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Recombinant von Willebrand Factor (vonicog alfa) reduces platelet inhibition caused by antiplatelet drugs and has potential as an acute haemostatic agent

Michael J R Desborough,[1,2]* Joanne L Mitchell,[3*] Polly Whitworth,[4,5] Ashifa Al Juwaiser,[6] Tanya Sage,[7] Neline Kriek,[7] Gemma Little,[7] Sakthivel Vaiyapuri,[6] Jonathan M Gibbins,[7] Alexander P Bye[6,7]

[1]Department of Clinical Haematology, Oxford University Hospitals NHS Foundation Trust, Oxford, UK

[2]Radcliffe Department of Medicine, University of Oxford; NHS Blood and Transplant, Oxford, UK

[3]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

[4]Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, Oxford, UK.

[5]Oxford Cardiovascular Clinical Research Facility (CCRF), John Radcliffe Hospital, Oxford, UK.

[6]School of Pharmacy, University of Reading, Reading, UK

[7]Institute for Cardiovascular and Metabolic Research, Harborne Building, University of Reading, Reading, UK

* Authors contributed equally

Corresponding Author:

Dr Alexander P Bye

School of Pharmacy, University of Reading

Hopkins Building, Whiteknights, Reading, Berkshire, UK

Email: a.bye@reading.ac.uk

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Authorship Contributions

MJD designed the study, collated data and wrote the manuscript. JLM performed research and analysed data. PW coordinated recruitment and collated data; AAJ, NK, TS and GL performed

research; SV and JMG designed the study; APB designed the study, performed research, analysed data, and wrote the manuscript.

Disclosure of Conflicts of Interest

This work was partially funded by an Investigator Initiated Research Grant from Takeda. The study was proposed by the authors and the design, data collection, analysis, interpretation and write up were conducted independently. MJRD has received speakers' fees and advisory board fees from Takeda that are unrelated to this work.

Data Sharing

The data that support the findings of this study are available from the corresponding author, APB, upon reasonable request.

Patients taking antiplatelet drugs who experience major bleeding, such as intracerebral haemorrhage (ICH), have an increased risk of death or disability.[1] There are approximately 2.9 million deaths worldwide per year from (ICH)[2] and a quarter of these patients are taking antiplatelet drugs.[3]

Strategies to reduce the elevated risk of death, such as platelet transfusion[4] and tranexamic acid,[5] have so far proven ineffective. One potential strategy for reducing the antiplatelet drug effect is increasing plasma von Willebrand Factor (VWF) levels. VWF facilitates platelet-collagen and platelet-platelet interactions, especially under high shear conditions present in arteries.[6]

Desmopressin (1-deamino-8-D-arginine vasopressin; DDAVP) is a vasopressin analogue that stimulates release of endogenous VWF from Weibel-Palade bodies present in vascular endothelial cells. It enhances platelet function and is under investigation in clinical trials for antiplatelet drug-associated ICH[7] and to reduce perioperative bleeding risk.[8] However it takes 60 to 90 minutes to take effect, produces highly variable changes in VWF levels, and is associated with side effects such as hyponatraemia and hypotension.[8]

Direct elevation of plasma VWF levels via infusion with vonicog alfa, a purified recombinant VWF (rVWF) product, may offer significant advantages due to its instantaneous and predictable effect. Vonicog alfa is approved in the US and Europe to treat von Willebrand disease (VWD) [9] and contains a high proportion of ultra-high molecular weight multimers due to its synthesis in the absence of ADAMTS-13[10] which may enhance is haemostatic activity.

This study reports the *in vitro* efficacy and mechanism of action of rVWF as a platelet function-enhancing haemostatic agent in blood samples from patients receiving antiplatelet therapy.

The effect of rVWF was studied using an *in vitro* thrombus formation assay due to the shear-dependent nature of the contribution of VWF to primary haemostasis. Thrombus formation was measured by perfusing citrated whole blood through type I collagen-coated (100 µg/mL type I) microfluidic flow chips (Vena8, Cellix Ltd) for 6 minutes at an arterial shear rate ($1000s^{-1}$), selected to reflect physiological shear conditions in small arteries and arterioles, where VWF-mediated platelet adhesion is most relevant. Samples were then fixed with 10% formyl saline (Sigma-Aldrich) and stained with 4µg/ml $DiOC_6$ (Thermo Fisher Scientific). Blood samples were treated with rVWF (vonicog alfa, supplied by Takeda) with concentrations expressed as U/ml (VWF:RCo). The volume of thrombi were measured by acquiring z-stack image series using an A1R confocal fluorescence microscope (Nikon).

We initially investigated concentration dependence of rVWF in blood samples donated by healthy subjects. Healthy subjects aged 21 to 65 were recruited to the study using procedures approved by the University of Reading Research Ethics Committee (UREC 20/20). Blood samples treated for 10 minutes with acetylsalicylic acid (aspirin, ASA), $P2Y_{12}$ antagonist cangrelor, or both, formed significantly smaller thrombi compared to vehicle-treated samples (Figure 1A). Addition of rVWF restored thrombus volume to vehicle-treated levels under all three conditions. rVWF was more potent at restoring platelet function of ASA-treated samples, with significant increase in thrombus volume even at the lowest concentration of rVWF tested (0.5U/ml). Thrombus volumes in cangrelor only or ASA + cangrelor-treated samples were significantly increased only at higher concentrations of rVWF (2 and 5U/ml).

We hypothesised that this enhancement might be due to the high proportion of high and ultra-high molecular weight multimers present in rVWF. High molecular weight multimers have high affinity for platelet GPIb and collagen, and consequently make the greatest contribution to primary haemostasis.[11] VWF multimer gels (Figure 2Ai) indicated that rVWF increased VWF multimer levels in the high and ultra-high molecular weight range (Figure 2Aii-iii). We compared the effect of rVWF to a non-recombinant VWF product with lower UHMWM content[12] and found that although thrombus volume was enhanced by both products and the effect of aspirin treated platelets appeared similar. thrombus volume remained significantly inhibited in the presence of $P2Y_{12}$ blockade (Supplementary Figure 1).

We then investigated the efficacy of rVWF in a cohort of patients (Figure 2B) receiving oral antiplatelet therapy with aspirin, a $P2Y_{12}$ antagonist or both (dual antiplatelet therapy, DAPT). Patients were recruited under Oxford Radcliffe Biobank research tissue bank ethics, HTA License Number 12217, Oxfordshire C REC: 09/H0606/5+5, project approval code SC/0173. All subjects provided informed consent in accordance with the Declaration of Helsinki. Patients (Supplemental table 1) were receiving antiplatelet therapy for ischaemic heart disease (17/22), suspected ischaemic heart disease (2/22; one with coronary artery spasm, one with atrial fibrillation and normal coronary arteries), or ischaemic stroke (3/22). Blood samples were collected into vacutainers containing 3.2% (w/v) sodium citrate. To define the therapeutic range for rVWF, we focussed our experiments on a narrower set of concentrations (0.5, 1, and 2 U/mL). Thrombus volumes were significantly increased after treating with 0.5 U/ml (100%), 1 U/ml (177%) and 2U/ml (217%) rVWF in samples from patients treated with aspirin only (Figure 2Bi). For patients receiving a $P2Y_{12}$ antagonist alone (Figure 2Bii) or DAPT (Figure 2Biii), rVWF caused a significant increase in thrombus volume at 1U/ml (252%) and 2U/ml (211%) but not 0.5U/ml. The magnitude of the improvements in platelet function compare favourably to those of a previous study in which DDAVP responses in patients with postoperative

bleeding was assessed using a similar thrombus formation assay, in which a modest but significant increase in thrombus surface coverage of 15% was observed after DDAVP infusion.[13]

To investigate the mechanism of action of rVWF, we first imaged rVWF localisation within thrombi. A concentration-dependent increase in VWF staining within platelet aggregates formed on collagen was observed following addition of rVWF to whole blood from healthy donors (Figure 3Ai and Aii). This suggests that rVWF promotes platelet-platelet interactions required for aggregation. This finding agrees with a previous report that elevated plasma VWF increases thrombus formation on collagen via enhancement of aggregate formation rather than by facilitating adhesion to collagen.[14] As both GPIb and integrin $\alpha_{IIB}\beta_3$ serve as receptors for VWF, we investigated the dependence of the rVWF effect on these receptors by inhibiting the interaction with GPIb and integrin $\alpha_{IIB}\beta_3$ using the blocking antibody AK2 and eptifibatide respectively. Both inhibitors ablated the rVWF-mediated increase in thrombus volume, indicating a dependence on both GPIb and integrin $\alpha_{IIB}\beta_3$ in mediating the interaction with rVWF. Integrin $\alpha_{IIB}\beta_3$ must adopt a high affinity confirmation that enables ligand binding, which can either be induced by activation of intracellular signalling processes, such as activation of PI3K, PKC and RAP1b[15] or via 'priming' in which binding of GPIb with VWF under high shear conditions initiates partial activation and inside-out activation of $\alpha_{IIB}\beta_3$.[16] As antiplatelet drugs inhibit TxA_2 and $P2Y_{12}$ receptor-mediated platelet signalling pathways, we hypothesised that the efficacy of rVWF might depend upon GPIb-mediated priming to induce integrin $\alpha_{IIb}\beta_3$ activation. We investigated activation of platelet signalling during thrombus formation by fixing thrombi formed on collagen after 60 seconds. Thrombi were then permeabilised and stained using an antibody raised against phosphorylated PKC substrates which can serve as marker of activatory platelet signalling (Supplementary Figure 2). This indicated that rVWF increased the proportion of platelets within the thrombi with low levels of platelet activation and staining negative for PKC activity, suggesting that rVWF-mediated platelet priming of integrin $\alpha_{IIB}\beta_3$ may contribute to the efficacy of rVWF. This is consistent with previous findings that exogenously added VWF can restore normal patterns of platelet aggregation on collagen surfaces by reinforcing integrin $\alpha_{IIb}\beta_3$-dependent platelet–platelet interactions.[17] A schematic model summarising this mechanism is shown in Figure 3D.

Reversal or reduction of antiplatelet effects in major haemorrhage remains an area of unmet need. A concentration of 1 U/mL (100 IU/dL) rVWF (vonicog alfa) was required to achieve significant correction of DAPT-associated inhibition *in vitro,* which would correspond to an infusion of approximately 50 IU/kg, a range already used clinically for major bleeding or peri-operative management in VWD.[18] Clinical trials of rVWF for patients with major bleeding who are taking antiplatelet drugs will be needed to determine whether the *in vitro* efficacy of rVWF can be

translated into clinical benefits by improving haemostatic efficacy without significant increase in thrombotic events.

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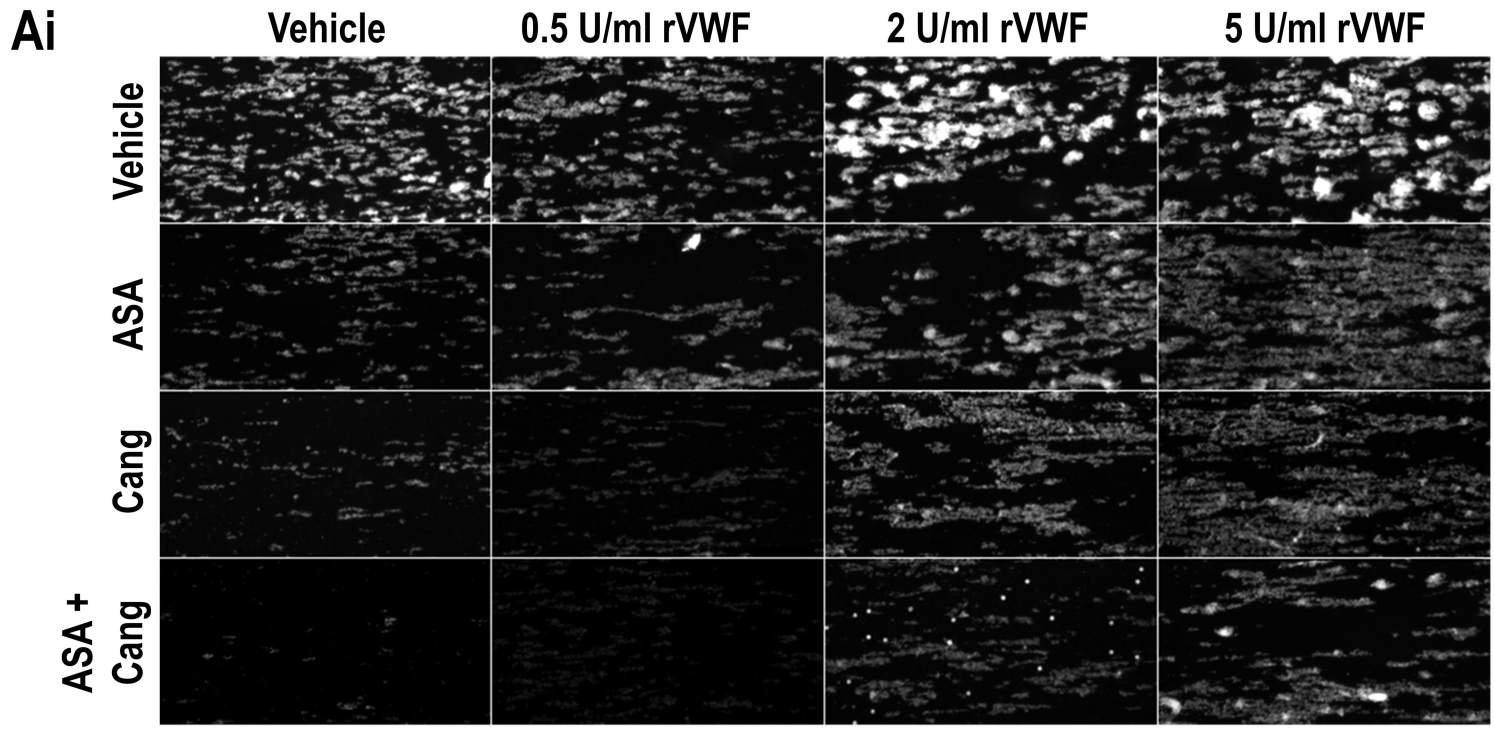
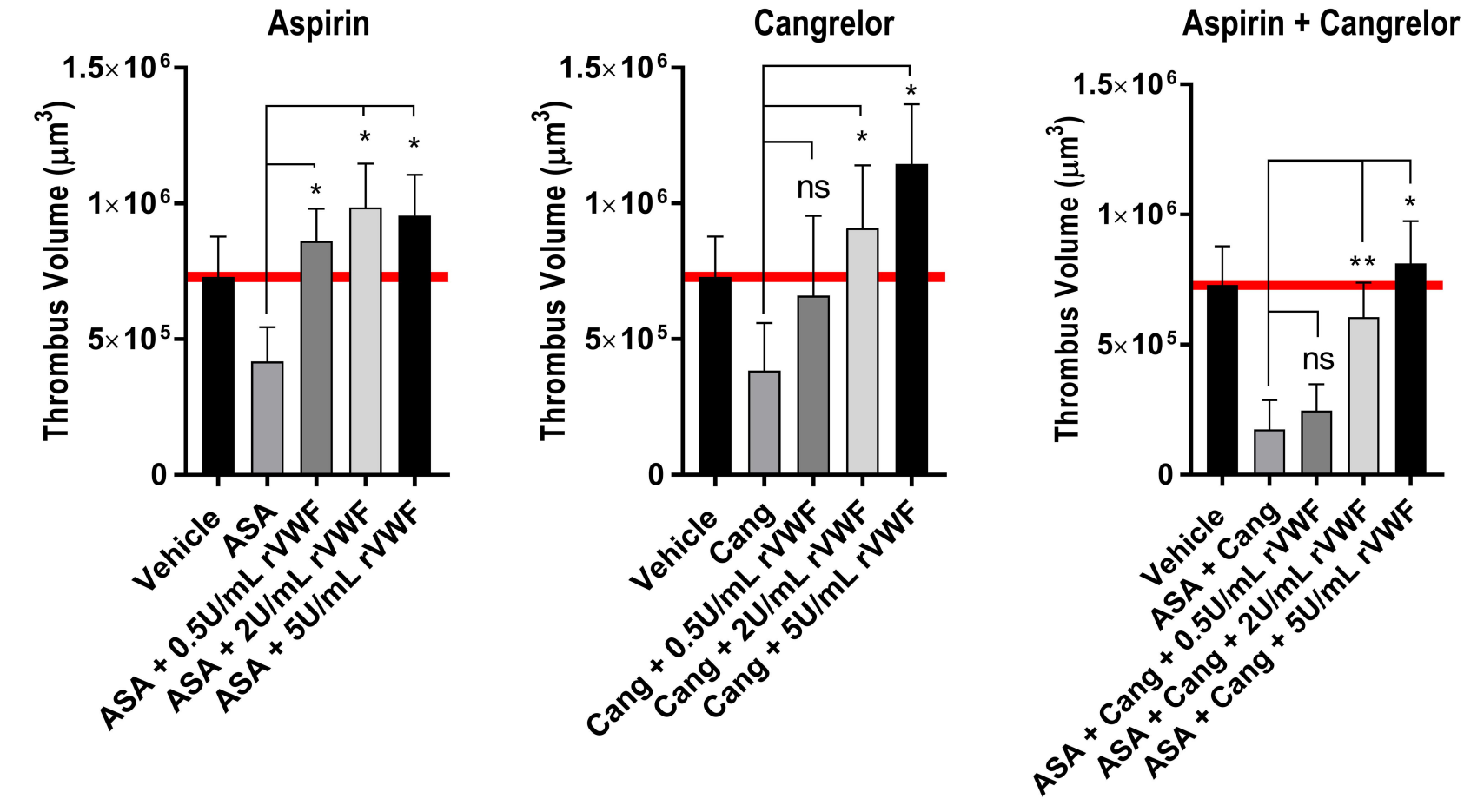
Figure Legends

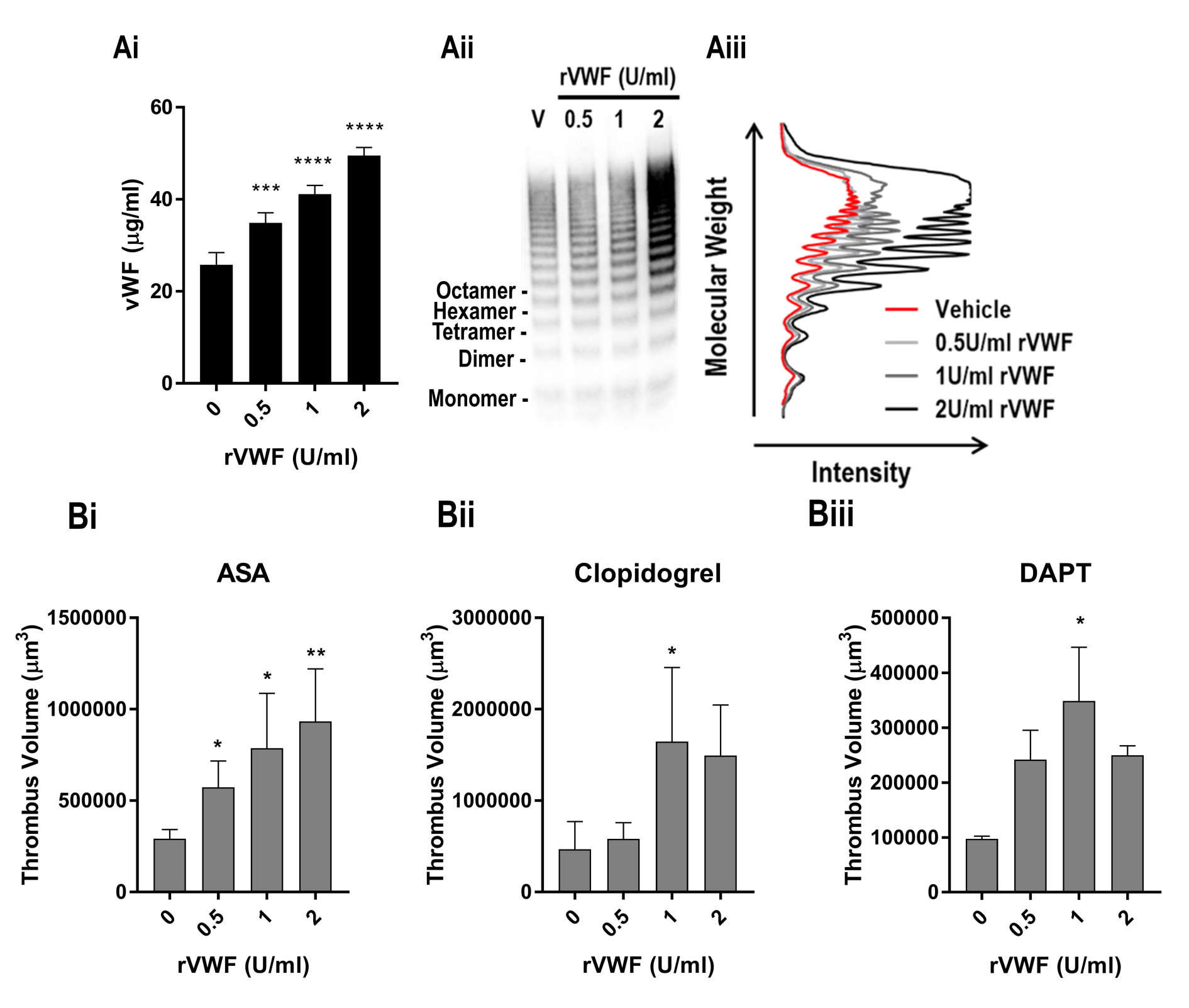
Figure 1. rVWF improves platelet function after antiplatelet treatment *in vitro*. Ai) Representative confocal z-stack images and Aii) thrombus volumes from healthy donor blood pre-treated with cangrelor (1 µM), ASA (100 µM), or both, and treated with 0, 0.5, 1, or 2 U/ml rVWF. Perfusion was at 1000 s^{-1} over type I collagen for 6 minutes. Red lines indicate mean thrombus volume of untreated control samples. *p < 0.05, **p < 0.01, **p < 0.001. 2-way ANOVA.

Figure 2. rVWF improves platelet function after antiplatelet treatment *ex vivo*. Ai) VWF antigen levels measured by ELISA in plasma samples spiked with rVWF. Aii) VWF multimer gel and Aiii) densitometry profiles showing increased high molecular weight multimers with rVWF. B) Thrombus volumes from patient samples obtained under treatment with ASA (Bi), clopidogrel (Bii), or DAPT (Biii) at 1000 s^{-1}. *p < 0.05, **p < 0.01, **p < 0.001. 2-way ANOVA.

Figure 3. rVWF enhances platelet aggregation by reducing dependence on intracellular signalling. Ai) Confocal fluorescence images of fixed and stained platelets (green) and VWF (red) on type I collagen after perfusion of whole blood with vehicle or 0.5, 2 or 5 U/ml rVWF at 1000s-1 for 6 minutes. Aii) Bar charts of fluorescence intensity of VWF staining within platelet aggregates after subtracting background fluorescence. Bi) Representative images of thrombi formed after pretreatment with vehicle, 20µg/ml AK2 or 10µM eptifibatide in the presence or absence of 1U/ml rVWF and Bii) mean thrombus volumes. C) A schematic model illustrating normal primary haemostasis whereby platelet activation is initiated by GPVI signalling, stimulating release of ADP and thromboxane A_2 (TxA_2) signalling, leading to activation of intracellular signalling pathways and integrin $\alpha_{IIb}\beta_3$-mediated fibrinogen bridging. (left); Antiplatelet drug inhibition: $P2Y_{12}$ antagonists and aspirin inhibit ADP and TxA_2 pathways, reducing activatory signalling and impairing platelet aggregation. Collagen-evoked GPVI signalling remains intact, but aggregate growth is limited (centre); and enhancement by rVWF: rVWF facilitates platelet-platelet interactions via a GPIb and integrin $\alpha_{IIb}\beta_3$-dependent mechanism. This enables aggregate growth despite reduced activatory signalling, supporting thrombus formation in the presence of antiplatelet effects (right). Bars represent the mean thrombus volume ± s.e.m. *p < 0.05, **p < 0.01, **p < 0.001. 2-way ANOVA.


Ai
Vehicle
0.5 U/ml rVWF
2 U/ml rVWF
5 U/ml rVWF
Vehicle
ASA
Cang
ASA + Cang


Aii
Aspirin
Cangrelor
Aspirin + Cangrelor
Thrombus Volume (μm3)
1.5×10^6
1×10^6
5×10^5
0
ns
*
**
Vehicle
ASA
ASA + 0.5U/mL rVWF
ASA + 2U/mL rVWF
ASA + 5U/mL rVWF
Cang
Cang + 0.5U/mL rVWF
Cang + 2U/mL rVWF
Cang + 5U/mL rVWF
ASA + Cang
ASA + Cang + 0.5U/mL rVWF
ASA + Cang + 2U/mL rVWF
ASA + Cang + 5U/mL rVWF


Ai
vWF (μg/ml)
60
40
20
0
***
****
****
0
0.5
1
2
rVWF (U/ml)
Aii
rVWF (U/ml)
V
0.5
1
2
Octamer -
Hexamer -
Tetramer -
Dimer -
Monomer -
Aiii
Molecular Weight
Intensity
Vehicle
0.5U/ml rVWF
1U/ml rVWF
2U/ml rVWF
Bi
ASA
Thrombus Volume (μm3)
1500000
1000000
500000
0
*
*
**
0
0.5
1
2
rVWF (U/ml)
Bii
Clopidogrel
Thrombus Volume (μm3)
3000000
2000000
1000000
0
*
0
0.5
1
2
rVWF (U/ml)
Biii
DAPT
Thrombus Volume (μm3)
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400000
300000
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*
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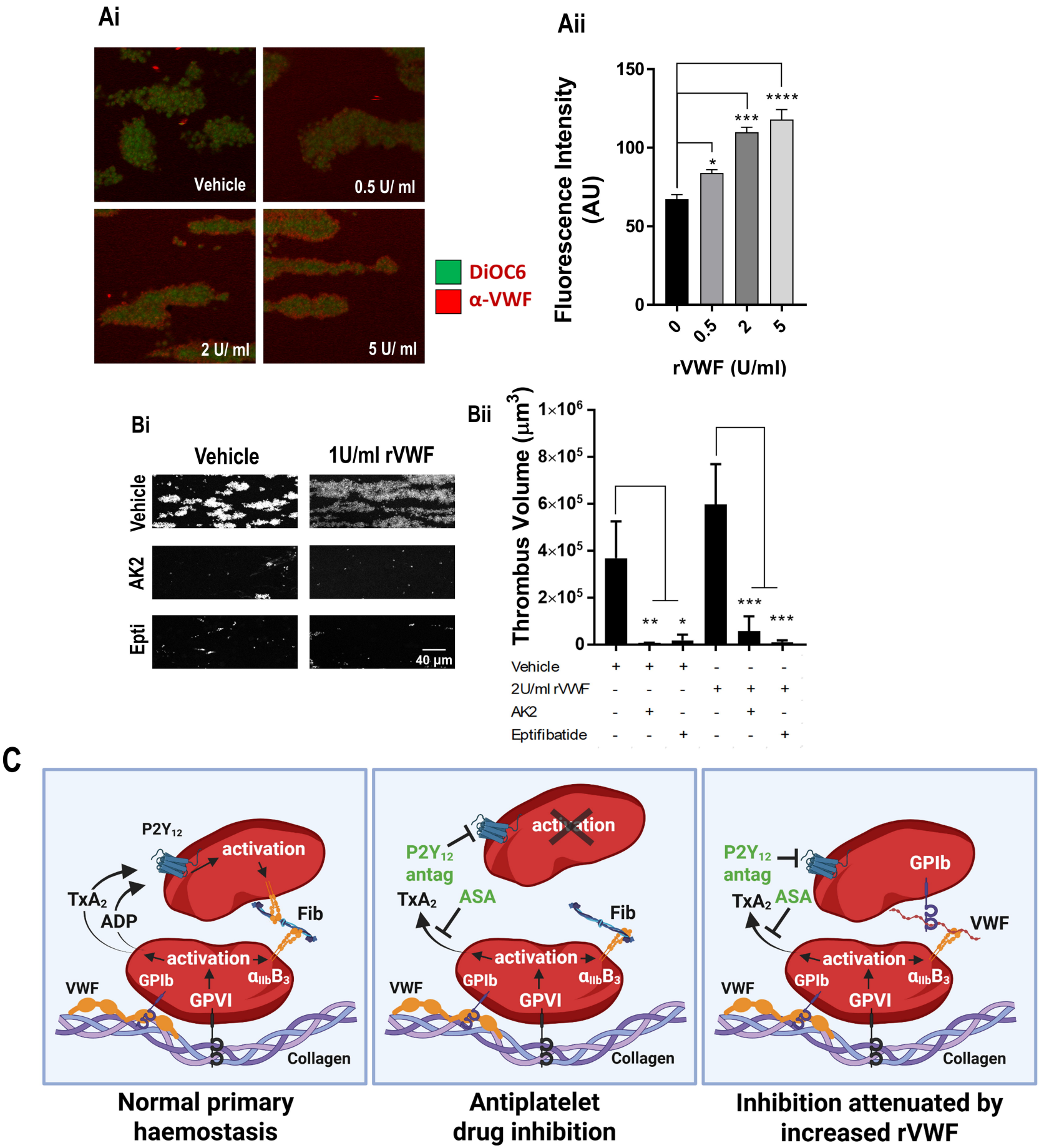
0.5
1
2
rVWF (U/ml)


Ai
Vehicle
0.5 U/ ml
2 U/ ml
5 U/ ml
DiOC6
α-VWF
Aii
Fluorescence Intensity (AU)
150
100
50
0
*
***
****
0
0.5
2
5
rVWF (U/ml)
Bi
Vehicle
1U/ml rVWF
Vehicle
AK2
Epti
40 µm
Bii
Thrombus Volume (µm3)
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
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Vehicle + + + - - -
2U/ml rVWF - - - + + +
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C
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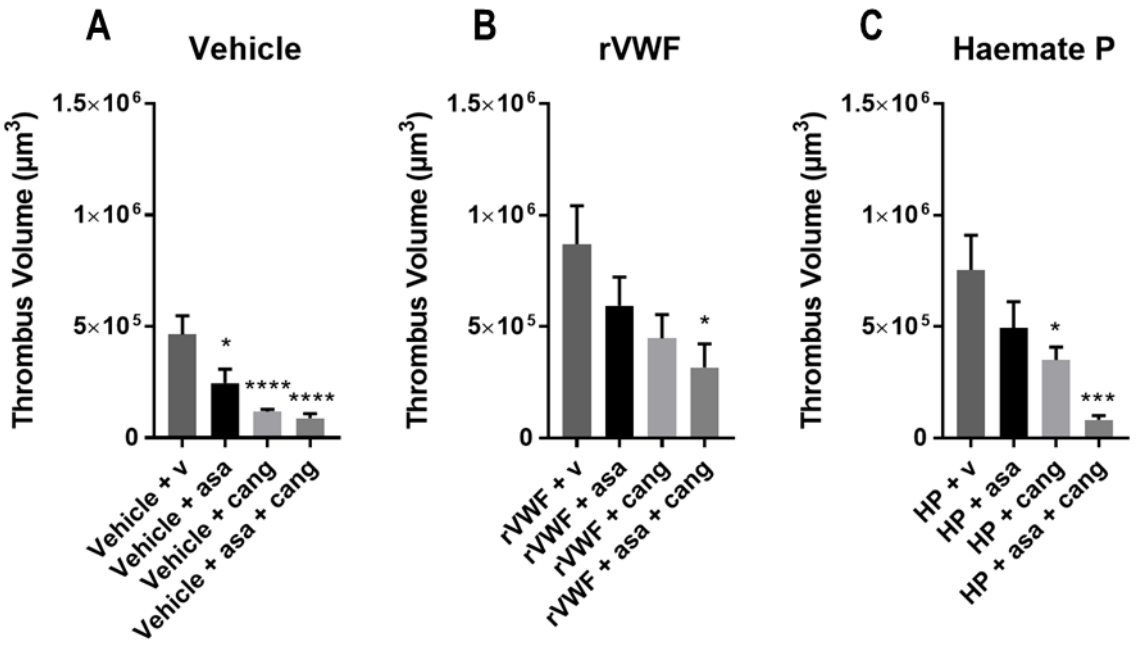
TxA2
ADP
Fib
activation
VWF
GPIb
αIIbB3
GPVI
Collagen
Normal primary haemostasis
P2Y12 antag
TxA2
ASA
Fib
activation
VWF
GPIb
αIIbB3
GPVI
Collagen
Antiplatelet drug inhibition
P2Y12 antag
GPIb
TxA2
ASA
VWF
activation
VWF
GPIb
αIIbB3
GPVI
Collagen
Inhibition attenuated by increased rVWF

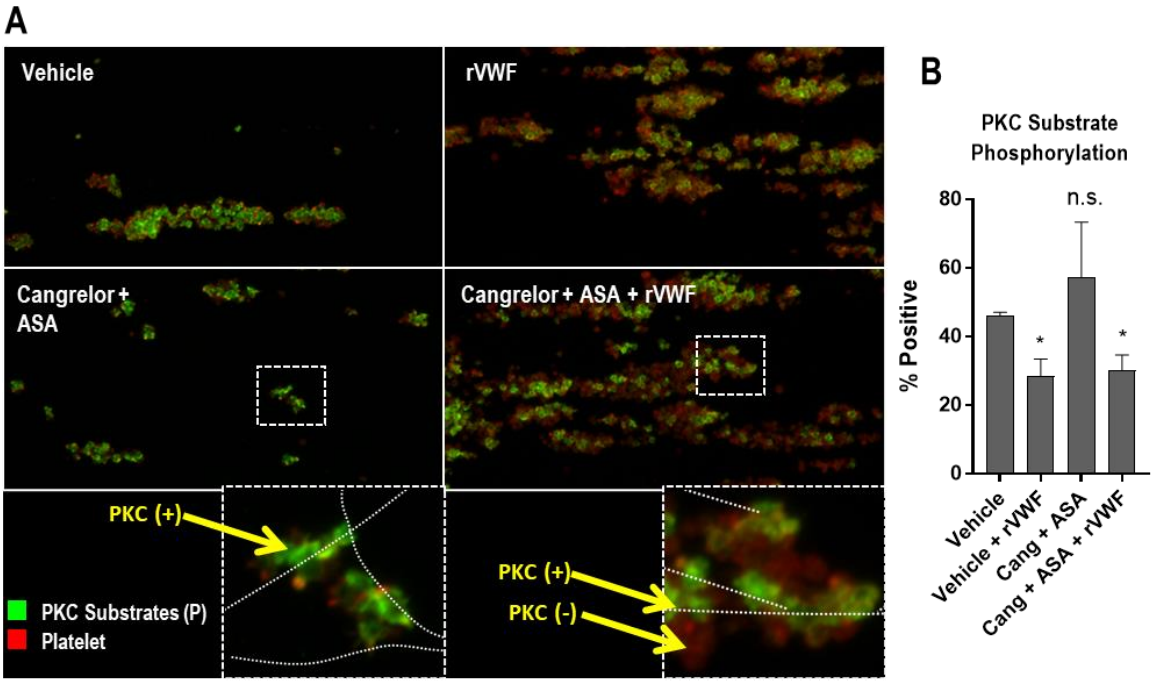
	Patients (n=22)
Age	66 (44 – 81)
Sex (Male)	15 (68%)
ASA only	15 (68%)
P2Y12 antagonist only	4 (18%)
Dual antiplatelet therapy*	3 (14%)
Hypertension	16 (72%)
High Cholesterol	11 (50%)
Diabetes	12 (56%)
Ischemic Heart Disease	19 (86%)
Smoker	6 (27%)
Haemoglobin (g/L), median (range)	142 (106 – 170)
Platelet count (x109/L), median (range)	233 (177 – 322)

* ASA plus clopidogrel, ticagrelor or prasugrel

Supplemental Table 1. Patient characteristics



Supplementary Figure 1. Effects of rVWF and non-recombinant VWF products on thrombus formation following antiplatelet treatment. Thrombus volumes from healthy donor blood pre-treated with cangrelor (1 µM), ASA (100 µM), or both, and treated with vehicle, 1U/ml rVWF or 1U/ml Haemate P. Perfusion was at 1000 s^{-1} over type I collagen for 6 minutes. Bars represent the mean thrombus volume ± s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. 2-way ANOVA.



Supplementary Figure 2. rVWF enables platelets with low levels of activatory signalling to join thrombi. A) Confocal fluorescence images of platelets (red) stained with phosphorylated PKC substrate antibody (green) after perfusion over type I collagen for 60 seconds. Yellow arrows indicate platelets positive or negative for PKC substrate phosphorylation; white dashed lines highlight collagen fibres in expanded views (bottom panels). B) Quantification of the percentage of aggregate volume staining positive for PKC substrate phosphorylation. *$p < 0.05$. 1-way ANOVA.