# Reticulated Platelets: Changing Focus from Basics to Outcomes

Bashar Hannawi[1]    Yousef Hannawi[2]    Neal S. Kleiman[1]

[1] Department of Cardiology, Houston Methodist DeBakey Heart and Vascular Center, Houston, Texas, United States
[2] Division of the Cerebrovascular Diseases and Neurocritical Care, Department of Neurology, The Ohio State University, Columbus, Ohio, United States

**Address for correspondence** Neal S. Kleiman, MD, 6565 Fannin Street, MS F-1035, Houston, TX 77030, United States (e-mail: nkleiman@houstonmethodist.org).

## Abstract

**Keywords**
► coronary artery disease
► stroke
► reticulated platelets
► immature platelets
► platelet function test
► anti-platelet drugs

Platelets play an essential role in the pathophysiology of atherothrombosis. Reticulated platelets (RPs) are the youngest platelet population in the circulation; their presence is an indicator of platelet turnover. Circulating levels of RPs are increased in patients with coronary artery disease and stroke. Preliminary indications are that the proportion of circulating RP is associated with the likelihood of ischaemic events such as acute coronary syndrome and stroke. Plausible mechanisms include: (1) increased participation of these platelets in thrombosis due to messenger ribonucleic acid that may be translated to active proteins, (2) lack of exposure to anti-platelet drugs since they are newly released from the bone marrow or (3) their presence is a non-specific marker of inflammation. In this state-of-the-art review, we discuss the implication of RP in coronary artery disease and in hypo-responsiveness to the most commonly used anti-platelet drugs.

## Introduction

Platelets are an essential component of the thrombotic process that follows plaque rupture and leads to acute coronary syndrome (ACS),[1,2] and acute ischaemic stroke (AIS).[3] Hence, anti-platelet regimens have become essential parts of the acute therapy and secondary prevention of atherosclerotic diseases.[4] Recently, the concept of anti-platelet drug resistance has emerged as a potential cause of thrombotic coronary artery events.[5] One of the plausible mechanisms contributing to this phenomenon includes the participation of immature or 'reticulated' platelets (RPs).[6,7] Here, we review the methodologies of RP assays, their currently known role in the pathophysiology of intravascular thrombosis associated with atherosclerosis and their role in the most common clinical complications of atherosclerosis, including ACS and AIS. We also discuss the role of RP in impaired platelet response to the most commonly used anti-platelet drugs.

## Reticulated Platelets: Definition and Measurements

### Definition and Functional Properties

Heterogeneity of platelet size and function has been recognized since the 1930s. Serious attention turned to differential platelet characteristics following the studies of Karpatkin.[8] The author separated platelets in the human circulation according to the size and weight and described 'large heavy' and 'small light' platelet populations. Similar platelet populations were then identified in rabbits; survival studies of radiolabelled platelets suggested that the large heavy platelets represented a younger population than the small light platelets. Metabolic studies pointed towards increased thrombotic activity in large heavy platelets. Adenosine diphosphate (ADP) and platelet factor 4 release following stimulation with ADP, thrombin or epinephrine were enhanced several fold in this group, compared with small light platelets.[9] Subsequently, Blajchman et al studied

newly released platelets in rabbits that had undergone bone marrow irradiation. Platelets harvested before the nadir platelet count occurred were considered to be old platelets, while those harvested during recovery of the counts were considered to be newly released. Newly released platelets were about 50% larger, had a measured survival time that was about threefold greater and contained greater amounts of membrane glycoproteins (GPs) than the older platelets. Bleeding times in the rabbits were also shorter when the platelet population consisted of younger platelets.[10] Since the late 1960s, a preponderance of evidence has indicated that larger platelets are more likely to be younger than smaller platelets, contain higher concentrations of thrombotic mediators and are more likely to participate in thrombosis. However, the literature does not universally support specifically identifying larger platelets as younger platelets, as some investigators have reported that quantitative differences between platelet populations (i.e. increasing size imparting increasing quantities of functional GPs) rather than qualitative differences (i.e. age alone) are responsible for functional differences.[11,12]

The term 'RPs' was first used to describe newly synthesized platelets by Ingram and Coopersmith. These investigators subjected dogs to acute blood loss and then sampled circulating platelets 5 to 7 days later. Using light microscopic techniques, they stained platelets with methylene blue, which was known to bind to ribonucleic acid (RNA) and had previously been used as a supravital stain to identify nucleated red blood cells that indicated accelerated erythropoiesis. They then studied platelets before and after phlebotomy. During the recovery phase after phlebotomy, reticular patterns were up to fivefold more common than prior to bleeding. Reasoning that these same patterns were analogous to those seen in juvenile red blood cells, they proposed that the RPs were recently released from the bone marrow.[13] More sophisticated identification of newly released platelets began in the early 1990s. Reticulated red blood cells were identified with flow cytometry using the fluorophore thiazole orange (TO), which binds to nucleic acids with particular affinity for RNA and fluoresces at 488 nm, increasing the fluorescence of RNA approximately 3,000-fold.[14] Interestingly, 'platelet contamination' was originally listed among the sources of erroneous reticulocyte determinations. In 1990, Kienast and Schmitz reported this technique after TO staining in thrombocytopenic patients who were found to have normal or increased numbers of megakaryocytes in the bone marrow. TO-positive staining was present in 26.9% of platelets in this population compared with 8.6% of platelets in control subjects. In contrast, patients with impaired platelet production did not have increased TO staining.[15] The implication was that TO-labelled platelets indicated increased platelet production in the bone marrow. This hypothesis was later confirmed in biotinylation experiments. Biotin binds covalently to free amino acids in the cell membrane of the circulating cells, but not to the membranes of cells sequestered in the bone marrow. Biotin injection can therefore be used to label cells that are already present in the circulation and this distinguishes them from cells that enter the circulation after the injection has been performed. In a series of experiments, circulating (biotin-positive but TO-negative) platelets were distinguished from newly synthesized (biotin-negative but TO-positive platelets), establishing that the nucleic acid-rich platelets were less than 24 hours old.[16,17] Accordingly, RP counts have been used clinically to distinguish between thrombocytopaenia due to platelet consumption from that to decreased platelet production,[18] and to monitor response to exogenous thrombocytopaenia.[19] It should be noted, though, that TO also stains dense granule contents. When platelets are incubated with high concentrations of TO or when the duration of incubation is prolonged, the finding of TO-positivity loses specificity and therefore platelets categorized as being 'reticulated' may not necessarily be newly synthesized.[20]

Much of our understanding of RPs is derived from the observation of platelet size heterogeneity with the assumption that the pool of larger platelets consists predominantly of RPs. Under conditions of stimulated thrombopoiesis, the bone marrow tends to produce larger platelets.[11] As platelets increase in size, they tend to produce more rapid and complete aggregation,[21] contain higher concentration of adenosine triphosphate, adenosine monophosphate and glycogen,[9] have greater lactate dehydrogenase activity and serotonin uptake and release,[11] produce more thromboxane B2[9,12,21] and express more GPIb and integrin $\alpha_{IIb}\beta_3$ GPIIb/IIIa.[22] These properties make larger platelets functionally more active.[11] Several studies have shown a significant correlation between mean platelet volume (MPV) and RP in patients with stable ischaemic heart disease (SIHD) and ACS.[23–26] Lakkis et al showed that in a group of patients with ACS, compared to healthy controls, MPV increased across the spectrum of ACS and was highest in patients with ST-elevation myocardial infarction (STEMI). This was accompanied with a significant increase in RPs.[24] The findings were later confirmed in a group of healthy volunteers stratified into tertiles according to RP counts. Individuals in the upper tertile had the highest MPV value.[26]

Until recently, the evidence for increased metabolic activity among RPs has been by association rather than by direct observation. Recently, important observations provided direct evidence that these findings in larger platelets, in fact, reflected the physiology of RPs which appear to play an extensive role in all the steps involved in thrombus formation. In 2002, Saving et al reported that RPs have higher expression of two important adhesion receptors GP-IV and $\alpha_6\beta_1$ (VLA-6).[27] GP-IV binds to thrombospondin, the major alpha-granule protein of platelets which in turns facilitates their adherence to the vascular endothelium. On the other hand, the VLA-6 facilitates the adhesion of activated platelets to laminin, one of three major components of sub-endothelial matrix (along with fibronectin and collagen). RPs were later found to have higher expression of P-selectin and procaspase activating compound-1 (an epitope on activated integrin $\alpha_{IIb}\beta_3$ ),[26,28] as well as GPIbα, the receptor for von Willebrand factor.[29] Additionally, other components of thrombotic signalling system cyclooxygenase-1 and -2 (COX-1 and COX-2) were shown to be up-regulated in

RPs.[26,30] While the contribution of COX-2 to the production of prostanoids may be minimal in normal individuals, this effect appears to be accentuated in patients with increased platelet turnover. In fact, NS-398, a selective COX-2 selective inhibitor, has a profound inhibitory effect on prostanoid synthesis (PGE2 and TxB2) in patients with immune thrombocytopaenia or peripheral blood stem cell transplantation.[31] Lador et al also examined the ability of RPs to respond to activation. Using flow cytometric triple staining, they identified RPs using TO, and studied expression of P-selectin and annexin V. After stimulation with ADP (10 μM), the proportion of RPs expressing P-selectin and annexin V rose fourfold and 1.5-fold, respectively, compared with mature platelets, implying greater reactivity among RPs.[32] Apart from their role in the formation of the platelet plug, RPs also seem to play an important role in the coagulation cascade through enhanced participation in pro-thrombinase complex assembly as indicated by the higher expression of both surface-bound α-granule factor V and factor X upon stimulation with thrombin.[29,33]

The mechanism underlying this enhanced ability may be related to the ability of messenger RNA (mRNA) present within RPs to undergo translation, resulting in protein synthesis. An early observation by Kieffer et al indicated that rough endoplasmic reticulum was more prominent in platelets taken from patients with idiopathic thrombocytopenic purpura than among platelets from healthy volunteers. These platelets were also able to incorporate radiolabelled methionine and leucine into their GP pool, implying that the immature platelet population had increased capacity for protein synthesis.[34] The literature is now replete with evidence that despite being anucleate, platelets have a vestigial pool of mRNA that has the ability to be translated into proteins,[35] although the functional significance of this RNA is still debated. Angénieux et al correlated the mRNA content in murine platelets with protein synthesis. Using diphtheria toxin, they induced thrombocytopaenia in mice which was followed by thrombocytosis. As expected, the proportion of circulating RPs increased dramatically as the platelet count rose. This finding was accompanied by the synthesis of proteins containing radiolabelled methionine and cysteine. Over a period of hours, RNA content decreased and, in parallel, protein synthesis decreased. This was accompanied by loss of about half of their ribosomal and beta actin RNA after 6 hours and > 90% after 24 hours.[36] Although these findings imply that the RNA observed in RPs is functional, the short duration of elevated RNA content raises important questions concerning the degree to which increased protein synthesis provides functional support of thrombosis.[36]

At least two experiments have so far confirmed that RPs are indeed more active in thrombosis compared to mature platelets. In the first experiment, plasma-rich thrombi were generated by perfusion of the whole blood from normal individuals over porcine carotid arterial segments under conditions of shear stress (shear rate: 3,350 s$^{-1}$) similar to those seen in significant coronary artery stenoses. The thrombi were then harvested by vortexing, and were then disaggregated and assessed with flow cytometry. The intensity of TO staining was greater in platelets harvested from thrombi compared to whole blood platelets. This finding was accompanied by a higher expression of the integrin β$_3$ chain in the platelets harvested from thrombi.[37] In the second experiment, plasma-rich platelets (PRPs) from healthy volunteers was stimulated with arachidonic acid (AA) or ADP and the relative composition of the non-aggregated platelet population was assessed. The investigators found that RPs contributed disproportionally to the composition of the thrombus. Furthermore, confocal microscopic examination showed that RPs are located in the core of aggregates.[38] These data suggest strongly that RPs play an essential role in platelet aggregation by forming a nidus which facilitates recruitment of older platelets.[38] Whether this observation remains valid in vivo, where other blood elements participate in thrombus formation, is unknown.

## Measurement of Reticulated Platelets

### Flow Cytometry
The standard for detecting the presence of RP is flow cytometry using a fluorescent dye, most commonly TO, which binds to nucleic acids.[15] The results are generally expressed as the percentage of RP (RP%) in the total platelet pool. This method has been criticized for its lack of standardization and for multiple technical issues such as the non-specific binding of TO, varying types and concentrations of fluorescent dye, varying incubation times and different gating and threshold settings used in analysis.[39] Consequently, RP analysis using flow cytometry is time-consuming, operator-dependent and subject to a large degree of variability in the reporting of a reference range, which has varied between 1 and 15%.[40,41]

### Immature Platelet Analysis
Although the terms 'RP' and "immature platelets" are used interchangeably in clinical practice, it is important to distinguish between the two, as significant but incomplete overlap exists in the populations measured. Sysmex (Kobe, Japan) developed a novel method to measure RP in blood samples using automated analysers (XE-2100, XE-5000 and XN). The technique is based on a fluorescent dye, most commonly a mixture of polymethine and oxazine, which penetrates cell membranes and stains platelet RNA. The stained platelets are then passed through a semiconductor diode laser to measure the resulting forward scattered light (cell volume) and fluorescence intensity (RNA content). A computerized algorithm then separates the mature platelets (represented as blue dots) from immature platelets (represented as green dots). RPs separated by this method are expressed as (immature platelet fraction [IPF%], or as an absolute number, i.e. immature platelet count [IPC, which is the product of IPF% and platelet count]). Using the Sysmex XE-2100 haematology analyser, a reference interval for immature platelets has been established for both IPF%, 0.5 to 3.3% (0.5–3.1% in men; 0.5–3.4% in women), and IPC, 1.25 to 7.02 × 10(9)/L (1.30–6.80 × 10(9)/L in men; 1.21–7.15 × 10(9)/L in women).[42] This method allows the measurement of immature platelets to be obtained along with complete blood count measurement, saving time with a minimal

added cost. Studies have shown modest positive correlation between immature platelet analysis and the traditional flow cytometry method. In patients with thrombocytopaenia of different aetiologies, the overall correlation was moderate to strong ($r = 0.57$ and $r = 0.65$) and was highest in the group of patients with thrombocytopaenia due to peripheral destruction.[43–45] We have demonstrated that this association tends to be highest ($r = 0.63$ vs. $r \leq 0.41$) in patients with peripheral thrombocytopaenia who have higher IPF% (8.3%) compared to patients with end-stage renal disease, stable coronary artery disease (CAD) and post-coronary artery bypass surgery, who have lower IPF% ($< 5\%$ for all). It is important to note that flow cytometry using TO generally reports a higher proportion of circulating RP compared to IPF% measured using this technique, most likely as a consequence of properties of non-specific binding of TO, to dense granules and deoxyribonucleic acid.[46]

## The Role of Reticulated Platelets in Thrombotic Diseases

### Reticulated Platelets and Coronary Artery Disease
RPs and immature platelet analysis have not clearly distinguished patients with the various acuity of CAD. The initial study by Lakkis et al evaluated 92 patients with CAD and showed a significant increase in RP% in patients with ACS compared to stable angina. Furthermore, RP% increased with the increasing acuity of ACS (10.53% in unstable angina, 15.99% in non-STEMI, and 18.94% in STEMI).[24] Concordant findings were demonstrated in a larger group of healthy volunteers ($n = 22$) and patients presenting with CAD ($n = 39$ with SIHD and $n = 359$ with ACS) using the Sysmex XE-2100.[25] IPF% steadily and significantly increased from a mean of 2.51% in healthy volunteers to 3.7% in patients with STEMI. These findings were independently confirmed by others in a separate study.[23] On the other hand, in a group of 280 patients coming to the emergency room with chest pain, immature platelets did not differentiate between patients with or without ACS.[47] However, it is important to note that the latter study included all patients coming to the emergency room including healthy subjects. It is known that RPs are increased in many conditions beside CAD and are makers of inflammation, infection and enhanced bone marrow activity. In a group of 190 patients with symptoms and signs suspicious for infection, RP% were sensitive and specific for diagnosing infection and changed dynamically with the progression and recovery of infection.[48] Thus, including all comers to the emergency room with chest pain of different aetiologies such as pneumonia, costochondritis or cholecystitis to name a few may have contributed to the elevated level of immature platelets and skewed these results.

Studies have also investigated the relationship between RPs and both short- and long-term cardiovascular outcomes. In patients admitted with ACS, a high IPF% measured in the first 24 hours of admission was an independent risk factor for hospital mortality (odds ratio [OR] = 2.42, 95% confidence interval [CI]: 1.08–5.43; $p = 0.032$) after adjustment for clinical variables including ST-elevation and troponin level.[23] In patients with STEMI, the proportion of RPs started to fall

4 hours after percutaneous coronary intervention (PCI).[49] Moreover, while RPs remain elevated for 30 to 60 days following myocardial infarction (MI), the level decreases after 1 year.[50] Thus, it is plausible that RPs may report long-term outcomes. Cesari et al measured RP levels at the time of presentation with ACS and found that RP levels independently predicted cardiovascular mortality at 1 year (OR = 4.15, 95% CI: 1.24–13.91; $p = 0.02$).[51] In another study of patients with both SIHD and ACS patients, we observed that RP levels predicted the major adverse cardiovascular events (MACE) of death, MI, unplanned re-vascularization and re-hospitalization for angina at a median of 31 months.[52] Patients with an IPC > 7,632 platelets/μL were more likely to experience a MACE (hazard ratio, 4.65, 95% CI: 1.78–12.16; $p < 0.002$). Interestingly, RP reactivity as measured by light transmission aggregometry (LTA) did not predict MACE. These findings suggest that RPs are better chronic predictors of events than LTA which is more likely to reflect short-term status. In a concordant observation, RPs also provided better prediction of MACE than either vasodilator-stimulated phosphoprotein phosphorylation or multi-plate electrode aggregometry in patients following PCI[53] (►**Table 1**).

### Reticulated Platelets in Acute Ischaemic Stroke
AIS results from embolic or thrombotic occlusion of a cerebral vessel that causes a focal cerebral ischaemic injury. According to the Trial of Org 10172 in Acute Stroke Treatment criteria, the causes of AIS have been classified in the following categories: large vessel atherosclerosis, cardioembolism, acute lacunar and cryptogenic or stroke of other determined aetiology.[54] Lipohyalinosis, resulting in cerebral small vessel occlusion, is thought to be the main mechanism related to acute lacunar infarct. Platelets are believed to play a small role in this syndrome.[55] Overall, initiation of anti-platelet therapy following AIS or transient ischaemic attack (TIA) is associated with a reduction in the rate of recurrent ischaemic strokes.[56] However, adding single or dual anti-platelet therapy did not result in further risk reduction compared to aspirin use alone.[57,58] More recently, in a randomized clinical trial performed in China, it was determined that the initiation of dual anti-platelet therapy within 24 hours of TIA or minor stroke resulted in a lower risk of stroke in the first 90 days.[59] These contrasting results may be due to the heterogeneous aetiologies of AIS and the potential relationship of AIS to platelet non-responsiveness.[60] Elevated MPV is associated with increased platelet turnover, platelet reactivity and resistance to anti-platelet therapy, as previously discussed in this review. MPV was a stroke predictor in patients with atrial fibrillation, after adjustment for other risk factors.[61] It was also found to be higher in non-lacunar stroke patients.[62] Moreover, elevated MPV was associated with worse acute stroke outcomes.[63]

Despite the relationship of MPV to AIS, studies investigating the role of immature platelets in stroke incidence, pathophysiology and outcomes are lacking. In one study, flow cytometric analysis of RP in patients with ischaemic stroke showed significantly high proportions of circulating RP in patients with cardioembolism compared to control patients.[64] In another study, the percentage of circulating RP

**Table 1** Studies of reticulated platelets and outcomes in coronary artery disease

| | | Cesari | Ibrahim | Freynhofer |
|---|---|---|---|---|
| RP method | | Sysmex XE-2100 | Sysmex XE-2100 | Sysmex XE-2100 |
| Population | SIHD | 0 | 38 | 198 |
| | US/NSTEMI | 104 | 47 | 164 |
| | STEMI | 125 | 4 | 124 |
| | Cardiogenic shock | 0 | 0 | 10 |
| | Total | 229 | 89 | 486 |
| Follow-up | | 1 y | Median 31 mo | Median 190 d |
| Outcomes | Death | 22 | 10 | 20 (18 cardiovascular) |
| | ACS | – | 11 | 21 |
| | Re-vascularization | – | 6 | 27 |
| | Angina | – | 7 | 6 |
| | Stent thrombosis | – | – | 10 |
| | TIA/Stroke | – | – | 4 |
| | MACE composite | 22 | 30 | 86 |
| RP | Correlation with MACE | Yes | Yes | Yes |
| | Cut-off | IPF > 3.3% | IPC > 7,632 | IPF > 3.35% |
| | Sensitivity | 63.6% | 70.7% | 67% |
| | Specificity | 77.3% | 82.1% | 51% |

Abbreviations: ACS, acute coronary syndrome; IPC, immature platelet count; IPF, immature platelet fraction; MACE, major adverse cardiovascular events; NSTEMI, non-ST-elevation myocardial infarction; RP, reticulated platelets; SIHD, stable ischaemic heart disease; STEMI, ST-elevation myocardial infarction; TIA, transient ischaemic attach; UA, unstable angina.

was elevated in the early and late stages following a stroke compared to control and a correlation with MPV was observed. However, the MPV was similar between patients with AIS and control patients, suggesting that RP may play a role beyond what can be measured by MPV alone.[65] Another recent report found that RPs are increased in both early (≤ 4 weeks) and late (≥ 3 months) symptomatic (TIA or AIS) compared to asymptomatic moderate or severe carotid stenosis, as measured by IPF% (5.78% vs. 3.48% and 5.11% vs. 3.48%, respectively). While there was a significant correlation between RPs measured using flow cytometry and by Sysmex XE-2100, RP% was not different between early or late symptomatic compared to patients who were asymptomatic. These findings suggest that the automated measurement of RPs may be more sensitive for RP quantification than is flow cytometry, possibly as a result of the multiple sources of error that can occur when flow cytometry is used as a clinical tool.[66] Further research is required to elucidate the role of RP in AIS and TIA.

## Role of Reticulated Platelets in Response to Anti-Platelet Drugs

### Reticulated Platelets and Response to Aspirin

Since its discovery more than 100 years ago, aspirin has remained one of the most widely used medicines worldwide. Aspirin reduces the future risk of MI, stroke and vascular death by approximately 25% in patients with acute MI or with a history of MI, stroke, or TIA.[67] Aspirin is an irreversible inhibitor of prostaglandin GH synthase-2 aka: COX-1 that inhibits prostaglandin GH synthase 2 production and subsequently the production of thromboxane (TxA2).[68]

Although aspirin has a short half-life (30 minutes), its irreversible inhibition of COX-1 renders platelets unable to form TxA2 and, therefore, unable to aggregate for their entire lifespan (7–10 days). This irreversible inhibition may explain why an aspirin dose as low as 40 mg per day can produce a cumulative and sustained inhibition of platelet function.[69] Previous findings indicate that the platelet pool recovers the ability to produce thromboxane B2 (TxB2) and aggregate as early as 4 hours after ingestion of 650 mg of aspirin in normal individuals.[70] In fact when tested in vitro, platelet aggregation in response to a single aggregating agent (either AA or collagen) occurred when 15 to 20% of platelets were aspirin-free. On the other hand, only 5% of aspirin-free platelets were needed to achieve platelet aggregation in response to the combination.[70]

About 100 billion new platelets are produced daily from megakaryocyte to maintain a sufficient platelet count.[71] Therefore, one might speculate that in patients with increased level of RPs, a higher turnover will be associated with enhanced early recovery of platelet function. DiMinno et al[72] first described the relationship between enhanced platelet turnover and aspirin resistance. These investigators found that the platelets of normal and diabetic individuals taking aspirin once a day (regardless of the dose 100, 330 or

1,000 mg) were able to aggregate and form measurable amounts of TxB2 after 12 to 15 hours. In vitro incubation of PRP with aspirin abolished platelet aggregation and TxB2 production indicating that recovery of platelet function is a result of aspirin-free new platelets entering the circulation. In healthy individuals, platelet aggregation and TxB2 production were diminished when aspirin dosing was increased to four times daily, but this was not the case for patients with diabetes. However, in vitro incubation of PRP from diabetic patients with aspirin abolished platelet aggregation and TxB2 production, indicating the higher platelet turnover in diabetic patients compared to normal individuals. These findings were again demonstrated in both normal individuals,[73] and in patients with CAD.[74]

The most convincing evidence that enhanced platelet turnover plays a role in decreased response to aspirin comes from studies of patients with essential thrombocythaemia, a disease characterized by immune-mediated platelet destruction and naturally increased platelet turnover. In this patient population, serum TxB2 level and urinary TxM excretion were significantly higher in patients on aspirin therapy (100 mg/day) compared to aspirin-treated healthy volunteers.[75] However, the addition of aspirin (50 μM) in vitro completely suppressed production of thromboxane to levels similar to aspirin-treated healthy volunteers.[75] One plausible mechanism is that platelet turnover results in the release of new platelets unaffected by aspirin, which is especially important when the turnover rate is high. However, although in a study of patients with diabetes, twice daily aspirin dosing (75 mg) led to a reduction of AA-induced whole blood aggregation compared to 75 or 320 mg once daily, the proportion of RP did not discriminate between patients who benefited from twice daily dosing.[76] Another explanation of how platelet turnover results in aspirin resistance is the observation that circulating RP possess uninhibited COX-1 as well as COX-2 activity. In a study of 60 healthy aspirin-treated volunteers stratified into tertiles according to their RP levels, we observed that post-aspirin thromboxane levels were higher in the upper tertile compared to the lower tertile and that that these levels decreased when specific COX-1 or COX-2 inhibitors were added ex vivo.[26] In a different study, the effect of RPs on platelet aggregation appeared to be independent of platelet turnover. In patients with SIHD receiving dual anti-platelet therapy (aspirin with either clopidogrel or prasugrel) following PCI, a correlation was observed between RP and platelet aggregation and was of similar magnitude during early ($<$ 2 hours of thienopyridine load) and late (24 hours later) phases of dosing.[77]

### Reticulated Platelets and P2Y12 Antagonists

Clopidogrel, the most commonly used P2Y12 antagonist, is a pro-drug that must be metabolized by hepatic CYP450 enzymes to inhibit the binding of ADP to platelet P2Y12. The platelet response to clopidogrel has wide biological variability between individuals.[78] RPs have recently garnered interest as a plausible explanation for hypo-response to clopidogrel. In a group of healthy individuals, hypo-responsiveness to clopidogrel was found in 60% of subjects

in the upper tertile of RP values compared to only 10% of subjects in the lower tertile.[79] An IPF cut-off of 3.6% predicted clopidogrel hypo-responsiveness with a sensitivity and specificity of 85.7 and 81.8%, respectively. These findings were further extended to patients with diabetes,[80] SIHD on dual anti-platelet therapy[81] and post-PCI for SIHD[77,82] or for ACS.[82,83] Among a group of patients who underwent elective PCI, IPC was the strongest independent predictor of anti-platelet response to P2Y12 inhibitors. In fact, 7% of on-clopidogrel platelet reactivity was explained by IPC.[28] On the other hand, when platelet function tests were performed 30 to 90 days after PCI for either SIHD or ACS, neither IPF nor IPC could be correlated with platelet aggregation.[84] Moreover, while both platelet aggregation and turnover indices were significantly elevated at time of STEMI and decreased with time over 3 months period, there was no significant correlation between the two.[49] This difference in observations is likely due to the heterogeneity in these study designs, including the differences in patient populations, platelet function test used, agonist concentration, timing of blood collection and the definition of hypo-response to anti-platelet therapy (►Table 2).

In a sub-study of TRITON-TIMI 38 trial, prasugrel, a new P2Y12 inhibitor, resulted in improved cardiovascular outcomes compared to clopidogrel. However, hypo-response to prasugrel was reported to be as high as 27%.[85] When platelet function was evaluated 2 to 4 days after PCI in 62 patients with STEMI who were started on prasugrel, high platelet reactivity was found in 11.3% of patients and persisted at the 30-day follow-up.[86] Furthermore, RP were strongly correlated with platelet reactivity in patients with ACS treated with prasugrel.[86,87] In contrast to these findings, RP could not be correlated with platelet aggregation in another study of patients with ACS and treated with prasugrel.[50] However, the small number of patients, higher use of integrin $\alpha_{IIb}\beta_3$ antagonists and, most importantly, the very low platelet reactivity (median platelet reactivity unit value of 25) may have accounted for this finding. As prasugrel irreversibly binds to P2Y12 and has a half-life of about 7 hours, the RP newly introduced into circulation will be unexposed to the active metabolite at the end of its once daily administration. In fact, P-selectin, a marker of platelet activation, was found to be higher in RP just before treatment with prasugrel compared to 2 hours after—the daily maintenance dose.[87] These properties may be lessened when ticagrelor, a reversible active inhibitor of P2Y12 with a longer half-life (11 hours) than the active metabolite of prasugrel (7 hours), that is administered twice daily, is used.[87] Both ticagrelor and its active metabolites interact equipotently with the P2Y12, which would be expected to result in more complete suppression of P2Y12 function. Most studies of platelet reactivity in patients with ACS treated with ticagrelor have very low residual platelet reactivity, which limits inter-individual variability, and thus might explain the lack of significant correlation with RP levels.[50,87,88] This hypothesis was supported in a recent study by Armstrong et al demonstrating that RPs play a role in hypo-response to clopidogrel but not ticagrelor. These investigators stained PRP with TO

**Table 2** Studies of reticulated platelets implication on anti-platelet function testing

| Author | Population | No. of patients | Anti-platelet | RP method | RP value | Platelet function | Agonist | ↓ Response to anti-platelet definition | Correlation with RP | Time[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| Guthikonda et al[26] | Healthy | 60 | ASA | Flow cytometry | RP% (4, 8, 12) lower, middle, upper tertiles | LTA | Collagen 1.0 μg/mL, AA 1.5 mM, ADP (5, 20 μM) | ≥70% by AA | + | 24 h |
| Würtz et al[91] | SIHD | 124 | ASA | XE-2100 | IPF 2.2% IPC 5.2 | VerifyNow MEA | Collagen 1.0 μg/mL, AA 1 mM | Not studied | + only with IPC (MEA) | 1 h |
| Grove et al[92] | SIHD | 177 | ASA | XE-2100 | IPC 6.8 | VerifyNow MEA | AA 1.0 mM, ADP 10 μM, collagen 1.0 μg/mL | Upper tertile of platelet aggregation | + only with IPC | 1 h |
| Ibrahim et al[79] | Healthy | 29 | Clopidogrel | XE-2100 | IPF (2.1, 3.3, 4.9) % in the lower, middle and upper tertiles | LTA VASP-P | ADP (2, 5, 20 μM) | Aggregation ≥50% in response to 5 μM ADP | + | NR |
| Freynhofer et al[82] | Post-PCI (SIHD, ACS) | 102 | Clopidogrel | NR | NR | VASP-P | NR | PRI > 50% | + | |
| Guthikonda et al[81] | SIHD | 90 | ASA, clopidogrel | Flow cytometry | RP% (1.3, 3.1, 17.9) % in the lower, middle and upper tertiles | LTA | Collagen 1.0 μg/mL, AA 1.5 mM, ADP 5 μM | ASA: Aggregation ≥20% by AA. Clopidogrel: Aggregation ≥50% by ADP | + for ASA and clopidogrel | 12–24 h |
| Cesari et al[83] | ACS | 372 | ASA, Clopidogrel | XE-2100 | IPF 3.9% | LTA | ADP 10 μM, AA 1 mM | Aggregation > 20% by AA and/or > 70% by clopidogrel | + | 4–6 h |
| Mijovic et al[80] | Diabetics | 79 | ASA, clopidogrel | Cell-DYN Sapphire | RP% Diabetic: 3.17% Non-diabetic: 2.39% | ADP test MEA TRAP | ADP 20 μL, ASPI 20 μL, TRAP 20 μL | %DPAadp = 100 × (1-ADP/TRAP). %DPAaspi = 100 × 1-ASPI/TRAP) | + with clopidogrel none with ASA | NR |
| Funck-Jensen et al[49] | STEMI | 48 | ASA, clopidogrel | XE-2100 | Not reported | VerifyNow MEA | Collagen 1.0 μg/mL, AA 1 mM, | MEA (AA > 300, ADP > 416) AUC, | No correlation | ≤1 h |

(Continued)

**Table 2** (Continued)

| Author | Population | No. of patients | Anti-platelet | RP method | RP value | Platelet function | Agonist | ↓ Response to anti-platelet definition | Correlation with RP | Time[a] |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  |  | ADP (6.4, 20) µM | VerifyNow ASA > 550 ARU P2Y12 > 230 PRU |  |  |
| Perl et al[86] | STEMI | 62 | Prasugrel | Flow cytometry | Not reported | VerifyNow MEA | ADP | MEA: ≥ 47 AU*min, VerifyNow: ≥208 PRU | + | NR |
| Vaduganathan et al[88] | NSTEMI | 53 | Ticagrelor | Flow cytometry | RP% (21.5, 23.8) % at 2–4, and 30 d | VerifyNow MEA | ADP | MEA: ≥47 AU*min, VerifyNow: ≥208 PRU | No correlation | NR |
| Eisen et al[50] | AMI | 35 | Ticagrelor, prasugrel | Flow cytometry | RP% (17.5, 14.9, 10.5) % at 2–4 d, 30–60 d and 1 y | VerifyNow | NA | ≥208 PRU | No correlation | NR |
| Bernlochner et al[87] | ACS | 124 | Ticagrelor, prasugrel | XE-5000 | IPF 3.8 vs. 3.7% IPC 8.6 vs. 9.2 in ticagrelor vs. prasugrel group | MEA | ADP | > 468 AU*min | + with prasugrel, none with ticagrelor | 6–48 h |
| Verdoia et al[84] | Post-PCI (SIHD) | 386 | Clopidogrel, ticagrelor | XE-2100 | IPF (3.5 vs. 3.6) % in diabetics vs. not IPC (7 vs. 7.4) in diabetics vs. not | MEA | AA, Collagen, ADP, PE1, TRAP-6 | ASA: > 862 AU*min Clopidogrel: > 417 AU*min | No correlation | NR |
| Stratz et al[28] | Post-PCI (SIHD) | 300 | Clopidogrel, prasugrel | XE-2100 | Not reported | MEA | ADP 6.4 µM | > 468 AU*min | + with clopidogrel, none with prasugrel | Day 1[b] |

Abbreviations: %DPA, percentage of decrease in overall platelet aggregability; AA, arachidonic acid; ADP, adenosine diphosphate; AMI, acute myocardial infarction; ARU, aspirin reaction units; ASA, aspirin; AU*min, aggregation units × minute; AUC, area under the aggregation curve; ACS, acute coronary syndrome; IPC, immature platelet count; IPF, immature platelet fraction; LTA, light transmission aggregometry; MEA, multiple electrode aggregometry; NR, not reported; NSTEMI, non-ST-elevation myocardial infarction; PCI, percutaneous coronary intervention; PRI, platelet reactivity index; PRU, P2Y12 reaction units; RP%, percent reticulated platelets; RP, reticulated platelets; SIHD, stable ischaemic heart disease; STEMI, ST-elevation myocardial infarction; VASP-P, vasodilator stimulated phosphoretin phosphorylation.
[a]Time between drug administration and blood sample collection.
[b]On day 1 prior to intake of first maintenance dose.

and then stimulated aggregation with ADP and found that RPs disappeared from the non-aggregated PRP in patients with CAD treated with aspirin and clopidogrel but not ticagrelor[38] (►**Table 2**). Similarly, RPs have been reported not to affect platelet reactivity in patients receiving cangrelor, an intravenous P2Y12 antagonist. Stratz et al observed a significant correlation between RPs and platelet aggregation in patients loaded with either clopidogrel or prasugrel. However, the correlation was not observed but in patients who received or ticagrelor or during cangrelor infusion in patients undergoing PCI to treat SIHD.[89]

## Future Directions

Similar to their role in thrombotic diseases and response to anti-platelets drugs, RPs appear to play a role in thrombotic complications in patients undergoing moderate to high risk non-cardiac surgical procedures. In a recent study of 730 patients (16.9% with CAD and 5.2% with cerebrovascular disease), IPF was higher both immediately and 24 to 72 hours following surgery in patients with compared to without modified MACE (combination of MACE, pulmonary embolism and deep venous thrombosis). An IPF value > 5.4% was associated with a 2.5 higher likelihood of experiencing a modified MACE during hospitalization. Therefore, RPs may be helpful in identifying a sub-group of patients at risk for increase adverse cardiac events who might benefit from intensive risk modifications immediately after non-cardiac surgery.[90]

## Conclusion

RPs may be a useful marker for predicting worse cardiovascular outcomes. This is pathophysiologically due to their inherited properties that lead to a higher degree of aggregation, and their reflection of a pool of platelets unresponsive to anti-platelet therapy. However, studies evaluating the role of RPs in response to anti-platelet therapy have shown inconsistent results. This heterogeneity is largely due to the differences in study design including differences in patient population, platelet function test used, agonist concentration, timing of blood collection and definition of hyporesponse to anti-platelet therapy. Until platelet function testing becomes more standardized, we believe that prospective studies are needed to focus on RPs as a predictor of clinical outcomes and to individualize anti-platelet therapy.

Conflict of Interest
None.

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