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Haematologica 2020 [Epub ahead of print]

Citation: Soumaya Jadoui, Ophélie Le Chapelain, Véronique Ollivier, Ali Mostefa-Kara, Lucas Di Meglio, Sébastien Dupont, Angèle Gros, Mialitiana Solo Nomenjanahary, Jean-Philippe Desilles, Mikaël Mazighi, Bernhard Nieswandt, Stéphane Loyau, Martine Jandrot-Perrus, Pierre H. Mangin, and Benoit Ho-Tin-Noé. Glenzocimab does not impact glycoprotein VI-dependent inflammatory hemostasis.
Haematologica. 2020; 105:xxx
doi:10.3324/haematol.2020.270439

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Glenzocimab does not impact glycoprotein VI-dependent inflammatory haemostasis

**Running head:** Glenzocimab and inflammatory haemostasis

Soumaya Jadoui\textsuperscript{1}\*, Ophélie Le Chapelain\textsuperscript{1}\*, Véronique Ollivier\textsuperscript{1}, Ali Mostefa-Kara\textsuperscript{1}, Lucas Di Meglio\textsuperscript{1}, Sébastien Dupont\textsuperscript{1}, Angèle Gros\textsuperscript{1}, Mialitiana Solo Nomenjanahary\textsuperscript{1}, Jean-Philippe Desilles\textsuperscript{1,2}, Mikaël Mazighi\textsuperscript{1,2}, Bernhard Nieswandt\textsuperscript{3}, Stéphane Loyau\textsuperscript{1}, Martine Jandrot-Perrus\textsuperscript{1}, Pierre H Mangin\textsuperscript{4}, Benoit Ho-Tin-Noé\textsuperscript{1}\#.

\textsuperscript{1}Université de Paris, LVTS, Inserm U1148, F-75018 Paris, France. 
\textsuperscript{2}Rothschild Foundation Hospital, Paris, France. Department of Interventional Neuroradiology.
\textsuperscript{3}University Hospital Würzburg, Rudolf Virchow Center for Experimental Biomedicine, Würzburg, Germany.
\textsuperscript{4}Université de Strasbourg, INSERM, EFS Grand-Est, BPPS UMR-S1255, FMTS, F-67065 Strasbourg, France.

\*equal contribution

#Correspondence:
Benoît Ho-Tin-Noé, PhD.
Laboratory of Vascular Translational Science, INSERM U1148, Hôpital Bichat, 46 rue Henri Huchard
75877 Paris Cedex 18 France.
Fax: + 33 (0) 1 40 25 86 02.
Tel: + 33 (0) 1 40 25 86 00.
benoit.ho-tin-noe@inserm.fr
Glycoprotein VI (GPVI), the main platelet receptor for collagen, has emerged as a new target for antithrombotic therapy because its genetic deficiency or pharmacological blocking inhibits platelet aggregation and experimental thrombosis without increasing bleeding time\(^1-^5\). While these data have stimulated the development of new antiplatelet drugs targeting GPVI, recent findings have indicated that GPVI is essential for repair of neutrophil-induced vascular injury in various inflamed organs and tissues\(^6-^9\). It thus appears important to assess and anticipate the yet uninvestigated risk of inflammation-induced bleeding under GPVI antagonists, especially considering that inflammation is a component of various thrombotic diseases. In that respect, it is worth noting that neutrophil mobilization is a predictor of hemorrhagic transformation of ischemic stroke\(^10\) and contributes to intraplaque hemorrhage\(^11\), which is known to precipitate plaque rupture and the clinical expression of atherosclerosis.

Among the newly developed drugs targeting GPVI, ACT017 (Glenzocimab, Acticor Biotech) is a humanized antibody fragment (Fab) that has already completed its phase I clinical trial in healthy volunteers\(^12\) and just entered a phase II trial in stroke patients (Acute Ischemic Stroke Interventional Study “ACTIMIS”, NCT03803007). ACT017 binds to human GPVI and inhibits the procoagulant activity and aggregation of collagen-stimulated platelets, as well as platelet adhesion and thrombus formation onto collagen surfaces under arterial flow conditions\(^1,13,14\). The inhibitory action of ACT017 occurs without causing thrombocytopenia or depletion of GPVI, and is not associated with spontaneous bleeding events or increased bleeding time\(^14\). Nevertheless, whereas preclinical bleeding time tests can help evaluate the risk of bleeding associated with trauma or surgery, they may not predict the risk of bleeding associated with inflammation\(^15\). Here, using the cutaneous reverse passive Arthus reaction (rpA) as a model situation where GPVI plays a major role in inflammatory haemostasis, we investigated whether ACT017 increases the risk of inflammation-induced bleeding.
We first assessed the contribution of GPVI to the prevention of inflammation-induced bleeding by platelets in the brain and lungs. In agreement with previous results obtained with an antibody causing depletion of mouse GPVI\textsuperscript{16,17}, there was no cerebral haemorrhage in any of the GPVI\textsuperscript{-/-} mice subjected to transient (90 min) middle cerebral artery occlusion (Figure 1A). In contrast, cerebral haemorrhage occurred in all mice that had been rendered severely thrombocytopenic by the mean of a platelet-depleting antibody (Figure 1A). Genetic deficiency in GPVI was not associated with an increased bleeding risk in the model of acute lung injury induced by inhalation of \textit{P. aeruginosa} endotoxin either (Figure 1B). In the cutaneous rpA, as predicted by previous reports\textsuperscript{6,7,9}, GPVI\textsuperscript{-/-} mice developed skin bleeding at the inflammatory reaction site, a bleeding phenotype that was seen neither in GPVI\textsuperscript{+/+} mice nor in GPVI\textsuperscript{+/+} mice, which expressed half of normal GPVI surface levels (Figure 1C-D). Taken together, these results are consistent with evidence that GPVI is dispensable for haemostasis in the inflamed brain and lungs\textsuperscript{6,16–18} but primarily involved in the prevention of bleeding in the rpA-inflamed skin. Notably, they further indicate that 50 % of normal GPVI surface levels are sufficient for haemostasis during the cutaneous rpA.

The ability of ACT017 to inhibit collagen/GPVI interactions and their functional consequences has been previously demonstrated in humans and in nonhuman primates\textsuperscript{14}. However, it has not been tested in hGPVI mice. We thus verified the activity of ACT017 against GPVI from hGPVI mice. Like its murine precursor Fab 9O12\textsuperscript{1}, ACT017 added to whole blood from hGPVI mice caused a drastic reduction in platelet adhesion and aggregation onto collagen fibers under arterial and venous flow conditions (Figure 2A-B, Supplemental Movie). We next tested whether hGPVI mice treated with therapeutic (16 mg/kg) or higher doses of ACT017 (32 and 64 mg/kg) were sensitized to inflammation-induced bleeding during the cutaneous rpA. No bleeding occurred in ACT017-treated hGPVI mice, whatever
the dose of ACT017 used (data not shown and Figure 2C-D). There was no bleeding either when ACT017 at the highest dose tested (64 mg/kg) was given through a continuous infusion over the 4 hours of rpA (data not shown). The absence of bleeding in hGPVI mice treated with ACT017 was in contrast to the petechial bleeding observed in GPVI−/− mice (Figure 1) and in platelet-depleted GPVI+/+ and hGPVI mice (Figures 1C-D and 2C-D), which is known to be a consequence of neutrophil recruitment. Absence of bleeding in rpA-challenged hGPVI mice treated with ACT017 was not due to altered neutrophil recruitment as this was comparable to that in hGPVI mice (Figure 2E). Interestingly, the latter result indicates that ACT017 does not impact neutrophil recruitment, at least in this model. Importantly, ACT017 did not alter platelet recruitment to the inflamed skin either (Figure 2F). The latter result underscores a major difference between the impact of genetic deficiency in GPVI and that of GPVI blocking by ACT017. In fact, bleeding in rpA-challenged GPVI−/− mice was previously shown to be associated with reduced platelet recruitment at the reaction site. Solid tumors represent another inflammatory situation in which platelets were shown to continuously prevent leukocyte-induced bleeding and recent data have suggested that GPVI could be central to this function. Like in the cutaneous rpA, acute treatment of hGPVI mice bearing skin tumors with ACT017 did not cause tumor bleeding. The absence of effect of ACT017 on tumor vessel stability was in contrast to that of acute depletion of platelets, which caused tumor bleeding (Figure 2F).

In conclusion, in addition to confirming that GPVI is not required for inflammation-associated haemostasis in the brain and lungs, our results show that pharmacological blockade of GPVI by ACT017 does not impair GPVI-dependent inflammatory haemostasis. There are several non-exclusive reasons that could explain why pharmacological inhibition of GPVI by ACT017 does not impair the vasculoprotective recruitment of platelets during the cutaneous rpA. First, it was shown previously that GPVI can cooperate with other platelet receptors like
integran alpha2beta1 to provide residual collagen-dependent platelet activation when its collagen binding site is blocked pharmacologically\textsuperscript{20}. Furthermore, while ACT017 blocks the interactions between GPVI and collagen, it remains unknown whether ACT017 has similar blocking effects towards the other ligands of GPVI. Besides collagen, fibrinogen and a number of adhesive proteins of the vessel wall have been reported as GPVI ligands (e.g. laminin, fibronectin and vitronectin) and could thus provide redundant binding mechanisms. Moreover, were collagen to be one of the ligands supporting the adhesion of platelets to inflamed skin vessels, it is interesting to note that despite a drastic reduction in platelet adhesion and aggregation onto fibrillar collagen in the presence of ACT017 (Figure 2A-B, Supplemental Movie), residual platelet adhesion was observed at both arterial and venous blood flow. Considering that ACT017 has no effect on platelet recruitment during the cutaneous rpA, as well as previous results showing that individual platelets and platelet monolayers ensure haemostasis at sites of mild inflammatory vascular injury\textsuperscript{15}, such residual interactions with collagen could be sufficient for inflammatory haemostasis. Previous studies have shown that platelets are particularly efficient in maintaining vascular integrity in inflamed organs, as platelet counts as low as 10\% can support this function\textsuperscript{15}. Consistent with this notion, GPVI\textsuperscript{+/-} mice with half of normal GPVI surface levels showed normal haemostasis during the cutaneous rpA (Figure 1C-D). All in all, our results indicate that the highly favorable safety profile of ACT017 suggested by previous results in bleeding time assays and by the absence of adverse bleeding events in the phase I clinical trial\textsuperscript{12} also applies to inflammatory situations. Whether the safety profile of ACT017 still holds true when combining it with other drugs like recombinant tissue-type plasminogen activator remains to ascertain, but the absence of effect of ACT017 on platelet recruitment to the inflamed vasculature suggests there is a realistic chance for it to be maintained.
Disclosures

M. Jandrot-Perrus is the founder of Acticor Biotech. The other authors report no conflicts.

Acknowledgments

This work was supported by grants from INCA (2016-09/435/NI-KA), La Fondation ARC (PJA 20151203107), La Fondation pour la Recherche Médicale (grant #DPC20171138959), and by public grants overseen by the French National Research Agency (ANR) as part of the Investments for the Future program (PIA) under grant agreement No. ANR-18-RHUS-0001 (RHU Booster) and BPI (CMI2 project TherAVC2.0). Soumaya Jadoui was awarded a grant from la Société Française d’Hématologie.
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Figure 1. Contribution of GPVI to inflammation-associated haemostasis. The contribution of GPVI to inflammation-associated haemostasis was determined in 3 different models of acute inflammation. A. Representative images of brain sections taken 24 hours after GPVI+/+, GPVI−/−, GPVI−/−, and platelet-depleted mice were subjected to 90 min transient middle cerebral artery occlusion (tMCAO). Note that tMCAO caused bleeding only in platelet-depleted mice. The images are representative of n = 6 mice per group. B. Representative images of the bronchoalveolar lavage fluid from GPVI+/+, GPVI−/−, and platelet-depleted mice collected 24 hours after LPS inhalation. The images are representative of n = 8 mice per group. C-D. Effect of partial or complete glycoprotein VI deficiency on inflammatory bleeding during the cutaneous reverse passive Arthus reaction (rpA). C. Representative images of the skin of GPVI+/+, GPVI+/−, GPVI−/−, and platelet-depleted mice after 4 hours of rpA. The images are representative of n = 7-10 mice per group. Bar = 500 µm. D. Skin haemoglobin content after 4 hours of rpA. # indicates a significant difference (p<0.05) from the rpA GPVI+/+ group, n=14-20 skin biopsies per group. Inset : Representative histogram of flow cytometry analysis of GPVI surface levels in GPVI+/+, GPVI+/−, and GPVI−/− mice, as assessed using the JAQ1 antibody to mouse GPVI.

Figure 2. Impact of ACT017 on GPVI-dependent haemostasis. A-B. Citrated whole blood from hGPVI mice was labeled with the fluorochrome DiOC6, incubated or not with ACT017 (80 µg/mL) for 10 min, and perfused at a wall shear rate of 1500 s−1 or 100 s−1 for 3 min over a collagen-coated surface. Bar = 50 µm. A. Representative images of platelet coverage at the end of the perfusion. B. Mean surface areas covered by platelets calculated from 20 different fields taken with a 20 x objective along channels from 4 different runs (5 fields per run). C. Representative images of the skin of hGPVI mice treated or not with ACT017 (64 mg/kg) after 4 hours of rpA. The images are representative of n = 4-9 mice per group. Bar = 500 µm. D. Skin haemoglobin content after 4 hours of rpA. # indicates a significant difference (p<0.05) from the rpA hGPVI group, n=6-18 skin biopsies per group. E-F. Skin myeloperoxidase (E) and platelet factor 4 (PF4) (F) content after 2 hours of rpA, as assessed by ELISA. n=12 skin biopsies per group.
**Supplemental Figure 1. Impact of ACT017 on platelet-dependent prevention of tumor bleeding.** Haemoglobin content and macroscopic aspect of B16F1 tumors from hGPVI mice treated or not with ACT017 (64 mg/kg) or a platelet-depleting antibody. Tumors were excised 8 hours after injection of the treatments. # indicates a significant difference (p<0.05) from the hGPVI group, n=8-10 tumors per group. Bar = 500 µm.

**Supplemental Movie Legend.** Citrated whole blood from hGPVI mice was labeled with the fluorochrome DiOC6, incubated or not with ACT017 (Glenzocimab, 80 µg/mL) for 10 min, and perfused at a wall shear rate of 1500 s⁻¹ over a collagen-coated surface. The video shows the comparison of platelet deposition onto collagen in the presence or absence of Glenzocimab.