-induced aggregation did not differ between the three groups.

Summary

Aspirin resistance measured with different methods has been associated with clinical endpoints in several small studies. Although larger, more reliable studies are needed it seems that association between increased platelet activity as well as true aspirin resistance measured with specific methods (AA-induced aggregation and TxB_2 generation) and increased risk of atherothrombotic endpoints exists. The current situation calls for exact determination of aspirin resistance and recommendations for its measurement. Larger, prospective studies using these definitions are also needed in the future to determine the relevance of aspirin resistance and mechanisms behind it.

5.2 Reasons for variability in aspirin efficacy

Aspirin resistance and the reasons behind it have recently been widely studied. Several extensive reviews have also been published (see Patrono 2003, Cattaneo 2004, Wong et al 2004, Rocca & Patrono 2005, Szczeklik et al 2005, Hankey & Eikelboom 2006, Wang et al 2006). The reasons for poor response to aspirin treatment can be roughly categorized to clinical, cellular and genetic reasons (Table 6).

Table 6. *Potential mechanisms of aspirin resistance – including both TxA_2-dependent and independent factors.*

Clinical factors	**Cellular factors**	**Genetic factors**
• Patient non-compliance	• Alternate pathways of TxA_2 production	• COX-1 polymorphisms
• Under-dosing	- Insufficient suppression of COX-1	• GP IIb/IIIa receptor polymorphism
• Drug-drug interactions	- Over-expression of COX-2	• Collagen receptor polymorphism
• Non-absorption	- Extra-platelet sources of TxA_2	• vWf receptor polymorphisms
• Acute atherothrombotic syndrome	- Generation of 8-iso-$PGF_{2\alpha}$	
• Increased platelet turnover	• Alternate pathways of platelet activation	
• Hypercholesterolemia	- Up-regulation of TxA_2-independent pathways (thrombin, ADP, collagen)	
• Hyperglycaemia/ diabetes	- Failure to inhibit catecholamine-mediated platelet activation	
• Gender	- Erythrocyte-induced platelet activation	
• Tolerance	• Resolvins	

Modified from Bhatt (2005) and Wang et al (2006).

Clinical factors

Poor compliance to aspirin has been suggested to explain aspirin resistance. It has been reported that up to 40% of patients with cardiovascular disease have poor compliance to antiplatelet treatment (Carney et al 1998). Others have reported frequencies between 9 and

16% (Cotter et al 2004, Schwartz et al 2005). In a recent study 3% of patients undergoing PCI were found non-compliant to aspirin despite oral assurance (Tantry et al 2005). However, poor compliance does not explain the entire phenomenon as in a study by Grundmann et al (2003) 34% of patients with cerebrovascular disease were found to be resistant to aspirin despite supervised aspirin dosing. Underdosing has also been proposed as a mechanism of aspirin resistance. However, data on the subject is conflicting. Drug-interactions with non-steroidal anti-inflammatory drugs (NSAID), especially ibuprofen, have been reported and could thus partly explain poor response to aspirin (Catella Lawson et al 2001). Inadequate systemic bioavailability in the case of aspirin cannot be explained by metabolic differences as most of aspirin's effects take place in presystemic circulation (see Hankey & Eikelboom 2006). However, hypotheses exist on decreased absorption of aspirin in stomach and upper intestine in relation to anti-acid medication. The formulation of the aspirin preparation has been suggested to affect absorption and thus aspirin efficacy (Maree et al 2005b). Indeed, in a study of 129 patients with cerebrovascular disease the use of enteric-coated aspirin correlated with an increased proportion of aspirin resistant patients when compared with uncoated preparation (Alberts et al 2004).

Different disease states, such as hyperglycemia, hypercholesterolemia, acute coronary syndromes and heart failure, have been associated with increased platelet activation or decreased aspirin efficacy (Fitzgerald et al 1986, Szczeklik et al 1996, Davi et al 1999, ATC 2002, Sane et al 2002, Friend et al 2003). Increased TxA_2 levels have been shown in patients with inflammation or atherosclerosis (Catella et al 1986, Ñakamura et al 2001). In addition, smoking has been associated with aspirin resistance, increased platelet activity and altered Tx metabolism (Lassila et al 1988, see Cambria-Kiely & Gandhi 2002, see Hankey & Eikelboom 2006).

Unexplained findings of changed response to aspirin after coronary artery or carotid surgery have been reported (Zimmermann et al 2003, Payne et al 2004). In patients whose aspirin response prior to surgery has been normal, postoperative Tx formation and enhanced platelet activation have been detected despite addition of *in vitro* aspirin (Zimmermann et al 2003). Increased platelet turnover has been suggested to explain part of the phenomenon, but it does not explain the lack of effect of additional *in vitro* aspirin dosing. Possibly increased thrombin activity or catecholamine surge in the preoperative phase could counteract the phenomenon of enhanced platelet activation despite aspirin.

Loss of the antiplatelet effect with long-term use, a phenomenon called tolerance, has been detected in patients using aspirin. Patients initially responding well to aspirin develop decreased response during follow up (Helgason et al 1994, Pulcinelli et al 2004). The reasons for this phenomenon have been postulated to be progressing atherosclerosis and reduction of compliance over time (see Hankey & Eikelboom 2006).

Cellular factors

It can be hypothesized that non-platelet sources of TxA_2, such as endothelial cells, macrophages, monocytes and the kidneys, might be a reason for aspirin resistance. Aspirin resistant Tx formation could result from extra-platelet sources of TxA_2, transient expression of COX-2 and generation of 8-iso-$PGF_{2\alpha}$ (Weber et al 1999, Cipollone et al 2000).

Platelet activation via pathways which are not TxA_2-dependent has been postulated to be behind aspirin resistance, which could be labelled *functional aspirin resistance*. Platelets

can also be activated by other agonists and factors such as collagen (GP Ia/IIa, GP VI), shear stress and vWf (GP Ib/V/IX), ADP (ADP-receptors), thrombin and epinephrine. More enhanced responses to agonists such as collagen and ADP has been found in aspirin resistant patients than in aspirin responders (Kawasaki et al 2000, Macchi et al 2003). Increased levels of catecholamines, especially in stressful situations with increased adrenergic surge, could activate platelets and decrease response to aspirin (Hjemdahl et al 1994, Mustonen & Lassila 1996, Christiaens et al 2002).

Interactions with platelets and erythrocytes contribute to platelet activity. Presence of erythrocytes has been shown to induce TxA_2 formation and the release reaction of platelets (Santos et al 1991, Valles et al 1991). Santos et al (1997) described that aspirin initially decreased the erythrocyte-induced enhancement of platelet activity but after 2-3 weeks this inhibition was lost.

Resolvins, a family of bioactive omega-3 fatty acid metabolites, have anti-inflammatory properties and their generation is enhanced by COX-2 acetylation by aspirin, thus offering an explanation for aspirin's anti-inflammatory properties (Serhan et al 2002, Arita et al 2005). It has been suggested that deficiency of these products could influence the therapeutic failure of aspirin (see Wang et al 2006).

Genetic factors

Mutations or polymorphisms of genes coding for COX-1, different factors of Tx synthesis, GP receptors, vWf receptor and the $P2Y_1$-receptor have been suggested to explain aspirin resistance at least partially (see Hankey & Eikelboom 2006).

Summary

Different clinical, cellular and genetic factors have been suggested to explain variability in responses to aspirin medication (Table 6). Larger studies on clinical relevance of aspirin resistance measured with platelet function tests are to be performed to establish its clinical relevance. However, as atherothrombotic disease is the leading cause of mortality in developed countries it is a phenomenon that might prove to have widespread consequences.

5.3 Dosing

Aspirin has been shown to be effective as an antiplatelet agent at low doses between 50 and 100mg/d. Low-dose aspirin has been shown to reduce MI or death in patients with unstable and chronic angina, reduce stroke and death in patients with cerebrovascular manifestations of atherothrombotic disease, prevent thrombotic complication in patients with peripheral arterial disease as well as reduce post-operative thrombotic complications in patients undergoing carotid endarterectomy (see Patrono et al 2004).

Even low-dose aspirin has been shown to inhibit the COX-1 enzyme by over 95% and this effect has been proven stable over time (Roth et al 1975, Patrignani et al 1982, Fitzgerald et al 1983). As a low-dose of 80mg/d has been shown to inhibit serum Tx levels by 95%, an additional benefit was found with a 160mg dose increasing the inhibition of Tx to 99% (Cerletti et al 2003). Theoretically, higher doses could be beneficial as the effects of aspirin unrelated to TxA_2 have been shown to be dose dependent (see Patrono et al 2004). However,

these mechanisms are not exactly understood and are believed to be much less important than inhibition of COX-1 (see Patrono et al 2004).

The variable extent of platelet inhibition has been observed with different aspirin doses when measured with platelet function test. An increase from <100mg/d to >300mg/d causes enhancement of the antiplatelet effect (Toghi et al 1992, Hart et al 2003, Lee et al 2005).

Doses between 300-1200mg/d in patients with cerebrovascular disease have been shown to be clinically effective and no difference was found between varying doses (Farrel et al 1991). Similar results were found in another study including patients with previous TIA or stroke and comparing doses of 30 and 283mg/d and patients with unstable angina or undergoing CABG with doses of 100-1300mg (Dutch TIA study group 1991, see Patrono et al 2004). In patients undergoing carotid endarterectomy lower doses of 81-325mg/ were superior to 650-1300mg/d (Taylor et al 1999).

In a meta analysis by the Antithrombotic Trialist´s Collaboration low doses of aspirin seemed as effective as higher doses (ATC 2002) (Fig 10). In addition, there is evidence that doses of approximately 300m/d produce fewer GI side effects than those of 1200mg/d (Farrel et al 1991). However, conflicting data exist on the differences in incidence of adverse effects with different aspirin doses (Derry & Loke 2000).

Nonetheless, inadequate dosing has been proposed as the reason behind decreased response to aspirin in some patients. It has been suggested that increasing the daily aspirin dose of aspirin resistant patients could offer a solution for the problem. Indeed, several authors have reported that stepwise increases in aspirin dose could overcome a diminished response to aspirin (Helgason et al 1994, Cerletti et al 2003).

Summary

At the population level increasing the dose of aspirin does not seem to increase its clinical benefits. However, it is possible that in some individuals with decreased response to aspirin higher doses might be beneficial. Nevertheless, further larger intervention studies are needed before definite conclusions can be drawn.

5.4 Non-platelet sources of TxA_2

Increased levels of TxA_2, and thus the possibility of platelet activation despite aspirin medication, have been detected in aspirin resistant patients. As aspirin inhibits the COX-1 enzyme responsible for TxA_2 synthesis in platelets non-platelet sources of TxA_2 have been suggested to be behind the phenomenon. In fact, TxA_2 can also be produced by circulating monocytes, endothelial cells and macrophages within atherosclerotic plaques (Ziegler et al 2004). AA can be converted to prostaglandin G_2/H_2 by COX-2 and is then converted to TxA_2 by thromboxane synthase, which is present in high quantities in monocytes and macrophages. This reaction has been suggested to be pronounced in inflammatory states, where COX-2 production has been upregulated, eg. atherosclerosis (Ziegler et al 2004). Normally, COX-2 is undetectable, but it is induced by cytokines, endotoxins, growth promoters and tumor promoters and can be expressed in different tissues including atherosclerotic vessels (Baker et al 1999).

Other factors suggested possibly to affect aspirin independent TxA_2 synthesis are oxygen free radicals as they catalyze the formation of F_2-isoprostanes from AA (Patrono et al 2005). Increased levels of F_2-isoprostanes, which are prostaglandin F_2-like compounds, have been found in smokers and patients with CAD, hypercholesterolemia and diabetes (Morrow et al 1995, Davi et al 1997 and 1999, Cipollone et al 2000). In addition, platelet activation is amplified by increased levels of F_2-isoprostanes (Cipollone et al 2000). F_2-isoprostanes, especially 8-iso-prostaglandin$F_{2\alpha}$, have been suggested to act as precursors for Tx and be formed by mechanisms catalysed by thromboxane synthase. The formation of F_2-isoprostanes from AA by oxygen free radicals is independent of COX-1, thus bypassing the inhibitory effect of aspirin (Cipollone et al 2000).

Summary

TxA_2 can be produced by monocytes, macrophages and endothelial cells via COX-2 and induce platelet activity. F_2-isoprostanes, which are generated via a non-enzymatic pathway from AA, contribute to the formation of TxA_2 and in addition they possess platelet activating properties. Thus, extra-platelet sources of TxA_2 are potential contributing factors to aspirin resistance measured as inability of aspirin to block Tx formation.

5.5 Expression of COX isoenzymes

Earlier assumption in aspirin's pharmacodynamics is that COX-1, not COX-2, is present in platelets and as platelets are anucleated they cannot produce new COX-1 when this enzyme is irreversibly inhibited by aspirin. However, in later studies COX-2 has been found to be present to some extent also in platelets and megakaryocytes, thus enabling the formation of TxA_2 in aspirin treated platelets (Weber et al 1999, Rocca B et al 2002). This transient expression of COX-2 causes proportional increases in platelets containing COX-2 in situations of increased platelet turnover, such as surgery, inflammation and bleeding.

A variant of COX-1, "COX-3", has been described. It is encoded by the COX-1 gene and has been detected in canine heart, aorta and brain (Chadrasekharan et al 2002). As it is more potently inhibited by aspirin than is COX-1, it seems an unlikely candidate for aspirin-resistant COX activity (see Szczeklik et al 2005).

Summary

COX-2 can be found in newly formed platelets and could contribute to aspirin resistance by enabling COX-1 independent TxA_2 formation. Inadequate COX-1 inhibition has also been suggested to explain aspirin resistance; this could be caused by inadequate aspirin dosing or increased expression of COX-1 possibly caused by genetic differences.

5.6 Diabetes

Platelet hyper-reactivity has been reported in patients with diabetes. There are also reports that aspirin is not as effective in diabetics as in non diabetics (ATC 2002). It has been suggested that cross talk between circulating inflammatory mediators, the vessel wall and platelets may contribute to the failure of aspirin to adequately prevent cardiovascular events in diabetic patients.

Diabetic patients have been reported to have decreased aspirin efficacy in both *in vitro* studies as well as large clinical trials (Davi et al 1999, ATC 2002, Sacco et al 2003). However, negative findings have also been published (Albert et al 2005). Different reasons for decreased sensitivity to aspirin in diabetic patients have been proposed. As increased platelet activity and decreased response to aspirin have also been reported in patients with hypercholesterolemia, it has been suggested that metabolic differences in diabetes and metabolic syndrome might explain decreased aspirin efficacy in diabetics (Meade & Brennan 2000, Friend et al 2003). Poor response to aspirin in diabetic patients associates with decreased levels of high density lipoproteins (HDL) or increased levels of low-density lipoproteins (LDL) and total cholesterol (Sacco et al 2003, Watala et al 2004, Mehta et al 2006). In concordance with metabolic syndrome as an explaining factor, obesity and insulin resistance have been associated with increased platelet reactivity and decreased aspirin efficacy (Westerbacka et al 2002, Tamminen et al 2003). The exact mechanism by which modification of cholesterol metabolism would affect aspirin efficacy and increase platelet activity are unknown.

Diabetic patients have increased levels of 8-iso-prostaglandin$F_{2\alpha}$ which could explain decreased aspirin response (Davi et al 1999, Mezzetti et al 2000). This was discussed more extensively in section 5.4.

Increased levels of HbA_{1c} and protein glycation in general associate with decreased aspirin efficacy in diabetics (Sacco et al 2003, Watala et al 2004 and 2005). It has been suggested that glucose and aspirin compete with each other in the course of non-enzymatic modifications and thereby increased glucose levels in blood decrease aspirin efficacy (Watala et al 2005). Thus, it has been suggested that increased aspirin doses would improve its efficacy especially in diabetics (Abaci et al 2005).

Endothelial dysfunction and inflammation as well as increased oxidative stress in diabetics cause progression of atherosclerosis and increase thrombogenity, both of which might explain decreased aspirin efficacy (Roffi & Topol 2004). Hyperglycaemia, hyperinsulinemia and increased oxidative stress induce inflammatory reactions by increasing expression of leukocyte and endothelial cell adhesive molecules and proinflammatory cytokines (De Vriese et al 2000, Marfella et al 2000, Biondi-Zoccai et al 2003). Insulin has been shown to down-regulate platelet activation and aggregation, an effect that is absent in insulin-resistant obese subjects, thus insulin resistance could be responsible for increased platelet reactivity (Trovati et al 1988, Westerbacka et al 2002, Tamminen et al 2003).

Circulating thrombogenic factors such as vWF, tissue factor, factors VII and VIII as well as plasminogen activator inhibitor are elevated in patients with diabetes (Bazzan et al 1998, Rao et al 1999, Diamant et al 2002, Biondi-Zoccai et al 2003). In diabetics platelet function, including procoagulation, is upregulated and levels of microparticles are increased (Davi et al 1989, Konieczkowski & Skrinska 2001).

Increased levels of inflammatory marker CD40 ligand have been shown to predict cardiovascular risk and are elevated in diabetic patients (Varo et al 2003). Platelets are the primary source of CD40, which is a ligand for GP IIb/IIIa receptor (Henn et al 1998, Prasad et al 2003). CD40 ligand is a proinflammatory cytokine which mediates thrombosis by inducing tissue factor expression. It has been shown to stabilize arterial thrombi in an *in vivo* model (Andre et al 2002). CD40L also has pro-atherosclerotic functions by mediating generation of reactive

oxygen species (ROS), generation and expression of adhesive molecules, macrophage chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) (Henn et al 1998, Miller et al 1998, Urbich et al 2002).

Summary

Increased platelet activity and non-response to aspirin has been reported in insulin-resistant patients. Enhanced COX-independent 8-iso-prostaglandin$F_{2\alpha}$ excretion and subsequent Tx formation together with circulating inflammatory mediators and high glucose levels have been suggested as the underlying mechanisms. Enhanced microparticle formation and shedding have been detected in atherosclerosis and inflammation and might contribute to increased COX-2 expression, platelet activity and aspirin resistance in patients with diabetes (Barry et al 1997 & 1998).

5.7 Genotype

Epidemiological studies have suggested that as much as one third of the variation in efficacy of antiplatelet drugs could be explained by genotype (O'Donnell et al 2001).

The association of aspirin resistance with genotypic differences of COX-1 and different platelet receptors have been studied. In addition, hundreds of single nucleotide polymophisms (SNP´s) of genes involved in the Tx biosynthetic pathway have been identified (see Hankey & Eikelboom 2006). However, there is no data available on their role in aspirin resistance.

To date, 14 COX-1 gene polymorphisms have been identified (Halushka et al 2003, Hillarp et al 2003, Maree et al 2005a). Ten of these are in exons, seven of which predict amino acid substitutions. Previous reports have suggested a correlation between COX-1 gene polymorphisms and aspirin resistance (Halushka et al 2003, Maree et al 2005a).

GP IIb/IIIa is responsible for aggregation of platelets and becomes active after initial activation of platelets. Aspirin has also been postulated to influence GP IIb/IIIa by interfering with COX-1 dependent signaling events. Interestingly, PLA1/A2 polymorphism of GP IIb/IIIa has been associated with aspirin resistance as well as increased risk for atherothrombotic complications (Weiss et al 1996, Walter et al 1997, Andrioli et al 2000, Szczeklik et al 2000). GP Ib, GP Ia and GP VI polymorphisms have also been associated with increased risk for MI (Gonzalez-Conejero et al 1998, Mikkelsson 1999 & 2001, Moshfegh et al 1999, Croft et al 2001, Ollikainen et al 2004). $P2Y_1$-receptor polymorphism has been associated with aspirin resistance by mechanisms not exactly known (Jefferson et al 2005).

Summary

Genetic variance of COX-1 and platelet GP receptors could partly explain aspirin resistance.

5.8 Gender

It is know that males are at 2-3 times higher risk for CAD compared to females. Exact reasons for this are unknown, but known differences can be divided into those related to the hormonal milieu and those that cannot be explained by hormonal differences (Levin 2005).

In recent meta-analysis of studies of aspirin efficacy in the prevention of atherothrombotic events sex differences were found. In prevention of MI aspirin exerted no effect in females, but the incidence of stroke was decreased with aspirin treatment. Findings in males were opposite as aspirin treatment caused decrease in MI, but did not affect incidence of stroke (Berger et al 2006). Similar results were also obtained in the Hypertension Optimal Therapy trial (Kjeldse et al 2000).

The frequency of aspirin resistance has been reported to be higher among females. In a study by Gum et al (2001) it was found that patients who had impaired aspirin efficacy determined by ADP- and AA-induced aggregation were more likely to be females compared with aspirin sensitive patients. Alberts et al (2004) found in their study of 129 patients with cerebrovascular disease that women and older people were less likely to achieve aspirin response measurable with PFA-100®.

Decreased aspirin efficacy in females has been shown in *in vitro* studies (Harrison & Weisblatt 1983, Escolar et al 1986, Spranger et al 1989). Reasons proposed to explain this phenomenon have included differences in metabolism of aspirin, platelet-subendothelium interaction, testosterone and estrogen levels as well as levels of anti-inflammatory mediator and the possible Tx generation-regulator 15-epi-lipoxin A4 (Escolar et al 1986, Spranger et al 1989, Durand & Blache 1996, Chiang et al 2006).

On the other hand, females have been shown to have enhanced platelet activity compared to males (Johnson et al 1975, Faraday et al 1997, Becker et al 2006). This might also partially explain increased frequency of aspirin resistance determined by platelet function tests in females. The finding implies that in females even though platelets are inhibited by aspirin, the inhibition is inadequate due to significantly increased basal platelet activity. This hypothesis is suggested by a study of 571 men and 711 women whose platelet function was assessed by PFA-100® and platelet aggregation induced by AA, ADP, and epinephrine. It was found that basal platelet activity was increased in females, but they had better response to aspirin than males when measured with aspirin specific AA-induced aggregation. Despite the aspirin efficacy in inhibiting AA-induced aggregation the general platelet activity of females measured with platelet function tests remained higher than that of males (Becker et al 2006).

Summary

Females have been reported to have increased platelet activity and decreased aspirin efficacy compared to males. However, data are scarce and conflicting reports have been published. The observations that aspirin resistance appears to be more frequent in females might be biased and as such at least partly explained by increased basal level of platelet activity.

5.9 Drug-drug interactions

Some NSAIDs have been shown to compete with aspirin for the serine residue of COX-1, thus preventing aspirin function (Catella-Lawson et al 2001). If NSAID with reversible action binds to COX-1 instead of irreversible aspirin, TxA_2 generation is suppressed momentarily, but is recovered within hours. In a study by Catella-Lawson et al (2001) it was shown that if aspirin is administered at least 2 h prior to ibuprofen TxA_2 generation remains inhibited for 24 h. However, if ibuprofen is administered prior to aspirin, or as often as three times per day, TxA_2 generation is inhibited reversibly.

Ibuprofen has been the most studied of the NSAIDs in relation to drug-interactions with aspirin and it seems to decrease its efficacy (Catella-Lawson et al 2001, Ray et al 2002, MacDonald & Wei 2003). Conflicting results have also been published (Kimmel et al 2004). Diclofenac, however, has not been shown to impede aspirin efficacy (Catella-Lawson et al 2001, MacDonald & Wei 2003).

NSAIDs had no cardio-protective effect in a large study by Ray et al (2002). In fact, the more extensive inhibition by COX-2 of some NSAIDs instead of selective inhibition of COX-1 has been postulated to increase the risk of atherothrombotic events as vasodilatory effects of prostaglandin I_2 are inhibited but TxA_2 formation is not (MacAdam et al 1999).

As aspirin is absorbed in the stomach and upper intestine some of it is hydrolysed to an inactive form by mucosal esterases. It has been postulated that use of protone pump inhibitors for acid suppression could cause enhanced hydrolysation and thus inactivation of aspirin, although conflicting data exist (Lichtenberger et al 1996, Iñarrea et al 2000).

Summary

Interaction between aspirin and ibuprofen seems likely on account of previous studies. A large, prospective, controlled trial is nevertheless needed, since clinical recommendations for avoidance of certain concomitant medication cannot be given based on current knowledge.

6 Variability in clopidogrel efficacy on top of ongoing aspirin treatment

6.1 Definition, prevalence and relevance of non-response to clopidogrel

Definition

In this study the term non-response to clopidogrel has been used to describe the inability of clopidogrel to cause the expected inhibition of platelet aggregation. After publication of the original articles presented here, the term clopidogrel resistance has become more established (see Cattaneo 2004, see Nguyen et al 2005, see Wang et al 2006). However, in the original articles we chose to use the less decisive term due to the continuous nature of the phenomenon. In the literature non-response and poor response to clopidogrel have been used as synonyms of clopidogrel resistance. It has been proposed that the term *clopidogrel resistance* would be used to describe the inability of clopidogrel to cause the expected platelet inhibition in laboratory measurements and the term *treatment failure* to describe failure of clopidogrel to prevent adverse clinical events (see Cattaneo 2004, see Nguyen et al 2005, see Wang et al 2006).

Clopidogrel non-response has been studied by several different methods. Nonetheless no uniform method has been established to determine non-response to clopidogrel. It is even argued that the existence of clopidogrel resistance as well as aspirin resistance is uncertain (see Cattaneo 2004). The general problem is that there is no effective, easily reproducible, reliable and easily executable method to define the phenomenon (see Cattaneo 2004, see Nguyen et al 2005, see Wang et al 2006). Platelet aggregation induced by ADP has been used widely, however its limitations are that it is labour-consuming, no uniform cut-off value has been established and the time chosen to measure platelet aggregation, agent used to anticoagulate

the blood samples as well as the concentration of the agonist used cause variation in results (Andre et al 2003, see Cattaneo 2004, see Nguyen et al 2005, see Wang et al 2006).

When defining clopidogrel resistance the cut-off levels of inhibition of ADP-induced platelets have varied between 0 and 40% in different studies (Gurbel et al 2004, Angiolillo 2004a). In addition, clopidogrel resistance has been defined not only by inhibition of ADP-induced aggregation, but also by GP IIb/IIIa receptor expression (Angiolillo et al 2004b, Gurbel et al 2004) and VASP phosphorylation (Schwarz et al 1999, Grossmann et al 2004, Aleil et al 2005). Additionally, expression of P-selectin, platelet/endothelial cell adhesion molecule-1 (PECAM-1) and vitronectin receptor as well as formation of platelet-monocyte aggregates have been used to study clopidogrel effect in flow cytometry (Serebruany et al 2005).

Prevalence

Järemo et al (2002), Gurbel et al (2003a) and Müller et al (2003b) were the first to report the phenomenon of clopidogrel resistance in patients with atherothrombotic disease. Järemö et al (2002) studied 18 patients with stable angina pectoris who were to undergo elective PCI, and found considerable interindividual variability in inhibition caused by 300mg clopidogrel loading doses administered after the procedure. Gurbel et al (2003a) assessed the clopidogrel efficacy in CAD patients undergoing PCI on ongoing aspirin treatment. They defined clopidogrel resistance as less than 10% decrease of 5 μM ADP-induced aggregation after clopidogrel administration when compared with baseline. By this criterion 31% of the patients were found to be clopidogrel resistant, but continuation of the medication decreased the number of clopidogrel resistant patients to 15%.

After the steady state in platelet inhibition has been achieved the phenomenon of clopidogrel non-response has been shown to be quite stable over time (Gurbel & Bliden 2003). Those patients who were non-responders after 300mg loading doses and 5 days of ongoing clopidogrel treatment 75mg/d remained so also after 30 days. Thus the clopidogrel efficacy did not change after 5 days, but remained at the same level.

Different studies using variable methods and dosing report the prevalence of clopidogrel resistance to vary between 4 and 55% in patients with CAD (Angiolillo et al 2004b & 2005a, Gurbel et al 2004, Grossman et al 2004, Wenaweser et al 2005). In larger study of 1001 CAD patients considered for PCI, the prevalence of non-response to clopidogrel was found to be one third (Hochholzer et al 2005). Similarly, 30 % prevalence has been reported by several other studies (Gurbel & Bliden 2003, Aleil et al 2005, Gurbel et al 2005a).

Müller et al (2003b) reported that 4 h after 600mg clopidogrel loading doses 5% or 11% of patients had less than 10% inhibition of their ADP-induced aggregations and were thus defined as non-responder to clopidogrel. The variation in prevalence or non-response was caused by different ADP (5 or 20μM) concentrations used to induce platelet aggregation. As the prevalence of non-responders reported by Müller et al was lower than in previous studies executed with loading doses of 300mg, it seemed that increasing the clopidogrel loading doses decreased the number of non-responders.

It has been pointed out that clopidogrel resistance is not an on-off phenomenon, but that the platelet inhibition follows a normal, bell-shaped distribution (Serebruany et al 2005). If clopidogrel resistance is defined as more than two standard deviations from the median, its

prevalence would be approximately 5%. This is naturally a fact that needs to be remembered when considering clopidogrel as well as aspirin efficacy.

Relevance

The purpose of studies of drug resistance is to understand the phenomenon of clinical treatment failure. If we understand the mechanisms behind treatment failure, it would be possible to prevent greater proportions of both thrombotic and bleeding events taking place during treatment with the antiplatelet medication of today. Even despite the dual antiplatelet regimen with aspirin and clopidogrel the incidence of adverse cardiac events after stent placement is 1.2-1.5% (Bertrand et al 2000). In some small studies clopidogrel resistance has been shown to be associated with atherothrombotic events. Comparable with this have been previous reports in relation to GP IIb/IIIa inhibitors and aspirin, where associations between measurable platelet inhibition and adverse clinical effects in patients undergoing PCI have been shown (Steinhubl et al 2001, Chen et al 2004).

Müller et al (2003b) studied the prevalence of non-responders to clopidogrel after increased loading doses of clopidogrel of 600mg on top of aspirin. Retrospective analysis of these patients revealed that the 5 patients of 105 who developed stent thrombosis were non-responders to clopidogrel according to ADP-induced aggregation. Thus the investigators speculated that an association between clinical outcomes and non-response measured by laboratory test existed. However, the study was small, conducted retrospectively and was not designed to assess the association of clinical endpoints with measurement of clopidogrel response.

Matetzky et al (2004) studied patients undergoing PCI and divided them into four quartiles according to the inhibition of ADP-induced aggregation by clopidogrel. They found that 40% of patients in the first quartile with lowest clopidogrel-induced platelet inhibition suffered from adverse cardiac events within the 6-month follow up period compared to 7% of patients in the second quartile and no patients in third and fourth quartiles. The study was quite small with only 60 patients, but the results were very interesting and among the first to show such correlation between clinical treatment failure and laboratory measurement of non-response to clopidogrel.

In another study of 1684 stented patients, 16 patients who suffered from subacute stent thrombosis were shown to have increased platelet activity despite combined aspirin and thienopyridine treatment compared to 30 control patients without stent thrombosis (Barragan et al 2003). However, in another study the platelet activity of 73 patients with CAD was assessed and no correlation between stent thrombosis and ADP-induced aggregation was found (Wenaweser et al 2005).

The incomplete correlation between clinical efficacy and ability of clopidogrel to inhibit platelet aggregation might be explained by other beneficial effects of clopidogrel. Other mechanisms besides $P2Y_{12}$-receptor inhibition might explain the discrepancy between inhibition of platelet activation and beneficial effects of clopidogrel, which are not always parallel. Clopidogrel causes decreased plasma tissue factor activity (Savi et al 1994a), endothelial passivation (Jacubowski et al 2005) vascular smooth cell effects (Wihlborg et al 2004), and anti-inflammatory effects, which are postulated to be related to effects on lymphocytes (Wang et al 2004).

Clopidogrel attenuates the post PCI increase of CRP and decreases the expression of activated-platelet-dependent inflammatory markers, especially CD40 ligand and CD62 P-selectin

(Savcic et al 1999, Quinn et al 2004, Vivekananthan et al 2004). CD40 ligand stimulates vascular inflammation, which causes platelet-leukocyte interactions and induces tissue factor expression. Clopidogrel has been shown to decrease platelet-leukocyte conjugate formation in CAD patients Xiao & Théroux 2004).

Despite contradictory findings the majority of studies imply that clopidogrel non-response measured with laboratory test correlates with clinical efficacy. However, these findings need to be reinforced in larger prospective study settings.

Summary

In most studies clopidogrel resistance has been defined as the inability of clopidogrel to inhibit ADP-induced aggregation. Recently more specific methods, such as VASP phosphorylation, have been introduced to measure clopidogrel efficacy. Clopidogrel resistance measured in laboratory studies has not yet reliably been shown to associate with clinical endpoints. Thus large, prospective studies are needed to establish such relationships.

6.2 Reasons for variability in clopidogrel efficacy

Clinical factors

Several mechanisms have been suggested to be behind non-response to clopidogrel (Table 7). Clinical mechanisms of non-response are caused mostly by inefficient availability of the active metabolite. This could be caused by inability to prescribe clopidogrel in a sufficient way or poor patient compliance. Patient compliance is an important factor in resistance to any drug, as described earlier in the case of aspirin. Drug-drug interactions, especially with atorvastatin, have been proposed as a significant cause behind decreased clopidogrel activity. Other factors shown to decrease the effect of clopidogrel are variability in hepatic cytochrome P450 3A4 activity and intestinal absorption of clopidogrel (Lau et al 2004, Taubert et al 2004).

Severity of atherothrombotic disease correlates with the clopidogrel efficacy. Patients with higher Braunwald angina scores had less inhibition of platelet aggregation by clopidogrel (Soffer et al 2003). Also, diabetes and high body mass index (BMI) have been suggested to modify the clopidogrel efficacy (Mehta et al 2001, Steinhubl et al 2002, Angiolillo et al 2004a and 2005b).

Cellular factors

Increased levels of ADP in clopidogrel non-responders have been suggested, but not very much studied. As ecto enzymes NTPDase-1 and -2 (CD39/ectoADPase and CD39L1/ecto ATPase, respectively) modulate platelet activation via deletion and formation of ADP they may have important roles in individual sensitivity to clopidogrel together with other regulators of thrombogenity (Table 8)(Marcus et al 2005, Robson et al 2005). As ATP, also regulated by ectonucleotidases, acts as an antagonist to platelet purinergic receptors (Hechler et al 1998b), variability in its quantity may affect individual responses to clopidogrel. Increased ADP receptor $P2Y_1$ function has been proposed to cause ADP-induced aggregations independent of $P2Y_{12}$-receptor inhibition. The $P2Y_1$-receptor can mediate transient platelet aggregation even in the absence of $P2Y_{12}$-receptor activation (Hechler et al 1998a). Thus, it has been postulated that variability in activity of $P2Y_1$-receptor could cause at least part of the clopidogrel resistance (see Nguyen et al 2005).

As platelets are activated via different pathways depending on the agonist, the inhibition of the ADP pathway by $P2Y_{12}$ antagonists leaves possibilities for the platelets to be activated by eg. collagen, Tx, catecholamines and especially the potent agonist thrombin, which will be further discussed later. Stress reactions and elevated levels of epinephrine, which are known to have synergistic effects on platelet activation with other agonists, may also interfere with platelet activity and response to medication (Hjemdahl 1994, Mustonen & Lassila 1996, Mustonen et al 2001). Epinephrine can also produce some features of $P2Y_{12}$ signalling by repressing cAMP levels through its α2A-receptor (see Conley & Delaney 2003). Thus, failure to inhibit catecholamine-mediated platelet activation could cause ADP-independent platelet aggregation.

Individual variability of $P2Y_{12}$-receptor density has been proposed as an explanation for variable response to clopidogrel. There is no available data on $P2Y_{12}$-receptor occupancy rate. Mills et al (1992) studied ADP-receptors found that the number of binding sites decreased 60% after clopidogrel treatment. In a review article Nguyen et al (see 2005) postulated that the results of Mills et al reflect that the surplus 40% of the receptors consist of other ADP-receptors such as $P2Y_1$ and P2X1 as well as the $P2Y_{12}$-receptors left uninhibited by clopidogrel. The mean number of $P2Y_{12}$-receptors per platelet would be approximately >300 molecules. Differences in amounts of $P2Y_{12}$-receptors could possibly cause clopidogrel non-response, especially if the doses are barely adequate. Defects in signalling pathways downstream from the receptor are also plausible mechanisms for drug resistance.

Table 7. *Potential mechanisms of non-response to clopidogrel.*

Clinical factors	**Cellular factors**	**Genetic factors**
• Patient non-compliance	• Alternate pathways of platelet activation	• $P2Y_{12}$-receptor polymorphisms
• Under-dosing	- Up-regulation of $P2Y_{12}$ -independent pathways (thrombin, TxA_2, collagen)	• $P2Y_1$-receptor polymorphisms
• Drug-drug interactions	- Failure to inhibit catecholamine-mediated platelet activation	• CYP3As polymorphisms
• Variable absorption	- Greater extent of $P2Y_1$ -dependent platelet aggregation	
• Body mass index	• Increased release of ADP	
• Insulin resistance	• Interindividual differences in $P2Y_{12}$ -receptors or in their number	
• Severity of atherothrombotic disease	• Defect in signalling pathways downstream from receptor	

Modified from Nguyen et al 2005 and Wang et al 2006

Genetic factors

Intrinsic mechanisms of clopidogrel non-response are postulated to be caused by individual variations in genotype and platelet activation pathways. Genotypes of the $P2Y_{12}$-receptor, the target of clopidogrel, and of liver CYP3A enzymes responsible for metabolism of clopidogrel have been suggested as explanations for decreased clopidogrel response (Fontana 2003b, Lau 2003).

Summary

Several clinical, cellular, metabolic and genetic factors have been suggested to influence the variability in clopidogrel efficacy and are summarized in Table 7.

6.3 Clopidogrel dosing – loading, maintenance and timing of pre-treatment

Dosage – loading

The commonly used dose of once-daily 75mg clopidogrel was originally chosen since it inhibited platelet aggregation to an equal extent as twice-daily 250mg ticlopidine, a previously used ADP-receptor antagonist (CAPRIE 1996). Later, loading doses of 300mg were introduced for patients undergoing PCI as the steady state of antiplatelet efficacy with 75mg/d clopidogrel took several days to be achieved (Steinhubl et al 2002). After several larger studies the 300mg loading dose became the standard (CURE 2001, Steinhubl et al 2002). As non-responsiveness to clopidogrel became of interest later, inadequate dosing became one of the reasons proposed for deficient platelet inhibition.

Higher loading doses of clopidogrel, 375, 450, 600 and 900mg, have also been studied. The loading dose most commonly compared with 300mg has been 600mg. The higher loading has increased efficacy in inhibiting platelet aggregation (Müller et al 2001, Gurbel et al 2005b). Decreased α-degranulation, GP IIb/IIIa activation and p-selectin levels have also been shown after the higher loading dose (Müller et al 2001, Seyfarth et al 2002, Angiolillo et al 2004b). In addition, the 600mg dose has a similar safety profile with the commonly used 300mg loading dose (Kastrati et al 2004, Hochholzer et al 2005, Patti et al 2005).

A 900mg loading dose has not been shown to be superior to 600mg, since limitations in clopidogrel absorption seem to block further appearance of the active metabolite in blood (Beckerath et al 2005b). The challenge for future research is to find a dose that would be at the same time effective and safe so that patients would not be exposed to bleeding complications.

Inefficient dosing has been proposed as a reason for limited response to clopidogrel and indeed, increased loading doses have been found to decrease the number of non-responders to clopidogrel (Gurbel et al 2005a). After 600mg loading doses of clopidogrel Müller et al (2003b) reported the prevalence of non-response to clopidogrel to be 5% compared with 30% prevalence reported after 300mg loading doses by others (Gurbel et al 2003a).

Angiolillo et al (2004b) found that large interindividual variation of platelet responses were not reduced by increased loading dose. However, at the same time they reported that the proportion of responders to clopidogrel increased in parallel with elevated dosage. Large variability in platelet activity even after 600mg was also seen by Hochholzer et al (2005) in a larger study of over 1000 patients, in which platelet activity was measured with receptor surface expression and platelet aggregation.

Clopidogrel resistance in the above mentioned studies has been defined as the inability to cause an antiplatelet effect measurable in a laboratory setting. Decreased inhibition of ADP-induced aggregation by clopidogrel has also been shown to correlate with incidence of recurrent cardiovascular events during a 6-month follow-up in relatively small study (N=60) by Matetzky et al (2004). In the clinical setting of the prospective, randomized ARMYDA-2 trial (N=255) the efficacy of 600mg clopidogrel loading dose was demonstrated to be superior to 300mg in relation to clinical endpoints (Patti et al 2005). In that trial, higher loading doses of clopidogrel reduced the risk of MI by 50%.

Conflicting results have been reported by Wolfram et al (2006) who found no difference in adverse cardiac events within 30 days of PCI in 445 patients receiving either 300 or 600mg loading doses of clopidogrel. Although this was a larger study than ARMYDA-2 it was conducted retrospectively from patient records and is therefore not directly comparable, and patient groups were also more variable.

Dosage – maintenance

In healthy individuals when daily doses of 25, 50, 75, 100 and 150mg clopidogrel were studied the plateau response was reached at 75mg dose (Thebault et al 1999b). However, supplementary 600mg clopidogrel loading doses in patients with ongoing, long-lasting treatment with 75mg/d clopidogrel has been shown to enhance the antiplatelet effect (Kastrati et al 2004). This suggests that increasing the maintenance dose could result in more effective platelet inhibition by clopidogrel.

The data comparing different clopidogrel maintenance regimens in patients with atherothrombotic disease are sparse. Müller et al (2001) studied the efficacy of both increased loading and maintenance dosing in a small population of patients undergoing PCI. A group of patients (N=10) received 300mg loading doses and 75mg/d maintenance doses and second group of patients (N=10) 600mg loading dose followed by 150mg/d maintenance dose. The platelet aggregation induced by ADP and platelet α-degranulation was measured. Significant improvement of efficacy was found with the higher loading dose as previously described, but after 48h of treatment with the higher maintenance dose (150mg/d) the platelet activation seemed to remain at a lower level when compared with the other group. Due to the short study period, it cannot positively be concluded whether this diminished platelet activity at 48 h was due to the initial higher loading dose or the increased maintenance dose.

Timing of pre-treatment

In healthy volunteers treatment with 75mg has been shown to reduce ADP-induced aggregation within 2 h (Savcic et al 1999, Thebault et al 1999a). However, it takes 3 to 7 days for maximal platelet inhibition to be achieved (Thebault et al 1999b). Therefore, loading doses of 300-900mg clopidogrel have been introduced.

Inhibition by loading dose of clopidogrel (300mg) has been shown to decrease platelet aggregation in 2-3 h after administration in healthy volunteers and patients (Savcic et al 1999, Helft et al 2000, Gurbel et al 2003b). However, the primary analyses of the clinical CREDO trial suggested only >6 h pre-treatment to be effective in decreasing adverse effects (Steinhubl et al 2002). In further analyses of the data the preferable timing for the 300mg loading dose was found to be over 12h (Steinhubl et al 2006).

Inhibition of platelet activity seems to be achieved more rapidly with 600mg loading doses compared to 300mg clopidogrel (Gurbel et al 2005b, Angiolillo et al 2004b). In line with this Hochholzer et al (2005) claimed that the full antiplatelet effect of 600mg measured by platelet aggregation as well as levels of release of p-selectin and expression of activated GP IIb/IIIa is achieved 2 h after administration and no further effect was seen during 10 h of monitoring. In correlation with that in the ISAR-REACT study a 600mg loading dose seemed to achieve its potential within 2h in decreasing the adverse post-PCI events, as no difference was found between groups receiving the pre-treatment 2->12h prior to procedures (Kandzari et al 2004). However, further studies are needed to confirm these results.

Summary

The previously discussed studies imply that inadequate dosing might explain some of the non-response to clopidogrel, but large variability in platelet activity remains. Increased loading doses of 600mg seemed generally beneficial, but its effect on non-response to clopidogrel remains unclear. Larger doses of clopidogrel (900mg) have not been found to cause further advantage. Studies of larger maintenance doses also remain to be carried out. As the onset of action of clopidogrel takes at least 2h even a with high loading dose, platelet GP IIb/IIIa inhibitors continue to play an important role in acute atherothrombotic situations in addition to utilization in patients with limited response to dual antiplatelet medication.

6.4 CYP 450 and drug-drug interactions

Clopidogrel is an inactive prodrug which requires *in vivo* conversion by liver enzymes to an active metabolite. The cytochrome P450 (CYP) 3A system is responsible for the majority of clopidogrel metabolism. Especially CYP 3A4 and to lesser extent CYP 3A5 have major roles, but also CYP 2B6, CYP 2C19, CYP 2C9 and CYP 1A2 have been suggested to take part in converting clopidogrel to its active form (Savi et al 1994b, Richter et al 2004, Turpeinen et al 2005).

Due to the large number of CYP enzymes involved in metabolism of clopidogrel there is an increased risk for interactions with concomitant medication in patients with atherothrombotic disease. This might partly explain the large individual variability of clopidogrel responses.

Other drugs metabolized by CYP 3A4 have been suggested as possible competitors for the formation of the active metabolite. Thus far atorvastatin has been the only drug more extensively studied in relation to drug-drug interactions. Lau et al (2003) showed an association between atorvastatin treatment and reduced platelet inhibition by clopidogrel in 44 patients undergoing PCI. They also found that other inhibitors of CYP 3A4 decreased the clopidogrel effect as the cytochrome inducer rifampisin enhanced inhibitory effects of clopidogrel on platelet activation (Lau et al 2003 and 2004). Two *in vitro* studies have confirmed these results (Clarke & Waskell 2003, Neubauer et al 2003) However, several other *in vitro* studies have been unable to identify significant interaction between clopidogrel and atorvastatin (Serebruany et al 2001, Müller et al 2003a, Gorchakova et al 2004, Mitsios et al 2004, Piorkowski et al 2004, Serebruany et al 2004, Smith et al 2004, Vinholt et al 2005).

The effects of possible drug-drug interactions have also been retrospectively assessed in two clinical studies. Post hoc analyses of the CREDO trial showed no difference between clinical clopidogrel efficacy in patients receiving either CYP 3A4 or non-CYP 3A4 metabolized statins

(Saw et al 2003). Primary end points of clopidogrel treatment at 28 days and 1 year in this study were death, MI or stroke. However, as a limitation of the study the patient distribution was uneven as only 158 patients received non-CYP 3A4 metabolized statins compared with 1001 patients who received CYP 3A4 metabolized statins. Although not significant, the incidence of adverse effects was smaller in the group with non-CYP 3A4 metabolized statins. In retrospective analyses of the Maximal Individual Therapy of Acute Myocardial PLUS (MITRA PLUS) registry no association between statin type and clopidogrel efficacy was found (Wienbergen et al 2003). In patients with coronary syndromes with clopidogrel treatment 833 patients also had atorvastatin and 1203 patients had other statins (simvastatin, pravastatin, cerivasatin, lovastatin, fluvastatin) at the time of discharge. Between these groups there were no differences in atherothrombotic endpoints during follow up. However, this study is limited by the fact that no distinctions were made between CYP 3A4 metabolised statins and those metabolised via other CYP enzymes.

A recent prospective study of 1651 patients with coronary syndromes was unable to find clopidogrel-CYP 3A4 statin interaction (Mukherjee et al 2005). The use of a combination of statin and clopidogrel was associated with lower 6 month incidence of adverse effects than either clopidogrel or statin alone. There were no significant differences in event rates of patients when clopidogrel was combined with either CYP 3A4 metabolised or non-CYP 3A4 metabolized statin.

In the PROVE-IT study (Pravastatin or Atorvastatin Evaluation and Infection Therapy) high dose atorvastatin was proven to be superior to standard dose pravastatin in patients of whom most (72%) had also clopidogrel or ticlopidine therapy (Cannon et al 2004). In yet another study of 1001 patients no correlation between clopidogrel efficacy (loading dose 600mg) and statins was found, neither were there correlation with any other medication used (Hochholzer et al 2005).

Thus, it seems that there is no clinically relevant interaction between clopidogrel and atorvastatin. Results by Lau and colleagues (2003) have been questioned because of their use of non-conventional methods to assess platelet activation, small study size, patients also receiving GP IIb/IIIa inhibitor and the uncontrolled use of aspirin and other CYP 3A4 inhibitors (see Poulsen et al 2005). However, the other studies can also be criticized for similar limitations as well as that some considered all statins and did not differentiate between CYP 3A inhibitors and others, the other potential CYP 3A4-inhibiting drugs were not taken into account (eg. ca-channel blockers, benzodiazepines and antidepressants) (Turgeon et al 2006). In some clinical studies sample sizes have been insufficient, especially to prove the absence of drug-drug interactions. In addition, most studies have assessed the effect of clopidogrel to inhibit platelet activation measured with different laboratory tests; however, the reduced antiplatelet effect has not been shown to be of clinical relevance.

Cholesterol levels have been shown to correlate with platelet activity (Rauch et al 2000). Thus, careful treatment of cholesterol itself will decrease platelet reactivity and reduce blood thrombogenicity in patients with atherothrombotic disease (Rauch et al 2000).

Smoking has been shown to correlate with increased clopidogrel efficacy. It has been suggested this phenomenon can be explained by activation by liver enzymes by the polycyclic aromatic hydrocarbons in cigarette smoke (Zevin et al 1999, Matetzky et al 2004).

Summary

Conflicting reports have been published on possible interaction of clopidogrel with atorvastatin, which could interact with clopidogrel due to competitive metabolism by the CYP 3A system. Drug-drug interactions between clopidogrel and other drugs have not been studied. As clopidogrel is a prodrug requiring metabolism by liver enzymes possible interactions may prove to be clinically important. In addition the large number of CYP enzymes participating in metabolism of clopidogrel enhances the possibility of interactions.

Table 8. *Factors which participate in thromboregulation and thus possibly affect efficacy of clopidogrel.*

Thromboregulator	Released	Action	Aspirin sensitive
Thromboxane	Yes	Platelet activation, recruitment and secretion; vasoconstriction	Yes
Prostacyclin	Yes	Inhibition of platelet responsiveness; vasodilatiation	Yes
NO	Yes	Inhibition of platelet responsiveness; vasodilatation	No
NTPDase-1	No, membrane associated	Removes ADP, ATP, decreasing purinergic signalling	No
NTPDase-2	No, membrane associated	Converts competitive antagonist ATP of ADP-receptors to agonist ADP	No
ADP	Yes	Acts on purinergic receptors; may be involved in erythrocyte prothrombotic activity	No platelet effects; partial effect on erythrocytes

Modified from Marcus (2005) and Robson (2005). NO=nitric oxide, NTPDase=nucleoside triphosphate diphosphohydrolase/ ecto-adenosine phosphatase (ADPase)/CD39

6.5 Genotype

Polymorphisms of several platelet receptors, such as GP IIb/IIIa and GP VI, have been shown to correlate with enhanced platelet activity (Michelson 2000, Joutsi-Korhonen et al 2003). Furthermore, genetic variation in receptors has been associated with increased risk for atherothrombotic complications (Moshfegh et al 1999, Mikkelsson et al 1999 and 2001, Croft et al 2001, Ollikainen et al 2004). Polymorphisms of both the $P2Y_{12}$-receptor and CYP 3A system have been proposed to explain variable clopidogrel efficacy.

Originally five $P2Y_{12}$-receptor polymorphisms were identified. Four of these were shown to be in complete linkage disequilibrium, thus determining haplotypes H1 and H2 (Fontana et al 2003a) (Table 9). In 98 healthy volunteers H2 haplotypes seemed to be associated with increased ADP-induced platelet response (Fontana et al 2003a). However, in another study of 200 healthy individuals no association between platelet activation and $P2Y_{12}$-receptor polymorphisms was found (Hetherington et al 2005).

$P2Y_{12}$-receptor polymorphisms have been shown to correlate with PAD in two independent studies. In a case-control study of 184 PAD patients and 330 age matched controls the $P2Y_{12}$-receptor H2 haplotype was more frequent in patients with atherothrombotic disease (Fontana et al 2003b). In another study of patients with advanced PAD $P2Y_{12}$-receptor polymorphisms associated with the risk of thrombotic neurological events. When 137 clopidogrel treated patients were studied carriers of the T allele of the C34T polymorphism had a 4-fold risk for occurrence of atherothrombotic events (Ziegler et al 2005).

Association between $P2Y_{12}$-receptor polymorphisms and clopidogrel response have been studied both in patients with atherothrombotic disease and in healthy individuals. In 96 healthy volunteers clopidogrel efficacy was not found to associate with H1/H2 haplotype (Fontana et al 2006). In another study 416 patients with CAD were genotyped according to H1/H2 haplotype as well as the C34T polymorphism and ADP-induced platelet aggregation was assessed after administration of 600mg clopidogrel loading dose. Neither the haplotype nor the C34T polymorphism were associated with clopidogrel response (von Beckerath et al 2005a). In accordance, in a smaller study of 36 patients with 300mg loading doses and 83 patients with ongoing 75mg/d clopidogrel the $P2Y_{12}$-receptor haplotypes were not associated with clopidogrel response (Angiolillo et al 2005c).

As the CYP 3A system plays an important role in metabolism and activation of clopidogrel its polymorphisms have been proposed to explain variability in platelet inhibition exerted by clopidogrel. Extensive genetic differences in CYP 3A5 have been shown as well as remarkable variability in its function (Lee et al 2003). More than 30 CYP 3A4 SNPs have been identified, however their functional role has not been shown (Lamba et al 2002). Despite several studies an association between CYP 3A4 activity and genotype has not been found (Turgeon et al 2006). Neither are there data on association of clopidogrel response and CYP 3A4 genotype. A CYP 3A5 gene polymorphism distinguishing expressor (*1) and non-expressor (*3) alleles has been shown to influence total CYP 3A activity (Kuehl et al 2001). A recent study showed that in clopidogrel treated patients atherothrombotic events occurred more frequently in patients with the non-expressor genotype (Suh et al 2006).

In addition, polymorphisms of other platelet receptors have been studied in an endeavour to explain variability in clopidogrel responses. T allele carriers of the GP Ia receptor C807T polymorphism had increased platelet responses compared with C allele homozygotes despite dual antiplatelet medication with aspirin and clopidogrel (Angiolillo et al 2005d). This finding highlights the importance of other activation pathways behind resistance to the ADP-receptor antagonist clopidogrel. Conflicting results on the role of the GP IIb/IIIa $PL^{A1/A2}$ polymorphism in clopidogrel resistance have been reported (Dropinski et al 2005, Angiolillo et al 2004c).

Summary

Several polymorphisms of the $P2Y_{12}$-receptor have been determined. $P2Y_{12}$-receptor genotype does not seem to correlate with clopidogrel response in patients with atherothrombotic disease. However, a correlation between clopidogrel efficacy and CYP 3A5 genotype was found in a single study. Thus far the subject of association between alleles of either $P2Y_{12}$ or the CYP 3A system and clopidogrel response has been inadequately studied.

Table 9. *Description and allele frequency of the previously described polymorphisms of the $P2Y_{12}$-receptor. The H1/H2 haplotype is defined by the i-C139T, i-T744C, i-ins801A, G52T polymorphisms.*

Name of SNP	Amino Acid	Region	Allele Frequency	
i-C139T	…	Intron	C 86.2%	T 13.8 %
i-T744C	…	Intron	T 86.2%	C 13.8 %
i-ins801A	…	Intron	Insertion of an A 13.8 %	
G52T	G/G	Exon 2	G 86.2%	T 13.8 %
C34T	N/N	Exon 2	C 72.5%	T 27.5%

Reproduced from Fontana et al (2003b), with permission.

6.6 Thrombin

Platelet activation due to thrombin has been suggested as a potential reason behind non-response to clopidogrel in some individuals. Thrombin is a potent platelet activator and normally such activations cause the release of ADP from dense granules. Thus ADP-receptors, including $P2Y_{12}$, become functional and take part in thrombin-induced aggregation.

$P2Y_{12}$-receptor antagonism has been shown to decrease thrombin-induced platelet activation, especially at lower thrombin concentrations (Adam et al 2003, Nylander et al 2003 & 2004). At concentrations of 0.25-2nM $P2Y_{12}$-antagonism caused an inhibition of 70-86%, while at concentrations of 100nm the inhibition was 15-20% and the inhibitory effect was present even at extreme thrombin concentrations in flow cytometric studies (Nylander et al 2003). However, $P2Y_{12}$-antagonism does not inhibit platelet activation caused by high thrombin concentrations in aggregations with washed platelets or in whole blood (Kim et al 2002, Adam et al 2003, Nylander et al 2003). Thus, the $P2Y_{12}$-receptor plays a role in thrombin-induced activation, but complete aggregatory reactions can be caused with high thrombin doses despite full $P2Y_{12}$-receptor antagonism.

The activation of platelets by thrombin in spite of $P2Y_{12}$-antagonism has also been reported in patients with CAD. Thrombin receptor activating peptide (TRAP)-induced platelet aggregation and degranulation was not inhibited by combination treatment with aspirin and clopidogrel, suggesting that platelet aggregation can not totally be inhibited in the presence of high levels of thrombin (Müller et al 2001).

The different roles of PAR receptors could explain the thrombin activation despite $P2Y_{12}$-antagonism. Activation of PAR-1 has been shown to be sufficient for complete ADP release (Covic et al 2002). At thrombin concentrations (>1.5nM) higher than those needed for PAR-1 activation and complete ADP release PAR-4 takes part in platelet activation (Covic et al 2000, Nylander & Mattsson 2003). Thus Nylander et al (2003) have suggested that at higher thrombin concentrations the role of ADP would be significantly diminished. However, they reported that activation of PAR-4 was inhibited by $P2Y_{12}$-antagonists (Nylander et al 2003). Again, at variance with flow cytometric measurements reported by Nylander et al (2003), a study by Adam et al (2003) showed no inhibition of PAR-4 related activation by $P2Y_{12}$-agonists in platelet aggregation experiments. Interestingly, PAR-1 genotype has recently been associated with increased platelet activity which cannot be overcome with clopidogrel treatment (Smith et al 2005).

Summary

Non-response to clopidogrel could, at least partly, be explained by the fact that in an environment where excess thrombin is generated the thrombin receptor PAR-4 could cause platelet activation. Interestingly, large variability in PAR-4 activation responses have been detected (Adam et al 2003).

Thus, in acute thrombotic setups or in thrombophilic patients, thrombin could be generated despite antiplatelet medication and could bypass the inhibition of other platelet activation pathways and cause activation via the PAR-4 receptor.

6.7 Diabetes and increased BMI

Both diabetics and non-diabetics benefit from clopidogrel and its efficacy exceeds that of aspirin treatment (CAPRIE 1996, Bhatt et al 2002b). In the CREDO and CURE studies patients with diabetes tended to benefit from clopidogrel slightly less than non-diabetics (CURE 2001, Steinhubl et al 2002). In the CURE (2001) study 2840 diabetic patients were investigated and showed no significant difference in incidence of adverse events when treated with the combination of clopidogrel and aspirin (14.2%) or aspirin alone (16.7%) (Mehta et al 2001). Also in the CREDO trial the benefit from combination (aspirin and clopidogrel) treatment in diabetic patients was smaller than in non-diabetic patients (relative risk reduction 11% and 33%, respectively) (Steinhubl et al 2002). However, only subgroup analyses of these clinical studies concerning the clopidogrel efficacy in diabetics versus non-diabetics and especially in association with insulin resistance have been published. Thus, diabetics seem to require potent antithrombotic medication especially during PCI or unstable angina. Despite their impaired prognosis in comparison with non-diabetics, patients with diabetes are reported to equally benefit from GP IIb/IIIa antagonists (Marso et al 1999).

The decreased clopidogrel efficacy in diabetic patients has also been proven with laboratory measurements of platelet activation (Angiolillo et al 2005b). Different explanations for decreased clopidogrel efficacy in diabetic patients have been suggested. Increased thrombogenic and arteriosclerotic activity could explain the phenomenon, as previously discussed in relation to aspirin. Glucose levels and formation of platelet microaggregates have been studied in attempts to explain the variability of drug efficacy.

Despite the fact that increased blood glucose concentration have been shown to enhance platelet aggregation and platelet-subendothelium interaction, high concentrations of glucose did not decrease clopidogrel efficacy (De La Cruz et al 2004). Another study assessed the formation of microaggregates in diabetic patients and healthy subjects (Matsuno et al 2005). In diabetic patients platelet activity was increased and microaggregates formed more easily than in controls. However, the $P2Y_{12}$-receptor antagonism *in vivo* and *in vitro* inhibited the formation of microaggregates, unlike in healthy controls. Thus, diabetic patients were more sensitive to clopidogrel in relation to inhibition of microaggregate formation, but as the baseline platelet activity in diabetics was increased compared to non-diabetics the clinical significance of this finding is questionable.

In patients with high body mass index the benefit from combination treatment with clopidogrel and aspirin has been postulated to be reduced (Angiolillo et al 2004a). This could be explained by enhanced platelet function, increased levels of platelet cytosolic Ca^{2+} and plasma

catecholamines in overweight individuals (Scherrer et al 1991, Takaya et al 1997). Angiolillo et al (2004a) have suggested increased loading doses to overweight patients, especially because CYP 3A4 activity has been shown to be reduced in overweight individuals (Kotlyar et al 1999). However, in subanalysis of the CAPRIE study (1996) there seemed to be no need for weight-adjusted dosing with the maintenance dose of 75mg/d studied. Benefits of increased loading doses in obese patients have not been studied.

Correlation between decreased response to clopidogrel and obesity could to some extent be explained by the fact that obesity is closely related to metabolic syndrome which has been associated with decreased clopidogrel efficacy.

Summary

Diabetic patients seem to gain less benefit from clopidogrel treatment than non-diabetics. The exact reason for this is unknown.

AIMS OF THE STUDY

Atherothrombotic disease is major cause of mortality in developed countries. Antiplatelet medication is one of the cornerstones in its treatment. A considerable proportion of patients suffer from atherothrombotic events despite antiplatelet medication with aspirin, clopidogrel or their combination. This study was aimed to clarify the individual variability of platelet activity and responses to antiplatelet medication and its mechanisms by using different platelet function tests.

The study focused on the following specific issues:

1) To compare the feasibility of different laboratory tests in detecting individual variability of platelet function and *in vitro* response to antiplatelet medication.

2) To assess the *in vitro* efficacy of ongoing aspirin in patients with coronary artery disease (CAD) and the efficacy of short term as well as steady-state treatment of clopidogrel on top of ongoing aspirin in CAD patients who were to undergo elective PCI.

3) To clarify whether genotype of platelet receptors is associated with individual variability in platelet functions and response to antiplatelet medication with aspirin or the combination of aspirin and clopidogrel as well as to determine clinical and metabolic factors which affect individual responses to antiplatelet medication with aspirin or the combination of aspirin and clopidogrel.

SUBJECTS, MATERIALS AND METHODS

1 Subjects (I-IV)

Blood was collected from either healthy volunteers (**I**) or patients undergoing elective PCI with angiographically proven CAD (**II-IV**). Twenty-one healthy, non-smoking volunteers, who denied taking any medication during the past ten days, participated in the first study (**I**). Hundred and one patients undergoing elective PCI without treatment with insulin, warfarin, heparin or antiplatelet agents other than aspirin participated in the following studies (**II-IV**).

Clinical endpoint data were collected from patients and confirmed from medical records 12 months after the PCI (unpublished data).

The protocols of these studies followed our institutional guidelines and informed consent was obtained from each subject. The studies which included patients (**II-VI**) were approved by the Ethics Committee of Helsinki University Central Hospital and complied with the Declaration of Helsinki.

2 Blood samples and anticoagulants (I-IV)

Blood was collected either with a free-flowing technique (**I**), via venipuncture or into evacuated tubes, from the antecubital vein (**II-IV**). The blood for platelet function assessments was collected in several anticoagulants depending on the platelet function test used: 1) in trisodium citrate (**I-IV**), 2) in acidic citrated dextrose (**I**) and 3) in either hirudin (**II-IV**) or 4) in D-phenylalanyl-1-prolyl-1 arginine chloromethyl ketone (**I**), as monomer-induced aggregation depends on Mg^{2+}, which is chelated by citrate (Siljander et al 1999).

3 Medication and time of specimen collection (II-IV)

The patients (**II-IV**) were being treated with a daily morning dose of aspirin at the time of admission to the outpatient clinic. The average dose was 100mg/d. Compliance with aspirin treatment was controlled by salicylate measurements and biological response in the form of plasma TxB_2. Blood samples were collected 2-120 h before the PCI.
An additional platelet inhibitor, clopidogrel (methyl (S)-(2-(2-chlorophenyl)-2-(4, 5, 6, 7,-tetrahydrothieno (3,2-c) pyridin-5-yl) acetate, hydrogen sulphate) was orally administered prior to the PCI. All blood samples were obtained before the actual procedure; 1) before the administration of clopidogrel, to study the effect of aspirin alone (**II-IV**) and either 2a) 2.5±0.5 h after the administration of a loading dose of 300mg clopidogrel to study the early combined effect of aspirin and clopidogrel (**III**) or 2b) after a loading dose of 300mg followed by a daily dose of 75mg for five days to study the combined effect of aspirin and ongoing clopidogrel (**IV**).

4 Whole Blood Perfusions (I)

4.1 Preparation for Perfusion and Labelling of Platelets

To standardize rheological conditions hematocrit was adjusted in the blood for the perfusions, if required (Turitto et al 1980). Depending on the hematocrit the final plasma concentration of citrate was adjusted (Sakariassen et al 1989). Platelet-rich plasma (PRP) was separated from blood after centrifugation and platelets in PRP were labelled with ^{3}H-serotonin. At this low serotonin concentration platelet membranes are labelled without the contribution of the $5HT_2A$-receptor (unpublished data). Before the perfusions the labelled PRP and the remaining blood cells were recombined and incubated (Sakariassen et al 1989, Mustonen et al 1996). Coverslips for perfusion procedure were coated either with collagen fibrils or collagen monomers (Siljander et al 1999).

4.2 Perfusion procedure

A parallel-plate perfusion chamber was used under a shear rate of 1600 s^{-1} (Turitto 1975, Hall et al 1998). Blood was prewarmed at 37^{o}C and perfused in a single passage through the chamber. Experiments were done in either duplicates or triplicates. After perfusion the ^{3}H-scintillation activities of the coverslips were analysed using liquid scintillation counter (Mustonen et al 1996). Intraindividual reproducibility of the perfusion was assessed on two subsequent days. The coefficient of variation was 20% in perfusions in PPACK-anticoagulated whole blood over collagen monomers (N=6), and 16% over collagen fibrils (N=14).

5 Platelet aggregations (I-IV)

Blood was prepared for aggregation experiments in PRP by centrifuging. The PRP platelet count of was adjusted to 300 x 10^{6}/mL with platelet-poor plasma, also prepared by centrifuging.

For aggregations with gel filtrated platelets (**I**), PRP separated after centrifugation from blood, apyrase and PGE_1 added to PRP to prevent activation while a platelet pellet was separated from plasma by further centrifugation. Platelets were then resuspended and eluted through a Sepharose CL-2B column (Timmons et al 1989). Prior to the experiments 2 mM $MgCl_2$ was added to enhance GP Ia/IIa functions and to provide physiological concentration of divalent cations for the platelets.

Aggregations were induced with 1) soluble collagen monomers (**I-IV**), 2) collagen fibrils (**I**), 3) adenoside 5´-diphosphate ADP (**II-IV**), 4) arachidonic acid (**II-IV**) and 5) epinephrine (**II-IV**). The additional effect of subthreshold concentrations (0.2 µM) of ADP or epinephrine was determined in some experiments by adding the agonist to PRP prior to either the collagen monomers or fibrils (**I**).

A specific $P2Y_{12}$-receptor antagonist AR-C69931MX (ARMX) (generously provided by Astra Zeneca, Loughborough, UK) and $P2Y_1$-receptor antagonist adenosine 3,5 diphosphate (A3P5P, 200µM) (Sigma, St. Louis, MO, USA) were used in certain ADP-induced aggregations (**IV**).

Aggregation was traced with a traditional, turbidometric aggregometer, either a Payton turbidometric aggregometer (Payton Associates Inc.; Buffalo, NY, USA) (**I**) or PPACKS-4 aggregometer (Helena Laboratories, Beaumont, Texas, USA) (**II-IV**). Maximal aggregation (**I-IV)**, rate (**I**) or slope (**II-IV**) and lag time (**I**) were assessed. The inhibition of platelet aggregation in response to clopidogrel and ARMX was calculated as a reduction of maximal ADP-induced aggregations (**III, IV**).

6 Platelet function analyser PFA-100® (I-IV)

PFA-100® (Dade-Behring AG, Düdingen, Switzerland) is a platelet function analyser that measures primary hemostasis in whole blood, under capillary flow conditions, with a high shear rate (4000 1/s). Platelets interact with a collagen mesh of the same origin as used in perfusion studies (**I**) spiked with either ADP (CADP) or epinephrine (CEPI), resulting in occlusion of an aperture in the respective cartridge. The results are expressed as closure times. In preparation for platelet function analyses the hematocrit was adjusted when applicable.

The PFA-100® method has been suggested to be useful in the assessment of the antiplatelet effects of aspirin, and the closure time of an epinephrine-stimulated cartridge should be prolonged in aspirin-treated patients (Mammen et al 1998, Homoncik et al 2000, Gum et al 2001, see Favaloro 2002). The manufacturer of the PFA-100® refers to 170 s as the limit of aspirin efficacy with a sensitivity of 95%. In our laboratory the intraindividual method- and donor-dependent variability of PFA-100® in CADP and CEPI was found to be 7% and 11%, respectively (3 repeated samples from 8 healthy donors over 3 weeks).

7 Definition of responders and poor responders to aspirin and clopidogrel (II-IV)

Non-responders to aspirin were defined with two alternative methods: 1) for the PFA-100® method, patient samples with a closure time shorter than 170 s in the CEPI cartridge (Mammen et al 1998, Homoncik et al 2000, Gum et al 2001, see Favaloro 2002) and 2) for the AA-induced aggregation method, patient samples with an aggregation slope >12% during the first minute of the test, as this cut-off level dichotomized patients as clear non-responders (maximal aggregation >80%) and responders (maximal aggregation < 40%).

Poor response to clopidogrel was defined by inhibition of ADP-induced platelet aggregation. Aggregations were performed before and after clopidogrel administration and if the inhibition of aggregation was less than 10% (post value subtracted from the pre value), these patients were defined as poor responders to clopidogrel.

8 Genotype Analyses (II, IV)

DNA was extracted from blood samples using a non-enzymatic method (Lahiri et al 1991). Genotypes for GP IIIa, GP VI, GP Ib (HPA2/Kozak) and GP Ia (C807T/HPA5) polymorphisms were determined. Genomic regions containing variant sequences were amplified via PCR and

then incubated with restriction enzymes. Genotypes were determined by staining the DNA fragments with ethidium-bromide and separation by agarose gel electrophoresis (for details see Mikkelsson et al 1999, 2001 and 2002, Moshfegh et al. 1999, Ollikainen et al 2004). COX-1 genotypes were also assessed. Fragments containing the single nucleotide polymorphisms (SNP) -A842G, C22T, C714A and C644A were amplified with primers and the resulting PCR products digested with enzymes and separated on agarose gel. The -A842G SNP is in complete linkage disequilibrium with the C50T SNP, therefore this polymorphism was not studied.

Genotypes for the $P2Y_{12}$-receptor G52T polymorphism and the CYP 3A5 enzyme A6986G polymorphism were determined. The G52T polymorphism has been previously reported to be part of the H1/H2 haplotype (Fontana et al 2003a). Genomic regions containing variant sequences were amplified via PCR and then incubated with restriction enzymes. Genotypes were determined by staining the DNA fragments with ethidium-bromide and separation by agarose gel electrophoresis (van Schaik et al 2002, von Beckerath 2005a).

9 Laboratory Analyses (II-IV)

Several laboratory measurements were performed: blood cell counts, levels of von Willebrand factor (vWF), fibrinogen, glycosylated hemoglobin (Hb A1c), C-peptide, C reactive protein (CRP), blood glucose levels, activated partial thromboplastin time (APTT), epinephrine and norepinephrine (HPLC-EC) (Scheinin et al 1991), plasma thromboxane B_2 (Tx B2) (EIA, Cayman Chemical, USA) (Ojanen et al 2003) and salicylate levels (liquid chromatography)(Brandon et al 1985).

10 Statistical Analyses (I-IV)

The data are presented as mean ± SD. Spearman's non-parametric test was used to assess the correlations between variables. The differences between groups were assessed with Mann-Whitney U-tests and Wilcoxon tests were used for paired samples. The level of significance used was 0.05. The genotype effects were studied with stepwise binary logistic regression analyses with appropriate covariate factor data (hematocrit, age, sex, smoking and diabetes, as well as C-peptide, fibrinogen and vWF levels) (**II**). The efficacy of antiplatelet medication was studied by using previously determined parameters of poor ASA response and by comparison between intraindividual samples before and after clopidogrel treatment (**II-IV**).

RESULTS

1 Platelet function tests and individual variability in platelet functions (I)

1.1 Individual variability in platelet functions

Large variability between donors was observed in whole blood perfusions, PFA-100®-system and platelet aggregations (Table 10). Collagen monomer-induced platelet activation was more sensitive to individual differences in platelet function than collagen fibril-induced activation.

Table 10. *Interindividual variability detected in different platelet function tests, as well as the coefficient of variation for duplicates or triplicates.* ***(I)***

Platelet function test		Variation between donors	CV (%)
Whole blood perfusions			
	MC/PPACK	45-fold	29 (10.8-53)
	FC/PPACK	3-fold	15.7 (1.9-47.7)
	FC/citrate	7-fold	16.6 (2.7-44.2)
Platelet function analyses PFA-100®			
	CEPI/PPACK	2-fold	13.8 (3.5-39.2)
	CEPI/citrate	3-fold	13.8 (0.6-47.8)
	CADP/PPACK	4-fold	11.8 (0-32.4)
	CADP/citrate	2-fold	13.1 (1.3-44.2)
Aggregations			
MC/PPACK	lag time (s)	3-fold	
	rate (1/min)	7-fold	
	max (%)	3-fold*	
FC/PPACK	lag time (s)	2-fold	
	rate (1/min)	2-fold	
	max (%)	1.3-fold*	

CV=coefficient of variation between duplicate or triplicate samples, MC= collagen monomers, FC= collagen fibrils, CEPI=collagen/epinephrine cartridge, CADP= collagen/ADP-cartridge, *= the calculation of variation is limited by the fact that maximal aggregation can not reach values above 100%

1.2 Associations between platelet function tests

Perfusions and PFA-100® (I)

Results of the perfusions correlated with those of PFA-100®. The epinephrine-enhanced closure times in PFA-100® correlated with platelet deposition on collagen fibrils in citrated blood (r=

-0.45, p<0.05) and collagen monomers (r= -0.49, p<0.05). The ADP-enhanced closure times correlated with platelet deposition on collagen fibrils both in PPACK- and citrate-anticoagulated blood (r= -0.47, p<0.05 and r= -0.43, p<0.05 respectively).

Platelet aggregation and PFA-100® (I-IV)

The platelet aggregations induced by collagen fibrils or collagen monomers did not correlate with PFA-100® closure times in healthy donors (N=21) (r=-0.05-0.26, P=0.17-0.96).

Association between PFA-100® and platelet aggregations was found in CAD patients (N=101) with either aspirin or aspirin and clopidogrel medication. (**I-IV***)* When platelet functions of patients with ongoing aspirin treatment were studied CEPI closure times associated with extent of aggregations induced by ADP and epinephrine (r=-0.23 and r=-0.24, P<0.030, respectively) (**II**). Measurements from PFA-100® and platelet aggregations correlated with each other better in patients who had ongoing platelet suppression by combinations of aspirin and clopidogrel medication. In these patients closure times in CEPI cartridge associated not only with those previously mentioned aggregation studies but also with the extent of collagen-induced aggregation (r=-0.31, P=0.03, N=50) (**III**). In these patients closure times in CADP associated with collagen and ADP as well as epinephrine induced aggregation (r=-0.28, P=0.05, r=-0.29, P=0.41, r=-0.34, P=0.02, respectively) (unpublished data).

1.3 Factors associating with variability in platelet functions (I)

Donors with C807T genotype of GP Ia/IIa had 8-59% larger deposition of platelet on collagen in perfusion studies than the ones with wild type, but this was not statistically significant (P=0.60-0.71).

Lag times of platelet aggregations, both monomer- and fibril-induced, correlated with the genotype. The donors homozygous to the C allele presented longer lag time, by 30% in monomer-induced and by 22% in fibril-induced aggregations, than the donors heterozygous to the platelet receptor GP Ia gene (90±16 s vs. 69±18 s, and 84±14 s vs. 69±12 s, respectively, both p<0.04).

2 Variability in aspirin efficacy (II-IV)

2.1 Prevalence of poor aspirin response

Response to aspirin treatment evaluated by AA-induced aggregation

When platelet aggregation was induced by AA 5% of the patients had a slope steeper than 12%/min and were categorized as aspirin non-responders (Fig 11). Two of these patients had both low salicylate and high Tx B_2 levels suggesting pharmacokinetic resistance or possible non-compliance. Non-responders to aspirin according to AA-induced aggregations also had shorter closure times in the PFA-100® CEPI-cartridge than the responders (193±90 vs. 257±69 s, P=0.03). In patients with sustained AA-induced aggregation limited platelet inhibition by aspirin could also be detected by epinephrine- or ADP-induced platelet aggregation, as they had enhanced responses to these agonists in comparison with responders (epinephrine 76±13 vs. 62±14 %, P=0.04 and ADP 91±7 vs. 84±7 %, P=0.02). There were no significant differences in collagen-induced aggregations between responders and non-responders.

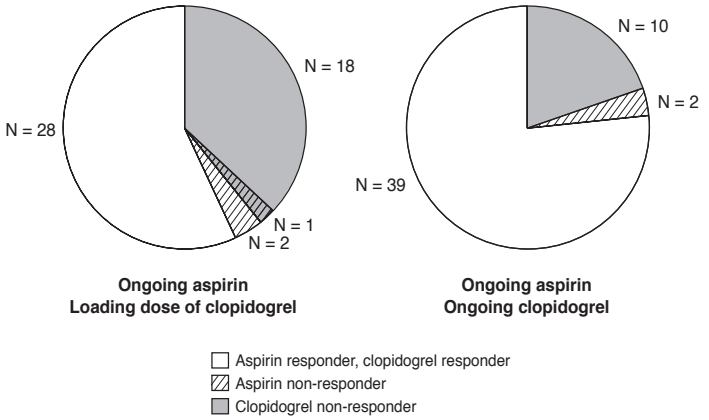


Figure 11. *Prevalence of non-response to antiplatelet medication in 101 patients with coronary artery disease.* ***A)*** *Prevalence of non-response to ongoing aspirin and 300mg loading dose of clopidogrel 2.5 h after administration of the latter (N=49).* **B)** *Prevalence of non-response to ongoing aspirin and ongoing clopidogrel (300mg loading dose followed by daily 75mg for five days) (N=51)* ***(II-IV).***

Response to aspirin treatment evaluated by PFA-100®

When whole blood was applied to the CEPI cartridge 21% of the patients had closure times shorter than 170 s and were categorized as aspirin non-responders (Fig 12). One of these patients had low salicylate and high Tx B_2 levels suggesting pharmacokinetic resistance or possible non-compliance. The population of non-responders had enhanced maximal platelet aggregation induced by ADP when compared with responders to aspirin (84±6% vs. 87±9%, P=0.01). No difference between the non-responders and responders to aspirin according to PFA-100® was observed when aggregation was elicited by AA, epinephrine or collagen.

2.2 Methodological aspects of measurement of aspirin response

Aggregations and PFA-100® detect different populations as non-responders

The present study (**II-IV**) revealed that the common methods used to detect aspirin response, AA-induced aggregation and PFA-100®, indicate different populations as being aspirin-resistant with a modest overlap. Of the five non-responders according to AA-induced aggregation only two were non-responders according to PFA-100® (Fig 12). PFA-100® detected 21% as aspirin non-responders, while AA-induced aggregation detected only 5% as non-responders.

Compliance to aspirin treatment

All patients affirmed aspirin intake, when assessed by questioning on the day of PCI. Salicylate levels measured varied between 0-31846 ng/ml (mean 2479±3770 ng/ml). Thirteen patients had plasma salicylate levels which were under the detection limit, possibly due to the short half life of salicylate or poor compliance (Fig 13). However, most patients had low plasma TxB_2 levels compatible with aspirin use. Tx B_2 levels varied between 40-455 pg/ml (mean 143±76

pg/ml). The Tx B_2 levels of the 13 patients with no measurable salicylate varied between 56-455 pg/ml (mean 212±119 pg/ml) and those with measurable salicylate levels between 40-331 pg/ml (mean 133±62 pg/ml)(P=0.01). There were two patients with no measurable levels of salicylate and higher Tx B_2 levels (436 and 455 pg/ml) than in salicylate positive patients indicating possible non-compliance.

3 Variability in clopidogrel efficacy administered on top of aspirin

3.1 Prevalence of poor clopidogrel response

Limited early response to loading dose of clopidogrel on top of aspirin (III)

Although at the population level clopidogrel exerted inhibitory effect, in a subgroup of patients clopidogrel treatment did not inhibit platelet aggregation. There were 2 patients in whom ADP-induced aggregation did not attenuate after administration of clopidogrel. Furthermore, we identified 20 patients in whom maximal aggregation decreased by less than 10%. As ADP-induced aggregations were used to define limited clopidogrel response, 40% (N=20) of the study population were defined as poor responders to clopidogrel (Fig 11). The effect of clopidogrel was not associated with the time between drug administration and blood sample collection (P=0.6).

Limited response to ongoing clopidogrel on top of aspirin (IV)

In the whole population clopidogrel exerted inhibition of platelet activity measured both with platelet aggregations and PFA-100®. However, in a subgroup of patients clopidogrel treatment was found not to inhibit platelet aggregation. There were 10 patients (20%) in whom maximal aggregation was decreased by less than 10% (Fig 11). These patients were defined as poor responders to clopidogrel according to limited inhibition of ADP-induced aggregation. Patients who had poor response to aspirin according to AA-induced aggregation (N=2) responded to clopidogrel better than responders to aspirin when measured with ADP (5 μM) -induced aggregations (41±14 vs. 19±10 %, P<0.033).

Efficacy of loading dose of clopidogrel in non-responders to aspirin (III)

In the group of patients receiving only the loading dose of clopidogrel (N=50) 9 patients presented measurable response to AA in aggregation studies (measurable slope). Of the 9 patients, 4 patients benefited from a loading dose of clopidogrel, whereas 5 patients did not. These 5 patients, 10% of the entire patient group, did not achieve any measurable antiplatelet effect from either aspirin (measured with AA-induced aggregation) or clopidogrel (measured with ADP-induced aggregation). Of these 9 patients 3 had aggregation slope >12%/min and where thus labelled non-responders to aspirin. One of the non-responders to aspirin was also non-responder to clopidogrel.

In patients with poor response to aspirin according to PFA-100® (N=14), clopidogrel inhibited ADP-induced maximal platelet aggregation by more than 10% in 10 patients. Thus, 4 patients remained poor responders to both aspirin and clopidogrel.

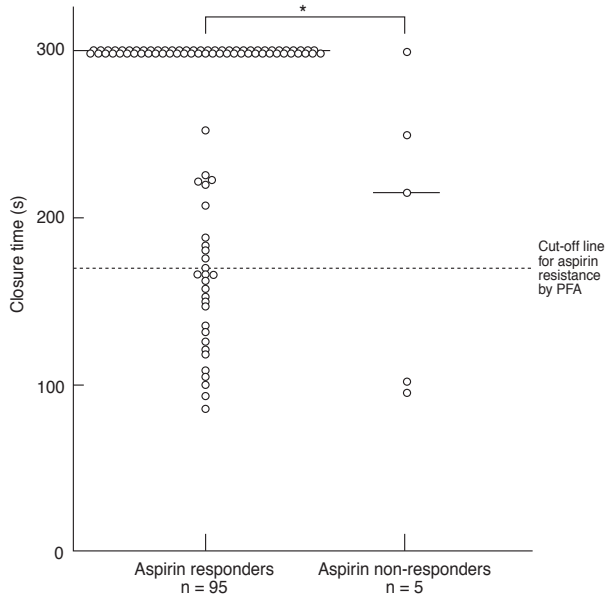


Figure 12. *PFA-100® and AA-induced aggregations detect different populations as aspirin non-responders. PFA-100®-closure times in aspirin responders and non-responders by AA-induced aggregation in 101 patients with coronary artery disease. The closure time 170s has been used as cut off line for aspirin resistance assessed by PFA-100®. The maximal measured closure time by PFA-100® is 300s. Short horizontal lines represent the mode of closure times* ***(II).***

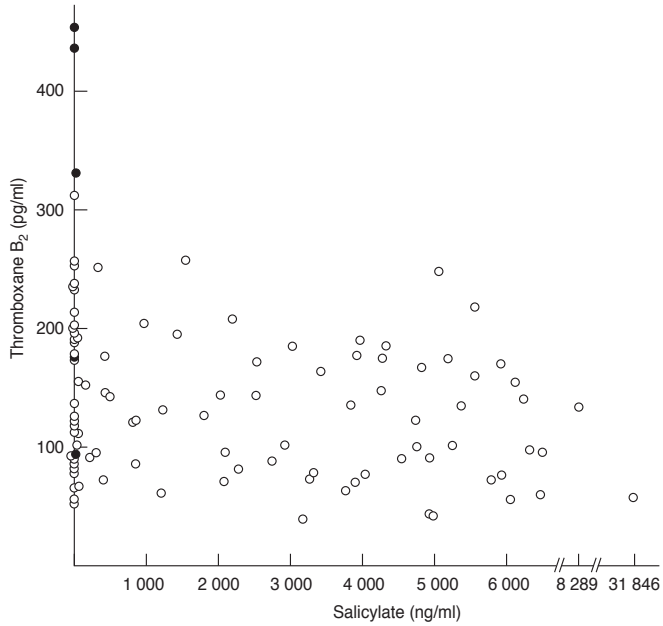
4 Factors associating with variability in efficacy of antiplatelet medication

4.1 Factors associating with variability in aspirin efficacy

Poor response to aspirin associates with genotype (II)

According to AA-induced aggregation, 60% of the aspirin non-responders in the sample of 101 patients carried the rare G allele for the COX-1 -A842G polymorphism in comparison with only 17% of aspirin responders (P=0.02). In other words, 2.5% of the patients homozygous for the common A allele were aspirin non-responders, whereas as many as 16% of the rare G allele carriers were aspirin non-responders (P=0.02). None of the other three common COX-1 polymorphisms studied associated with aspirin response with either method. Plasma Tx B_2 levels did not correlate with different COX-1 polymorphisms.

Patients heterozygous for the GP VI C13254T polymorphism had shorter closure times in PFA-100® than did patients homozygous for the common C allele (218±86 vs. 262±65 s, P=0.02). Of the aspirin responders according to PFA-100®, 13% had the rare T allele whereas this allele was carried by 38% of the non-responders (P=0.01).



	Salicylate (ng/ml)	Thromboxane B_2 (pg/ml)
Non-responders to aspirin	0	455
	0	435
	46	331
	0	178
	25.6	92

Figure 13. *Thromboxane and salicylate levels of 101 coronary artery disease patients of which 5 were defined as non-responders to aspirin according to AA-induced aggregation (●).Thromboxane and salicylate levels of 5 non-responders are given in the table. The mean thromboxane and salicylate levels in the whole patient group were 143±76 pg/ml and 2479±3770 ng/ml, respectively* ***(II)****.*

Other factors explaining variation in aspirin efficacy (II)

Other factors related to non-response to aspirin according to AA-induced aggregation were increased Tx B_2-levels, type II diabetes, previous AMI and surprisingly, low levels of fibrinogen. In addition, levels of vWF did not influence aspirin response. Non-responders to aspirin according to AA-induced aggregation had higher Tx B_2-levels than the responders (298±159 vs. 135±60 pg/ml, P=0.02). Of the 5 non-responders, 2 had type II diabetes in contrast to 10 of the responders to aspirin (N=95) (P=0.05). Four of five non-responders had suffered previous AMI in contrast with 39% of responders (37 of 95) (P=0.07). Non-responders to aspirin had lower fibrinogen levels (3.1±0.3 g/L) than responders (3.8±0.6 g/L) (P=0.01).

When aspirin response was measured with PFA-100® non-responders had higher Tx B_2-levels

compared to responders (186±90 vs. 131±68 pg/ml, P=0.00). Interestingly, 36% of females were non-responders in comparison with 16% of males (P=0.04). Moreover, females had an overall decreased response to aspirin as their PFA-100® closure times were shorter than those of males (225±79 vs. 263±66, P=0.02) (4.4 odds ratio, 1.3-15.4 95% confidence interval). The plasma levels of salicylate and TxB_2 did not differ according to gender.

4.2 Factors associating with variability in clopidogrel efficacy

Differences at the receptor level (IV)

The $P2Y_{12}$-receptor inhibition by clopidogrel correlated with the inhibition exerted by ADP-receptor antagonist AR-C69931MX (ARMX) (r=0.43, P=0.00). Adding the $P2Y_1$-receptor antagonist A3P5P before and after clopidogrel, as expected, inhibited the extent of aggregation having synergistic effect with either one of the $P2Y_{12}$-receptor antagonists clopidogrel or ARMX. Inhibition of $P2Y_1$-receptor by A3P5P attenuated the ADP-induced aggregation to a similar extent in both non-responders and responders to clopidogrel, thus suggesting that clopidogrel resistance is a $P2Y_1$-independent phenomenon.

Clopidogrel non-responders did not achieve as great inhibition of ADP-induced aggregation by *in vitro* $P2Y_{12}$-receptor antagonist ARMX as clopidogrel responders (25±7 vs. 32±7%, P<0.02), suggesting a common reason behind attenuated response to clopidogrel and ARMX. There were no differences in basal platelet activity between clopidogrel responders and non-responders (P>0.55).

Efficacy of loading dose of clopidogrel and insulin resistance (III)

Attenuated early response to a loading dose of clopidogrel correlated with insulin resistance. Patients with poor long-term glucose balance measured with glycosylated hemoglobin (Hb A1c) and elevated insulin levels measured with C-peptide, did not benefit from clopidogrel as much as the normoglycemic patients. Clopidogrel-induced inhibition of aggregation triggered by ADP was lower in patients with high levels of glycosylated hemoglobin (Hb A1c) or C-peptide (r = -0.33, P=0.03, r= -0.36, P=0.01). The patients with limited responses (change in aggregation <10%) to clopidogrel had higher serum C-peptide levels than patients who responded well to clopidogrel. As expected, increased C-peptide levels correlated with increased body mass index (r=0.51, P=0.00).

However, response to ongoing clopidogrel was not associated with Hb A1c, C-peptide or blood glucose levels (P> 0.15). So the association was lost during prolonged medication, suggesting interference at the time of early metabolism.

Other factors associating with clopidogrel efficacy (III, IV)

The higher the level of catecholamines the less the early prolonging effect of clopidogrel loading dose in closure times of CADP (r = -0.37, P=0.01) (**III**). Epinephrine levels also correlated with the efficacy of ongoing clopidogrel. The higher the level of epinephrine the less the prolonging effect of ongoing clopidogrel in closure times of CEPI (r = -0.30, P=0.03) (**IV**).

There was no association with the antiplatelet efficacy of clopidogrel and any statin used

(P>0.1) (Table 11) (**III, IV**). Neither did the genotypes of liver enzyme CYP 3A5 or $P2Y_{12}$ associate with clopidogrel efficacy (**IV**).

Table 11. *Mean inhibition of ADP (2 and 5μM)-induced aggregation by either loading dose of clopidogrel or ongoing clopidogrel in patients with different lipid lowering medication.*

Statin	**Loading dose of clopidogrel (300mg)**			**Ongoing clopidogrel (300mg + 75mg/ for 5 days)**		
	N	ADP 2 μmol/L	ADP 5 μmol/L	N	ADP 2 μmol/L	ADP 5 μmol/L
Atorvastatin	8	21±12	15±8	18	24±9	22±9
water-soluble statins	3	4±13	6±12	10	23±14	19±12
lipid-soluble other than atorvastatin	20	19±16	19±16	18	23±10	19±9
No statin	19	17±10	15±11	5	14±16	19±20

None of the differences were significant **(III-IV)**.

5 Poor responses to antiplatelet medication and clinical treatment failure (unpublished data)

When 101 CAD patients assessed in studies **III** and **IV** were followed up for 12 months 19% (N=19) had undergone repeat PCI, 3% (N=3) CABG and 4 % (N=4) had died. Three out of the 20 patients with poor response to clopidogrel loading dose died during the follow up whereas there was no deaths is in patient group with measurable clopidogrel effect (P=0.03). Re-PCI within the first 12 months seemed to be more common in patients with poor response to aspirin (2 out of 5) than in patients with measurable response (17 out of 95, 18%) (P=0.22). (See also table 12).

Table 12. *Frequency of adverse events in responders and non-responders to aspirin, measured with either AA-induced aggregation of PFA-100® as well as loading dose or ongoing clopidogrel*

Clopidogrel: loading dose 300mg

Endpoint	**Responders N=30**		**Non-responders N=20**		**P**
	%	N	%	N	
Re-PCI	27	8	25	5	0.90
CABG	0	0	5	1	0.22
Death	0	0	15	3	0.03*
Combined endpoints	27	8	35	7	0.53

Clopidogrel: ongoing treatment 75mg/d

Endpoint	Responders N=41		Non-responders N=10		P
	%	N	%	N	
Re-PCI	12	5	10	1	0.85
CABG	5	2	0	0	0.48
Death	2	1	0	0	0.62
Combined endpoints	20	8	10	1	0.480

Aspirin response: assessed with AA-aggregations

Endpoint	Responders N=95		Non-responders N=5		P
	%	N	%	N	
Re-PCI	18	17	40	2	0.22
CABG	3	3	0	0	0.69
Death	4	4	0	0	0.62
Combined endpoints	23	22	40	2	0.39

Aspirin response: assessed with PFA-100®

Endpoint	Responders N=78		Non-responders N=21		P
	%	N	%	N	
Re-PCI	22	17	10	2	0.21
CABG	4	3	0	0	0.36
Death	5	4	0	0	0.29
Combined endpoints	28	22	10	2	0.08

(Unpublished data). PCI=percutaneous coronary intervention, CABG=coronary artery bypass grafting, AA=arachidonic acid

DISCUSSION

Despite current antiplatelet regimens with aspirin, clopidogrel or their combination, several patients suffer from atherothrombotic events, the incidence of which are enhanced by increased platelet activity, inflammation, lipid levels and vasoactivity. Therefore, growing interest has been focused on this treatment failure and the phenomena of aspirin and clopidogrel resistance have become subjects of increasing research. The terms non-response or resistance to either of these antiplatelet drugs have been used to describe lack of platelet inhibition in laboratory tests. On the other hand, treatment failure describes the occurrence of adverse effects in patients despite antiplatelet medication. The goal of this area of study is to determine whether drug resistance measurable in laboratory tests associates with treatment failure and could thus be used as a predictor of risk of atherothrombotic events. Identifying these patients at greater risk, and more importantly understanding the mechanisms behind the increased risk, would enable us to tailor individual antithrombotic treatment to these patients.

1 Individual variability of platelet functions

As platelet activity varied markedly between individuals, we studied the usability of different laboratory methods in detecting this variance (**I**). When compared with PFA-100®, perfusions induced by collagen fibrils and platelet aggregations, platelet deposition on collagen monomers was found to be the most sensitive method for assessing individual variability. However, the measurement of platelet deposition on collagen monomers might be influenced by the fact that it is mostly GP Ia/IIa-dependent and thus does not entirely correspond to the normal function of platelets. PFA-100® was found to be less sensitive to individual variability but was proved to be valuable as it was shown to correlate with sophisticated functional assays of perfusion, and has better availability, usability and reproducibility than traditional methods. Individual variability in platelet functions has been postulated to associate with increased risk for atherothrombotic events (see Michelson 2004). The large individual variability in platelet functions could also partly explain differences in responses to antiplatelet medication. Thus, in the future, platelet function tests might prove to be useful in detecting patients at increased risk for adverse events and in need of targeted antiplatelet medication. However, optimal function test are yet to be developed as some of the sensitivity and most of the versatility of the methods are lost when traditional but laborious methods are substituted by modern apparatus.

2 Measurement of aspirin resistance

Due to the variety of methods used to detect aspirin resistance and the number of studies published during the past few years, an effort to unify the terminology and methodology has recently been made (see Cattaneo 2004, see Hankey & Eikelboom 2006). It has been suggested that aspirin resistance measured with TxA_2-dependent methods would be defined as pharmacological or "true" aspirin resistance and that methods which measure platelet activity inducible also via TxA_2-independent pathways would be labelled functional or "unproven" aspirin resistance (see Cattaneo 2004, see Wong et al 2004).

Apart from two studies in the 1990´s the interest in this question was not provoked until recently (Helgason et al 1994, Mueller et al 1997, Buchannan et al 2000, Peters et al 2001). In the beginning aspirin resistance was determined by several methods, including tests which were either independent of or dependent on TxA_2 production. We measured the antiplatelet effect of aspirin with TxA_2-dependent AA-induced aggregation and PFA-100®, which despite

also measuring TxA_2-independent pathways of platelet activation had been reported to detect aspirin use in previous studies (Feuring et al 1999, Homoncik et al 2000). In the present study the two methods were observed to indicate different populations as being aspirin-resistant with a modest overlap (**II**). Of the 5 non-responders according to AA-induced aggregation only 2 were non-responders according to PFA-100®. Similar findings were reported by Gum et al (2003).

Measurement of AA-induced aggregation directly evaluates the capacity of COX-1 to produce TxA_2 subsequent to platelet aggregation (Gum et al 2003). Therefore, this method, although time-consuming, is a valuable determinant of aspirin efficacy. In addition, sustained AA-induced aggregation has been associated with clinical endpoints in aspirin-treated patients (Gum et al 2003). This tendency could also be detected in our studies (unpublished data). It has also been confirmed by others (Wenaweser et al 2005).

Despite the fact that PFA-100® has been reported to detect aspirin use, other mechanisms compensate and surpass the need for TxA_2 production when platelets are challenged in the PFA-100® system. It is well accepted that aspirin does not affect shear stress-induced platelet aggregation, one of the important features in the PFA-100® system (see Cattaneo 2004). Data are too scanty to draw any conclusions about clinical associations. The presence of collagen and epinephrine in the cartridges could pinpoint collagen, vWF and adrenergic receptors as possible modulators of differences in aspirin sensitivity (Poujoul 1998, Chakroun et al 2004). In fact, it has been shown that under flow conditions such as those of the PFA-100® analyzer, platelet aggregation is mainly mediated by specific interactions between vWF and GP Ib as well as GP IIb/IIIa, and between collagen and GP Ia/IIa as well as GP VI. VWf has been shown to play an important role in PFA-100® (Chakroun et al 2004). Thus, PFA-100® has been found useful for detecting variation in defects of vWf, such as von Willebrands disease, as well as in general platelet activity, which is not necessarily related to specific function of aspirin (Cattaneo et al 1999, Favaloro et al 1999).

Measurement of TxB_2 formation from serum, plasma or urine has been suggested as a TxA_2-dependent method of measuring aspirin efficacy (Eikelboom et al 2002). In our study, plasma Tx levels were associated with enhanced platelet functions and aspirin resistance measured with AA-induced aggregation, and more so with PFA-100® (**II**). This could indicate that basal systemic Tx acts as a synergistic preactivating agonist, as has been suggested for epinephrine and thrombopoietin (see Akkerman 2006). A limitation of our study is, however, the lack of data on serum TxB_2 production which would better reflect the Tx-dependent platelet responses.

Thus, it seems that division of aspirin resistance into at least two different types is justified. The type directly related to aspirin's pharmacological mechanism of action, inhibition of TxA_2 formation, could possibly be labelled as *pharmacological resistance* as Wong at al (see 2004) suggested. However, despite the fact that current methods of measuring AA-induced aggregation and formation of TxA_2 seem the best possible options at this time, both have limitations leaving clinicians without a definitive test for pharmacological aspirin efficacy. Measurement of Tx levels ignores the fact that while aspirin might block COX-1-induced Tx formation in platelets, it could be formed in other cells or in platelets by COX-1-independent mechanisms, thus making a patient appear aspirin resistant in contrast to reality. In addition, increased levels of TxB_2, the stable metabolite of TxA_2, could also reflect enhanced F_2-isoprostanoid function and not specifically formation of TxA_2 from AA via COX enzymes

(Cipollone et al 2000). The recent findings of Frelinger et al (2006) also support the role of COX-independent Tx formation in aspirin resistance, as in aspirin treated patients a residual AA-induced aggregation could not be inhibited by additional *in vitro* aspirin.

In conclusion, AA-induced aggregations, preferably in combination with serum TxB_2 measurements, are at the moment best available method in detecting true, *pharmacological aspirin resistance,* whereas PFA-100® could be used to assess the general platelet activity, but is not specific to aspirin efficacy.

3 Prevalence of aspirin resistance

In this study PFA-100® detected 21% as aspirin resistant patients, while AA-induced aggregation detected only 5% as aspirin-resistant (**II**). This is in accordance with previous and later reports by others (Table 5). The variability in prevalence of aspirin resistance in previous studies can be explained by the variety of methods as well as differences in severity of atherothrombotic disease in the patients studied. In addition, variable doses of aspirin have been used. The present results are comparable to studies with similar patients and methodologies (Gum et al 2003). However, AA-induced aggregation has seldom been used to detect aspirin resistance, instead studies with PFA-100® or combination of aggregations with different agonists are more common. Likewise, PFA-100®, ADP- and collagen-induced aggregations are not specific to the mechanism of action of aspirin, but measure general platelet activity (see Cattaneo 2004, see Hankey & Eikelboom 2006).

4 Reasons for variability in aspirin efficacy

Several reasons for aspirin resistance, such as non-compliance, inadequate dosing, drug-interactions, diabetes, gender, genotype and increased TxA_2 production have been suggested. As recent studies found 2-3% of CAD patients non-compliant to aspirin (Tantry et al 2005, Frelinger et al 2006), in this study the compliance was assessed with questioning as well as salicylate measurement. In line with these previous findings, 2 of our patients had increased AA-induced aggregation, elevated Tx levels and no measurable salicylate in their plasma, suggestive of poor compliance or aspirin resistance (**II**). Nonetheless, the whole phenomenon of aspirin resistance is not explainable by poor compliance as we found measurable salicylate levels in patients with aspirin resistance measured with AA-induced aggregation (**II**). In addition, aspirin resistance has been reported despite supervised aspirin administration (Grundmann et al 2003).

Until further information is gained on factors affecting aspirin resistance also possible drug interactions should be considered. More information is needed on possibilities to overcome aspirin resistance with increased dosing. In light of current limited information it seems that some of the patients could benefit from increased dosing, but inadequate dosing is unlikely to explain the whole phenomenon. Patients with apparent states of increased platelet turnover, such as during post-operative periods, could be the ones to benefit from increased and more frequent dosing (Zimmermann et al 2003).

Interestingly, we found that type II diabetes seemed to be associated with non-response to aspirin measured with platelet function tests (**II**). Previously diabetics have been reported to gain less clinical benefit from aspirin than non-diabetics (ATC 2002, Sacco et al 2003).

Increased platelet activity and non-response to aspirin have been reported with insulin-resistant patients (Westerbacka et al 2002, Tamminen et al 2003), and enhanced COX-independent 8-iso-prostaglandin$F_{2\alpha}$ excretion and subsequent Tx formation have been suggested as the underlying mechanisms (Davi et al 1999). Increased inflammatory reactions and different inflammatory markers such as CD40 ligand in diabetics have been proposed to affect platelet activity (Varo et al 2003). However, in this study CRP was measured from half of the patients, but was not found to associate with aspirin response (**III**).

Females more frequently responded poorly to aspirin than males when measured with the high-shear dependent PFA-100® method (**II**). This phenomenon has also previously been reported both in clinical trials as well as laboratory measurements of aspirin resistance and platelet activity (Harrison & Weisblatt 1983, Escolar et al 1986, Gum et al 2003, Berger et al 2006). As female gender was found to be associated with shortened closure times in PFA-100®, but not with AA-induced aggregation, it is possible that this finding might reflect the increased basal level of platelet activity in females and not specifically the sensitivity to aspirin. Indeed, in a study of 711 women and 571 men it was found that though females had increased levels of platelet activity compared to males, they gained better inhibition by aspirin (Becker et al 2006). However, despite good inhibitory effects, the platelet reactivity of females remained increased compared to males (Becker et al 2006). In concordance with this is also our finding that individual variability in platelet activity is vast and thus might explain some of the variability in responses to antiplatelet medication (**I**).

Non-response to aspirin measured with AA-induced aggregation was associated with the rare G allele of the -A842G polymorphism of COX-1. On the other hand, the rare T allele of the C13254T polymorphisms of GP VI was common in non-responders to aspirin according to PFA-100®. This finding might reflect the differences in methods of measuring aspirin efficacy. As AA-induced aggregation is specific to COX-1 inhibition by aspirin the finding of an association between COX-1 polymorphism and aspirin resistance suggests that genotype influences individual's drug response (**II**). COX-1 polymorphisms might modulate the generation of TxA_2 in platelets and increased levels of TxA_2 have been suggested to influence aspirin resistance. In addition, extra-platelet formation of TxA_2, transient expression of COX-2 in platelets and formation of TxA_2 by COX-independent methods, such as via 8-iso-prostaglandin$F_{2\alpha}$ have been reported (Davi et al 1997, Weber et al 1999, Rocca et al 2002, Ziegler et al 2004). In contrast to AA-induced aggregations, PFA-100®, which measures platelet activity inducible also by TxA_2-independent mechanisms, associated with polymorphisms of GP VI. GP VI is a platelet receptor which mediates adhesion to collagen under high shear conditions, such as PFA-100®. The observations that different platelet function tests used for assessing the aspirin efficacy associated with two different polymorphisms is in concordance with the nature of these methods; AA-induced aggregation as a measurement of TxA_2-dependent platelet activation associated with COX-I polymorphisms and the PFA-100® as a measurement of more general platelet function, inducible by several different pathways including high shear rates associated with polymorphisms of GP VI receptor responsible with shear-induced activation and adhesion to collagen.

In conclusion, individual variability in aspirin efficacy has been shown, but the mechanisms behind it are yet to be resolved. The phenomenon seems multifactorial and this study showed that genotype is one of the underlying factors.

5 Measurement of clopidogrel resistance

Aspirin resistance became a hot topic due to increased knowledge of platelet functions, better availability and usability of tests to measure it and because clopidogrel with acceptable efficacy and safety profile was introduced as the first feasible option for aspirin. This situation also evoked interest in clopidogrel efficacy. Clopidogrel acts as an inhibitor of ADP-induced aggregation by blocking the $P2Y_{12}$-receptor. Thus its efficacy has most often been studied with assessment of ADP-induced platelet activity. Most recently measurement of intracellular activation such as VASP phosphorylation has been suggested to be more specific for determination of clopidogrel efficacy, but it too has limitations (Schwarz et al 1999, see Conley & Delaney 2003). In previous studies, patients have been regarded as resistant if clopidogrel has been unable to inhibit ADP-induced activation significantly. Different cut-off points for this dichotomy have been used. This has been objected to, since inhibition exerted by clopidogrel has been shown to follow a normal bell-shaped curve (Serebruany et al 2005). However, in our studies a strict criterion of 10% change in maximal aggregation induced by ADP was used to illustrate the minimal effect of clopidogrel in certain patient groups (**III, IV**). Despite this dichotomy to responders and non-responder in our study, the continuous nature of the phenomenon has been acknowledged.

6 Prevalence of clopidogrel resistance after loading dose in addition to ongoing aspirin

As clopidogrel administered on top of aspirin has proven beneficial in acute coronary syndromes and in a PCI setting we set out to study the variability in inhibition exerted by clopidogrel in CAD patients. Thus, a study involving 50 elective PCI patients using permanent aspirin was designed to study the efficacy of short-term clopidogrel in a setup mimicking an acute PCI setting (**III**). In healthy subjects a loading dose of clopidogrel (300 mg) was found to inhibit ADP (5 μM)-induced platelet aggregation by 70% within 2 h (Savcic et al 1999). However, in our study 40% of patients showed persistent aggregation to ADP (**III**). Therefore, we concluded that under acute PCI the inhibition exerted by the clopidogrel loading dose (300 mg) will not be complete in all patients and further antiplatelet medication, such as GP IIb/IIIa inhibitors, are needed. This prevalence of 40% is in concordance with studies by others, where prevalence of 30% is most often reported. The increased proportion of limited response in our study can be explained by the fact that we specifically determined clopidogrel efficacy shortly (2.5h) after administration. This was done to assess its usefulness in acute situations. Consequently, the clinical clopidogrel efficacy administered prior to PCI has been reported to be decreased compared to administration more than 6 h prior to the surgical procedure (Steinhubl et al 2002).

7 Prevalence of clopidogrel resistance in patients with ongoing treatment

As limited response to clopidogrel was frequently found shortly after administration, we then wanted to study the effect of an ongoing combination of aspirin and clopidogrel (**IV**). In addition, despite the fact that prolonged clopidogrel administration had been reported to improve its efficacy, recurrent thrombotic events still occur in patients with atherothrombotic disease (Steinhubl et al 2002, Gum et al 2003). We found 20% of patients with stable CAD to

have limited response to clopidogrel when measured with platelet function tests. Thus, it seems that there are significant individual differences in responses to antiplatelet medication.

8 Reasons for variability in the clopidogrel efficacy

Previously suggested explanations for variability in effectiveness of clopidogrel have been under-dosing, drug-interactions, insulin resistance, differences in the genotypes of platelet receptors or enzymes responsible for liver metabolism of clopidogrel, differences in metabolism and availability of antiplatelet drugs, increased levels of ADP, enhanced baseline function of $P2Y_1$-receptor, defects in signalling pathways downstream from the ADP-receptors and platelet activation via other pathways such as collagen, thrombin, or Tx.

Over-expression or -activity of $P2Y_1$-receptors has been reported to cause hyper-reactivity of platelets (Hechler et al 2003). Thus such state could be speculated to cause poor responses to clopidogrel. However, in our study the extent of $P2Y_1$-dependent ADP-induced aggregation did not vary between clopidogrel responders and resistant patients. Interestingly, the patients with poor response to *in vivo* clopidogrel were the ones with the least *in vitro* response to ARMX, suggesting that the limited response to clopidogrel emerges at or downstream of the receptor and not, for example, at the metabolism of the prodrug clopidogrel to its active metabolite. Indeed, unlike Lau et al (2002), but in accordance with Müller et al (2003a), we did not find impaired clopidogrel responses to associate with any statin. Enhanced basal platelet activity has also been suggested to explain limited responses to clopidogrel (Aleil et al 2005). However, in our study the basal platelet activity did not associate with clopidogrel response (**IV**).

The extent of clopidogrel response did not correlate with the polymorphisms of the CYP3A5 enzyme, which is responsible for most of liver metabolism of the prodrug (Savi et al 1994b). The different $P2Y_{12}$-receptor G52T polymorphism did not seem to explain the individual variability in responses to clopidogrel, a finding in concordance with previous studies (von Beckerath et al 2005a) (**IV**).

As activation of platelets by thrombin has been suggested to overcome inhibition of the $P2Y_{12}$-receptor at high concentrations, high thrombogenic burdens in patients with atherothrombotic disease could explain non-response to clopidogrel. However, in our study the responders and poor responders did not differ in their F_{1+2} levels while on aspirin before the induction of clopidogrel treatment (**IV**).

We found that the inhibition of ADP-induced aggregation by clopidogrel was not complete in CAD patients. Addition of *in vitro* $P2Y_{12}$-receptor antagonist ARMX further attenuated platelet functions on top of *in vivo* clopidogrel (**IV**). However, clopidogrel and ARMX have different modes of action, which complicates the direct comparison of these drugs. In addition, it could be argued that the results of these studies might have been influenced if larger doses of ARMX or clopidogrel would have been used. In latest studies which contradict the original ones, increasing the concentration of ARMX above 100 nM has been beneficial. ARMX, named cangrelor, has been proposed in clinical use as an intravenous drug with acceptable efficacy and safety profile (see Storey 2001, Greenbaum et al 2006). In previous studies ARMX has also been found to be more effective in inhibiting platelets than clopidogrel. However, in these comparisons a fixed dose of clopidogrel was used and there are no studies comparing larger doses of clopidogrel with ARMX. Recently, conflicting reports have appeared on the efficacy

of 600mg clopidogrel loading doses compared with 300mg. Larger loading doses intensify platelet inhibition, decrease the lag of effect after drug administration and reduce the number of patients with poor clopidogrel response (Müller et al 2001, Gurbel et al 2005b). However, the effect of increasing the daily clopidogrel dose has been poorly studied. Kastrati et al (2004) showed that loading dose of 600mg on top of ongoing 75mg/d clopidogrel caused additional inhibitory effect of platelet activation, which suggests to the fact that further benefits might be achieved by increasing daily dosages. In our study the effect of clopidogrel was studied in patients on aspirin treatment after 300mg loading doses followed by 75mg daily doses of clopidogrel, which represents the stable state drug effect with the current dosing regimen. Under-dosing is a possible contributor to clopidogrel resistance, but increased loading and especially maintenance doses of clopidogrel have not yet been widely studied.

When efficacy of the 300mg clopidogrel loading dose on top of ongoing aspirin was assessed in CAD patients who were to undergo PCI, increased body mass index and levels of C-peptide were found to be associated with decreased response to clopidogrel (**III**). However, when patients where treated with ongoing dual antiplatelet medication, clopidogrel response did not correlate with increased glycosylated hemoglobin levels, nor C-peptide levels (**III**). A possible explanation for this could be that these factors cause a delay, rather than suppression, in effect of clopidogrel. Activity of CYP 3A4 has been shown to be reduced in overweight patients and obesity is often related to type II diabetes (Kotlyar & Carson 1999).

In conclusion, varying clopidogrel efficacy has been found. The adequate dosing and timing of the clopidogrel treatment decrease the prevalence of non-response to clopidogrel. As in the case of aspirin, the reasons behind this phenomenon are many. It is seems that certain patient groups eg. diabetics would benefit from monitoring of their antiplatelet treatment.

9 Limitations of the study

This thesis consists of studies on the *in vitro* efficacy of antiplatelet medication with aspirin or the combination of aspirin and clopidogrel. While, the phenomenon of resistance to antiplatelet medication has been shown in patients with CAD the clinical significance of these findings remain to be confirmed in larger, prospective studies. Interestingly, the COX-1 -A842G polymorphism associated with decreased aspirin efficacy. However, most of the patients were either homozygous for the common A allele or heterozygous for the -A842G polymorphism. Only one patient was homozygous for the rare G allele for the -A842G polymorphism, precluding any conclusions on the functional role of this genotype. The limited prevalence of rare -A842G genotypes produced relatively small material for the study and thus, these results need to be confirmed in a larger study. We found no drug interactions which could have explained decreased efficacy of antiplatelet medication. However, this could have been influenced by limited study size.

10 Variability in efficacy of antiplatelet medication

Patients with atherothrombotic disease have variable inhibition of platelet function as measured *in vitro*. Taking notice of this is vital as the response to certain medications might vary from inadequate to excessive in different patients, thus exposing them to risk of an atherothrombotic event or bleeding, respectively. Some patients treated with either aspirin or the combination of aspirin and clopidogrel have suffered from clinical events and the risk of major bleeding in

these patients varies from 1-2%, but can be significantly higher in certain patient groups. Restrictions in the efficacy of antiplatelet medication suggest a need for enhanced or alternative antiplatelet medication in aspirin- or clopidogrel-resistant patients. Further studies in larger patient populations are needed to confirm our results. In addition, larger prospective studies are needed to determine how these platelet responses in functional tests associate with patient outcome. In the future, platelet function tests or pharmacogenetics may be applied to identify patients at high risk of cardiovascular events and in need of specifically targeted antiplatelet therapy.

CONCLUSIONS

This study was designed to assess the individual variability of platelet activity and responses to antiplatelet medication. It was found that a considerable number of CAD patients were non-responders either to aspirin, clopidogrel or both when measured with platelet function tests.

1) Large individual variability in platelet functions was found. Different methods of assessing platelet function under shear conditions correlated with each other. PFA-100® and whole blood perfusions are useful in assessing individual variation of platelet activity.

As platelet aggregation tests and PFA-100® represent dissimilar environments and use different platelet activating agents the results between them are not comparable. Indeed, they detected two different populations as being aspirin resistant. AA-induced aggregations, unlike PFA-100®, can be used to detect pharmacological aspirin resistance. However PFA-100® detects enhanced platelet activity, also considered as functional aspirin resistance. PFA-100® failed to detect variability in responses to short-term clopidogrel and thus cannot be used for monitoring of its efficacy.

2) There seems to be a patient population that fails to achieve full platelet inhibition by aspirin treatment or combination of aspirin and clopidogrel. Considering the worldwide prevalence of atherothrombotic disease size this patient group is considerable. In future platelet function test might prove beneficial in tailoring individual antiplatelet medication.

3) Mechanisms behind decreased aspirin efficacy or combination of aspirin and clopidogrel are multifactorial. Both genetically determined factors such as gender and platelet receptor genotype as well as acquired factors such as type II diabetes influence platelet activity and efficacy of antiplatelet medication. In future, special care could be used when administrating antiplatelet medication to patients with known risk factors for resistance towards antiplatelet medication.

ACKNOWLEDGEMENTS

This study was undertaken in the Wihuri Research Institute and further carried out at the Coagulation Disorders Unit of the Division of Hematology, Department of Medicine, Helsinki University Central Hospital. I want to express my thankfulness to Professor Petri Kovanen for the research facilities at the Wihuri Research Institute and to Docent Riitta Lassila for those at Coagulation Disorders Unit.

I would not have been able to accomplish this work without the help of a number of people to whom I wish to express my deepest gratitude:

My supervisor Docent Riitta Lassila for her vast knowledge of platelets, continuous support and efficient way of working. The fact that the Coagulation Disorders Unit was generated and consolidated during the period of my research and that it has been only one of the things achieved by her during this time, reflects her interest, expertise and dynamics. Her never-ending enthusiasm, which she also channels into motivating us "youngsters", has even caused my friends from other fields to congratulate me for my luck.

Professor Heikki Vapaatalo and Professor Christian Gachet for their valuable advice and their encouraging support at the final stage of this study. Professor Vapaatalo put his enormous academic experience at my disposal in the search for the final structure of the thesis. Professor Gachet delivered most profound and updated information especially on ADP and ADP-receptor antagonism.

For Julio Reséndiz MD, Docent Jussi Mikkelsson MD, Docent Kari Virtanen MD, Pia Siljander PhD for important discussions, methodological tutorage, and their contributions during the years as well as Professor Juhani Heikkilä MD, Professor Pekka Karhunen MD, Leena Viiri MSc, Professor Jürg H Beer MD, Docent Martti Syrjälä MD, Docent Janne Backman MD, Esko Kankuri MD, Ulla Wartiovaara MD for their ideas and valuable collaboration in the original studies.

Sorella Ilveskero MD for her guidance during my first steps in the world of science and in many practical issues during these years, and the other members of the thrombosis group, Seija Peltonen MD, Pirjo Mustonen MD, Petteri Kauhanen MD, Eeva-Maija Weselius MD and Birgitta Salmela MD.

Ms. Marja Lemponen for teaching me the technical performance of all the platelet function tests, being my right hand in the laboratory and good company in the coffee-room, without forgetting the important roles of Ms. Tuula Järvenpää and Ms. Maria Kellokoski as well as the personnel at Wihuri Research Institute.

Heini Huhtala MSc for help with statistics, Jodie Painter MD for revision of the English language, Ms. Helena Schmidt for helping me with the figures, Ms. Sari Hirvonen for repacking my reference list.

My family for their caring. My father Mauri for encouragement during the times of bewilderment and uncertainty, sharing during joys and successes, listening during the times of anxiety and

distress. My mother Outi for her mind for balance and for all help given whenever I needed it. My brother Antti as well as Anni for their friendship and warmth.

My friends for their friendship, relaxing times away from the platelets and encouragement. Noora for right words in the critical moments. Anniina for taking care of this lonely thesis-writer especially at times when mostly needed. Kikka for encouraging me to take time off for a cup of coffee with good friends whenever possible. Tuula for listening, understanding and support. Anne, Heidi and Maukka for refreshing horse rides in the woods.

Helsinki University Central Hospital Research Funds, Finnish Society of Angiology, Einar and Karin Stroems Fund, Aarne Koskelo Foundation, Maud Kuistila Foundation, Finnish Heart Research Foundation**,** Aarne and Aili Turunen Foundation and Finnish-Norwegian Medical Foundation for financial support.

Tampere, 13th of December 2006

Aino Lepäntalo

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