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Newly formed reticulated platelets undermine pharmacokinetically short-lived antiplatelet therapies

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Abstract

Objective—Aspirin together with thienopyridine $P2Y_{12}$ inhibitors, commonly clopidogrel, is a cornerstone of antiplatelet therapy. However, many patients receiving this therapy display high on-treatment platelet reactivity (HTPR) which is a major therapeutic hurdle to the prevention of recurrent thrombotic events. The emergence of uninhibited platelets following thrombopoiesis has been proposed as a contributing factor to HTPR. Here we investigate the influences of platelet turnover on platelet aggregation in the face of different dual antiplatelet therapy strategies.

Approach and Results—Traditional light transmission aggregometry, cytometry, advanced flow cytometric imaging and confocal microscopy were used to follow the interactions of populations of platelets from healthy volunteers and patients with stable cardiovascular disease. Newly formed, reticulated platelets over-proportionately contributed to, and clustered at, the core of forming aggregates. This phenomenon was particularly observed in samples from patients treated with aspirin plus a thienopyridine, but was absent in samples taken from patients treated with aspirin plus ticagrelor.

Conclusions—Reticulated platelets are more reactive than older platelets, and act as seeds for the formation of platelet aggregates even in the presence of antiplatelet therapy. This is coherent with the emergence of an uninhibited subpopulation of reticulated platelets during treatment with aspirin plus thienopyridine, explained by the short pharmacokinetic half-lives of these drugs. This phenomenon is absent during treatment with ticagrelor, due to its longer half-life and ability to act as a circulating inhibitor. These data highlight the importance influences of pharmacokinetics on anti-platelet drug efficacies especially in diseases associated with increased platelet turnover.

Keywords

aspirin; $P2Y_{12}$; anti-platelet therapy; platelet

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Subject codes

platelets; pharmacology; acute coronary syndromes; thrombosis

Introduction

Platelets are central to the processes underlying atherothrombotic events and consequently are the target of well established prophylactic therapy. The drug dosing regimen referred to as dual antiplatelet therapy (DAPT) typically comprises aspirin combined with a $P2Y_{12}$ receptor antagonist, commonly the thienopyridine compound clopidogrel. 1–4 The reoccurrence of thrombotic events during therapy represents a major therapeutic hurdle and is associated with high on-treatment platelet reactivity (HTPR).3, 4 However, the causes of HTPR and thrombotic complications are complex and require deeper investigation to improve anti-thrombotic therapies.3–5

One potential contributing factor to HTPR is an increased rate of platelet turnover. There are a notable number of pathological states linked to HTPR where platelet turnover and the circulating levels of newly formed immature platelets are increased. In particular, a recent study has associated elevated immature platelet counts, a measure of platelet turnover, with adverse cardiovascular outcomes.6 This relationship is clearly demonstrated in patients with chronic renal failure requiring haemodialysis whose reticulated platelet proportion increases threefold.7 Additionally there is a strong association between poor clopidogrel responsiveness and increased thrombotic risk.8, 9 Nonetheless a pathophysiological mechanism has yet to be identified.

Aspirin and clopidogrel, the most widely used $P2Y_{12}$ receptor antagonist, irreversibly bind their respective targets, but are short-lived in the circulation. This suggests that as standard daily doses of either drug are quickly cleared newly formed platelets subsequently entering the circulation will remain uninhibited until the next dose is taken. We have recently demonstrated that uninhibited platelets can act as seeds for aggregate formation during antiplatelet therapy.10 Therefore, in patients with pathologies in which platelet production is increased larger subpopulations of these uninhibited platelets will arise. Compounding this, newly produced immature, or reticulated, platelets appear to be generally more reactive.11

The more recently developed non-thienopyridine $P2Y_{12}$ antagonist ticagrelor is, unlike thienopyridines, pharmacokinetically long-lived with a circulating half-life of about 8 hours. With standard twice daily dosing it consistently circulates at inhibitory concentrations. Also unlike thienopyridines, ticagrelor acts directly as a reversibly-binding antagonist of $P2Y_{12}$ receptors, and may consequently provide more thorough anti-thrombotic cover than short-lived thienopyridines.12

Here we compare pharmacokinetically different anti-thrombotic regimens in healthy volunteers, and examine their relationship with uninhibited platelets. In a previous *in vitro* study we mimicked the *in vivo* interaction of differently inhibited platelet populations by separately labelling and recombining platelets.10 In this *ex vivo* study we have for the first time directly examined the functionality of reticulated platelets, those most likely to be

uninhibited, in samples from patients with stable cardiovascular disease and receiving DAPT. Finally we describe a mechanism by which newly formed reticulated platelets may promote HTPR and potentially explain the reported increased effectiveness of ticagrelor over thienopyridines.13, 14

Methods

Materials and Methods are available in the online-only Data Supplement.

Results

In vitro modelling identifies differential inhibition between thienopyridines and ticagrelor

Drug-free platelets were added to platelets pre-incubated with drug to model the *in vitro* functional consequences of platelet turnover during DAPT treatment comprising aspirin +prasugrel or aspirin+ticagrelor. This modelling demonstrated that for samples treated with prasugrel active metabolite (PAM) responses to ADP returned with increases in the proportion of inhibitor-free platelets. In contrast, aggregatory responses remained inhibited in samples treated with ticagrelor (Figure 1A & 1B). Analysis by flow cytometry and confocal microscopy of aggregates formed from labelled platelet subpopulations indicated that drug-free platelets were over-proportionately recruited to the formed aggregates in samples treated with aspirin+PAM. This was evidenced by clear clustering of uninhibited platelets at the cores of the formed aggregates. In contrast, analysis of samples treated with aspirin+ticagrelor did not show this bias (Figure 1C). Quantitative analyses of aggregate images obtained by confocal microscopy demonstrated a significantly bigger core volume when drug-free platelets were mixed with aspirin+PAM-treated samples (95±18 μm^3) than with aspirin+ticagrelor-treated samples (24±4 μm^3; Figure 1D & 1E).

Ticagrelor but not prasugrel therapy inhibits drug-free platelets upon *in vitro* transfusion

Having identified pharmacological differences between PAM and ticagrelor *in vitro*, we tested differences between prasugrel and ticagrelor in their ability to inhibit drug-free platelets in a larger inhibited environment using an *ex vivo* approach in healthy volunteers who had either taken aspirin+prasugrel or aspirin+ticagrelor for 7 days. Blood was collected at estimated peak concentrations of prasugrel active metabolite (30 minutes after last dose) and ticagrelor (120 minutes after last dose), as well as at 6 hours after the last dose and PRP was made. Drug-free platelets from healthy volunteers were then combined with PRP collected from treated subjects to model various rates of platelet turnover, ranging from no turnover (x=0%) to full turnover of new platelets (x=100%), and aggregation responses determined. In samples derived from volunteers treated with aspirin+prasugrel, responses to ADP recovered as the proportion of drug-free platelets was increased, whereas responses remained strongly inhibited in samples from volunteers who had received aspirin+ticagrelor (Figure 2A & Supplemental Figure II). In aspirin+ticagrelor samples the addition of naïve drug-free platelets also produced a smaller increase in the response to AA than in aspirin +prasugrel samples (Figure 2C & Supplemental Figure II).

Following LTA, PRP underwent high-throughput flow cytometric imaging to assess aggregate structures. Aggregates (mean=104 per individual sample) were blindly assessed

for the proportion of drug-free core aggregates relative to total aggregates (Figures 2E & Supplemental Figure II). Following stimulation by ADP, aggregates formed in PRP derived from volunteers treated with aspirin+prasugrel contained a higher proportion of drug-free cores than those formed in PRP derived from volunteers treated with aspirin+ticagrelor (Figure 2B). In contrast, no such difference in proportion was observed between volunteer groups for aggregates formed following AA stimulation (Figure 2D). Further confocal imaging of aggregates from populations comprising 20% drug-free and 80% inhibited platelets confirmed in response to ADP the formation of platelet aggregates with a core of drug-free platelets in aspirin+prasugrel-treated samples, but not in aspirin+ticagrelor-treated samples (Figure 3a). Moreover quantitative analysis of these images demonstrated fewer drug-free platelets were recruited (relative volumes 0.14±0.02 μm^3 vs 0.32±0.04 μm^3 respectively $p<0.001$; Figure 3C) and smaller drug-free cores formed (17±3 μm^3vs 70±12 μm^3; $p<0.001$; Figure 3D) in aspirin+ticagrelor-treated samples compared to aspirin +prasugrel-treated samples. Differences in distribution of platelet subpopulations following stimulation by AA were less pronounced (Figure 3B, 3E & 3F) but were similarly observed at ‘plasma peak’ time-points (Supplementary Figure III).

Reticulated platelets are more reactive than older platelets and locate to the core of aggregates

To monitor the reactivity of newly formed platelets, also called reticulated platelets due to the presence of mRNA, platelets were labelled *ex vivo* with the nucleic dye, thiazole orange (TO).15 We devised a gating strategy (Supplemental Figure IV) to determine the proportional usage of these newly formed platelets relative to older non-reticulated platelets16 during aggregate formation. Using PRP from untreated healthy volunteers stimulated by AA (1 mmol/L) or ADP (20 μmol/L) until 40% of platelets were aggregated the relative composition of the non-aggregated platelet population was assessed by flow cytometry. Following aggregation there was a significant reduction in the relative proportion of reticulated platelets indicating that reticulated platelets contributed over-proportionately to aggregate formation (Figure 4A & 4B). Subsequent examination of formed aggregates by flow cytometric imaging confirmed the presence of reticulated platelets in the majority of aggregates (Figure 4C).

Reticulated platelets undermine platelet inhibition by DAPT in patients taking thienopyridines but not in patients taking ticagrelor

Differential effects of thienopyridine- and ticagrelor-mediated inhibition on reticulated platelet populations were assessed in patients with established stable coronary artery disease who received DAPT comprising either aspirin+clopidogrel or aspirin+ticagrelor. Patients were assessed for pharmacological efficacy by testing of platelet reactivity using LTA. All patients had a final aggregation to ADP (20 μmol/L) of <43%, with those receiving ticagrelor considerably lower (Supplemental Table I). TO stained PRP was incubated with vehicle, AA or ADP for 5 min and reticulated proportion of the non-aggregated platelets assessed by flow cytometric imaging. In both therapy groups, upon stimulation by AA, reticulated platelets disappeared from the non-aggregated platelet population; reducing from 10.9±0.3 % to 4.0±0.4 % ($p<0.001$) in aspirin+clopidogrel patient samples and from 11.1±0.5 % to 5.4±0.5 % ($p<0.001$) in aspirin+ticagrelor patient samples (Figures 5A &

5B). This indicated an over-proportional recruitment of reticulated platelets into the formed aggregates. In line with above data from AA-stimulated samples, stimulation by ADP of samples from patients receiving aspirin+clopidogrel caused a significant reduction in the relative population of reticulated platelets in the non-aggregated population, from 10.9±0.3 % to 5.0±0.6 % (Figure 5B, $p<0.01$). However, in contrast to these observations, stimulation with ADP of PRP from patients receiving aspirin+ticagrelor did not result in a drop of the reticulated platelet population (11.1±0.5 % to 10.1±0.8 %; Figure 5A, $p>0.05$). The absence of a change in proportion indicates a proportionally equivalent recruitment of reticulated and non-reticulated platelets to aggregates. Qualitative analysis of the imaged formed aggregates from each patient group demonstrated that in samples from patients receiving aspirin+ticagrelor there were few reticulated platelets dispersed throughout the aggregate (Figure 5C) whereas in samples from patients receiving aspirin+prasugrel there were increased numbers of reticulated platelets primarily located in the core of the formed aggregates (Figure 5D).

Discussion

In our previous study, we demonstrated through *in vitro* modelling that drug-free platelets can act as seeds for aggregate formation during antiplatelet therapy.10 Here we have studied the impact of platelet turnover, including the influences of reticulated platelets, during standard dual antiplatelet therapy in both healthy volunteers and stable cardiovascular patients. Furthermore, we have compared thienopyridines with ticagrelor and from our results provide a potential pathophysiological mechanism that unites previous, but separate, associations between differential effectiveness of $P2Y_{12}$ receptor inhibition, HTPR, immature platelet counts and thrombotic risk.3, 6, 13, 14 We directly demonstrate that following stimulation reticulated platelets are over-proportionately recruited to aggregates where they can act as seeds for larger aggregate formation and by interplay with drug pharmacokinetics provide a causative mechanism for observed HTPR.

Key to explaining our observations is an understanding that the formation of a drug-free, uninhibited, subpopulation of platelets during DAPT occurs as a result of platelet turnover and drug pharmacokinetics. In terms of standard therapy, aspirin is a short-lived but irreversible inhibitor of platelet COX-1. Similarly, the thienopyridines, prasugrel or clopidogrel acting through their active metabolites, are pharmacokinetically short-lived and irreversible antagonists of platelet $P2Y_{12}$ receptors. When used as DAPT this combination of aspirin plus thienopyridine produces inhibition of circulating platelets. However, as we model *in vitro* and demonstrate *ex vivo,* neither aspirin nor prasugrel (or prasugrel active metabolite) appears present in circulating blood at sufficient levels to inhibit the responses of exogenous platelets added *in vitro*. One potential explanation for this observation is that these drugs are present in effective inhibitory concentrations only within the portal circulation and so inhibit platelets as they pass through, as has been suggested for aspirin for over 30 years.17 This would explain also why platelets newly released from the bone marrow are either poorly, or not, inhibited by aspirin and thienopyridines.18, 19 One should not overlook, however, the alternative explanation that because of their short half-lives within the circulating blood the active forms of thienopyridines may have insufficient time to interact with exogenously added platelets in our test system. In contrast, as expected,

ticagrelor as a longer lasting (plasma half-life of about 8 hours) direct acting reversible antagonist of $P2Y_{12}$ receptors 12 inhibited the responses to ADP of exogenously added platelets.

As well as modelling these interactions with regard to standard tests of platelet reactivity4, our imaging techniques demonstrated that exogenously added uninhibited platelets were clustered at the cores of aggregates formed in response to ADP in samples from volunteers receiving aspirin+clopidogrel, consistent with their ability to act as seeds for aggregate formation. As previously hypothesised12 the longer half-life and reversible binding of ticagrelor, in contrast to the irreversible binding of prasugrel, means it is present and able to act upon the exogenous drug-free, uninhibited, platelet subpopulation. It can be noted that ticagrelor might in addition act pleiotropically upon adenosine uptake to influence platelet function20, 21, but we did not test this possibility. It was notable that the recovery of the response to AA caused by the addition of exogenous drug-free platelets was blunted in samples prepared from individuals receiving aspirin+ticagrelor compared to those receiving aspirin+prasugrel. This is consistent with $P2Y_{12}$ blockade reducing the amplifying effects of TxA_2 produced in response to AA22–26, and confirmed our *in vitro* observation that circulating ticagrelor, unlike prasugrel, may provide additional compensation for the loss of COX-1 inhibition noted in individuals with elevated platelet turnover 26, 27.

In our *in vitro* and *ex vivo* models we stained or labelled un-inhibited platelets to allow determination of their function as a subpopulation. Examination of the definitive drug-free population in patients is less straightforward. Newly formed immature platelets are also called reticulated platelets due to the presence of residual cytosolic mRNA. Dyes such as TO, which stain nucleic acids, are routinely used for determining the percentage of reticulocytes (including platelets) in blood samples.28 Accepting that the emergence of a drug-free platelet subpopulation occurs as a result of platelet turnover and the associated release of newly formed platelets, analyses of newly formed platelets in samples can be used to inform on the behaviour of drug-free platelets. We have demonstrated under our particular conditions that TO staining of PRP identifies those platelets with the highest mRNA content (manuscript under review). We therefore utilised this approach to track newly formed platelets during aggregate formation. In samples from healthy volunteers not taking anti-platelet drugs reticulated platelets were over-proportionally recruited to the formation of aggregates, indicating that under normal physiological conditions they are important drivers of the aggregation process. This finding concurs with previous reports by ourselves, and others, that newly formed reticulated platelets possess inherently greater reactivity and have a greater propensity for recruitment to thrombi.29

Finally, we sought to determine whether such a mechanism was also present in patient samples. We recruited patients with established, stable coronary artery disease taking clopidogrel or ticagrelor plus aspirin, and confirmed drug efficacy to ensure patients exhibiting HTPR were not included in our analyses. As in our *in vitro* and *ex vivo* modelling, the behaviour of reticulated platelets matched that of uninhibited platelets. In samples taken from patients receiving aspirin+clopidogrel there was a significant loss in the proportion of reticulated platelets from the non-aggregated single platelet population following ADP stimulation, whereas strikingly, in patients receiving aspirin & ticagrelor no

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proportional change was observed. Similarly, examination of the formed aggregates revealed that in response to ADP reticulated platelets were clustered in the core and present in greater proportion in samples from patients receiving clopidogrel+aspirin than in samples from patients receiving ticagrelor+aspirin.

Our results support a mechanism through which newly formed uninhibited reticulated platelets play a key role in the limiting the effectiveness of particular antiplatelet therapies. Furthermore, we provide functional evidence and unique images substantiating the observed impact of subpopulations of reticulated drug-free platelets on the formation of platelet aggregates under recommended clinical testing settings.30, 31 Together these are consistent with emerging evidence establishing a link between reticulated platelets and platelet responsiveness to short-lived $P2Y_{12}$ antagonist (thienopyridines)11, 32 but not ticagrelor.33 Whilst there have been recent indications of similar short-term efficacy for prasugrel and ticagrelor in patients with acute myocardial infarction34, no comparisons or sub-group analyses has yet been conducted in conditions of high platelet turnover. Moreover our data suggests that *in vivo* the use of ticagrelor rather than clopidogrel or prasugrel may mitigate incomplete inhibition of TXA_2 formation by prophylactic aspirin4, consistent with our previous reports regarding the importance of $P2Y_{12}$ receptors in amplifying responses to platelet produced TXA_2.35–38

In conclusion, we demonstrate a functional mechanism for newly formed reticulated platelets to drive thrombus formation even during standard DAPT. Furthermore our study demonstrates that ticagrelor may be more efficacious than thienopyridine (prasugrel or clopidogrel) therapy for mitigating HTPR associated with the generation of new platelets during standard anti-thrombotic regimens. In turn this illustrates the importance of considering platelet turnover and the pharmacological inhibition of the reticulated platelet subpopulation in attaining optimal anti-thrombotic potential. Finally given the central role for platelet turnover in our model, patients with conditions such as diabetes and chronic kidney disease, where increased platelet turnover has been identified or suspected, may particularly benefit from such considerations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	Arachidonic acid

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ADP	Adenosine Diphosphate
COX	Cyclooxygenase
DAPT	Dual anti-platelet therapy
HTPR	High on treatment platelet reactivity
LTA	Light transmission aggregometry
PAM	Prasugrel active metabolite
PRP	Platelet rich plasma
TO	Thiazole Orange
TXA_2	Thromboxane A2

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Highlights

1. We demonstrate a functional mechanism by which newly formed reticulated platelets can drive thrombus formation even during standard DAPT.
2. Study reveals underlying reasons to consider platelet turnover and drug pharmacokinetics in the selection of appropriate antiplatelet therapies for optimal anti-thrombotic protection.

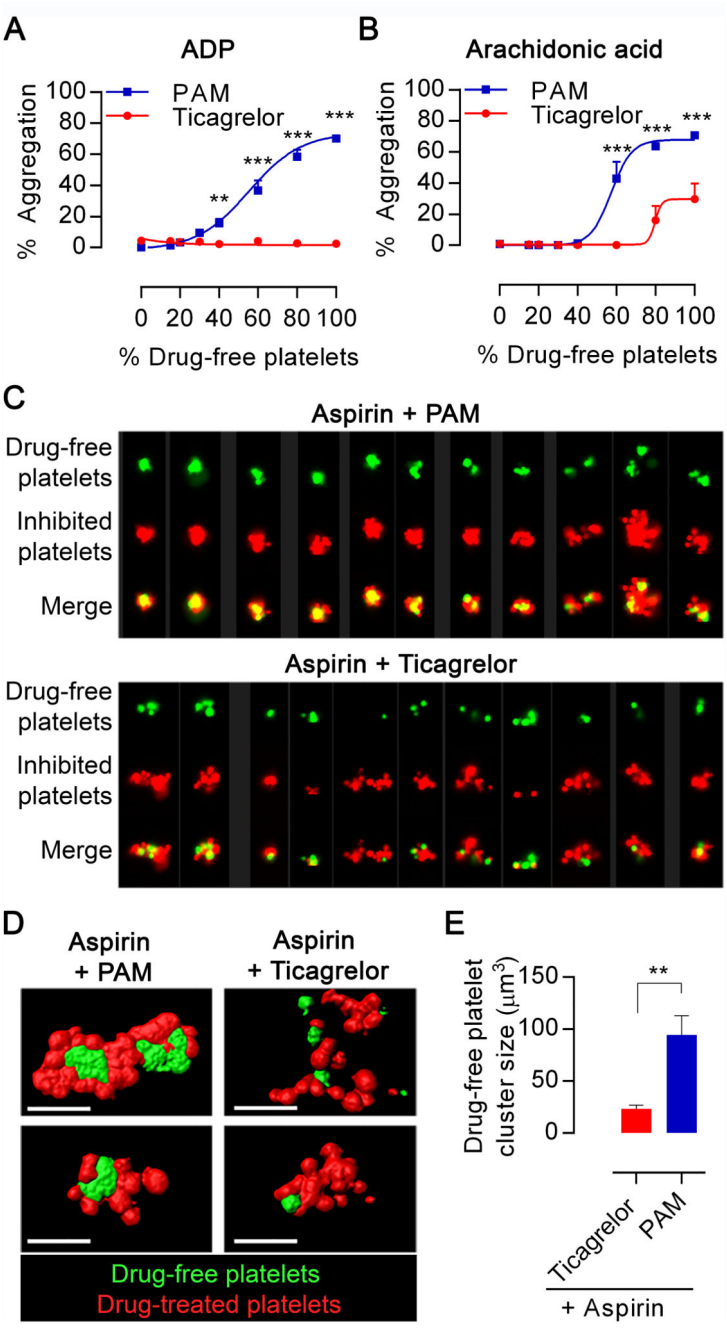

Figure 1. Ticagrelor reduces aggregation and prevents formation of drug-free platelet cores during aggregate formation *in vitro*.

PRP derived from blood pre-incubated with aspirin (30μM) and PAM (3μM) or ticagrelor (1.35μM) was mixed in a range of proportions with PRP from blood pre-incubated with respective vehicles or with ticagrelor (1.35μM) to reflect mid-dose t=6hour levels. Aggregation in response to (A) ADP 20μM or (B) AA 1mM was determined by LTA. Data presented as mean±SEM and compared by two-way ANOVA (n=4, **p<0.01, ***p<0.001). (C) Multiple images captured by ImageStreamX of aggregates (mixtures of 85% aspirin +PAM-pretreated platelets or aspirin+ticagrelor pretreated platelets plus 15% uninhibited

platelets). Each panel contains columns with following image sets: drug-free (green), inhibited platelets (red), merged image. (D) Representative confocal images of aggregates (left) formed from mixtures comprising 85% aspirin+PAM-pretreated platelets or aspirin +ticagrelor-pretreated (green) and 15% uninhibited platelets (red). (E) Images were analysed for size of the uninhibited platelet particles. Data is presented as mean±SEM and compared by t-test (n=4, **p<0.01).

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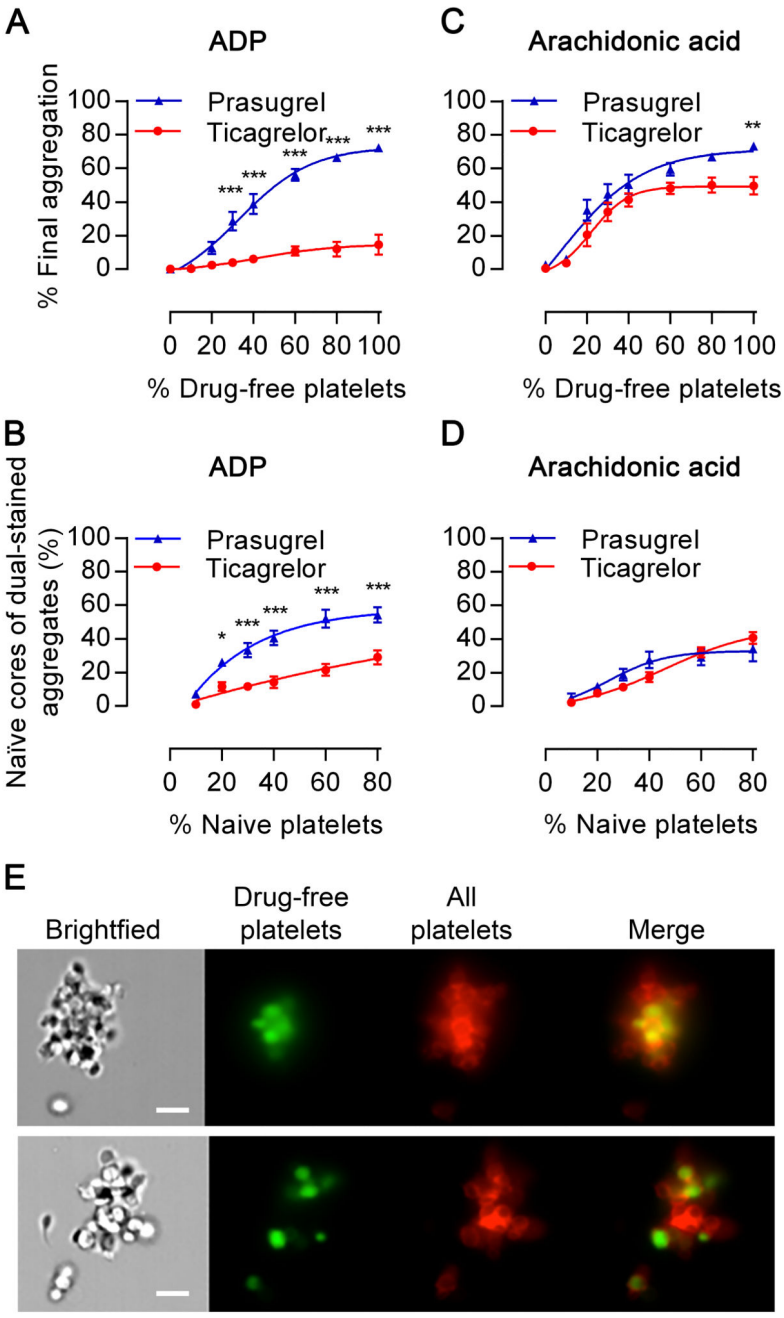

Figure 2. Drug-free platelets restore *ex vivo* aggregation responses differentially in presence of prasugrel or ticagrelor.

PRP isolated from individuals 6h after receiving aspirin+prasugrel or aspirin+ticagrelor was mixed with increasing proportions of drug-free platelets and then stimulated. Final aggregation of samples stimulated with (A) ADP (20 μM) or (C) arachidonic acid (1 mM). (B, D) Aggregates (%) where cores comprise naïve platelets were blind scored and calculated from flow cytometric images of platelet aggregates from corresponding samples. 104 aggregates assessed per individual sample, with data presented as mean±SEM and compared by two-way ANOVA (n=10 samples, *p<0.05, **p<0.01, ***p<0.001). (E)

Representative flow cytometric imaging (x60 objective) of aggregates formed in response to ADP (20μM) from 80%:20% mixtures of aspirin+prasugrel-inhibited PRP or aspirin +ticagrelor-inhibited PRP obtained 6 hours after the last drug dose was administered (red) and drug-free platelets (green). Scale bars equal to 7μm.

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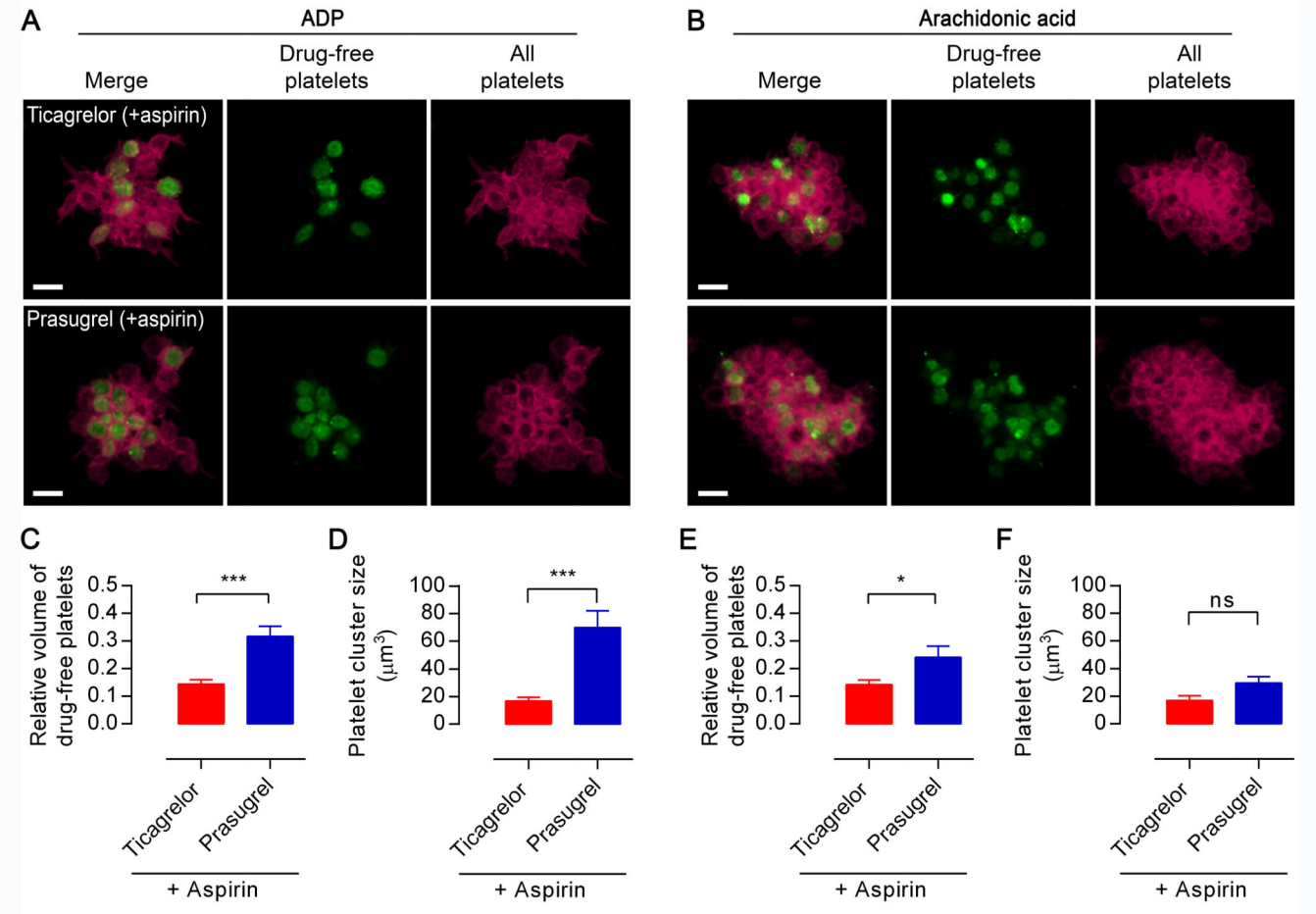

Figure 3. Drug-free platelets form cores within aggregates in presence of prasugrel but not ticagrelor.

Representative confocal images of (A) ADP-stimulated or (B) AA-stimulated aggregates formed from 80%:20% mixtures of aspirin+prasugrel-inhibited PRP or aspirin+ticagrelor-inhibited PRP obtained 6 hours after the last drug dose was administered (red) and drug-free platelets (green), conditions as in Figure 2. Images were analysed for (C, E) volume of the drug free platelet particles relative to the total aggregate volume and (D, F) average size of drug-free platelet clusters. Scale bars represent 5 μm. Data is presented as mean±SEM and compared by t-test (n=7-10; *p<0.05, ***p<0.001).

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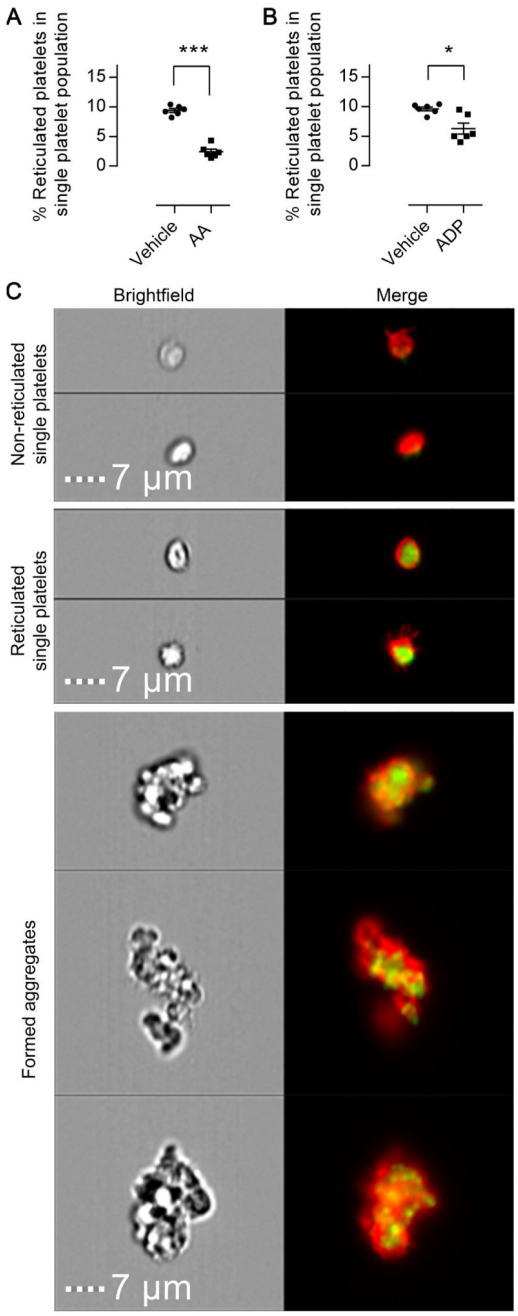

Figure 4. Reticulated platelets display elevated reactivity in response to both arachidonic acid and ADP.

The proportion of reticulated platelets among non-aggregated platelets was assessed by flow cytometry in platelet rich plasma incubated with vehicle, (A) arachidonic acid, or (B) ADP. (C) Representative flow cytometric images of non-reticulated and reticulated (mRNA stain green) single platelets (red), as well as aggregates formed in response to ADP. Scale bars equal to 7μm. Data presented as individual data points with overlaid mean±SEM and compared by paired t-test (n=6; *$p<0.05$, ***$p<0.001$).

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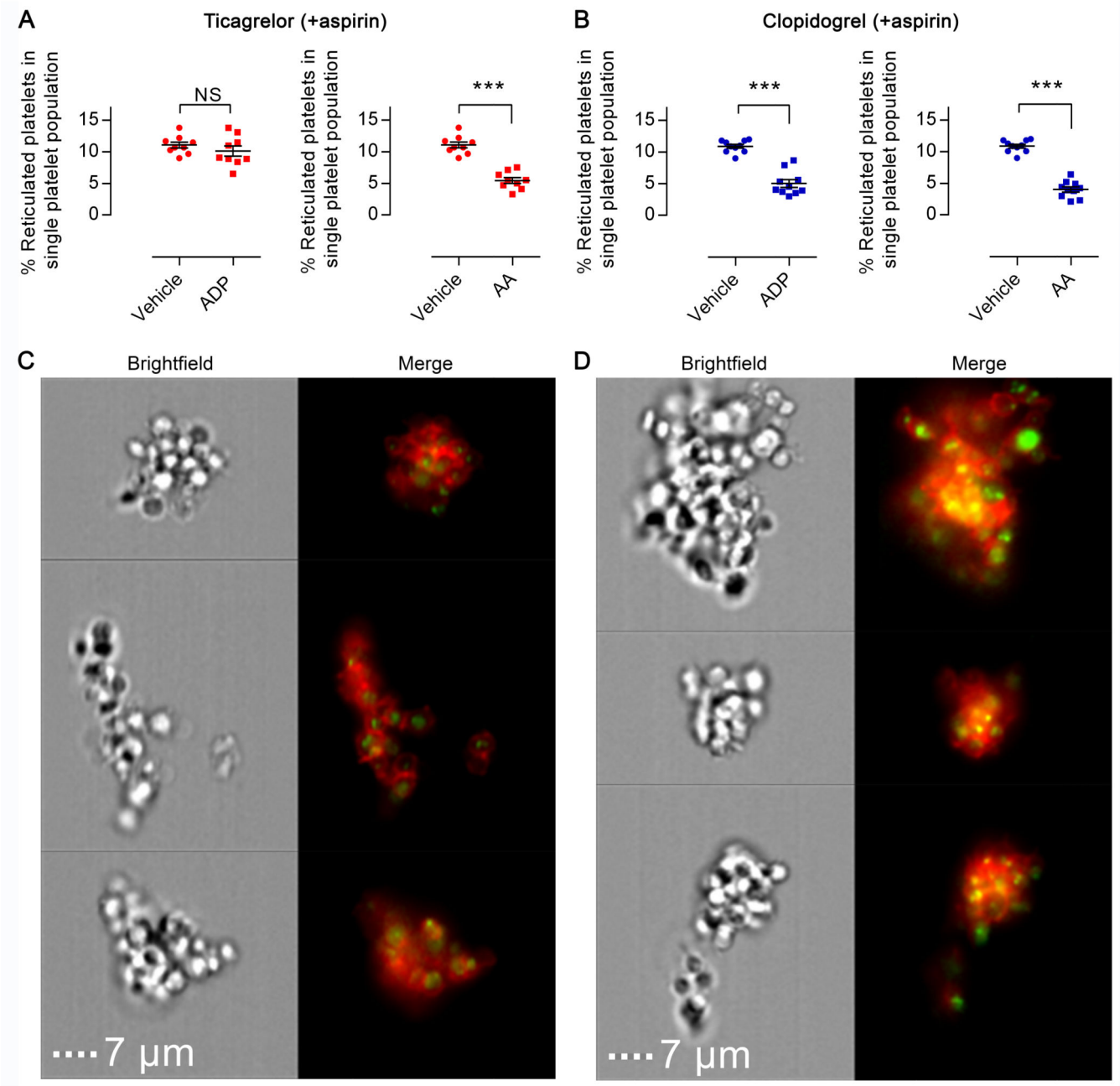

Figure 5. In patients the response of reticulated platelets to ADP is inhibited to a greater extent by ticagrelor than by clopidogrel.

The reticulated platelet subpopulation among non-aggregated single platelets was assessed by flow cytometry in PRP incubated with vehicle, ADP, or arachidonic acid. Samples were obtained from patients taking (A) ticagrelor or (B) clopidogrel (in addition to aspirin). Representative flow cytometric images of ADP-stimulated platelet aggregates (platelets red; mRNA green) formed in samples from patients taking aspirin plus (C) ticagrelor or (D)

clopidogrel. Scale bars equal to 7μm. Data presented as individual data points with overlaid mean±SEM and compared by paired t-test (n=9-10; ***p<0.001).

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