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

Patients taking antiplatelet drugs who experience major bleeding, such as intracerebral hemorrhage (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 to increase 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 analog 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 (min) to take effect, produces highly variable changes in VWF levels, and is associated with side

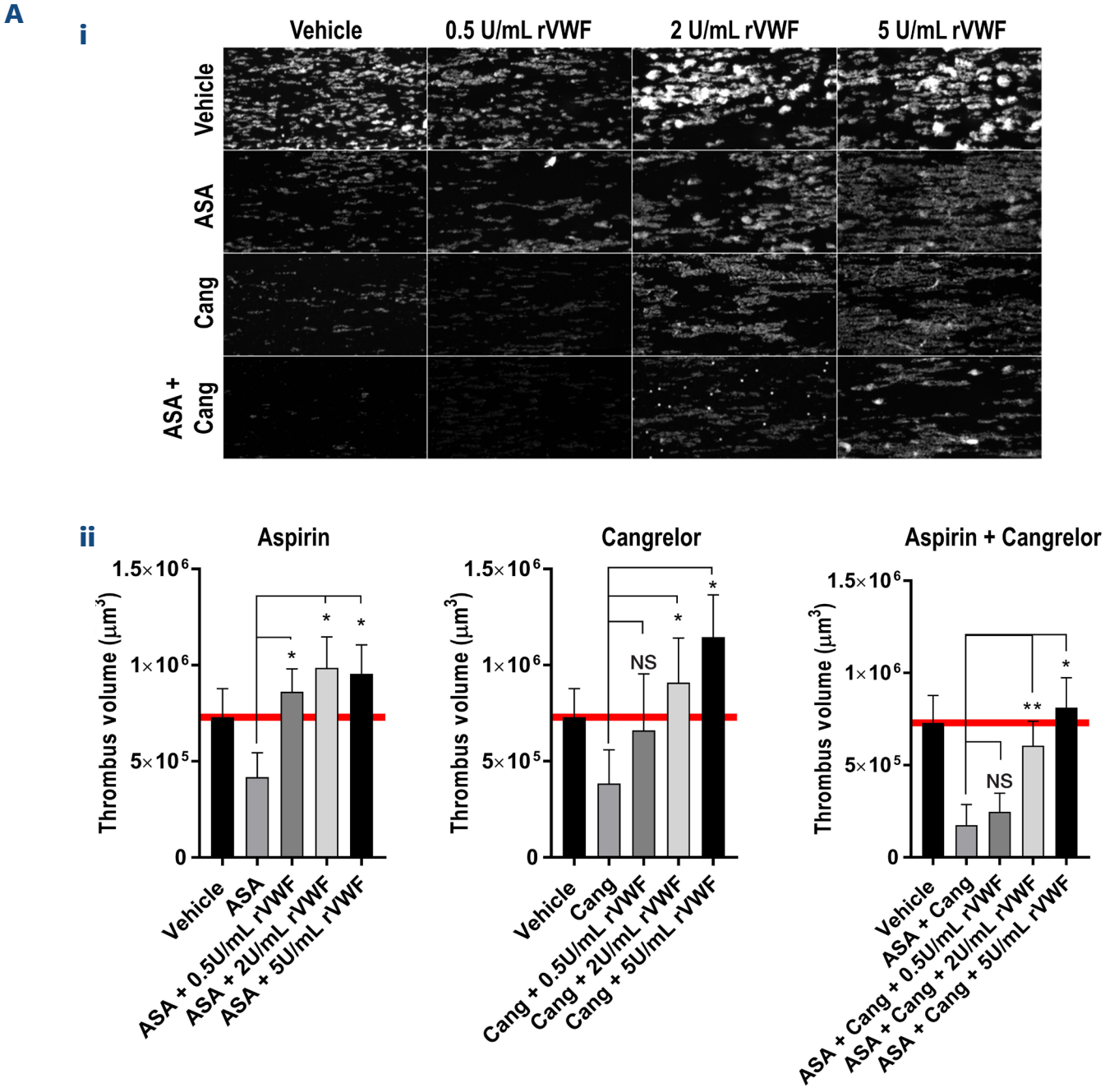

Figure 1. Recombinant Von Willebrand Factor improves platelet function after antiplatelet treatment *in vitro*. (Ai) Representative confocal z-stack images and (Aii) thrombus volumes from healthy donor blood pre-treated with cangrelor (1 μM), acetylsalicylic acid (aspirin, ASA) (100 μM), or both, and treated with 0, 0.5, 1, or 2 U/mL recombinant Von Willebrand Factor (rVWF). Magnification 20X. Perfusion was at 1000 s^{-1} over type I collagen for 6 minutes. Red lines indicate mean thrombus volume of untreated control samples. 2-way ANOVA: *P<0.05, **P<0.01, NS: not significant.

effects such as hyponatremia 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 its hemostatic activity. This study reports the *in vitro* efficacy and mechanism of action of rVWF as a platelet function-enhancing hemostatic 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 hemostasis. 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 min 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 4 μg/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 was 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 years were recruited to the study using procedures approved by the University of Reading Research Ethics Committee (UREC 20/20). Blood samples treated for 10 min 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 (0.5 U/mL). Thrombus volumes in cangrelor only or ASA + cangrelor-treated samples were significantly increased only at higher concentrations of rVWF (2 and 5 U/mL).

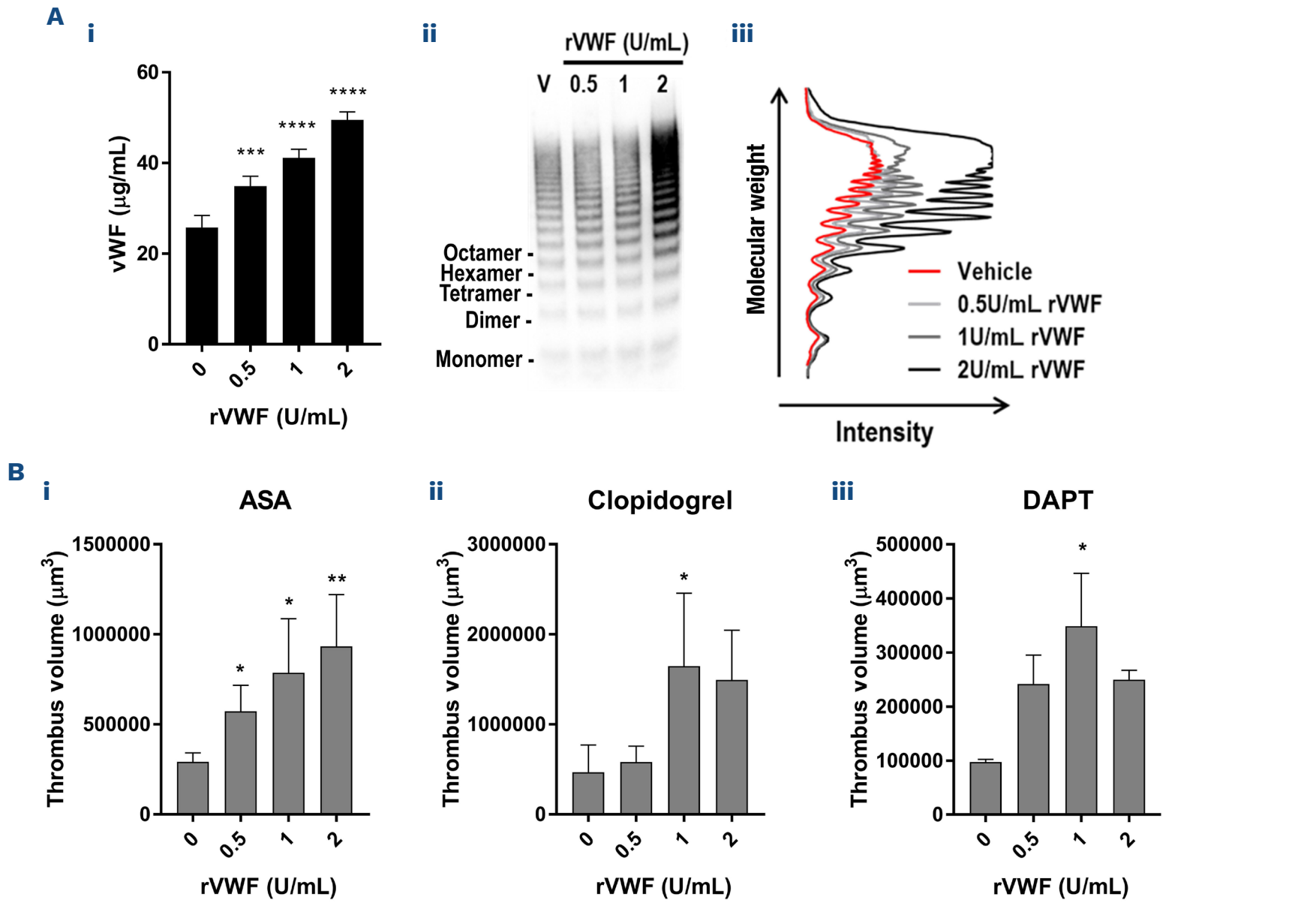

Figure 2. Recombinant Von Willebrand Factor improves platelet function after antiplatelet treatment *ex vivo*. (Ai) Von Willebrand Factor (VWF) antigen levels measured by ELISA in plasma samples spiked with recombinant VWF (rVWF). (Aii) VWF multimer gel and (Aiii) densitometry profiles showing increased high molecular weight multimers with rVWF. (B) Thrombus volumes from patient samples obtained under treatment with acetylsalicylic acid (aspirin, ASA) (Bi), clopidogrel (Bii), or dual antiplatelet therapy (DAPT) (Biii) at 1000 s^{-1}. 2-way ANOVA: **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

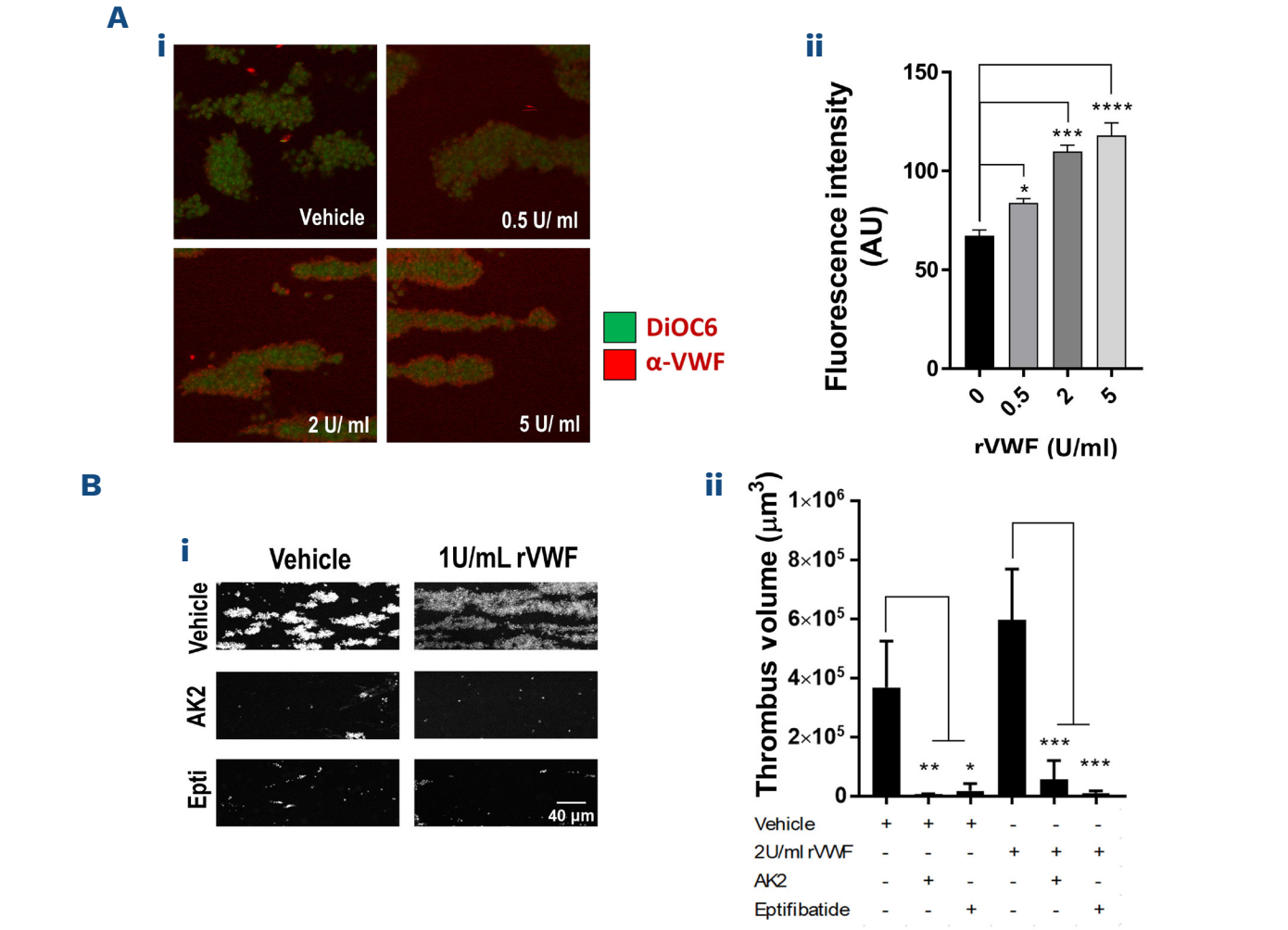

Figure 3. Recombinant Von Willebrand Factor enhances platelet aggregation by reducing dependence on intracellular signaling. (Ai) Confocal fluorescence images of fixed and stained platelets (green) and Von Willebrand Factor (VWF) (red) on type I collagen after perfusion of whole blood with vehicle or 0.5, 2, or 5 U/mL recombinant VWF (rVWF) at 1000 s^{-1} for 6 minutes. Magnification 20X. (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, 20 µg/mL AK2 or 10 µM eptifibatide in the presence or absence of 1 U/mL rVWF and (Bii) mean thrombus volumes. (C) A schematic model illustrating normal primary hemostasis whereby platelet activation is initiated by GPVI signaling, stimulating release of ADP and thromboxane A_2 (TxA_2) signaling, leading to activation of intracellular signaling 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 signaling and impairing platelet aggregation. Collagen-evoked GPVI signaling remains intact, but aggregate growth is limited (center), 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 signaling, supporting thrombus formation in the presence of antiplatelet effects (right). Bars represent the mean thrombus volume ± standard error of mean. 2-way ANOVA: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

We hypothesized 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 hemostasis.[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 ultra-high molecular weight von Willebrand Factor multimer (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 (*Online Supplementary Figure S1*).

We then investigated the efficacy of rVWF in a cohort of patients receiving oral antiplatelet therapy with aspirin, a $P2Y_{12}$ antagonist or both (dual antiplatelet therapy, DAPT) (Figure 2B). 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 principles of the Declaration of Helsinki. Patients were receiving antiplatelet therapy for ischemic heart disease (17/22), suspected ischemic heart disease (2/22; one with coronary artery spasm, one with atrial fibrillation and normal coronary arteries), or ischemic stroke (3/22) (*Online Supplementary Table S1*). Blood samples were collected into vacutainers containing 3.2% (w/v) sodium citrate. To define the therapeutic range for rVWF, we focused 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 2 U/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 1 U/mL (252%) and 2 U/mL (211%) but not 0.5 U/mL. The magnitude of the improvements in platelet function compare favorably to those of a previous study in which DDAVP responses in patients with post-operative 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 localization 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 study reporting 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 signaling 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 TxA2 and $P2Y_{12}$ receptor-mediated platelet signaling pathways, we hypothesized that the efficacy of rVWF might depend upon GPIb-mediated priming to induce integrin $\alpha_{IIB}\beta_3$ activation. We investigated activation of platelet signaling during thrombus formation by fixing thrombi formed on collagen after 60 seconds. Thrombi were then permeabilized and stained using an antibody raised against phosphorylated PKC substrates which can serve as marker of activatory platelet signaling (*Online Supplementary Figure S2*). 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 3C.

Reversal or reduction of antiplatelet effects in major hemorrhage 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 in VWD for major bleeding or peri-operative management.[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 hemostatic efficacy without significant increase in thrombotic events.

Authors

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] and Alexander P. Bye[6,7]

[1]Department of Clinical Haematology, Oxford University Hospitals NHS Foundation Trust, Oxford; [2]Radcliffe Department of Medicine, University of Oxford and NHS Blood and Transplant, Oxford; [3]Institute of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham; [4]Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, Oxford; [5]Oxford Cardiovascular Clinical Research Facility (CCRF), John Radcliffe Hospital, Oxford; [6]School of Pharmacy, University of Reading, Reading and [7]Institute for Cardiovascular and Metabolic Research, Harborne Building, University of Reading, Reading, UK

**MJRD and JLM contributed equally as first authors.*

Correspondence:
A.P. BYE - a.bye@reading.ac.uk

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Contributions

MJD designed the study, collated data and wrote the manuscript; JLM performed research and analyzed data; PW co-ordinated recruitment and collated data; AAJ, NK, TS and GL performed research; SV and JMG designed the study; APB designed the study, performed research, analyzed data, and wrote the manuscript.

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Data-sharing statement

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

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