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BRIEF REPORT

# Impact of antiplatelet therapy on hemostatic plug formation in the setting of thrombocytopenia

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## Abstract

**Background:** Antiplatelet therapy (APT), mainly aspirin and P2Y12 receptor inhibitors, reduces the incidence of recurrent arterial thrombosis but also increases bleeding risk. Therefore, management of APT in patients with thrombocytopenia, itself an independent risk factor for bleeding, is a clinical challenge with few evidence-based guidelines. Data are lacking on the combined impact of thrombocytopenia and APT on hemostasis.

**Objectives:** To systematically investigate the combined effect of thrombocytopenia and APT in mouse models of hemostasis and thrombosis.

**Methods:** Platelet-depleted mice were repleted with donor platelets inhibited with aspirin and/or clopidogrel at low ($<1 \times 10^8$/mL) or normal (>2) platelet counts. Hemostasis was assessed in the saphenous vein laser injury model, and thrombosis was assessed in the carotid artery ferric chloride model.

**Results:** In the saphenous vein laser injury model, neither single nor dual APT significantly increased bleeding compared with vehicle at platelet counts $>2 \times 10^8$/mL. However, for platelet counts <1, clopidogrel prolonged the time to the first hemostatic plug, and dual APT prolonged the time to the first plug and total bleeding time compared with vehicle and aspirin treatment. In the carotid artery ferric chloride thrombosis model, clopidogrel was entirely protected against platelet-rich thrombus formation, while aspirin had minimal effect.

**Conclusion:** Our experimental data suggests that for severe thrombocytopenia, single APT provides an appropriate balance of antithrombotic effect and limited bleeding, with clopidogrel demonstrating a greater antithrombotic effect but slightly increased bleeding compared with aspirin.

KEYWORDS
aspirin, clopidogrel, dual antiplatelet therapy, intravital microscopy, thrombocytopenia

**Essentials**

- Thrombocytopenia (TP) and antiplatelet therapy (APT) are independent risk factors for bleeding.
- This study addresses a lack of experimental data on safety of APT in the setting of TP.
- The P2Y12 inhibitor clopidogrel and dual APT (aspirin + clopidogrel) increased bleeding in mice with TP, while aspirin alone did not increase bleeding; clopidogrel and DAPT but not aspirin strongly protected against arterial thrombosis.
- In patients with TP, aspirin may be safe but P2Y12 inhibitors may be required with higher thrombosis risk; more clinical trials are needed for combined TP and APT.

## 1 | INTRODUCTION

Antiplatelet therapy (APT) is the mainstay of secondary prevention following a primary thrombotic event such as myocardial infarction or ischemic stroke [1] but increases the risk of hemorrhage. The most common antiplatelet agents are aspirin (ASA) and P2Y12 receptor inhibitors (clopidogrel [Clop], prasugrel, and ticagrelor), given as single APT (SAPT) or dual APT (DAPT) treatment. In patients with acute coronary syndrome (ACS) who undergo percutaneous coronary intervention and/or stent placement, DAPT is recommended for 1 to 12 months postprocedure, followed by SAPT indefinitely [2]. Bleeding risk is greater with DAPT compared with SAPT [3] and with the more potent, newer-generation P2Y12 inhibitors (prasugrel and ticagrelor) compared with Clop [4].

Thrombocytopenia (TP; $<150 \times 10^9$ platelets/L) is an independent risk factor for bleeding. While severe spontaneous bleeding is rare with platelet counts $>10 \times 10^9$/L [5], significant periprocedural bleeding can occur with platelet counts $<50 \times 10^9$/L [6]. TP can be hypoproliferative (most often associated with chemotherapy) or caused by enhanced platelet clearance/destruction, such as in immune TP (ITP), HIT, chronic liver disease, infection, or following cardiac procedures such as coronary artery bypass grafting [7]. Surrounding procedures, TP is classified as baseline (preprocedural) or incident (peri/postprocedural). Incident TP is observed in >10% of hospitalized ACS patients and is associated with bleeding and worse outcomes [8].

Importantly, TP often contraindicates robust APT, even in patients with a high risk of thrombotic complications [9]. In major trials investigating DAPT use for ACS, TP patients are typically excluded [6]. However, disorders with TP can also be associated with an increased risk of arterial and/or venous thrombosis, such as in ITP [10], and patients with hematological malignancies have a higher risk of arterial thrombosis despite the high incidence of TP [11]. To date, there are no evidence-based guidelines defining platelet count thresholds for safe use of APT. Here, we used mouse models to investigate the impact of varying severity of TP on bleeding associated with APT.

## 2 | METHODS

### 2.1 | Mice

C57BL/6 wild-type (WT) and human interleukin-4 receptor (hIL4R)α/glycoprotein (GP)Ibα transgenic mice [12] (on B6 background) were bred in-house. Male and female mice were equally used for experiments. All experimental protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.

### 2.2 | Platelet counts and activation assays

Platelet counts were determined in diluted whole blood by flow cytometry using an anti-GPIX Alexa Fluor 488 antibody (clone Xia.B4, Emfret Analytics). Light transmission aggregometry was performed by stimulating washed platelets in Tyrode's buffer (137 mM NaCl, 0.3 mM $Na_2HPO_4$, 2 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.3) containing 1 mM $CaCl_2$ with 1 mM arachidonic acid (Sigma) or 10 μM adenosine diphosphate (ADP; Thermo Fisher). The flow cytometry $\alpha_{IIb}\beta_3$ activation assay was performed by stimulating platelets in diluted whole blood with 20 μM ADP for 15 minutes in the presence of JON/A-PE antibody (Emfret Analytics).

### 2.3 | TP mouse model

TP mice were generated using the adoptive platelet transfer model [13]. Briefly, hIL4Rα/GPIbα mice were depleted of platelets and transfused with donor platelets from WT mice treated with water (vehicle) or APT (ASA, 20 mg/kg and Clop, 25 mg/kg, or both; Med-Vet International) 24 and 3 hours prior to blood collection. Recipient platelet counts were determined 30 minutes posttransfusion.

### 2.4 | Saphenous vein laser injury hemostasis model

Hemostasis was assessed in mice using the established saphenous vein laser injury model with some modifications. Briefly, the saphenous vein was exposed, and a 50 μm penetrating injury was created with a 10-millisecond laser pulse ablation. Platelets and fibrin were visualized with fluorescently conjugated antibodies. Injury sites were visualized for 10 minutes postablation. Time to first hemostatic plug formation was determined, as well as instances of rebleeding after plug reopening, which were combined for total bleeding time. For the determination of plug density and thickness, 4-dimensional imaging was performed and analyzed as recently described [14].

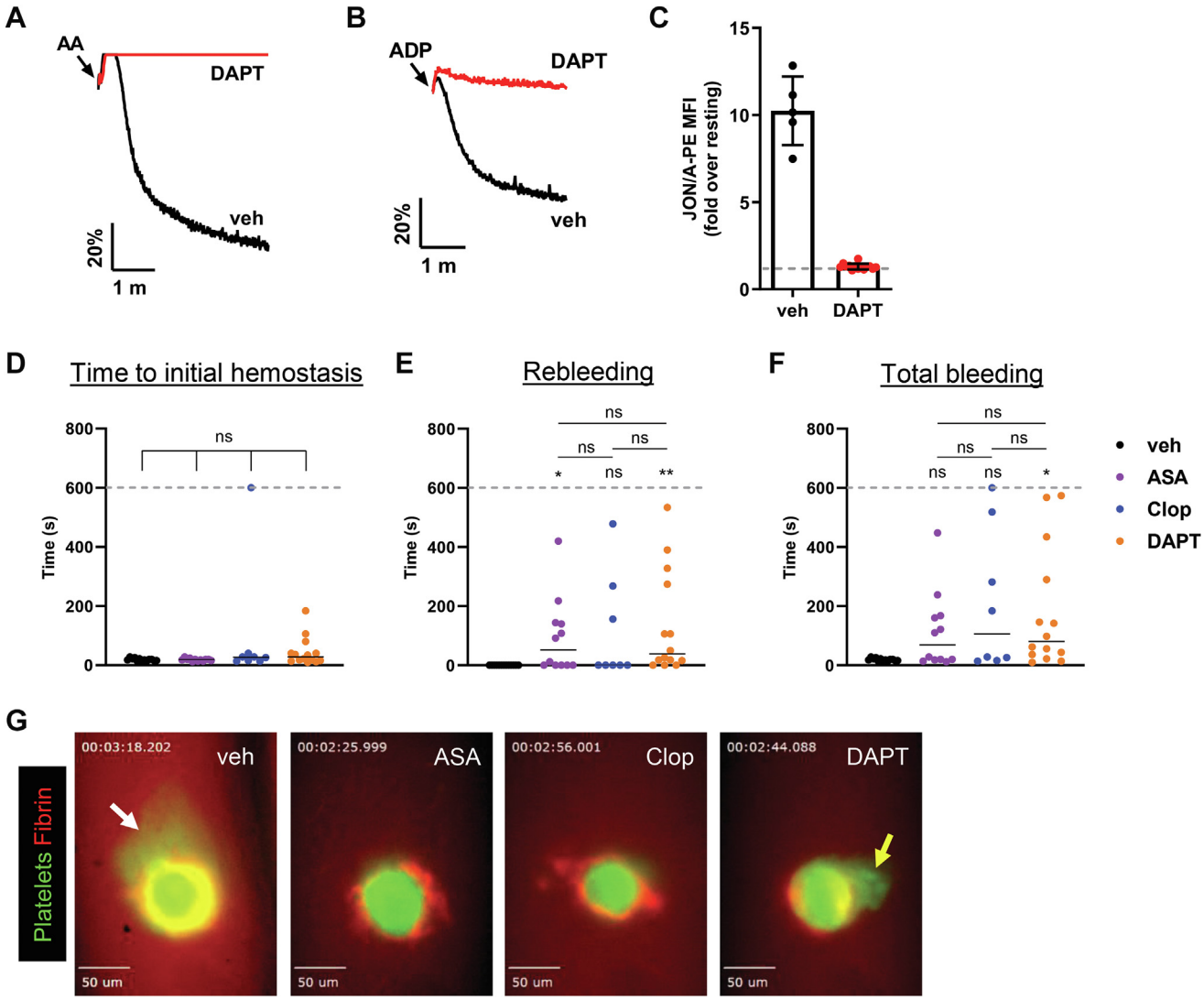


**FIGURE 1** Impact of antiplatelet therapy on bleeding in wild-type (WT) mice. C57BL/6 WT mice were gavaged twice with vehicle (veh), aspirin (ASA, 20 mg/kg), clopidogrel (Clop, 25 mg/kg), or dual antiplatelet therapy (DAPT). (A, B) Inhibition of platelets by ASA + Clop (DAPT) was tested by light transmission aggregometry following stimulation with arachidonic acid (AA, 1 mM) or adenosine diphosphate (ADP, 10 μM). Aggregation traces are representative of multiple experiments. (C) Inhibition of P2Y12-mediated $\alpha_{IIb}\beta_3$ activation was additionally tested in a flow cytometry assay. Platelets in diluted whole blood from veh- or DAPT-treated mice were stimulated with 20 μM ADP in the presence of JON/A-PE antibody for 15 minutes to detect the active conformation of $\alpha_{IIb}\beta_3$ integrin. Data are shown as mean ± SD. *n* = 5 veh and 14 DAPT. The dotted line represents a normalized value of 1 for the unstimulated (resting) condition. (D–F) Hemostasis was assessed in the saphenous vein laser injury model in WT mice treated with veh, ASA, Clop, or DAPT. Bleeding was determined by intravital microscopy using fluorescently conjugated antibodies against platelets and fibrin. Bleeding was categorized as (D) time to initial hemostasis (formation of first hemostatic plug), (E) rebleeding (bleeding occurring after formation of first plug), and (F) total bleeding (initial hemostasis + rebleeding). Data points represent individual injuries, with the median shown. The dotted line represents the maximum time of observation. *n* = 13 veh, 12 ASA, 8 Clop, and 14 DAPT. (G) Representative still-frames of hemostatic plugs formed in WT mice treated with veh, ASA, Clop, or DAPT. Intravascular platelet accumulation observed in veh-treated mice (white arrow) is absent in all APT-treated mice, and DAPT-treated mice demonstrate extravascular platelet accumulation caused by excessive bleeding (yellow arrow). Scale bar = 50 μm. Statistical significance was performed by the Kruskal–Wallis test with Dunn's multiple comparisons test (D–F). No statistical differences were observed between any groups in (D). $^*P < .05$, $^{**}P < .01$, ns, not statistically significant.

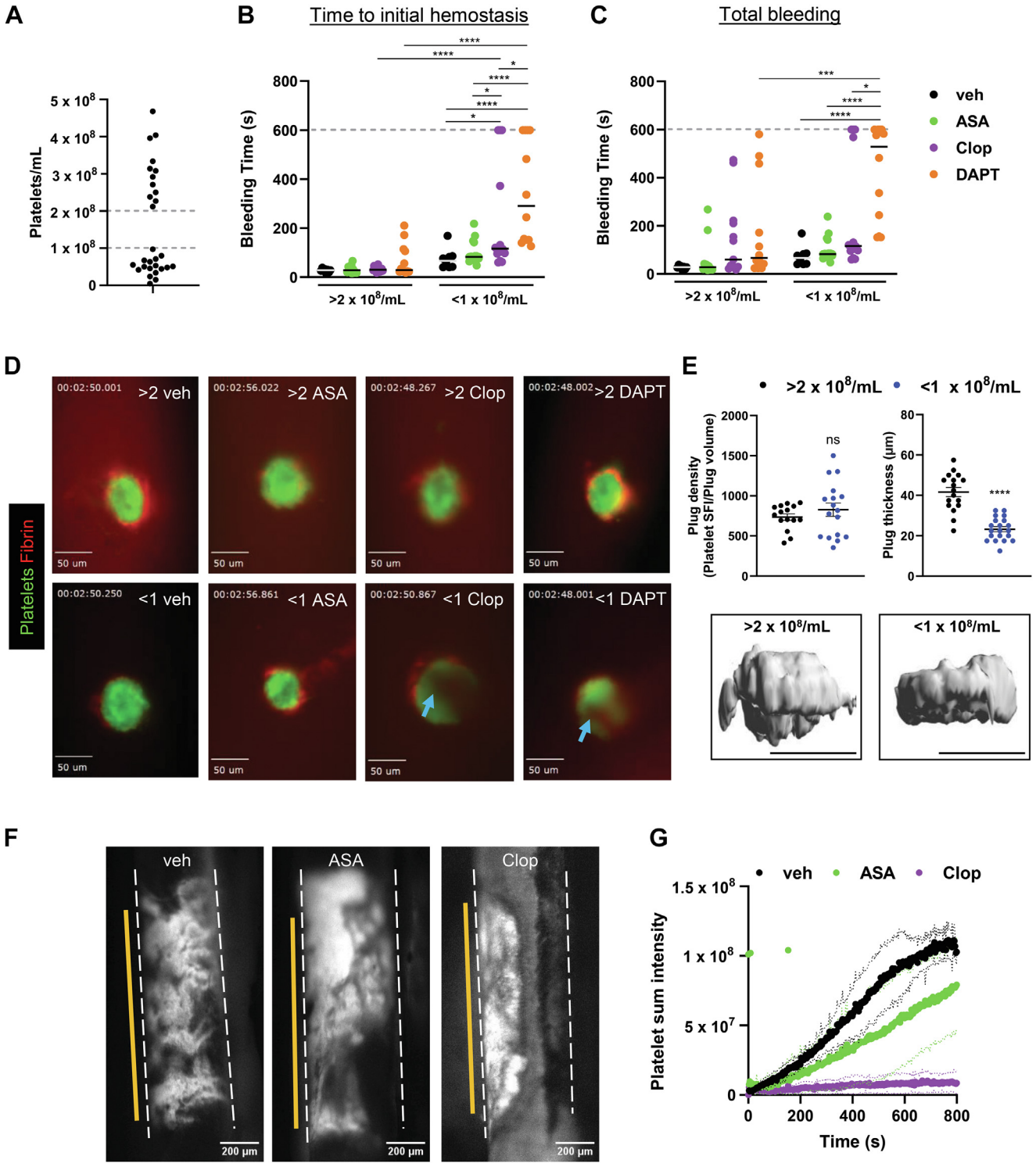


**FIGURE 2** Impact of antiplatelet therapy on bleeding in thrombocytopenic mice. Human interleukin-4 receptor (hIL4R)/GPIb recipient mice were depleted of platelets and transfused with donor platelets from wild-type (WT) mice treated with vehicle (veh), aspirin (ASA), clopidogrel (Clop), or dual antiplatelet therapy (DAPT) to achieve platelet counts >2 or <1 × $10^8$/mL. (A) Platelet counts in all transfused recipient mice. (B, C) Initial hemostasis and total bleeding times in hIL4R/GPIb mice transfused with veh, ASA, Clop, or DAPT at >2 or <1 × $10^8$/mL. Rebleeding times are omitted due to minimal rebleeding observed. Data points represent individual injuries, with the median shown. $n$ = 8 veh > 2, 11 ASA > 2, 13 Clop > 2, 12 DAPT > 2, 8 veh < 1, 16 ASA < 1, 14 Clop < 1, and 12 DAPT < 1 × $10^8$/mL. (D) Representative still-frames of hemostatic plugs. No intravascular platelet accumulation is observed, and active bleeding can be observed at injury sites in mice with <1 × $10^8$/mL Clop or DAPT-platelets (blue arrows). Scale bar = 50 µm. (E) Plug density and plug thickness were determined by 4-dimensional imaging in mice transfused with WT platelets at counts > 2 and <1 × $10^8$/mL. Data points represent values for individual injuries. Data are shown as mean ± SD. $n$ = 17 > 2 × $10^8$/mL and 20 < 1 × $10^8$/mL. Side views of representative 3-dimensional reconstructed platelet plugs used for analysis. Scale

### 2.5 | Carotid artery ferric chloride thrombosis model

Mice were anesthetized with 2% isoflurane and placed in a supine position, and the right carotid artery was isolated by blunt dissection. A 1 $mm^2$ piece of filter paper was soaked with ferric chloride ($FeCl_3$; Sigma, 10% solution in water) and applied to the lateral artery surface for 3 minutes, and the vessel was washed 3 times with saline. Platelet accumulation was visualized by injection of rhodamine 6G (50 μL of a 1 mg/mL solution in water; Sigma) on a Nikon SMZ25 fluorescence microscope equipped with a Lumencor Sola light engine and a 1.6× objective for 20 minutes.

### 2.6 | Statistical analysis

For statistical analysis, GraphPad Prism software (version 10.3 for Windows, GraphPad Software) was used. Comparisons between >2 groups were performed using the Kruskal–Wallis test with Dunn's multiple comparisons or 2-way analysis of variance with Tukey's multiple comparisons. $P < .05$ was considered statistically significant.

## 3 | RESULTS AND DISCUSSION

We first compared vehicle-treated WT mice with WT mice treated with ASA (20 mg/kg), Clop (25 mg/kg), or both (DAPT) in the saphenous vein laser injury hemostasis model. ASA and Clop were dosed for >90% inhibition of arachidonic acid and ADP-induced platelet activation, respectively, as measured in DAPT samples (Figure 1A–C) and single inhibitors (not shown). Bleeding time was defined as the time to first hemostatic plug (initial) or total bleeding (time to first plug + rebleeding time). Nearly all injury sites in APT-treated mice had normal initial hemostasis times (Figure 1D), and while ASA and DAPT slightly increased rebleeding events (Figure 1E), only DAPT caused significantly prolonged total bleeding times, which remained well below the maximum observation time (Figure 1F). Intravital imaging demonstrated that APT-treated mice had a loss of intravascular platelet accumulation at sites of injury (Figure 1G), which may contribute to plug instability and rebleeding. Importantly, these results demonstrated that the saphenous vein laser ablation model is suitable for investigating the exacerbation of bleeding with combined TP and APT.

Mice have ~3 to 5 times higher platelet counts than humans [15]. To compare platelet counts in the normal and TP ranges for humans, platelet-depleted hIL4Rα/GPIbα recipient mice were reconstituted with donor platelets from APT- (ASA, Clop, and DAPT) or vehicle-treated WT donor mice to achieve circulating platelet counts of >2 or $<1 \times 10^8$/mL (Figure 2A). At a platelet count $>2 \times 10^8$/mL, none of the APT treatments significantly prolonged initial or total bleeding compared with mice reconstituted with control platelets (Figure 2B, C). At $<1 \times 10^8$/mL, Clop prolonged initial bleeding compared with both control and ASA, and DAPT dramatically prolonged both initial and total bleeding time. When comparing platelet counts for each treatment, initial bleeding for Clop and both initial and total bleeding for DAPT were significantly prolonged for <1 compared with $>2 \times 10^8$/mL (Figure 2B, C). Plugs in mice with lower platelet counts or stronger APT (DAPT > Clop > ASA) appeared thinner, and prolonged bleeding was due to either reopening or failure to completely close off the center of the plug (Figure 2D). To measure platelet density and thickness of the hemostatic plugs, we performed 4-dimensional imaging of plug formation after injury in mice with >2 or $<1 \times 10^8$/mL control platelets [14]. While the resolution of our current imaging system is not sufficient to identify single platelets in the plug, we determined that plug density (ratio between platelet accumulation and plug volume) was not significantly altered in mice with low platelet counts (<1). However, plug thickness in mice with platelet counts $<1 \times 10^8$/mL was reduced by ~50% compared with mice with platelet counts $>2 \times 10^8$/mL (Figure 2E). These results suggest that APT exacerbates bleeding with TP by further destabilizing the thin center of the plug.

To investigate the impact of ASA vs Clop on experimental thrombosis, we performed the $FeCl_3$-induced carotid artery thrombosis model in platelet-depleted recipients transfused with ASA or Clop-treated platelets at $\sim 2 \times 10^8$/mL. Platelets were labeled *in vivo* with rhodamine 6G, and the sum intensity of the intravascular thrombi was measured over time. While mice transfused with Clop-treated platelets were entirely protected from thrombus formation, ASA-treated platelets formed robust thrombi (area under the curve vehicle vs ASA $P > .05$; Figure 2F, G). DAPT-platelets prevented thrombus formation similar to Clop alone (not shown).

The goal of this study was to provide experimental *in vivo* data on the synergistic impact between various antiplatelet strategies and TP during hemostasis. We found that at low platelet counts, ASA did not prolong bleeding, while Clop caused more bleeding than ASA but less than dual therapy. However, Clop had a stronger antithrombotic effect than ASA. Similar to previous work from our laboratory [16], very low platelet counts had minimal impact on hemostasis in the laser injury model. In the tail clip bleeding model, which causes a larger vascular injury, platelet depletion to <10% of normal causes uncontrolled bleeding [17], suggesting that TP-associated bleeding is

bar = 50 μm. (F) Representative intravital microscopy still-frames of thrombi formed after ferric chloride ($FeCl_3$) challenge to the carotid artery in hIL4R/GPIb mice transfused with veh, ASA, or Clop platelets at a final platelet count of $\sim 2 \times 10^8$/mL. Platelets were labeled by injection of rhodamine 6G (white color in images). Dotted lines represent vessel walls, and yellow lines represent the approximate location of the $FeCl_3$ application. (G) Sum fluorescence intensity analysis in the $FeCl_3$ thrombosis model in hIL4R/GPIb mice transfused with veh, ASA, or Clop platelets at $2 \times 10^8$/mL. Data are shown as mean ± SD. $n = 5$ veh, 3 ASA, and 3 Clop. Statistical significance was performed by (B, C) 2-way analysis of variance with Tukey's multiple comparisons and (E) the Student's *t*-test. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$, ns, not statistically significant.

dependent on injury size in a controlled experimental setting. Similar observations were made for P2Y12 inhibition [18]. We similarly observed fairly minimal bleeding with Clop at higher platelet counts, although bleeding increased with more severe TP. We also demonstrated that ASA spares severe bleeding during TP compared with Clop and DAPT. ASA was the key antiplatelet agent prior to the discovery of P2Y12 biology [19], but some studies showing a greater antithrombotic effect of P2Y12 inhibitors with fewer gastrointestinal bleeding complications may begin pushing ASA out of favor [20]. Other mouse studies also showed that ASA provides little to no protection from $FeCl_3$-induced arterial thrombosis [21], but this is difficult to interpret with no consensus on the mechanism(s) of $FeCl_3$-induced thrombosis [22,23], and it may be an inherently ASA-insensitive model.

Platelet thrombi have defined regions known as the "core" and "shell," mainly defined by activation by collagen/tissue factor/thrombin or ADP/thromboxane $A_2$, respectively [24]. In a hemostatic plug, the core could be defined as the ring of platelets that initially bind to the edges of the injury site, while the shell comprises the center of the plug as well as the intravascular platelets accumulating over the plug. In both APT and TP conditions, we observe a loss of intravascular accumulation, and APT additionally destabilizes the center of the plug, leading to rebleeding. Thus, thinner hemostatic plugs formed in TP conditions are more prone to reopening. A recent study investigated the synergistic impact of Clop and factor Xa inhibition with rivaroxaban, albeit in mice with normal platelet counts [25]. While Clop impaired platelet accumulation, rivaroxaban prevented fibrin formation while sparing platelet accumulation, and the combination prolonged bleeding times compared with single agents at the same concentrations. Interestingly, the authors also observed a dose-dependent response of Clop; while 25 mg/kg strongly impaired platelet aggregation and prolonged bleeding times, 5 mg/kg demonstrated a partial aggregation defect and only led to increased bleeding in the presence of high-dose rivaroxaban [25]. We would expect that partial P2Y12 inhibition in DAPT would cause less bleeding in the setting of TP but may also lose antithrombotic efficacy. These concepts could also extend to other drugs with platelet inhibitory effects, such as Bruton's tyrosine kinase inhibitors.

Despite a lack of large clinical trials, some recent studies are beginning to provide more information on antiplatelet strategies for patients with TP. An analysis of ITP patients in a French registry exposed to APT found that the frequency of bleeding in patients taking ASA at the time of ITP diagnosis was similar to nonexposed patients described in previous studies, even at platelet counts $<10 \times 10^9$/L [26]. Although, the authors questioned whether ASA would provide complete inhibition due to rapid platelet turnover. In cancer patients with severe TP and suffering from acute myocardial infarction, ASA was associated with improved survival without major hemorrhage [27]. However, a recent study in patients undergoing autologous stem cell transplants observed that ASA significantly increased bleeding risk once platelet counts dropped below $50 \times 10^9$/L [28]. It is likely that the impact of APT highly depends on the underlying condition causing TP.

Our study has several limitations affecting direct translation to patients. Mice have inherently higher platelet counts than humans ($>1 \times 10^9$/mL vs $1.5\text{-}4 \times 10^8$/mL, respectively), so we were required to manipulate platelet counts. The ideal model of hypoproliferative TP would be myeloablative therapy, such as with busulfan. Other strategies to generate TP mice include antibody-mediated platelet depletion [29] and toxin-induced TP in transgenic mice [30]. Similar to humans, the "cause" of TP could affect thrombosis risk, such as in ITP, where thrombosis rates are often higher than in reference populations [10]. Guidelines may, therefore, need to be assigned by disease rather than platelet count alone. We also used a venous bleeding model, but arterial assess site bleeding is common in cardiac surgery, and there are likely differences in hemostasis requirements for venous vs arterial bleeding, especially due to differences in shear rates.

In summary, our study demonstrated that ASA caused minimal excess bleeding in the setting of severe TP, while Clop and DAPT caused increasing amounts of bleeding, and DAPT significantly prolonged the time to the first hemostatic plug. Large trials are needed to provide evidence-based guidelines, which may depend on the condition causing TP. Until then, fear of major hemorrhage in TP patients may continue to prohibit appropriate antiplatelet management.

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## AUTHOR CONTRIBUTIONS

R.H.L. performed experiments, analyzed data, and drafted the manuscript. A.B.-K. performed experiments and analyzed data. S.R.J. performed experiments. W.B. designed studies and wrote the manuscript. All authors edited and approved the manuscript.

## RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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