Blocking platelet glycoprotein V cleavage reduces bleeding in mouse models of *MYH9*-related disease

Platelet adhesion and activation critically contributes to hemostasis and thrombosis and involves multiple receptor-ligand interactions. When a vessel is injured, platelets adhere to the exposed extracellular matrix, aggregate and form a hemostatic plug to prevent blood loss. Initial adhesion occurs via the glycoprotein (GP)Ib-V-IX complex binding to collagen-bound von Willebrand factor, retaining platelets near the vessel wall. This interaction enables GPVI to bind collagen, triggering signaling that activates integrin receptors for firm adhesion and thrombus growth. Thrombin then catalyzes coagulation factor activation and converts fibrinogen to fibrin, stabilizing the plug. GPV is part of the GPIb-V-IX complex,[1] interacts with collagen[2] and can be cleaved by thrombin.[3,4] Recently, we demonstrated that soluble GPV limits fibrin generation and blockade of GPV cleavage attenuates defects in hemostatic platelet function.[5] However, the impact of blocking thrombin-mediated GPV cleavage on inherited platelet bleeding disorders caused by mutations in cytoskeletal-regulatory proteins has not yet been studied. *MYH9*-related disease is one of the most frequent inherited platelet disorders caused by heterozygous mutations in the *MYH9* gene, which encodes the heavy chain of non-muscle myosin IIA, an actin-binding protein. These mutations result in macrothrombocytopenia, mild to moderate bleeding, and increased risk of renal failure, hearing loss, and pre-senile cataracts, depending on the mutation site.[6-8] Studies on megakaryocyte-specific myosin IIA-deficient mice revealed macrothrombocytopenia, prolonged bleeding time, and impaired clot retraction.[9] Mouse models with knock-in mutations Arg702Cys (R702C), Asp1424Asn (D1424N), and Glu1841Lys (E1841K)[10] exhibit macrothrombocytopenia, moderately prolonged bleeding times, decreased clot retraction, and non-hematologic defects, mirroring human patients. Impaired clot retraction and bleeding were linked to reduced platelet force generation, with fibrinolytic system interference improving the hemostatic function.[11] Based on our previous finding that interfering with GPV cleavage can enhance fibrin formation in the setting of defective hemostasis,[5] we investigated whether this approach could serve as a potential therapeutic strategy to mitigate hemostatic defects associated with *MYH9*-related disease.

To address this, we first studied fibrin formation using mouse blood in a collagen/tissue factor-induced thrombus formation assay under flow. Citrated whole blood was recalcified by co-infusion with 6.3 mM $CaCl_2$ (final concentration [f.c.]) and 3.2 mM $MgCl_2$ (f.c.) and perfused over the collagen/tissue factor spots for up to 6 minutes (min) at a shear rate of 1,000 s^{-1}. Before each experiment, blood samples were pre-labeled for 5 min at 37°C with Alexa Fluor™ (AF) 546-conjugated fibrinogen and with AF647-conjugated anti-GPIX derivative to stain platelets, and incubated with 10 μg/mL of the anti-GPV antibody, DOM/B,[5,12] to inhibit thrombin-mediated GPV cleavage. Fluorescent images were taken every 30 seconds and time to fibrin formation as well as fibrin surface area coverage were determined. Time to fibrin formation was more variable in samples from *Myh9*$^{R702C/+}$ mice compared to controls (time to fibrin formation >6 min: 2/8 for *Myh9*$^{R702C/+}$, 0/7 for control; Figure 1A, B). Anti-GPV treatment reduced the time to fibrin formation in samples of both control and *Myh9*$^{R702C/+}$ mice (Figure 1A, B). After 6 min of perfusing the blood over the coated surface, fibrin surface area coverage was significantly higher in *Myh9*$^{R702C/+}$ mouse samples pretreated with an anti-GPV antibody compared to untreated *Myh9*$^{R702C/+}$ mouse blood. The fibrin coverage reached levels comparable to the untreated control (Figure 1C). However, anti-GPV treatment did not affect procoagulant platelet formation on collagen under flow or thrombin generation under static *in vitro* conditions (*data not shown* and ref.[5]). We therefore speculate that cleavage of membrane-bound GPV occurs after platelet adhesion and the initiation of thrombin generation. Together, these results demonstrate that inhibiting thrombin-mediated GPV cleavage enhances fibrin formation in a mouse model of *MYH9*-related disease.

Next, control and *Myh9*$^{R702C/+}$ mice were intravenously (i.v.) injected with 100 μg of the anti-GPV antibody, DOM/B,[12] 5 min prior to a 1 mm amputation of the mouse tail tip to determine the hemostatic function. Wild-type littermates served as controls for heterozygous mice from each mutant strain to account for differences in genetic backgrounds. These experiments were approved by the local authorities, the District Government of Lower Franconia, under the approval numbers 2-1562 and 2-944. Bleeding times of *Myh9R*$^{702C/+}$ mice were prolonged compared to control mice, confirming previous data (Figure 2A).[11] Anti-GPV treatment significantly reduced the bleeding time of *Myh9*$^{R702C/+}$ mice, which was in the range of bleeding times of control mice treated with the anti-GPV antibody (Figure 2A). As bleeding symptoms can also be severe in other downstream mutations of *Myh9*, we also subjected mice with the D1424N mutation and E1841K mutation to the bleeding time model. We observed that anti-GPV treatment was highly effective in reducing the bleeding time in all *Myh9* mutant mouse models (Figure 2B, C). These results demonstrate that inhibiting GPV cleavage, which locally enhances thrombin activity and fibrin formation,[5] reverts the bleeding defect in mouse models of *MYH9*-related disease.

There are several current options for treating bleeding complications in *MYH9*-related disease patients.[13] Thrombopoietin

receptor agonists can be used short-term to increase platelet counts.[14] However, in *Myh9* mutant mouse models, clot retraction was markedly decreased and thrombi were more unstable, even after adjusting for platelet count, suggesting that reduced platelet-generated forces, rather than platelet number, play a central role in the increased bleeding tendency.[11] Desmopressin, combined with the antifibrinolytic agent tranexamic acid, is also administered short term to patients

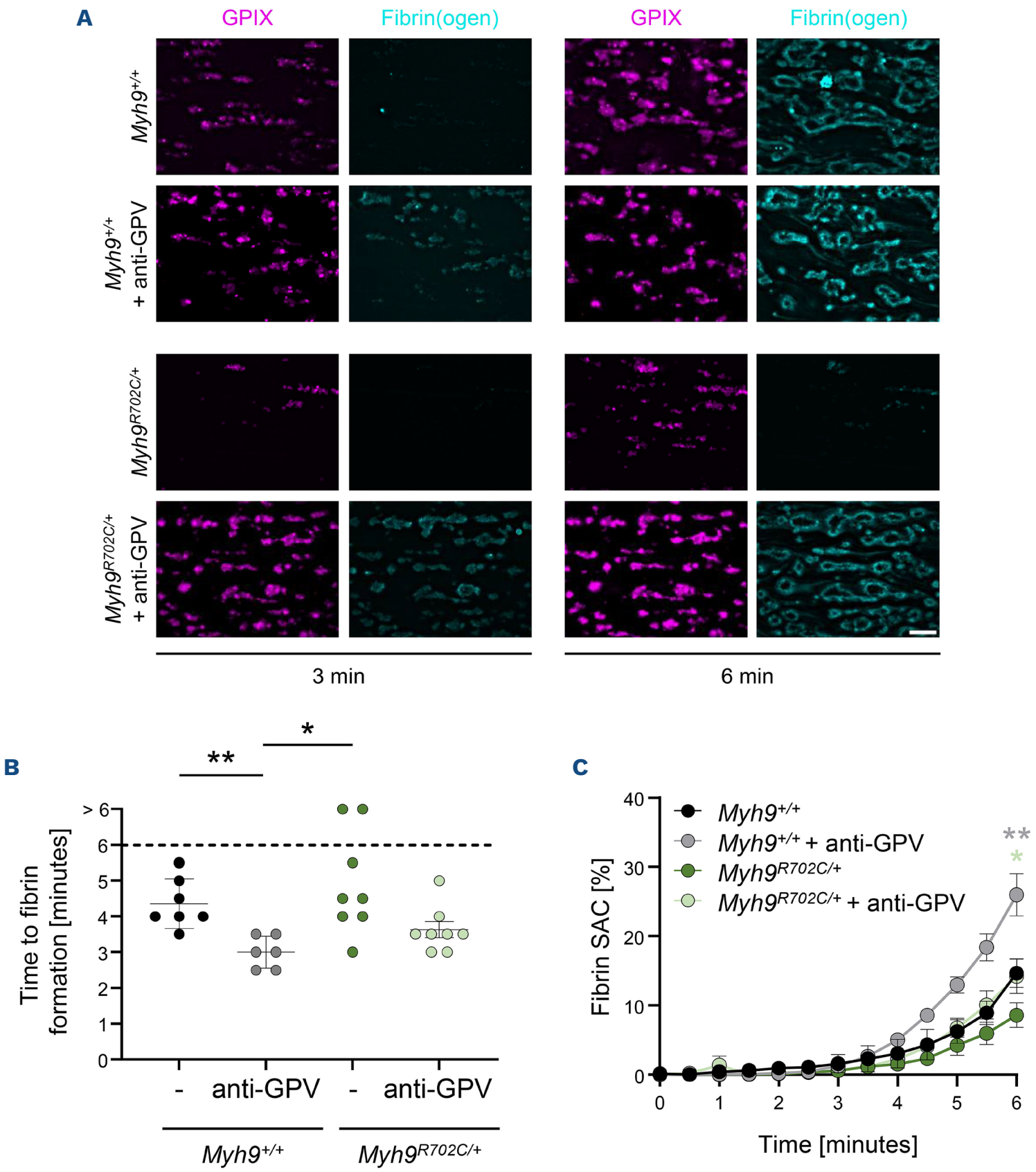

Figure 1. Anti-glycoprotein V antibody treatment increases fibrin generation in whole blood of *Myh9*$^{R702C/+}$ mice under flow *ex vivo*. Glass coverslips were coated with collagen type I (10 μL, 50 μg mL^{-1}) and tissue factor (10 μl, 10 pM) and blocked with 1% bovine serum albumine - phosphate-buffered saline. Citrated whole blood was recalcified by co-infusion with 6.3 mM $CaCl_2$ (final concentration [f.c.]) and 3.2 mM $MgCl_2$ (f.c.) and perfused over the collagen-tissue factor spots for up to 6 minutes (min) at a shear rate of 1,000 s^{-1}. Before each experiment, blood samples were prelabeled with AF546-conjugated fibrinogen and an anti-GPIX derivative to stain platelets. (A) Representative images of thrombus formation (anti-GPIX–AF647 antibody, magenta) and fibrin formation (fibrinogen-AF546, cyan) on collagen-tissue factor spots after 3 min and 6 min. Scale bar, 30 μm. (B) Quantification of time to fibrin formation. Each dot represents one animal. Mean ± standard deviation, Kruskal–Wallis test with Dunn's multiple comparison. *P<0.05; **P<0.01. (C) Quantification of fibrin formation over time; SAC: surface area coverage. Mean ± standard error of the mean, Mann-Whitney test of *Myh9*$^{+/+}$ *versus Myh9*$^{+/+}$ + anti-glycoprotein V (anti-GPV) (grey asterisk), and *Myh9*$^{702/+}$ *versus Myh9*$^{702/+}$ + anti-GPV (green asterisk). *P<0.05; **P<0.01.

to prevent bleeding before surgery. Tranexamic acid alone is also used to manage heavy menstrual bleeding or applied locally after dental surgery.[7] We recently demonstrated that treatment with tranexamic acid restored clot retraction and reduced bleeding in all three mouse models of *MYH9*-related disease (R702C, D1424N, E1841K).[11] In general, tranexamic acid has been shown to reduce bleeding complications, but its efficacy varies by clinical context, timing, and dose. While tranexamic acid does not increase the overall risk of thrombotic events it can induce nausea and may have rare side effects in certain scenarios.[15] Here, we provide an alternative, platelet-specific, antibody-based treatment strategy of *MYH9*-related disease. We have previously shown that platelet GPV, released by thrombin during platelet activation at sites of vascular injury, plays a crucial role in regulating fibrin formation and limiting thrombus growth.[5] Soluble GPV remains complexed with thrombin, modulates its activity and thereby limits fibrin formation. Consistent with the present study, we previously demonstrated that blocking GPV cleavage improves and restores hemostasis in mice

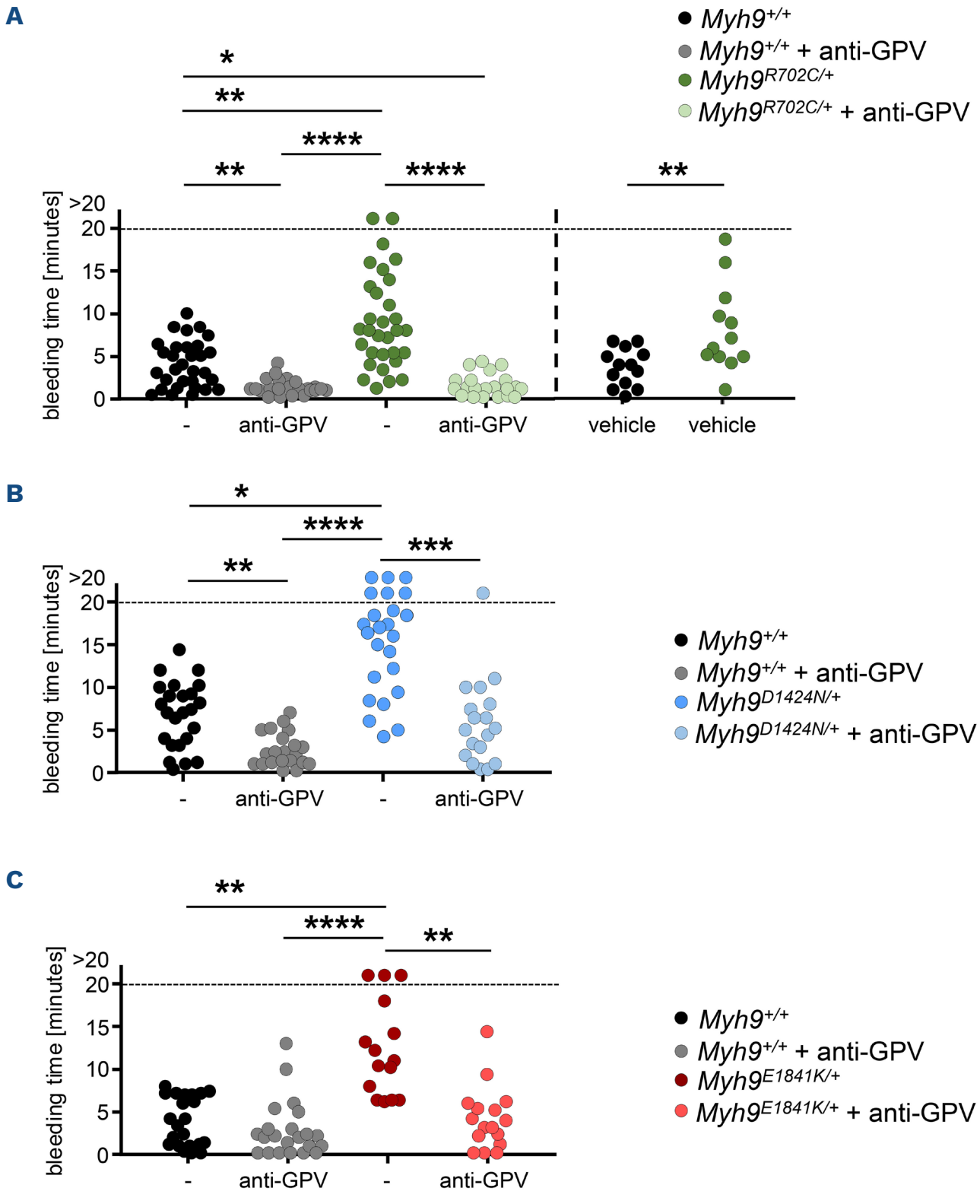

Figure 2. Anti-glycoprotein V antibody treatment improves hemostatic function in *Myh9* mutant mice. One mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gently absorbing blood with filter paper at 20-second intervals without making contact with the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. Otherwise, experiments were stopped after 20 minutes. Antibody DOM/B (100 μg per mouse), sodium chloride (-), or vehicle was injected intravenously 5 minutes before cutting the tail for bleeding time experiment. (A) *Myh9*$^{R702C/+}$, (B) *Myh9*$^{D1424N/+}$, and (C) *Myh9*$^{E1841K/+}$ mice, each compared to their corresponding *Myh9*$^{+/+}$ wild-type controls. Each symbol represents 1 individual. Statistics: Kruskal-Wallis test with Dunn's multiple comparison; vehicle: Mann-Whitney test; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. GPV: glycoprotein V.

with a signalling defect caused by platelet RhoA deficiency and in mice with Gray platelet syndrome caused by *NBEAL2* knock-out, respectively.[5] In this study, anti-GPV treatment effectively increased fibrin formation *ex vivo* and significantly reduced the *in vivo* bleeding time in point-mutated *Myh9* mouse models. Our results extend our earlier findings on the pro-hemostatic benefits of blockade of thrombin-mediated GPV cleavage by demonstrating that targeting GPV is also beneficial in mouse models of inherited platelet disorders. Specifically, we show that targeting GPV is beneficial using point-mutated *Myh9* mouse models that replicate clinical manifestations observed in patients with *MYH9*-related disease. We speculate that this approach might be also an option in improving hemostasis in acquired, drug induced, impaired platelet function to prevent bleeding during and after major surgery.

Authors

Patricia Öftering,[1*] Juliane Baumann,[1*] Sarah Beck,[1,2] Andreas Greinacher,[3] Bernhard Nieswandt[1,2#] and Markus Bender[1#]

[1]Institute of Experimental Biomedicine - Chair I, University Hospital Würzburg; [2]Rudolf Virchow Center for Integrative and Translational Bioimaging, Julius-Maximilians University of Würzburg, Würzburg and [3]Institute for Immunology and Transfusion Medicine, University Medicine Greifswald, Greifswald, Germany

[*]PÖ and JB contributed equally as first authors.

[#]BN and MB contributed equally as senior authors.

Correspondence:
M. BENDER - Bender_M1@ukw.de
B. NIESWANDT - bernhard.nieswandt@virchow.uni-wuerzburg.de

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SB and BN hold a patent on recombinant GPV for treating thrombotic diseases reporting sGPV (lacking a functional transmembrane domain) for use in the treatment or prevention of thrombotic diseases. WO 2017/109212; US patent no. US 11,028,144 B2. All other authors have no conflicts of interest to disclose.

Contributions

Experiments were performed by PÖ, JB and MB. Data analysis by all authors. Writing of the original draft by MB, BN and AG. Research supervision by MB and BN. Funding acquisition by MB and BN. All authors have critically revised and approved the final version of the manuscript.

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Data-sharing statement

Requests for materials should be addressed to either of the corresponding authors.

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