Central role of the platelet in arterial thrombosis

Platelet rich thrombus generation at the site of plaque rupture is the primary underlying factor responsible for the development of ischemic events in patients with cardiovascular disease. In the setting of pre-existing dysfunctional endothelium and inflammation, uncontrolled platelet activation leads to occlusive thrombus generation. Thrombus development also leads to embolization resulting in microvascular dysfunction observed in stroke and myocardial infarction (MI). Platelets are not only central to these thrombotic events, but also play important roles in the progression of atherosclerosis, coagulation and inflammation [1-3]. Therefore, pharmacologic strategies associated with superior platelet inhibition are expected to produce superior clinical outcomes by attenuating the occurrence of ischemic events in patients with cardiovascular disease (the “platelet hypothesis”).

Atherosclerotic plaque rupture and endothelial denudation that occur during acute coronary syndromes and percutaneous interventions result in the exposure of the subendothelial matrix. Following adhesion to the exposed subendothelial matrix, platelets are activated by shear and soluble agonists released at the site of plaque rupture. The binding of thrombin generated by exposed tissue factor, collagen and von Willebrand factor (vWF) (primary platelet activating factors) to specific platelet receptors leads to the release of major secondary agonists. Thromboxane (Tx) A2 is produced from arachidonic acid originating from membrane phospholipids. Cyclooxygenase (COX)-1 converts arachidonic acid to PGH2 that is subsequently converted to TxA2 by platelet TxA synthase. Adenosine diphosphate (ADP) is secreted from dense granules [1,2].

Amplification of aggregation by thromboxane A2 and ADP

TxA2 binds to thromboxane receptors whereas ADP binds to P2Y12 (Fig.) and P2Y1. These two secondary agonists are necessary for the propagation of platelet activation at the site of plaque rupture through paracrine mechanisms resulting in further platelet aggregation.
in sustained expression of activated GPIIb/IIIa receptors that possess fibrinogen binding sites. It has been proposed that phosphatidyl-inositol 3-kinase dependent signaling downstream of P2Y_{12} plays a critical role in the sustained activation of the GPIIb/IIIa receptor (Fig.) [3,4]. Stable platelet aggregation develops through fibrinogen and vWF binding.

Activation of platelets by ADP also leads to surface expression of P-selectin and CD40L that are important in platelet-leukocyte interactions and further amplification of inflammation and thrombin generation (Fig.) [1,2]. Platelet activation also results in the membrane exposure of phosphotidyl serine providing binding sites for coagulation factors. Large amounts of thrombin are produced that convert fibrinogen to fibrin leading to the formation of a fibrin network and a stable occlusive platelet-fibrin clot.

**Platelet inhibition by aspirin and P2Y_{12} blockers**

Aspirin irreversibly acetylates serine residue (ser529) in COX-1 preventing the binding of arachidonic to the catalytic site. Controversy exists regarding the clinical relevance of non-COX-1 mediated antiplatelet effects of aspirin [2]. In the ASPECT Study, a double crossover investigation of 3 different doses of aspirin (81, 162, and 325 mg daily), we observed dose-dependent inhibition of collagen-, ADP-, and shear-induced platelet aggregation. COX-1 activity was profoundly inhibited at all 3 doses [5]. ASPECT Study raised the question whether selected patients may benefit form >81 mg daily aspirin through improved inhibition of non-COX-1 pathways.

Clopidogrel is a second-generation thienopyridine that is converted to an active metabolite by the hepatic cytochrome P450 pathway [2]. The active thiol metabolite of clopidogrel forms a covalent disulfide bond with cys17 and cys270 residues present in the extracellular domains of P2Y12 and inhibits ADP binding. Pharmacodynamic studies have demonstrated a faster onset of effect and increased platelet inhibition associated with less nonresponsiveness after higher loading doses of clopidogrel (≥600 mg) as compared to a 300 mg loading dose [6,7].

New P2Y_{12} receptor antagonists are currently undergoing investigation [8-15]. Prasugrel is a third generation thienopyridine that is associated with greater active metabolite generation, superior inhibition of ADP-induced platelet aggregation and less response variability than clopidogrel [8]. Ticagrelor (AZD6140) is a novel oral cyclo-pentyl-triazolo pyrimidine (CPTP) non-thienopyridine agent that acts directly (requires no metabolic activation) and provides rapid, reversible, and
potent P2Y<sub>12</sub> receptor inhibition. The plasma t 1/2 is approximately 12 hours and thus requires twice daily dose administration [10]. In a platelet function substudy of Dose confirmation Study assessing anti-Platelet Effects of AZD6140 versus clopidogrel in NSTEMI (DISPERSE)-2, a randomized comparative trial of ticagrelor versus clopidogrel in patients presenting with acute coronary syndromes, ticagrelor provided a greater magnitude of platelet inhibition with less inter-individual variability than was observed with clopidogrel [12].

Cangrelor (ARC 69931 MX) is a parenterally administered direct acting ATP analogue that provides dose dependent, reversible P2Y<sub>12</sub> inhibition. At high doses, cangrelor achieves nearly 100% inhibition of ADP-induced aggregation with very limited inter-individual variability in response. The plasma t 1/2 of cangrelor is approximately 3.3 minutes and platelet function returns to normal rapidly (~60 min) following termination of an intravenous infusion [13,14].

PRT128 is an oral and parenteral, direct-acting, reversible P2Y<sub>12</sub> inhibitor. PRT128 has been demonstrated to be a more potent antithrombotic agent than clopidogrel in an animal model [15]. Like ticagrelor and cangrelor, PRT128 also shows promise as an effective and reversible drug for treating patients undergoing percutaneous coronary intervention (PCI).

Central role of P2Y<sub>12</sub> receptor signaling

The pivotal role of P2Y<sub>12</sub> mediated signaling in the generation of stable thrombi is supported by multiple lines of evidence:

1) studies demonstrating attenuation of platelet aggregation induced by multiple agonists through P2Y<sub>12</sub> blockade [2]

2) modulation of procoagulant activity and thrombin generation by clopidogrel and prasugrel [16-18]

3) modulation of P-selectin expression and soluble CD40L by clopidogrel treatment [19,20]

4) modulation of inflammation marker release such as C-reactive protein and tumor necrosis factor-α by clopidogrel [20,21]

5) association of adverse ischemic events with high on-treatment ADP-induced platelet reactivity [1,22]

6) superior clinical outcomes with respect to ischemia observed in patients treated with the more potent P2Y<sub>12</sub> receptor blockers [8,9,11]

7) recent observations of the clustering of adverse events in the initial 90 days after stopping clopidogrel among both medically treated and PCI-treated patients with acute coronary syndrome (ACS) [23].

Clinical trial data to support the importance of P2Y<sub>12</sub> in atherothrombosis

Dual antiplatelet therapy with aspirin and clopidogrel is the current standard of care to prevent thrombosis in patients with acute coronary syndromes and patients undergoing stenting, especially with drug eluting stents. Optimal platelet inhibition is dependent upon the degree of ischemic risk in the individual patient and is counterbalanced by the risk of bleeding.

In the Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial, clopidogrel was associated with an 8.7% relative risk reduction compared to aspirin for the occurrence of the composite endpoint of vascular death, MI, or stroke and further reduced re-hospitalization for ischemic events [24]. The addition of clopidogrel to aspirin was associated with significantly lower adverse vascular events in the Antithrombotic Trialists’ Collaboration meta-analysis [25]. Subsequent landmark clinical trials in high-risk patients have demonstrated that clopidogrel plus aspirin therapy is superior to aspirin therapy alone in reducing the odds of serious cardiovascular events including stroke, MI or vascular death.

In the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial, dual antiplatelet therapy was associated with a 20% reduction in relative risk for the composite endpoint of cardiovascular (CV) death, MI, or stroke compared to aspirin plus placebo [26]. In a subset analysis of the CURE study (PCI-CURE) patients who underwent PCI and received clopidogrel and aspirin pretreatment for up to 10 days and continued on long-term treatment, there was a ~30% reduction in the risk of MI before PCI and cardiovascular death or MI four weeks after PCI [27]. In the Clopidogrel for the Reduction of Events During Observation (CREDO) trial, there was a 26.9% relative reduction in the combined risk of death, MI, or stroke at 1 year in patients undergoing PCI treated with 12 months of dual antiplatelet therapy. Benefits of a clopidogrel loading dose (300 mg) were seen only when the loading dose was given more than 6 hours before PCI [28].

In the Clopidogrel as Adjunctive Reperfusion Therapy-Thrombolysis in Myocardial Infarction (CLARITY)-TIMI 28 study, 3491 patients within 12 hours of onset of STEMI received clopidogrel pretreatment (300 mg loading dose followed by 75 mg/day) or placebo in addition to aspirin and fibrinolytic therapy. Angiography was performed 2 to 8 days after enrollment. There was a 36% reduction in odds of the primary endpoint (composite of occluded infarct artery or death or recurrent MI before angiography) and a 20% reduction in the composite end point of cardiovascular death, reinfarction, or recurrent ischemia requiring urgent revascularization at 30 days in the clopidogrel group [29]. The PCI-CLARITY study which included 57% of patients from CLARITY-TIMI 28 who underwent PCI, showed that clopidogrel pretreatment was associated with a 46% reduction in the odds of cardiovascular death, recurrent MI or stroke within 30 days with no significant increase in the incidence of bleeding complications [30]. This benefit was observed regardless of GPIIb/IIIa inhibitor treatment or a loading dose of open-label clopidogrel at the time of PCI. It is also interesting to observe that patients who were pretreated with a daily dose of 75 mg clopidogrel and received an additional
loading dose of 300 mg at the time of PCI, had the maximum protection against death, reinfarction or stroke [31].

Dual antiplatelet therapy has also demonstrated efficacy in high-risk populations with established cardiovascular disease, as shown by a 12.5% relative reduction in the composite endpoint of MI, stroke, or CV death compared to aspirin alone in patients in the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization Management and Avoidance (CHARISMA) trial [32].

A recent meta-analysis revealed that a high clopidogrel loading dose (600 mg) during PCI was associated with a superior one month clinical outcome (cardiac death or nonfatal MI) without any significant increase in major or minor bleeding compared to a 300 mg loading dose [33].

In the TRial to assess Improvement in Therapeutic Outcomes by optimizing platelet InhibitionN with prasugrel (TRITON)-TIMI 38 trial, the third generation thienopyridine, prasugrel was compared to clopidogrel in patients with moderate to high risk acute coronary syndromes undergoing PCI. The prevalence of cardiovascular death, nonfatal myocardial infarction or nonfatal stroke was lower with prasugrel treatment compared to clopidogrel (12.1% vs. 9.9%). However, there were higher rates of bleeding in the prasugrel group [9]. TRITON is a landmark study that tested the platelet hypothesis and conclusively demonstrated that superior P2Y12 blockade produces superior reduction in ischemic events in moderate to high-risk ACS patients.

A recent meta-analysis of randomized clinical trials that compared the addition of clopidogrel to aspirin plus placebo for the treatment of coronary artery disease, demonstrated a reduction in all-cause mortality (6.3% vs. 6.7%, p = 0.023); a reduction in myocardial infarction (2.7% vs. 3.3%, p = 0.001); and a reduction in stroke (1.2% vs. 1.4%, p = 0.002) [34]. These data highlight the superior clinical efficacy resulting from P2Y12 blockade in patients with cardiovascular disease who are at risk for ischemic events.

In the DISPERSE-2 randomized comparative trial of ticagrelor versus clopidogrel in patients presenting with acute coronary syndromes, myocardial infarction was less frequent in patients receiving ticagrelor than clopidogrel. Ticagrelor is being compared to clopidogrel in the treatment of acute coronary syndromes in the ongoing PLatelet inhibition And Patient Outcomes (PLATO) trial [8]. Cangrelor is currently undergoing clinical evaluation in the Cangrelor versus standard therapy to Achieve optimal Management of Platelet Inhibition PCI (CHAMPION) trial [35].

Translational research supporting the importance of P2Y12 in atherothrombosis

Since ADP is an important secondary agonist that plays a critical role in the amplification of platelet aggregation and the genesis of a stable, occlusive thrombus, much interest has been focused on determining whether poor inhibition of ADP-induced platelet aggregation (non-responsiveness to clopidogrel treatment) and high on-treatment platelet reactivity correlate with the occurrence of adverse ischemic events. Matetzky, in a study of clopidogrel responsiveness

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ADP – adenosine diphosphate, MACE – major adverse cardiovascular events, PCI – percutaneous coronary interventions
in patients undergoing stenting for acute STEMI, found that patients who exhibited the lowest quartile of platelet inhibition had a 40% probability for a recurrent cardiovascular event within 6 months [36].

In the prospective PREPARE POST-STENTING (Platelet Reactivity in Patients And Recurrent Events POST-STENTING) Study of 192 consecutive patients undergoing elective stenting, we first demonstrated the relation of high on-treatment platelet reactivity to ADP measured by light transmittance aggregometry to ischemic event occurrence [37]. A higher rate of recurrent ischemia was observed in patients within the highest quartile of ADP-induced platelet aggregation as compared to patients within the lowest quartile. In a prospective study of patients followed for up to 2 years post-PCI, we demonstrated that on-treatment 20 μM ADP-induced platelet aggregation above a cutpoint was the most significant risk factor for the occurrence of ischemic events (odds ratio = 8.6, p < 0.0001) [38].

In the CLEAR PLATELETS (Clopidogrel Loading with Eptifibatide to Arrest the Reactivity of Platelets) Study, high periprocedural platelet reactivity to ADP was associated with the occurrence of in-hospital myocardial infarction [39]. Subsequent investigations by others have also demonstrated that PCI patients with high post-treatment platelet reactivity to ADP exhibit an increased risk of cardiovascular events [40-45].

The association of high on-treatment ADP-induced platelet aggregation to the occurrence of stent thrombosis has been explored in several studies. Barragan and colleagues demonstrated that poor clopidogrel responsiveness indicated by a high P2Y₁₂ receptor reactivity ratio measured by vasodilator-stimulated phosphoprotein (VASP) phosphorylation was associated with stent thrombosis [46]. In the CREST (Clopidogrel Effect on platelet Reactivity in patients with Stent Thrombosis) Study, we demonstrated elevated levels of ADP-stimulated expression of active GPIIIa/IIia expression by flow cytometry, increased ADP-induced aggregation and a high P2Y₁₂ reactivity ratio measured by VASP phosphorylation in patients with stent thrombosis compared to patients free of stent thrombosis [47]. Recent results of Buonamici, in the largest prospective study thus far (n = 804), have supported that high on-treatment platelet reactivity measured by aggregometry is an independent predictor of stent thrombosis (Tab.) [48].

Is there a platelet reactivity threshold predictive of ischemic events?

Recent data suggest that there may be a threshold of platelet reactivity as measured by light transmittance aggregometry after ADP stimulation of platelet rich plasma that predicts an increased risk of thrombotic events following PCI. The CLEAR PLATELETS Study results demonstrated that >50% mean platelet aggregation in response 5 μM ADP was a threshold for the occurrence of periprocedural myocardial infarction [39]. In the PREPARE-POSTSTENTING study, a threshold of ~50% periprocedural platelet aggregation in response to 20 μM ADP predicted the subsequent development of ischemic events following stenting within 6 months [37]. In the CREST study, ~40% platelet aggregation in response to 20 μM ADP was associated with the occurrence of stent thrombosis [47]. Finally, in a recent study by our group, patients treated with long term clopidogrel and aspirin prior to PCI had a threshold of ~40% preprocedural platelet aggregation in response to 5 μM ADP that was associated with the occurrence of ischemic events in the 12 months following stenting [40]. These studies may provide a "testable" level of platelet reactivity in future studies, similar to the international normalized ratio ranges established for warfarin therapy.

Limitations of measuring platelet function in isolation

The development of atherothrombosis is heavily influenced by platelet function, inflammation, and hypercoagulability ultimately leading to symptomatic occlusive thrombus generation in selected patients. Several events must occur in order for a stable thrombus to develop at the site of plaque rupture. Platelets must first adhere firmly to the subendothelium, and undergo sustained activation by secondary agonists. The coagulation cascade must be activated with sufficient kinetics to generate a clot having strong tensile strength to withstand the disruptive effects of blood flow. The majority of previous translational research studies focused on measuring platelet function in isolation either to evaluate the relation to adverse ischemic events, or to evaluate the efficacy of antiplatelet therapy. Since platelet function is intimately associated with thrombin generation and fibrin network formation, the measurement of platelet function in isolation may not be the optimal tool to assess thrombotic risk. Therefore, in addition to measuring platelet function, the measurement of platelet-fibrin interactions together with an analysis of thrombin generation kinetics may be more informative. In this regard it was demonstrated that high maximum platelet-fibrin clot strength, as measured by thrombelastography, was more predictive of long-term ischemic events than ADP-induced platelet aggregation measured by light transmittance aggregation [37].

Another study demonstrated a link between a prothrombotic state, characterized by ex vivo measurements of high platelet-fibrin clot strength, platelet reactivity, and inflammation characterized by the elevation of selected biomarkers. Moreover, the prothrombotic state identified prior to stenting strongly correlated with 2 year ischemic risk [49]. In another report the prothrombotic state was most prevalent in patients with symptomatic disease requiring PCI as compared to asymptomatic patients with long term quiescent coronary disease [50].

What are the reasons for thienopyridine treatment failure?

Despite significant clinical benefits associated with dual antiplatelet therapy in the treatment of high risk patients,
10 to 20% of treated patients will suffer from recurrent thrombotic events during long-term follow-up. In the TRITON Trial there was a high prevalence of treatment failure (~10%) even with the superior platelet inhibitor, prasugrel [9]. The explanation for the high rate of dual antiplatelet treatment failure remains an unresolved and underinvestigated critical issue. The clinical trials described above have been limited by a “one size fits all” approach that ignores the individual patient’s antiplatelet response. In addition to antiplatelet non-responsiveness, other potential reasons for treatment failure include: 1) non-compliance; 2) underdosing in selected patients; 3) premature discontinuation; and 4) other uninhibited pathways leading to platelet activation.

Current research is addressing the importance of uninhibited thrombin-induced platelet activation by the administration of specific protease activated receptor (PAR)-1 blockade. Oral PAR-1 antagonists may provide several advantages over thrombin inhibitors by having no influence on the enzymatic effect of thrombin in the coagulation cascade, the generation of the fibrin network and the stimulation of anticoagulant pathways (activation of protein C). These attributes make PAR-1 antagonism a unique antithrombotic target with potential limited bleeding side effects [51].

SCH-530348, a derivative of himbacine, is a specific, potent and reversible PAR-1 antagonist with a long half-life and no effect on bleeding time or other receptor signaling pathways in platelets. In a recently completed randomized, double-blind, placebo controlled, dose ranging Phase 2 study (TRA-PCI), 1030 patients undergoing coronary angiography and/or non-emergent PCI were treated with loading doses of 10, 20 or 40 mg of SCH-530348 together with aspirin, clopidogrel, and an antithrombotic agent (heparin or direct thrombin inhibitor) [52]. Following PCI, maintenance doses of 0.5, 1 or 2.5 mg were administered for 60 days along with aspirin and clopidogrel. Treatment with SCH530348 was not associated with a significant increase in the trial primary endpoint (TIMI major or minor bleeding) while slight reductions in the secondary endpoints of MACE and MI were observed. In a substudy, SCH530348 did not effect arachidonic acid, ADP- or collagen induced platelet aggregation, but was associated with >80% inhibition of 15 mM TRAP-induced platelet aggregation at both the 1 and 2.5 mg maintenance doses [52]. The results from TRA-PCI have provided the rationale for two large scale ongoing multinational, randomized, double-blind, placebo-controlled phase 3 studies: the Thrombin Receptor Antagonist in Secondary Prevention of atherothrombotic events (TRA20P-TIMI 50) and the Thrombin Receptor Antagonist in Acute Coronary Syndrome (TRA-ACS) trials.

Results from pharmacodynamic studies and translational research studies assessing platelet reactivity have highlighted the limitations of clopidogrel therapy. The data from translational research studies present strong arguments against the “one size fits all” approach that has been used in large-scale clinical trials. At one end of the spectrum, selected patients with excessively low on-treatment platelet reactivity may unnecessarily bleed while other patients with high platelet reactivity may experience ischemic events.

**Clopidogrel resistance and response variability**

The phenomena of clopidogrel response variability was initially reported by measuring platelet aggregation in patients undergoing coronary stenting [53]. Of great potential concern was the observation of nonresponsiveness (“resistance”), defined as ≤10% absolute change in ADP-induced platelet aggregation in a substantial percentage of patients. It was also demonstrated that clopidogrel nonresponsiveness was dependent on the time of platelet function measurements in reaction to drug administration and the clopidogrel dose [6,53]. Other investigators confirmed these results and it is now well established that 5 – 44% of patients may exhibit clopidogrel nonresponsiveness [54]. These data served as the rationale for the development of the new P2Y12 receptor blockers that have superior pharmacodynamic profiles.

The mechanisms responsible for clopidogrel response variability and resistance are incompletely defined. Several lines of evidence indicated that clopidogrel non-responsiveness is a pharmacokinetic problem associated with insufficient active metabolite generation that is influenced by limitations in intestinal absorption, and functional and genetic variability in the hepatic cytochrome P450 isoenzymes [55]. In addition, diabetes and body mass index were also implicated as contributors to the prevalence of clopidogrel nonresponsiveness [56,57].

**Underutilization, noncompliance and premature discontinuation**

Despite the proven benefits of thienopyridine therapy in acute coronary syndromes and stenting its use in the real world is still limited as reported in various registries. For example, in the Global Registry of Acute Coronary Events (GRACE), overall only 30% of patients with ACS received thienopyridines; use was 39.2% in the USA versus 24% in Europe [58]. In a recent population based cohort study from Canada, the lowest prescription fill rate for cardiac medications was for antiplatelet therapy. Only ~44% of patients with MI filled their antiplatelet prescription. One-year mortality was significantly higher in patients who did not fill their discharge prescriptions [59].

Stent thrombosis occurs in compliant and non-compliant patients. Among the former patients, response variability and resistance to antiplatelet therapy may play an important role. Premature discontinuation of antiplatelet therapy is an important risk factor, for the occurrence of stent thrombosis [60]. In a retrospective cohort study, clustering of adverse events in the initial 90 days after cessation of clopidogrel treatment among both medically treated and PCI-treated patients with ACS suggested a rebound hyperthrombotic period [23].
In an observational study drug eluting stent (DES) thrombosis occurred in 1.3% of patients (29/2229); 0.6% had subacute stent thrombosis (SAT) and 0.7% had late stent thrombosis (LST) at 9 month follow-up. Premature discontinuation of antiplatelet therapy was the main independent predictor of SAT and LST [61]. Moreover, a recent observational study assessed the association between clopidogrel use and long-term clinical outcomes in 4666 patients undergoing PCI with bare metal stents (BMS) or DES. Among patients treated with DES who were event free at 12-months, continued clopidogrel use was associated with lower death or MI at 24 months. However, and same difference was not observed in patients treated with BMS [62]. Therefore, current guidelines recommend long-term clopidogrel treatment (up to one year or beyond) following DES implantation [63].

Pharmacologic blockade of COX-1 and P2Y$_{12}$ have revolutionized the treatment of patients with coronary artery disease. The importance of P2Y$_{12}$ in the genesis of thrombosis has been confirmed by large scale clinical trials across the spectrum of acute coronary syndromes. Ongoing studies are evaluating the role of reversible and more potent P2Y$_{12}$ inhibitors than clopidogrel. Inhibitors of receptors other than P2Y$_{12}$ have the potential to overcome treatment failure associated with current dual antiplatelet therapy.

Translational research has identified high on-treatment platelet reactivity to ADP as a quantifiable and modifiable risk factor [22]. The determination of an on-treatment platelet reactivity target that optimally prevents thrombotic events and avoids bleeding risk remains an elusive and overall understudied goal at this time. Large prospective trials are needed to establish the role of individualized antiplatelet therapy guided by platelet function measurements. Importantly, platelet reactivity and other biomarker measurements in translational research studies may assist in identifying the high risk patient prior to the occurrence of the first thrombotic event. Based on the current evidence, platelet reactivity has the potential to become a standard of care risk factor measured in all patients with cardiovascular disease.

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