Dear Sirs,

Von Willebrand factor (VWF), a multimeric plasma protein, plays an important role in haemostasis, most importantly by stabilisation of coagulation factor VIII (FVIII) and by recruiting platelets to the vessel wall through interaction with glycoprotein (GP)Ib (1). Thrombin generation in the presence of platelets has been shown to depend on VWF (2). Increased hydrodynamic shear forces induce a conformational change in VWF from its globular into an elongated form, and this structural change is imperative for the interaction of VWF with GP Ib on platelets (reviewed in [3]). Alternatively, this conformational change is induced by surface binding of VWF, such as collagen in the wall of damaged blood vessels (4, 5). Likewise, it was hypothesised that VWF is activated after binding to fibrin, allowing its interaction with platelet GP Ib (2). Keuren et al. confirmed that plasma VWF is critical for platelet adhesion to a fibrin network (6). Given its importance in haemostasis, it is of no surprise that deficiencies and/or defects in VWF result in the most common inherited bleeding disorder von Willebrand disease (VWD). Apart from quantitative deficiencies in VWF present in type 1 and 3 VWD, structural and functional defects of VWF are classified as type 2 VWD. In one of its subtypes, type 2B VWD, a gain-of-function mutation in the VWF A1 domain induces the spontaneous binding of VWF to platelet GP Ib.

Previous results suggested that thrombin generation tests, in combination with routine FVIII and VWF measurements, could be of interest in the assessment of the individual bleeding risk in patients with VWD (7). We hypothesised that the activity of VWF is a major determinant of thrombin generation in the presence of platelets. Former studies did not find an increase in thrombin generation upon addition of VWF in VWD patient samples containing platelets using calibrated automated thrombography (CAT) (7) (and our unpublished results). This is attributed to the static environment of the CAT method (no shear rate is applied), leaving VWF in its globular non-active form. Indeed, the addition of botrocetin to platelet-rich plasma (PRP) was found to increase thrombin generation, and a neutralising anti-VWF antibody diminished thrombin generation (2). In this study, we have used a recombinant VWF corresponding to reported mutations in type IIB VWD, which has been described before (8). This gain of function mutant VWF-2B, binding GP Ib on platelets spontaneously, was incubated with PRP of a healthy control and tested in the classical CAT assay described before (9). Interestingly, VWF-2B increased the thrombin generation, illustrating that the activity of VWF greatly influences thrombin generation in platelet-rich plasma (Figure 1A). β2-glycoprotein I (β2GPI) is known to bind to the A1 domain of VWF, preferably when the A1 domain is in its active GPIb-binding conformation, and has been documented to inhibit VWF-dependent platelet adhesion (10). The increase in thrombin generation induced by VWF-2B was completely abolished in the presence of β2GPI (Figure 1A). Furthermore, addition of a monoclonal antibody Rag-35 (11) specifically binding to the A1 domain of VWF and thereby inhibiting the binding of platelets to VWF, completely abrogated the procoagulant effect of VWF-2B (Figure 1B). Taken together, our data illustrate that the procoagulant effect of VWF-2B results from the interaction of VWF-2B with GPIb on the platelets. This procoagulant effect of VWF-2B proved to be independent of the TF concentration, although the increase in peak height of the thrombin generation proved to be lower in the presence of high compared to low TF concentrations (28% vs 70%, respectively) (Figure 1B). Interestingly, incubation of platelets with VWF-2B resulted in a five-fold increased exposure of phosphatidyserine and a three-fold increased expression of P-selectin, as assessed by flow cytometry using fluorescently-labeled Annexin V and antibodies against P-selectin (data not shown).

In VWD type 2B patients, a gain-of-function mutation in the VWF A1 domain results in spontaneous binding of VWF to platelet GPIb. To confirm that thrombin generation is sensitive to the activation status of VWF, we compared the effect of adding platelets to platelet-poor plasma (PPP) from these patients (N=5) to PPP from healthy controls (N=10) on the thrombin generation. Furthermore, we have included patients with VWD type 2N (N=3), caused by an inherent VWF defect resulting in defective FVIII binding. Patient characteristics are shown in Table 1. Platelets were isolated from healthy controls (HCs), washed with Hepes buffer (136 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 2 mM MgCl2, 0.9 H2O (pH 6.6) containing 0.1 U/mL apyrase and acid-citrate-dextrose) and added to the different samples to end up with a final concentration of 3*10⁸ platelets/mL. Both for PPP and recomplemented PRP, all results were normalised to the results of normal pooled plasma (NPP) (all parameters of the NPP were set to 100). As demonstrated before (7), looking at the PPP thrombin gener-
ation curves, both the ETP and peak were significantly reduced in type 2B patients compared to HCs (ETP 31 vs 110; peak 22 vs 133). Additionally, lag time and TTP were significantly prolonged in type 2B patients compared to HCs (lag time 188 vs 105; TTP 157 vs 92). This diminished thrombin generation can probably be attributed to reduced FVIII levels in these VWD samples (average FVIII activity in VWD type 2B patients of 41%). In the reconstituted PRP, the differences between type 2B patients and HC were much less pronounced (ETP 108 vs 126; peak 87 vs 107; lag time 147 vs 105; TTP 131 vs 104). Subsequently, for each parameter of the thrombin generation, the ratio PRP/PPP was determined for all healthy controls and patients. * p<0.05 in comparison with HC samples; ** p<0.05 in comparison with HC and VWD type 2N samples (Mann-Whitney U test).

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Figure 1: Thrombin generation in PRP is sensitive to the activation status of VWF. A) Thrombin generation was initiated in PRP with 1 pM TF as described previously (9), in the absence and presence of 10 µg/ml VWF-2B and 25 µg/ml β2GPI. Results represent the mean of three different measurements. B) Thrombin generation was initiated in PRP with 1 (solid lines) or 5 (dotted lines) pM TF, in the absence and presence of 10 µg/ml VWF-2B and 150 µg/ml Rag-35 monoclonal antibody (mAb). Results represent the mean of three different measurements. C-F) Thrombin generation was initiated with 1 pM TF in PPP and reconstituted PRP from healthy controls, VWD type 2B and 2N patients. For both PRP and PPP, results were normalised to NPP. For the four parameters of thrombin generation, the ratio PRP/PPP was determined for all healthy controls and patients. * p<0.05 in comparison with HC samples; ** p<0.05 in comparison with HC and VWD type 2N samples (Mann-Whitney U test).
trations are much more affected by FVIII levels compared to PRP, we hypothesise that the increased PRP/PPP ratio for both the ETP and peak in type 2N VWD patients compared to healthy controls is caused by their reduced FVIII levels.

In this study we clearly demonstrate that thrombin generation is sensitive to the activation status of VWF, both by adding a gain-of-function mutant of VWF to the CAT assay and by studying VWD type 2B patient samples. Further research is warranted to determine whether this approach will be useful in testing the activation status of VWF and the diagnosis of VWD.

**Ethical approval**

All enrolled HC volunteers gave their full informed consent according to the Helsinki Declaration and its amendments; the study fulfilled all institutional ethical requirements and was approved by the Medical Ethical Committee of Maastricht University Medical Center.

**Author contributions**

B. de Laat and H. Kelchtermans designed the study. B. de Laat, L. Pelkmans, A. Misztaj and H. Kelchtermans performed the CAT assays. H. Kelchtermans, B. de Laat, and R. Al Dieri interpreted the data and prepared the manuscript. All authors read the manuscript, provided feedback and approved the final manuscript.

**Conflicts of interest**

None declared.

**References**