

## Theme Issue Article

# Glycoprotein Ib $\alpha$ and von Willebrand factor in primary platelet adhesion and thrombus formation: Lessons from mutant mice

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### Summary

The von Willebrand factor (VWF) receptor complex, glycoprotein (GP)Ib-V-IX, and its main ligand VWF play a key role in the adhesion process of platelets to sites of vascular injury. Recent studies in mutant mice have shed new light on the importance of

either molecule for the development of arterial and venous thrombosis. In this review, we summarize the most important aspects from these studies.

### Keywords

Thrombosis, glycoprotein, von Willebrand factor, mutant, mice

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### Introduction

Von Willebrand factor (VWF) is a glycoprotein that circulates in plasma as large multimers ranging from 500–20,000 kDa. The VWF monomer consists of 2,050 residues and contains a number of domains important for the function of the molecule, as well as cysteine residues for cross-linking into dimers and multimers via disulfide bonds (Fig. 1) (1, 2). Endothelial cells (ECs) and megakaryocytes are the only cells that synthesize VWF. In ECs, VWF is either constitutively secreted or targeted to storage granules, the Weibel-Palade bodies, for agonist-induced secretion (1). In contrast, only agonist-induced secretion from  $\alpha$ -granules is observed in platelets. Consequently, plasma VWF is essentially completely derived from ECs (3). VWF multimers released from storage granules are extremely large (4) and designated as ULVWF (ultra large von Willebrand factor). ULVWF is biologically hyperactive as it forms stronger bonds with its platelet receptor, GPIb $\alpha$  (5), and it binds better to extracellular matrix (ECM) (6) than do small plasma multimers. Upon secretion, ULVWF multimers are cleaved to less active smaller multimers by the protease ADAMTS13 (A Disintegrin-like And Metalloprotease with Thrombospondin type I repeats-13) under conditions of fluid shear stress (see below) (7, 8). Deficiency of ADAMTS13 – either familial (mutation in ADAMTS13 gene) or

acquired (inhibitory antibodies generated against ADAMTS13) – has been linked to the pathogenesis of thrombotic thrombocytopenic purpura (TTP), a disorder of thrombotic microangiopathy. Studies in mice lacking ADAMTS-13 (9, 10) confirmed previous findings in patients (11, 12), which suggested genetic and environmental factors in addition to ADAMTS13 deficiency that are required to trigger TTP. In circulation, VWF non-covalently binds coagulation factor VIII (FVIII), thus protecting it from proteolysis. Consequently, humans and mice lacking VWF are characterized by a marked decrease in plasma FVIII levels (13, 14). VWF facilitates primary adhesion (tethering) of platelets to the exposed ECM and it efficiently localizes FVIII to sites of vascular injury (see below).

VWF has two main receptors on the platelet surface: GPIb $\alpha$  in the GPIb-IX-V complex, and integrin  $\alpha$ IIB $\beta$ 3. In contrast to  $\alpha$ IIB $\beta$ 3, which is a prototypic integrin that requires inside-out activation for its ligands to bind, GPIb $\alpha$  is a constitutively active, single transmembrane receptor expressed in high copy numbers on the platelet surface. The importance of the GPIb-V-IX receptor and VWF for haemostasis is shown by the strong bleeding diathesis found in Bernard-Soulier syndrome (BSS) patients, who lack the GPIb-V-IX complex on the platelet surface, or in patients lacking VWF (15, 16). A similar bleeding phenotype was reported for mice deficient in GPIb $\alpha$  (17) or VWF (14). The

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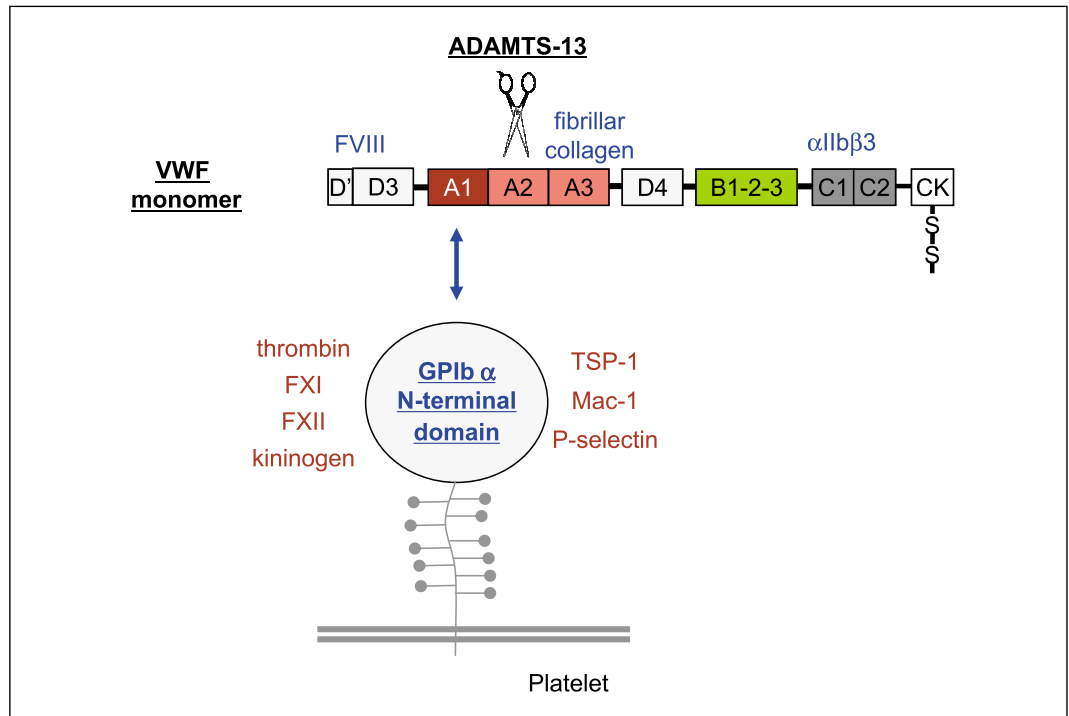
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**Figure 1: Schematic showing the domain structure and major binding partners (in blue) of a VWF monomer, the cleavage site for ADAMTS13 within the VWF monomer (A2 domain), and the ligands binding to the N-terminal domain of GPIb $\alpha$  (red). The GPIb $\alpha$ , GPIX, and GPV subunits of the GPIb-V-IX receptor are not shown. A-D) Individual domains within the VWF monomer; CK, "cysteine knot" domain at the C-terminal end of the monomer; -S-S-, disulfide bonds holding monomers together; TSP-1, thrombospondin-1; FVIII/XI/XII – coagulation factors**



GPIb-V-IX receptor complex consists of four gene products, GPIb $\alpha$ , GPIb $\beta$ , GPIX, and GPV (18–21). The N-terminal region of GPIb $\alpha$  is the major ligand-binding domain, with binding sites for VWF, thrombin (22, 23), high-molecular-weight kininogen (24), coagulation factors XI and XII (25, 26), TSP-1 (27), Mac-1 (28, 29), and P-selectin (30). The main binding partners for VWF and GPIb $\alpha$  are summarized in Figure 1. Engagement of GPIb-V-IX leads to various cellular changes such as calcium mobilization, the rearrangement of the cytoskeleton, granule release, and activation of the  $\alpha$ IIb $\beta$ 3 integrin (31, 32).

## Comparing the role of VWF and GPIb $\alpha$ in arterial and venous thrombosis

### Arterial thrombosis

The key role of GPIb $\alpha$  and VWF for thrombus formation under elevated shear conditions has long been demonstrated in flow chamber studies *in vitro* (33, 34). Based on these studies, the classical model of platelet adhesion to VWF or a thrombogenic surface was proposed in the late 1990s (35, 36). At shear rates of  $>1,000\text{s}^{-1}$ , human platelets completely depend on surface-expressed GPIb $\alpha$  for their transient adhesion to VWF in the ECM. The GPIb-VWF interaction, however, does not allow firm adhesion and aggregate formation, processes that require agonist-induced activation of  $\beta$ 1 and  $\beta$ 3 integrins. An important revision of this model was provided by Kulkarni et al. (37), who demonstrated that the GPIb $\alpha$ -VWF interaction is equally important for initiating platelet adhesion to the ECM and to already adherent platelets, i.e. that GPIb $\alpha$  on the platelet surface also mediates the reversible adhesion to VWF expressed on adherent platelets. In very recent work, a novel mechanism was identified that allows formation of platelet aggregates independent of platelet acti-

vation and  $\alpha$ IIb $\beta$ 3 integrin, but which seems to be entirely dependent on the GPIb-VWF interaction (38, 39). It is important to note that this latter mechanism operates at shear rates  $>10,000\text{s}^{-1}$ , conditions that are only found in partially stenosed arterioles and arteries (40, 41).

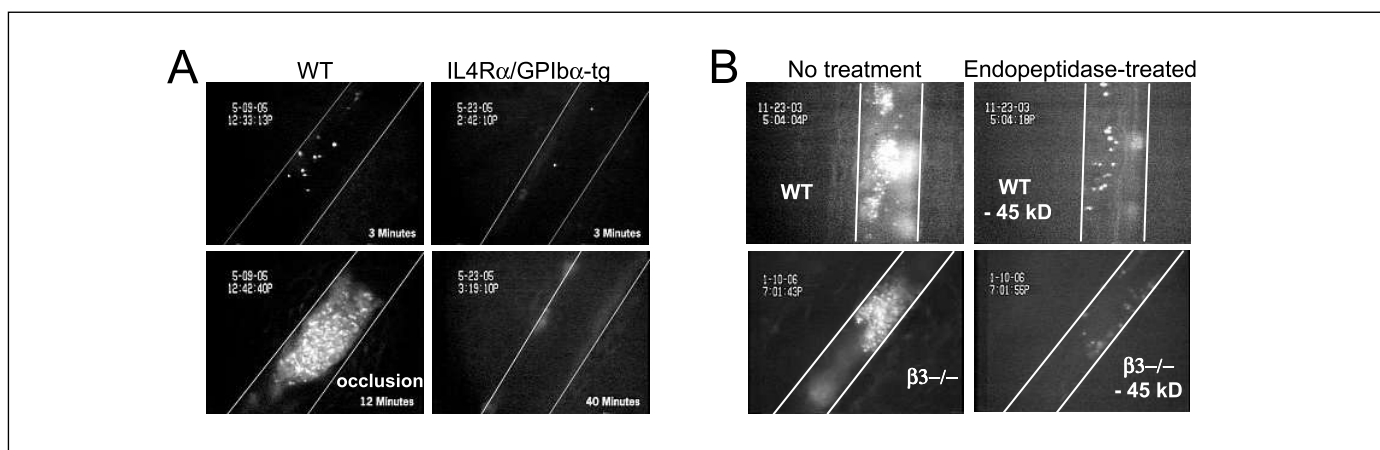
A variety of compounds blocking the interaction of VWF and GPIb $\alpha$  have been developed and tested for their effects on thrombus formation, both *in vitro* and *in vivo* (reviewed in [42–44]). They include monoclonal antibodies, VWF fragments containing the A1 domain, and snake venom proteins. The major findings from these studies are: i) inhibitors to both VWF or GPIb $\alpha$  have strong antithrombotic effects, especially in models of arterial thrombosis, ii) various inhibitors of the GPIb-VWF interaction showed only moderate effects on the bleeding time, suggesting that such agents have a broader therapeutic window than drugs with similar antithrombotic effects such as  $\alpha$ IIb $\beta$ 3 inhibitors, and iii) targeting GPIb $\alpha$  with divalent agents such as intact IgG or F(ab)<sub>2</sub> fragments leads to severe thrombocytopenia (shown in baboon, rats, and mice).

In contrast to these inhibitors, which may affect more than just the GPIb $\alpha$ -VWF interaction, studies in mice lacking VWF or (the extracellular domain of) GPIb $\alpha$  provide an excellent tool to address the importance of all binding partners of either molecule for the development of arterial thrombosis. VWF-deficient mice were first studied in a model of ferric chloride (FeCl<sub>3</sub>)-induced thrombosis in mesenteric arterioles (14, 45). As expected, strongly impaired thrombus formation was observed in VWF<sup>-/-</sup> mice within 10 minutes after vascular damage. However, platelet adhesion and thrombus formation in VWF<sup>-/-</sup> mice were only delayed but not absent, and 50% of the mice established an occlusive thrombus at the site of the vascular damage. Thrombus growth in the VWF-deficient mice often arrested, leaving an open channel through which blood could pass. The existence of

these channels suggests that VWF plays a particularly important role in platelet-platelet cohesion at very high shear rates as found in almost stenosed arterioles (45). The presence of open channels was also reported for thrombi forming in pigs with von Willebrand's disease (46), and a decrease in thrombus size was shown for VWF $^{-/-}$  mice studied in a model of laser-induced thrombosis in arterioles of the cremaster muscle (47). By applying the FeCl $_3$ -induced thrombosis model to mice lacking fibrinogen (45) or plasma fibronectin (48, 49), other key ligands for platelet adhesion, it was established that each molecule has its own specific impact on platelet plug formation in arterioles: VWF is important for platelet tethering to the ECM and to existing thrombi (especially in partially stenosed vessels), fibrinogen/fibrin is crucial for the stable adhesion of thrombi to the site of vascular damage, and fibronectin is a key ligand mediating firm platelet-platelet interactions in the growing thrombus (50).

The surprising observation that arterial thrombus formation was delayed but not absent in VWF $^{-/-}$  mice led to the conclusion that a ligand(s) other than VWF can mediate platelet adhesion under conditions of arterial shear flow *in vivo*. In order to determine if this VWF-independent adhesion is mediated by GPI $\alpha$ , we studied arterial thrombosis in transgenic mice expressing GPI $\alpha$  in which the extracellular domain was replaced by that of the human IL4 receptor (IL4R $\alpha$ /GPI $\alpha$ -tg mice) (51). Compared to mice lacking GPI $\alpha$  (17), which show hallmark characteristics of Bernard-Soulier Syndrome such as thrombocytopenia and giant platelets, IL4R $\alpha$ /GPI $\alpha$  mice are characterized by normal platelet size and a platelet count of  $\sim$ 70% of wild type. Like GPI $\alpha$  deficiency, lack of the extracellular domain of GPI $\alpha$  results in a severe bleeding phenotype (51). While the number of tethering platelets was reduced by  $\sim$ 80% in damaged arterioles of VWF $^{-/-}$  mice, virtually no tethering platelets were observed in IL4R $\alpha$ /GPI $\alpha$  mice (52). Consequently, thrombus formation was completely inhibited in the absence of GPI $\alpha$

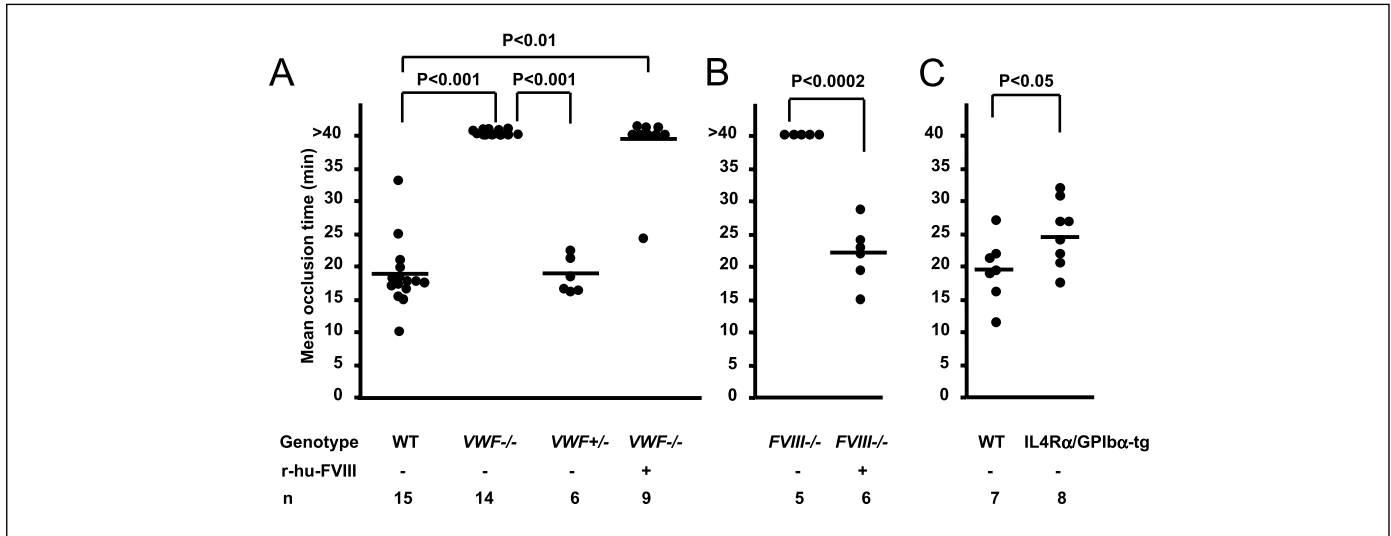
(Fig. 2A). These results demonstrate that GPI $\alpha$  contributes to arterial thrombosis by important adhesion mechanisms independent of the binding of VWF. Just like the  $\alpha$ IIB $\beta$ 3 integrin, GPI $\alpha$  utilizes more than one ligand expressed in the ECM and the growing thrombus to allow for reversible platelet adhesion (Fig. 1). Thrombospondin-1 is one likely candidate mediating GPI $\alpha$ -mediated platelet adhesion in the absence of VWF. It is expressed in the ECM and platelets, and it was shown to mediate GPI $\alpha$ -dependent platelet adhesion under flow conditions *in vitro* (27). Furthermore, *in-vivo* studies demonstrated impaired arterial thrombosis in mice deficient in thrombospondin-1. This defect, however, was attributed to an inhibitory effect of TSP-1 on ADAMTS13 and thus protection of VWF (53). Further studies in mutant mice lacking VWF in combination with other known ligands of GPI $\alpha$  such as TSP-1, P-selectin, or kininogen (see Fig. 1) will be required to uncover which ligand(s) in addition to VWF can successfully mediate GPI $\alpha$ -dependent platelet adhesion under arterial flow conditions. A key role for GPI $\alpha$  in the development of arterial thrombosis was also demonstrated in a model of FeCl $_3$ -induced thrombosis of the carotid artery (54), where impaired vessel occlusion, measured by a Doppler probe, was observed in IL4R $\alpha$ /GPI $\alpha$ -tg mice. Furthermore, a role for signaling through GPI $\alpha$  in the development of occlusive thrombi was suggested by applying the same thrombosis model to mice lacking six amino acids from the C-terminus of GPI $\alpha$  that are important for the binding of the signaling molecule 14-3-3 $\zeta$  (55). In contrast to rapid and stable reduction of blood flow in FeCl $_3$ -treated arteries of wild-type (WT) mice, fluctuating blood flow, indicative of embolization, was observed in the mutant mice. However, the defect in thrombus formation observed in these mutant mice was much less severe than that shown for IL4R $\alpha$ /GPI $\alpha$ -tg mice (54), indicating that GPI $\alpha$  signaling through 14-3-3 $\zeta$  plays a significant but minor role for GPI $\alpha$  function in thrombosis and haemostasis. In an interesting study



**Figure 2: Role of GPI $\alpha$  in arterial thrombus formation.**

A) Thrombus formation in mesenteric arterioles after ferric chloride (FeCl $_3$ ) injury. In wild-type mice (WT) many tethering platelets were observed 3 minutes after application of FeCl $_3$  and stable vessel occlusion is reached within 12 minutes. In contrast, platelet tethering was virtually absent in injured vessels of IL4R $\alpha$ /GPI $\alpha$ -tg mice (lacking the extracellular domain of GPI $\alpha$ ) and no thrombus formation was observed within the observation period. B) Platelet incorporation into growing WT

thrombi. Recipient WT mice were co-injected with untreated platelets labeled with calcein-green and O-sialoglycoprotein endopeptidase-treated platelets of the same genotype (lacking the 45 kD N-terminal domain of GPI $\alpha$ ) labeled with calcein-red. While deficiency in  $\beta$ 3 integrin (lower panel) did not impair incorporation of injected platelets into the growing thrombus, strongly impaired incorporation was seen with endopeptidase-treated platelets (WT or  $\beta$ 3-deficient). Reprinted with permission from Bergmeier et al. (52).



**Figure 3: Time to occlusion in FeCl<sub>3</sub>-treated mesenteric venules of mice lacking VWF, FVIII, or the extracellular domain of GPIb $\alpha$ .** A) Mean occlusion time (MOT) in WT and VWF mutant mice. While no difference in the MOT between WT and VWF<sup>+/-</sup> mice was observed, none of the VWF<sup>-/-</sup> mice occluded within the 40 minutes observation period. Infusion of recombinant FVIII into VWF<sup>-/-</sup> mice did

not normalize MOT. B) Infusion of recombinant FVIII into FVIII<sup>-/-</sup> mice normalized the markedly prolonged MOT observed in these mice. C) MOT in venules of IL4R $\alpha$ /GPIb $\alpha$ -tg mice was slightly but significantly longer than in WT (25 vs. 19.5 minutes, respectively). Reprinted with permission from Chauhan et al. (68).

by Strassel et al. (56), thrombosis was studied in mouse models of Bernard-Soulier syndrome induced by genetic deletion or intracellular truncation of the GPIb $\beta$  subunit of the receptor. While complete knockout of GPIb $\beta$  reduced the platelet surface expression of GPIb $\alpha$  to 3% of WT, the levels of both GPIb $\beta$  and GPIb $\alpha$  were reduced to 20% of WT in mice expressing the truncated form of GPIb $\beta$ . Interestingly, laser-induced thrombosis in arterioles was almost completely inhibited in GPIb $\beta$ <sup>-/-</sup> mice, but not mice expressing truncated GPIb $\beta$ . These data suggest that 20% remnant GPIb $\alpha$  expression is still sufficient to allow platelet adhesion to the ECM and the growing thrombus.

Our studies (52) further demonstrated that platelets lacking just the 45 kD N-terminal domain of GPIb $\alpha$  failed to incorporate into growing arterial thrombi in WT mice, even if these platelets were activated before infusion. In contrast, platelets lacking  $\beta$ 3 integrins were still incorporated into thrombi under these experimental conditions (Fig. 2B). Thus, platelet recruitment to the surface of a growing thrombus depends on a primary adhesion step mediated by GPIb $\alpha$ , while it does not necessarily require the activation of the major platelet integrin,  $\alpha$ IIb $\beta$ 3. A similar finding was reported by Dubois et al., who, using calcium flux as a measure for platelet activation, found a large population of unactivated platelets within thrombi forming in arterioles of the cremaster muscle (47). It still has to be established whether this adhesion is transient, and if not, whether it solely depends on the interaction of GPIb $\alpha$  with one of its ligands. As outlined above, such a mechanism of GPIb $\alpha$ -dependent but activation-independent aggregate formation has been described for shear rates  $>10,000\text{s}^{-1}$  *in vitro*, conditions that could be found on the surface of a growing thrombus. The increased adhesiveness of platelet VWF compared to plasma VWF may also contribute to this integrin-independent adhesion process (57).

### Venous thrombosis

While the importance of the GPIb-VWF interaction for arterial thrombosis is well accepted, its role in thrombus development under venous shear conditions has been unclear. In *in-vitro/ex-vivo* flow chamber studies, several investigators found evidence for (37, 58, 59) and against (60–62) a key role of VWF or GPIb $\alpha$  in thrombus formation at shear rates  $<500\text{s}^{-1}$ . These studies, however, were limited in that they used artificial adhesive surfaces and mostly anticoagulated blood. In animal models of venous thrombosis, a significant prolongation of the occlusion time was demonstrated for inhibitors to GPIb $\alpha$  (63–65) or VWF (66, 67). These studies, however, did not provide information on the effects of such treatment on initial platelet adhesion or thrombus stability.

To directly compare the role of VWF and GPIb $\alpha$  in venous thrombosis, we studied mutant mice in a model of FeCl<sub>3</sub>-induced thrombosis in mesenteric venules with shear rates  $<150\text{s}^{-1}$  (Fig. 3) (68). Compared to WT mice, VWF<sup>-/-</sup> mice were characterized by i) significantly reduced adhesion of single platelets at the site of vascular damage, ii) formation of small, but not occlusive thrombi (Fig. 3A), and iii) frequent embolization of the growing thrombi. Plasma clotting time and thrombus embolization but not growth was corrected by infusion of recombinant FVIII into VWF<sup>-/-</sup> mice, suggesting that impaired coagulation due to the low FVIII levels in VWF<sup>-/-</sup> mice ( $\sim 20\%$  of WT) accounts for the instability of the thrombi. In contrast, FVIII infusion restored vessel occlusion in injured arterioles of FVIII<sup>-/-</sup> mice (Fig. 3B). Compared to VWF<sup>-/-</sup> mice, IL4R $\alpha$ /GPIb $\alpha$ -tg mice showed a similar defect in transient platelet adhesion to the site of vascular injury, but they formed stable thrombi and their occlusion time was only slightly prolonged (Fig. 3C). Thus, VWF likely uses other adhesion receptors, such as  $\alpha$ IIb $\beta$ 3, besides GPIb $\alpha$  in thrombus growth under venous shear conditions. It is important to note that studying thrombosis in FeCl<sub>3</sub>-injured mesenteric ve-

nules is not a model for venous thrombosis due to vascular stasis and endothelial activation, conditions responsible for a large fraction of thrombotic events in patients. FeCl<sub>3</sub> exposes sub-endothelial matrix, which allows for the formation of platelet-rich thrombi. In contrast, stasis-induced thrombi contain platelets, leukocytes, red blood cells, and large quantities of fibrin. Thus, further studies in animal models of stasis-induced venous thrombosis (69) may be required to more accurately evaluate the role of VWF and GPIb $\alpha$  in this process.

## Role of ADAMTS13 and VWF processing in arterial and venous thrombosis

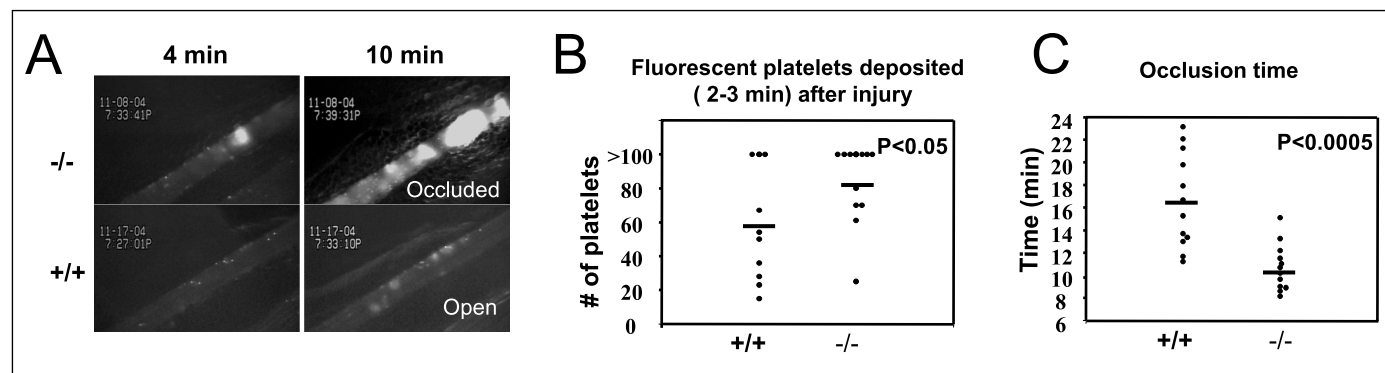
ADAMTS13 is a metalloproteinase that plays a key role in the processing of ULVWF by cleaving the peptide bond between Y1605 and M1606 of the A2 domain (Fig. 1) (70). Recently, mice lacking ADAMTS13 have been developed by two independent groups (9, 10). Both groups found that ADAMTS13-deficient mice are born in expected Mendelian distribution, are fertile and do not show any evidence of thrombocytopenia, haemolytic anemia or microvascular thrombosis. However, when crossed on a different background with high levels of plasma VWF and challenged with shigatoxin (a substance made by strains of *E. coli* and *Shigella* that is toxic to endothelial cells), ADAMTS13<sup>-/-</sup> mice showed a TTP like phenotype (9), suggesting that genetic and environmental factors in addition to ADAMTS13 deficiency are needed to trigger TTP.

Despite the lack of spontaneous TTP in ADAMTS13-deficient mice, these animals have a significant phenotype compared to WT mice. Using intravital microscopy, we have demonstrated that, after endothelial activation in veins, VWF-platelet strings form and remain intact for several minutes only if ADAMTS13 is absent (9). These results confirm previous *in-vitro* studies, which suggested that platelets align as beads on the released ULVWF string on the endothelial surface and then are cleaved by ADAMTS13 under flow conditions (71). Furthermore, we and others have found that complete deficiency of ADAMTS13 in mice is prothrombotic (10, 72). ADAMTS13-deficient mice

show severe thrombocytopenia when challenged with collagen and epinephrine and accelerated thrombus formation on collagen under flow (10). When we stimulated microvenules with calcium ionophore A23187, which induces secretion of Weibel-Palade bodies, platelet aggregation resulting in spontaneous thrombus formation was observed in ADAMTS13-deficient mice. The same phenotype was reproduced in WT mice by infusion of a polyclonal antibody that inhibits ADAMTS13 activity (72). These observations demonstrate that ADAMTS13 is also active under venous shear conditions, and that it inhibits thrombus formation in microvenules by cleaving ULVWF multimers released from Weibel-Palade bodies. In the FeCl<sub>3</sub> injury model in arterioles, we have shown that ADAMTS13 downregulates both platelet adhesion to the subendothelium and thrombus formation (Fig. 4). Furthermore, we have demonstrated that infusion of r-human ADAMTS13 into ADAMTS13-deficient or WT mice inhibits thrombus growth (72). In line with our observations, another study showed the role of ADAMTS13 in controlling the formation of activation-independent platelet aggregates under high shear stress *in vitro* (73). Our findings reveal that r-ADAMTS13 could potentially be used as a therapeutic agent to treat patients suffering from thrombotic disorders or perhaps even stroke.

## Conclusions

The studies reviewed here suggest that GPIb $\alpha$  plays a more important role than VWF in arterial thrombosis, while VWF is more important than GPIb $\alpha$  in venous thrombosis. Studies in mutant mice show that VWF is not the only ligand that can mediate GPIb $\alpha$ -dependent platelet adhesion and thrombus formation under arterial flow conditions *in vivo*. Further studies are required to determine which other ligand(s) of GPIb $\alpha$  are important for the receptors role in arterial thrombosis. The anti-thrombotic potential of an inhibitor to GPIb $\alpha$  will then be determined by its ability to block the binding of one or more of these ligands. The strongest antithrombotic effect is expected with soluble GPIb $\alpha$  constructs such as the recently published GPG-290 (74). However, complete inhibition of GPIb $\alpha$  function may re-



**Figure 4: Role of ADAMTS13 in arterial thrombus formation.** Vascular injury in mesenteric arterioles was induced by application of FeCl<sub>3</sub>. A) Representative images. Increased platelet adhesion and a decreased time to vessel occlusion are observed in ADAMTS13<sup>-/-</sup> mice. B) The number of deposited platelets per minute was determined in the

interval 2–3 minutes after injury. Significantly more adhering platelets were seen in ADAMTS13<sup>-/-</sup> mice. C) The occlusion time in ADAMTS13<sup>-/-</sup> arterioles was significantly shorter than in WT mice (10.6 vs. 16.7 minutes, respectively). Reprinted with permission from Chauhan et al. (72).

sult in a smaller therapeutic window, making it more difficult to control bleeding complications. VWF is an attractive target for antithrombotic therapy, as inhibitors could be designed to preferentially inhibit arterial thrombosis or to inhibit both arterial and venous thrombosis. Specific intervention with the GPIb-VWF interaction has the potential to prevent arterial occlusion, while maintaining the formation of stable mural thrombi. Only minor effects on the formation of venous thrombi are expected. In contrast, inhibition of VWF binding to both GPIb $\alpha$  and  $\alpha$ Ib $\beta$ 3 integrin will also reduce thrombus growth under venous shear conditions. Inhibiting occlusive thrombus formation by targeting the adhesive function of VWF should be safer than complete blockage of platelet aggregation and thrombus formation by  $\alpha$ Ib $\beta$ 3 inhibitors. However, inhibitors should not compromise the key role of VWF as a carrier and stabilizer of plasma FVIII, as this would likely increase the rate of embolization. Degradation of ULVWF multimers by infusion of recombinant ADAMTS13 represents another strategy to inhibit the formation of occlusive thrombi. Like inhibitors to VWF, recombinant

ADAMTS13 effectively limits thrombus growth in arteries without affecting thrombus stability. However, its mode of action may make it superior to VWF inhibition, as it does not cleave all VWF circulating in plasma but specifically targets the elongated active form of the molecule, thus keeping the basal levels and haemostatic activity of VWF intact.

It is important to note that the conclusions made in this review are based on thrombosis models in mice that may not perfectly simulate the human disease. Further studies in other animal models as well as carefully done clinical trials will be needed to validate the proposed mechanisms underlying arterial and venous thrombosis.

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