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## **Relevance of platelet desialylation and thrombocytopenia in type 2B von Willebrand disease: preclinical and clinical evidence**

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## **ABSTRACT**

Patients with type 2B von Willebrand disease (caused by gain-of-function mutations in the gene coding for von Willebrand factor) display bleeding to a variable extent and, in some cases, thrombocytopenia. There are several underlying causes of thrombocytopenia in type 2B von Willebrand disease. It was recently suggested that desialylation-mediated platelet clearance leads to thrombocytopenia in this disease. However, this hypothesis has not been tested *in vivo*.

The relationship between platelet desialylation and the platelet count was probed in 36 patients with type 2B von Willebrand disease (with p.R1341Q, p.R1306Q, and p.V1316M mutations) and in a mouse model carrying the severe p.V1316M mutation (the 2B mouse). We observed abnormally high elevated levels of platelet desialylation in both patients with the p.V1316M mutation and the 2B mice. *In vitro*, we demonstrated that 2B p.V1316M/von Willebrand factor induced more desialylation of normal platelets than wild-type von Willebrand factor did. Furthermore, we found that N-glycans were desialylated and we identified  $\alpha$ IIb and  $\beta$ 3 as desialylation targets. Treatment of 2B mice with sialidase inhibitors (which correct platelet desialylation) was not associated with the recovery of a normal platelet count. Lastly, we demonstrated that a critical platelet desialylation threshold (not achieved in either 2B patients or 2B mice) was required to induce thrombocytopenia *in vivo*. In conclusion, in type 2B von Willebrand disease, platelet desialylation has a minor role and is not sufficient to mediate thrombocytopenia.

## INTRODUCTION

Type 2B von Willebrand disease (VWD) is characterized by gain-of-function mutations in the gene coding for von Willebrand factor (VWF), which enhance the factor's ability to bind platelet glycoprotein Iba (GPIba). Patients with type 2B VWD display bleeding to a variable extent and, in some cases, thrombocytopenia. There are several underlying causes of thrombocytopenia described in type 2B VWD, including the incorporation of platelets bound to plasma VWF into circulating aggregates,<sup>1-3</sup> and defective platelet production.<sup>4,5</sup> In many cases multiple mechanisms may contribute to the development of thrombocytopenia and may lead to severe life-threatening bleedings. Identification of the causes of thrombocytopenia is crucial for the appropriate management of patients. A new and original concept relates to platelet desialylation a process in which terminal sialic acids are cleaved on the platelet surface and leads to accelerate platelet clearance and thrombocytopenia.

Sialic acids are terminal sugar components of glycoprotein oligosaccharide chains. Platelet desialylation is involved for physiological platelet aging *in vivo*. Indeed, the removal of platelet sialic acid exposes  $\beta$ -galactose residues (considered to be senescence antigens), and facilitates platelet uptake by Kupffer cells in cooperation with hepatocytes via the hepatic asialoglycoprotein receptor.<sup>6,7</sup> Over the past few decades, it has been shown that platelet desialylation is responsible for platelet clearance in many contexts, such as immune thrombocytopenia.<sup>8,9</sup> Recently, Deng *et al.* described a new mechanism for platelet clearance mediated by active VWF bound to GPIba.<sup>10</sup> More specifically, the researchers demonstrated that a VWF/botrocetin complex and VWF from a patient carrying a p.V1316M mutation led to  $\beta$ -galactose exposure *in vitro*. On the basis of these observations, Deng *et al.* predicted that this  $\beta$ -galactose exposure might be responsible (at least in part) for thrombocytopenia in type 2B VWD. However, this hypothesis had not previously been tested *in vivo*.

## METHODS

*Patients.* A total of 36 patients with type 2B VWD (from 17 unrelated families) and 35 healthy age- and sex-matched controls were enrolled. In accordance with the tenets of the Declaration of Helsinki, the study participants were informed about the anonymous use of their personal data, and gave their written, informed consent. The study was approved by the local investigational review board (Lille University Medical Center, Lille, France). The French National Reference Centre for Von Willebrand Disease database and biological resource center (plasma and DNA) were registered with the French National Data Protection Authority (reference: CNIL 1245379). Platelet counts and mean platelet volume (MPV) were measured with an automated analyzer (XN-10, Sysmex France).

*Mice.* The type 2B VWD knock-in mouse model (p.V1316M) has been described elsewhere.<sup>11</sup> Mice homozygous for the p.V1316M mutation are referred to hereafter as “2B mice”, and their control littermates are referred as wild-type (“WT”) mice. Platelet counts were determined with an automated analyzer (Scil Vet ABC Plus, Horiba Medical). Male and female mice were used indifferently. The study was approved by the local animal care and use committee (reference: APAFIS#1294-2015072816482568).

*Flow cytometry.* Platelet surface  $\beta$ -galactose exposure was determined by using FITC-conjugated *Ricinus communis* agglutinin I (RCA for platelet-rich plasma 12.5  $\mu$ g/mL, for washed mouse platelets 5  $\mu$ g/mL) and *Erythrina cristagalli* lectin (ECL, 10  $\mu$ g/mL). Samples in which RCA or ECL was incubated with  $\beta$ -lactose<sup>12</sup> (200 mM) were used as corresponding negative controls. Platelet surface  $\alpha$ -2,3-sialylation on O-glycans was determined by using biotinylated *Maackia amurensis* lectin II<sup>7,13</sup> (MALII, 10  $\mu$ g/mL) and phycoerythrin (PE)-streptavidin (10  $\mu$ g/mL). Briefly, platelet-rich plasma or washed platelets (5  $\mu$ L) were incubated with lectins for 30 min at room temperature in a final volume of 100  $\mu$ L. The reaction was stopped with PBS (400  $\mu$ L). In some experiments, washed murine platelets (100  $\mu$ L,  $10^5/\mu$ L) were treated with 10,000 U/mL of Peptide:N-glycosidase F (PNGase F) for 18h at 37°C or treated with 100  $\mu$ g/mL O-sialoglycoprotein endopeptidase (OSGE) for 30 min at 37°C. The platelets were washed and then stained with a lectin or an FITC-conjugated antibody against mouse GPIb $\alpha$ , GPVI or  $\alpha$ IIb $\beta$ 3. Surface neuraminidase-1 (NEU1) expression was measured with rabbit anti-NEU1 antibody (4  $\mu$ g/mL) and detected with Alexa Fluor 488 secondary antibody (6  $\mu$ g/mL). Lectin or antibody binding was determined using a flow cytometer (a BD Accuri system for mouse samples, and a Beckman Coulter Navios system for human samples).

*Statistics.* Statistical analyses were performed using Prism 6 for Mac software (version 6; GraphPad, Inc., San Diego, CA). If only two groups were compared, a Student's *t* test was used. For 3 or more groups, a 1-way analysis of variance (ANOVA) and Dunnett's post-test were used. Before performing these tests, a D'Agostino-Person normality test was used to determine whether data were normally distributed. Equality of variance was tested with an F test prior to Student's *t* test or with Bartlett's test prior to an ANOVA. Correlations were assessed by calculating Pearson's coefficient *r*.

## RESULTS

### Platelet desialylation in human and murine type 2B VWD *in vivo*

To examine the putative link between platelet desialylation and thrombocytopenia in type 2B VWD, we assessed  $\beta$ -galactose exposure at the platelet surface (*i.e.* a marker of sialic acid removal from glycoproteins). We first analyzed the platelet count and the extent of platelet  $\beta$ -galactose exposure (by measuring RCA binding) in 36 patients with type 2B VWD and 35 healthy controls. The mean  $\pm$  standard deviation (SD) platelet count was significantly lower in the patient group ( $217 \pm 70 \times 10^3/\mu\text{L}$ ) than in the control group ( $256 \pm 47 \times 10^3/\mu\text{L}$ ;  $p=0.012$ ). The amount of  $\beta$ -galactose (measured as the mean  $\pm$  SD fluorescence intensity, MFI, for RCA) was significantly higher in the patient group ( $7.0 \pm 2.6$ ) than in the control group ( $5.5 \pm 2.3$ ;  $p=0.011$ ). The individual platelet counts were weakly correlated with levels of surface-exposed  $\beta$ -galactose in patients with type 2B VWD ( $r^2=0.113$ ,  $p=0.048$ ) but not correlated in controls ( $r^2=0.095$ ,  $p=0.092$ ) (Figure 1A). Patients bearing the p.R1341Q mutation displayed a significantly lower mean platelet count and a significantly greater RCA MFI (Figure 1B-Ci). In contrast, the platelet count and RCA binding in patients bearing the p.R1306Q mutation did not differ significantly from the values observed for controls. Interestingly, patients carrying the severe p.V1316M mutation exhibited the lowest platelet count and the highest amount of  $\beta$ -galactose (2.1-fold more than controls) (Figure 1B-Ci). To take platelet size into account, we also measured the ratio between the RCA MFI and the MPV. The elevated level of RCA binding (relative to controls) was no longer observed for patients with the p.R1341Q mutation but was still observed for those bearing the p.V1316M mutation (Figure 3Cii). Given that the p.V1316M mutation in VWF was associated increased  $\beta$ -galactose exposure in our study ( $n = 2$  patients) and also *in vitro*,<sup>10</sup> we studied the possible role of desialylation in our recently engineered knock-in murine model of severe type 2B VWD (p.V1316M mutation). The murine disease mimics the human disease, and 2B mice display thrombocytopenia<sup>4,11</sup> (mean  $\pm$  SD platelet count:  $355 \pm 104 \times 10^3/\mu\text{L}$ ), relative to WT mice ( $803 \pm 159 \times 10^3/\mu\text{L}$ ). Platelets from 2B mice had a significantly greater amount of exposed  $\beta$ -galactose (MFI for RCA:  $7093 \pm 3156$ ,  $n=67$ ) than WT mice did ( $3154 \pm 1098$ ,  $n=61$ ;  $p<0.001$ , Figure 1Di). After taking platelet size into account by measuring the ratio between the MFI for RCA to the MFI for CD41 ( $\alpha\text{IIb}$  integrin), an (2-fold) elevation was still observed (RCA/CD41:  $0.358 \pm 0.181$  for 2B mice,  $n=67$ ;  $0.179 \pm 0.600$  for WT mice,  $n=61$ ;  $p<0.001$ ) (Figure 1Dii). Thus, our results evidenced a link between the platelet count and platelet desialylation in type 2B VWD.

### 2B VWF induces N-glycan-specific platelet desialylation

With a view to highlighting a direct link between the p.V1316M mutation in VWF and desialylation, we incubated plasma from 2B mice (p.V1316M) with WT platelets. In contrast



to VWF-deficient or WT plasma, 2B plasma induced  $\beta$ -galactose exposure (with a  $1.48 \pm 0.12$ -fold increase, Figure 2A) to the same extent as botrocetin treatment of WT plasma (Supplemental Figure 1), as previously described.<sup>10</sup> We confirmed that p.V1316M/VWF (0.2  $\mu\text{g}/\text{mL}$ ) induced desialylation (relative to WT/VWF) by incubating normal washed platelets with recombinant p.V1316M/VWF; we observed a  $1.85 \pm 0.15$ -fold relative increase (Figure 2B). We confirmed this result with another lectin (ECL) that is specific for  $\beta$ -galactose (Figure 2C). We then investigated how does gain-of-function VWF mediate platelet desialylation. Desialylation is due to the activity of neuraminidases NEU1, a sialidase catalyzing the removal of terminal sialic acids from sialyloconjugates. Relative to WT/VWF, p.V1316M/VWF treatment was able to induce the translocation of NEU1 to the platelet surface (Figure 2D). Platelet glycoproteins are commonly modified by complex carbohydrates including N-linked glycans (N-glycans) and mucin-type O-linked glycans (O-glycans).<sup>7,14</sup> Both N- and O-glycans are commonly “capped” by sialic acids. We next looked at whether the desialylation induced by the p.V1316M/VWF occurred on O-glycans and/or N-glycans. To this end, we used MALII lectin which recognizes  $\alpha$ -2,3-sialylation on O-glycans,<sup>7,13</sup> and a recent study found that the absence of O-glycans (in *C1galt1*<sup>-/-</sup> mice) reduced MALII binding but did not change RCA binding.<sup>7</sup> We first confirmed the specificity of MALII by using desialylated platelets showing a decrease of the MALII binding, as expected (Supplemental Figure 2). We found that p.V1316M/VWF did not change MALII binding to platelets, relative to WT/VWF (Figure 2E), suggesting that desialylation was not likely to be on O-glycans. We next looked at whether the desialylation induced by the 2B VWF occurred on N-glycans by using PNGase F - the most effective enzymatic method for removing N-linked oligosaccharides from glycoproteins. We first characterized the effect of PNGase F treatment on RCA, ECL and MALII binding on platelets. PNGase F treatment reduced the binding of RCA (to  $40 \pm 4\%$  of the control value, Figure 2F) and ECL (to  $50 \pm 10\%$ , data not shown) to WT platelets but not that of MALII. Given that RCA and ECL were sensitive to PNGase F, the platelets were then incubated with WT or p.V1316M/VWF to induce desialylation and were then treated (or not) with PNGase F during 18h. Platelet desialylation was still observed after 18h of incubation in the absence of PNGase F (RCA ratio:  $0.97 \pm 0.04$  for WT, and  $1.49 \pm 0.13$  for 2B;  $p < 0.01$ ) (Figure 2G). Strikingly, removal of N-glycans after p.V1316M/VWF treatment was associated with much lower RCA binding (Figure 2G,  $p < 0.001$ ). Indeed, RCA binding in the presence of WT or 2B VWF was similar after PNGase F treatment (RCA ratio:  $0.62 \pm 0.07$  for WT, and  $0.72 \pm 0.06$  for 2B). Taken as a whole, our results demonstrated for the first time that the p.V1316M mutation in VWF induces N-glycan-specific platelet desialylation.

## **2B VWF induces $\alpha$ IIb and $\beta$ 3 desialylation**

Many platelet glycoprotein and surface receptors contain sialic acid. GPIb $\alpha$  contains the highest levels, followed by integrin  $\beta$ 3, integrin  $\alpha$ IIb, GPV and GPVI/GPIb $\beta$ /GPIX, for the main receptors.<sup>7</sup> We first investigated whether GPIb $\alpha$  and GPVI were desialylated-targets by the p.V1316M/VWF. We found that treatment with OSGE, which removes GPIb $\alpha$  and, to some extent, GPVI but not  $\alpha$ IIb $\beta$ 3 did not change RCA and ECL binding (Figure 3A), suggesting that the desialylation induced by p.V1316M/VWF did not take place on GPIb $\alpha$  and GPVI. We then confirmed that p.V1316M/VWF did not induce GPIb $\alpha$  and GPVI desialylation by performing RCA pull-down experiments followed by western blotting (Figure 3B). This data confirms our results and rule out mouse GPIb $\alpha$  and GPVI as a specific platelet desialylation targets by p.V1316M/VWF. Taken as a whole our present results reveal the existence of desialylation of platelet proteins other than GPIb $\alpha$  and GPVI.<sup>7,15</sup> The next candidates were the integrins  $\alpha$ IIb and  $\beta$ 3 carrying sialic acid both on N- and O-glycans.<sup>7,15</sup> We performed RCA pull-down experiments by incubating normal washed platelets with recombinant WT/VWF or p.V1316M/VWF. Our results demonstrated that p.V1316M/VWF induced  $\alpha$ IIb and  $\beta$ 3 desialylation. Indeed, we observed a  $1.90 \pm 0.17$ -fold relative increase for  $\alpha$ IIb and a  $2.02 \pm 0.36$ -fold relative increase for  $\beta$ 3 compared to WT/VWF (Figure 3C). Taken as a whole, our results demonstrated for the first time that the p.V1316M mutation in VWF induces  $\alpha$ IIb and  $\beta$ 3 desialylation.

### **Thrombocytopenia in type 2B mice is independent of platelet desialylation**

Since desialylation is mediated by sialidases, we next treated 2B and WT mice with two sialidase inhibitors (DANA or oseltamivir phosphate) or Hank's balanced salt solution as a control. Treatment with each inhibitor was associated with significantly lower platelet desialylation *in vivo* (as measured by RCA binding) in 2B mice but not in WT mice (Figure 4A-B). After 6h of treatment, the level of RCA binding was much the same as in the WT mice (Figure 4A-B). Surprisingly, the platelet counts remained low and unchanged in 2B mice for up to 7 days after the infusion (168h, Figure 4A). Our observation was especially surprising because both sialidase inhibitors have been reported to correct platelet counts in immune thrombocytopenia, with a desialylation profile after a single administration of a lower dose than that used in our experiments.<sup>9,16</sup> Our findings indicate for the first time that the level of desialylation observed in type 2B VWD mice has only a minor role in platelet clearance or is not sufficient to induce thrombocytopenia. This lack of effect might be attributable to the type of desialylation. Although elevated platelet clearance has been reported in mice lacking N-glycan sialylation,<sup>17</sup> a recent study provided insights into the essential role of O-glycan sialylation in platelet clearance.<sup>7</sup> Furthermore, it has been reported that both mouse and

human platelets contain high levels of O-glycans, with more sialic acids on the latter than on N-glycans.<sup>7,18</sup>

### **Threshold of platelet desialylation and thrombocytopenia**

We then looked at whether the sialidase inhibitors' apparent lack of effect in 2B mice was due to a threshold effect, *i.e.* whether a minimum level of desialylation was required to significantly affect platelet count. Hence, WT mice were treated with various concentrations of neuraminidase, and the desialylation efficiency was monitored by RCA binding (Figure 5A). We observed a clear relationship between desialylation and the fall in the platelet count (Figure 5A). Importantly, the platelet count was not affected when the RCA ratio was around 2 or less (Figure 5B). We observed a strong and inverse correlation between the platelet count and RCA ratio ( $r^2=0.95$ ) and the circulating platelet count was about half the control value, corresponding to a RCA ratio of 10 (Figure 5C). This strongly indicates that the RCA ratio observed in patients and mice with the severe p.V1316M mutation (giving 2.1-fold and 2-fold differences, respectively) does not explain the low platelet count.

To investigate the threshold of platelet desialylation linked or not to thrombocytopenia, reference interval has to be determined in the WT population. With the parametric approach, the central 95% boundaries are specified by the mean  $\pm$  2SD, if the data follow a Gaussian (normal) distribution. After having confirmed if our values come from a Gaussian distribution by using a D'Agostino-Pearson omnibus normality, we calculated, in WT mice population, a mean platelet count of  $803 \times 10^3$  platelets/ $\mu$ L with SD=159 ( $n=129$ ) and mean- 2SD of  $485 \times 10^3$  platelets/ $\mu$ L. Under this value of platelet count, mice could be considered as thrombocytopenic. Based on graph Figure 5C, a minimal platelet count of  $485 \times 10^3$  platelets/ $\mu$ L corresponds to a RCA ratio of 6.2. Under this threshold, the thrombocytopenia observed is likely to be independent of desialylation, and above this threshold, the thrombocytopenia is likely to be desialylation dependent.

## DISCUSSION

In the present study, we tested the hypothesis whereby accelerated platelet clearance (and thus thrombocytopenia) in type 2B von Willebrand disease is caused by desialylation. Indeed, Deng *et al.* recently proposed a novel original mechanism of platelet clearance mediated by active von Willebrand factor.<sup>10</sup> More specifically, authors demonstrated that type 2B VWF led to platelet desialylation, and predicted that this process might be responsible (at least in part) for the thrombocytopenia observed in type 2B VWD. This hypothesis has yet to be tested *in vivo*.

We had access to blood samples from a cohort of 36 patients with three different mutations (p.R1341Q, p.R1306Q, and the severe p.V1316M) and from 35 healthy controls. We also studied our novel mouse model bearing a point mutation (p.V1316M) in the endogenous *Vwf* gene; we recently validated this mouse as a relevant model of type 2B VWD.<sup>4,11</sup>

Our present results indicated that even though the level of desialylation was abnormally high in both human and murine models, it was not sufficient to mediate thrombocytopenia. Indeed, treatments of 2B mice with the sialidase inhibitors oseltamivir (Tamiflu®) and DANA did not correct the thrombocytopenia.

How does gain-of-function VWF mediate platelet desialylation? In platelets, the sialidase activity is due to the activity of neuraminidases. Neuraminidase 1 (NEU1) is a lysosomal sialidase catalysing the removal of terminal sialic acids from sialyloconjugates. Furthermore, this NEU1 was found to be abundant in the granules of permeabilized platelets, and was able to translocate to the platelet membrane.<sup>9</sup> Plasma-membrane-bound NEU1 modulates a plethora of receptors by desialylation. At the plasma membrane, NEU1 has been shown to be required for signal transduction, and recent results have provided new insights in the molecular organization of membrane-bound NEU1. Indeed, the protein has two potential transmembrane domains that may anchor NEU1 to the membrane and control its dimerization and sialidase activity.<sup>19</sup> It has been reported that the sialidase NEU1 is involved in platelet desialylation.<sup>20</sup> In the present study, we found a translocation of NEU1 at the platelet surface after stimulation with the p.V1316M/VWF, suggesting a reorganization of platelet membrane. However, neither platelet activation nor P-selectin exposure were found after p.V1316M/VWF treatment (data not shown). This results are in agreement with previous reports. Indeed, patients with type 2B VWD have a bleeding tendency that is linked to the loss of VWF multimers and platelet dysfunction.<sup>21</sup> Platelet functions were diminished due to the inhibition of integrin  $\alpha\text{IIb}\beta\text{3}$  and of the small GTPase Rap1 by VWF/p.V1316M following exposure to platelet agonists. These data indicate that the type 2B mutation p.V1316M is associated with severe thrombocytopathy, and that the addition of 2B/VWF leads to platelet

inhibition rather than activation. The mode of action of NEU1 and its localization and also the potential link between NEU1 activation and platelet dysfunction require further investigations.

We determined which mode of desialylation was induced by type 2B VWF. We studied the binding of MALII lectin (which reportedly binds to  $\alpha$ -2,3-sialylation on O-glycans) in the light of a recent study showing that O-glycan desialylation is important for platelet clearance.<sup>7</sup> The level of MALII lectin binding did not change in the presence of type 2B/VWF (relative to WT/VWF). We next determined whether the desialylation induced by the 2B/VWF occurred on N-glycans. After the desialylation induced by 2B/VWF, we removed N-glycans by incubation with PNGase F. This treatment reduced RCA and ECL binding but not MALII binding. Taken as a whole, our results demonstrate for the first time that the p.V1316M mutation of VWF specifically induces desialylation on platelet N-glycans. One important question was the identification of targets. We ruled out desialylation of GPIIb $\alpha$  and GPVI. Indeed, we found that treatment with O-sialoglycoprotein endopeptidase (OSGE, which removes GPIIb $\alpha$  and, partially, GPVI) did not change RCA and ECL binding - suggesting that the desialylation induced by 2B/VWF did not occur on GPIIb $\alpha$  and GPVI. Interestingly, assessment of baseline desialylation of GPIIb $\alpha$ <sup>-/-</sup> platelets (using RCA) revealed an unexpected elevation in desialylation, relative to WT platelets.<sup>15</sup> Furthermore, treatment of GPIIb $\alpha$ <sup>-/-</sup> platelets with neuraminidase was associated with a 10-fold relative increase in RCA binding.<sup>15</sup> On the other hand, mouse GPIIb $\alpha$  contains the highest levels of sialic acid on O-glycans but no sialic acid was predicted on N-glycans.<sup>7</sup> The next obvious candidates were the integrins  $\alpha$ IIb and  $\beta$ 3 carrying sialic acid both on N- and O-glycans and in mouse platelets the integrins  $\alpha$ IIb $\beta$ 3 is one of the most sialylated glycoprotein on N-glycans.<sup>7</sup> We performed RCA pull-down and our results demonstrated for the first time that p.V1316M/VWF induced  $\alpha$ IIb and  $\beta$ 3 desialylation. Nowadays, no study demonstrated that the integrin  $\alpha$ IIb $\beta$ 3 is a target of desialylation.

We then looked at whether a minimum level of desialylation was required to significantly affect platelet count. To investigate the threshold of platelet desialylation linked or not to thrombocytopenia, reference interval has been determined in the WT population and the RCA value corresponding to the low platelet count was calculated. We found a RCA value of 6.2 suggesting that under this threshold, the thrombocytopenia observed is likely to be independent of desialylation, and above this threshold, the thrombocytopenia is likely to be desialylation dependent. However, this critical platelet desialylation threshold requires to induce thrombocytopenia *in vivo* was not achieved in either 2B patients or 2B mice with the p.V1316M mutation (giving 2.1-fold and 2-fold differences, respectively) and so does not explain the low platelet count.

To go further, the weak observed correlation between RCA binding and the platelet count in patients (Figure 1A) suggests that under stressful conditions where thrombocytopenia may be exacerbated (e.g. pregnancy or surgery), desialylation could be associated with other mechanisms involved in thrombocytopenia, such as platelet production defects in megakaryocytes,<sup>4</sup> and the accelerated uptake of VWF/platelet complexes in macrophages.<sup>1</sup> However, this speculative point requires further investigation.

In conclusion, elevated levels of desialylation are observed in patients with type 2B VWD and mice carrying the p.V1316M mutation. The desialylation primarily concerns N-glycans and does not involve GPIb $\alpha$  or GPVI but involved  $\alpha$ IIb and  $\beta$ 3 integrins. Although desialylation was observed under baseline conditions, it does not appear to be important contributor to thrombocytopenia in type 2B VWD.

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**Disclosures**

The authors have declared that no conflict of interest exists.

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## Figure legends

### Figure 1: Platelet desialylation in human and murine type 2B VWD *in vivo*

A) Analysis of the correlation between RCA binding and the platelet count in patients with type 2B VWD (n=36, left panel:  $r^2=0.113$ ,  $p=0.048$ ) and healthy controls (n=35, right panel:  $r^2=0.095$ ,  $p=0.092$ ). Distribution of platelet counts (B) and RCA MFI (Ci) or RCA/MPV (Cii) values in healthy controls and in patients with type 2B VWD, as a function of the mutation in the VWF A1 domain (p.R1341Q, n=30, p.R1306Q, n=4, p.V1316M, n=2). A one-way ANOVA was followed by Dunnett's test; \* $p<0.05$ . D) Distribution of RCA MFI (Di) or RCA/CD41 (Dii) values in WT (n=61) and 2B mice (n=67) (unpaired Student's *t* test; \*\*\* $p<0.001$ ).

### Figure 2: Platelet desialylation by 2B VWF *in vitro* occurs on N-glycans

A) A histogram of RCA lectin binding on WT mouse platelets in PRP treated with VWF-deficient plasma (VWF-dp), WT plasma or 2B plasma. The fold change in each experiment was calculated relative to the binding obtained with VWF-deficient plasma, set to 1. The mean  $\pm$  SD values (n=3 experiments) were compared using a one-way ANOVA and Dunnett's post-test. \*\* $p<0.01$ . B) A histogram of RCA lectin binding to washed WT mouse platelets treated with WT or 2B mouse VWF (p.V1316M). C) Histograms of RCA and ECL binding (for  $\beta$ -galactose exposure) on washed WT mouse platelets treated with 0.2  $\mu$ g/mL WT or 2B mouse VWF. The fold change in each experiment was calculated relative to the baseline value (in the absence of VWF), set to 1. The mean  $\pm$  SD values (n=4 experiments) were compared using a one-way ANOVA and Dunnett's post-test, \*\* $p<0.01$ ). D) Flow cytometric analysis of NEU1 expression on washed WT mouse platelets treated with 0.2  $\mu$ g/mL WT or 2B mouse VWF. \* $p<0.05$ . E) Histograms of MALII lectin binding (for  $\alpha$ -2,3-linked sialic acids on O-glycans) on washed WT mouse platelets treated with 0.2  $\mu$ g/mL WT or 2B mouse VWF. F) Flow cytometric analysis of WT platelets after treatment (or not) with PNGase F and staining with RCA and MALII lectin. The data are representative of 3 independent experiments. G) A histogram of RCA binding on washed WT mouse platelets treated with 0.2  $\mu$ g/mL WT or 2B VWF. Platelets were then treated (or not) with PNGase F. The mean  $\pm$  SD values (n=3 experiments) were compared using a one-way ANOVA and Dunnett's post-test. \*\*\* $p<0.001$ .

### Figure 3: Type 2B VWF induces desialylation of the integrin $\alpha$ IIb $\beta$ 3

A) Flow-cytometric analysis of WT platelets after being stained for GPIIb $\alpha$ , GPVI,  $\alpha$ IIb $\beta$ 3, RCA and ECL with or without (w/o) OSGE treatment. Graphs are representative of 3 independent experiments.

B) RCA pull-down and western-blot. Washed WT mouse platelets were treated with WT or

2B mouse VWF during 1 hour and then lysed and prepared to RCA pull-down. Samples were subjected to western blot to analyze: B) GPIIb $\alpha$  and GPVI, C)  $\alpha$ IIb and  $\beta$ 3. A histogram of  $\alpha$ IIb and  $\beta$ 3 in the pull-down expressed in desialylated form (from pull-down) / total form (from lysate). The mean  $\pm$  SD values (n=3 experiments) were compared using a one-way ANOVA and Dunnett's post-test, \*p<0.05; \*\*\*p<0.001).

**Figure 4: Effect of platelet desialylation on the platelet count**

A) Platelet RCA MFI (left) and whole-blood platelet counts (right) in 2B (red line) and WT (black line) mice were measured at the indicated time points before and after treatment with a sialidase inhibitor (DANA or oseltamivir phosphate) or HBSS as a control (2B: n=4 mice for the control, n=11 mice for DANA, n=6 mice for oseltamivir phosphate, WT: n=4 mice for the control, n=3 mice for DANA, n=3 mice for oseltamivir phosphate). The mean  $\pm$  SD values were compared using a one-way ANOVA and Dunnett's post-test, \*p<0.05, \*\*p<0.01, in a pre-/post-treatment comparison. B) A histogram of RCA lectin binding (top panel) and the platelet count (bottom panel) before and 6 hours after the injection of the drugs into WT and 2B mice. \*\*p<0.01.

**Figure 5: A threshold of platelet desialylation is required to affect the platelet count**

A) Relative whole-blood platelet counts (dashed red line) and platelet RCA MFI (solid black line) in WT mice 1 hour after *in vivo* treatment with neuraminidase (n=3 mice for each concentration, values are quoted as the mean  $\pm$  SD). The increase in RCA binding was calculated for each mouse as the ratio between the RCA MFI after treatment and the RCA MFI before treatment. B) The panel focuses on the stability of the relative platelet count and the increase in RCA binding after treatment with low doses of neuraminidase (0, 2.5 and 5 mU/g of body weight). C) The correlation between the relative platelet count and RCA binding after neuraminidase treatment ( $r^2=0.95$  in a linear regression).

Figure 1

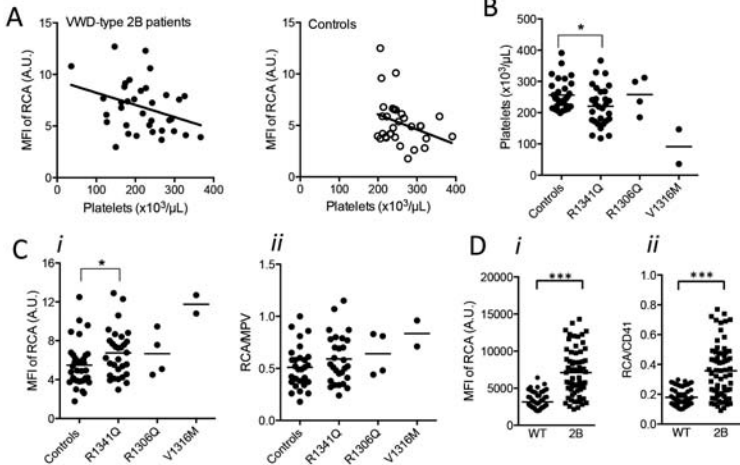
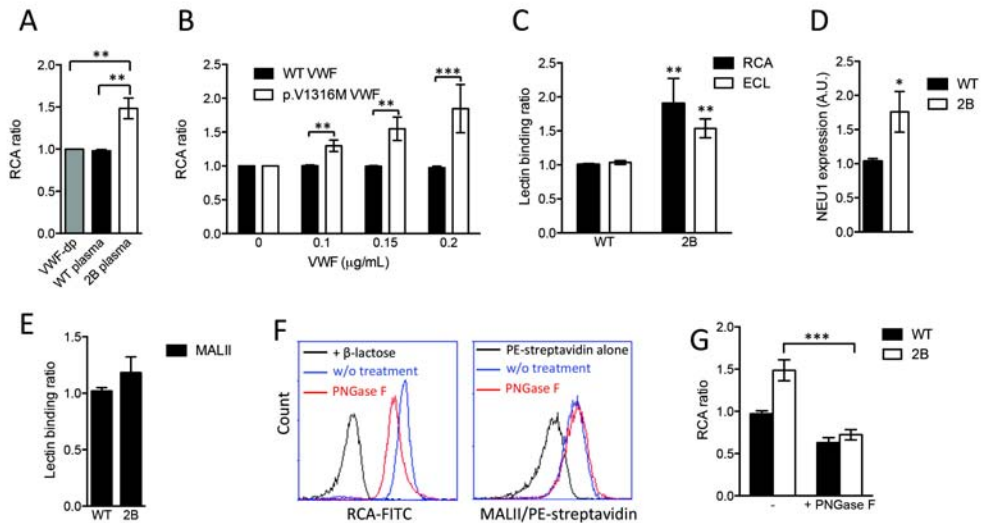
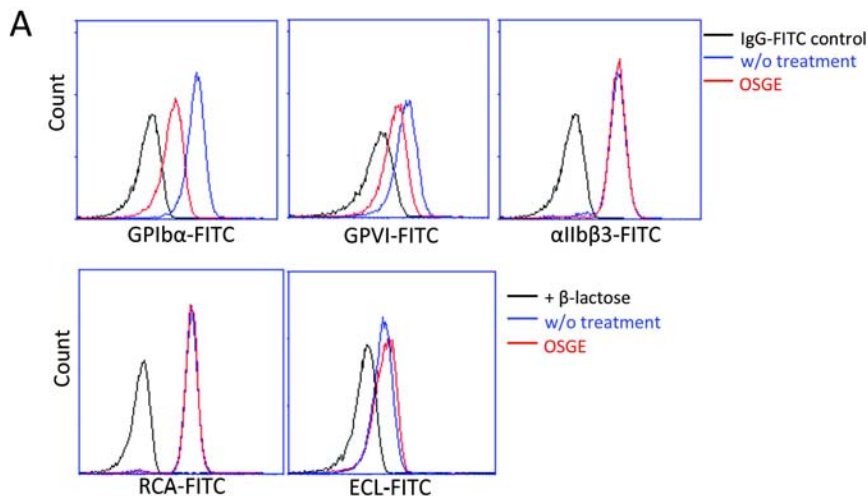


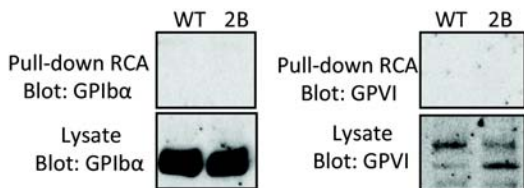
Figure 2



# Figure 3



**B**



**C**

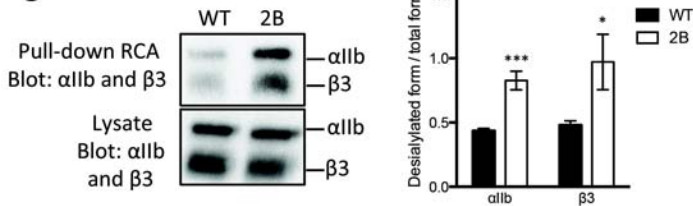
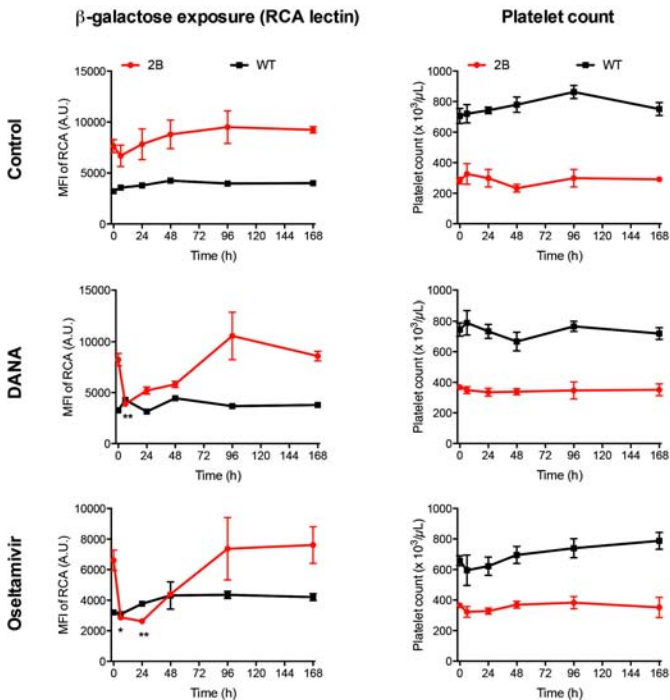


Figure 4

A



B

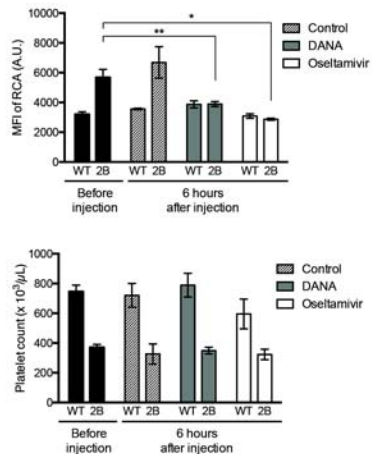
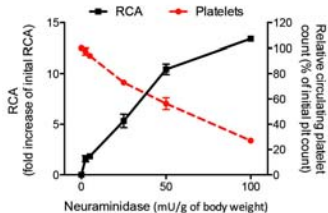
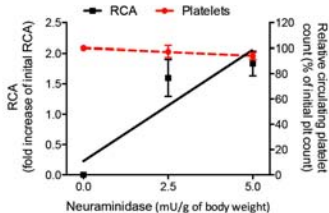


Figure 5

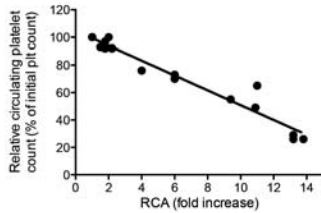
A



B



C





## Relevance of platelet desialylation and thrombocytopenia in type 2B von Willebrand disease: preclinical and clinical evidence

Dupont et al.

### Major Resources Table

#### Reagents

Name	Vendor or Source	Catalog #
<i>Ricinus communis</i> agglutinin I (RCA)	Vector Labs	FL-1081
<i>Erythrina cristagalli</i> (ECL)	Vector Labs	FL-1141
Biotinylated <i>Maackia amurensis</i> lectin II (MALII)	Vector Labs	B-1265
$\beta$ -lactose	Sigma-Aldrich	L3750
$\alpha$ 2-3,6,8 neuraminidase from <i>C. perfringens</i>	Sigma-Aldrich	N-3001
PE-streptavidin	Calbiochem	189737
PNGase F	New England Biolabs	P0704
O-sialoglycoprotein endopeptidase (OSGE)	Cedarlane	CLE100
DANA (N-Acetyl-2,3-dehydro-2-deoxyneuraminic Acid)	Santa Cruz	SC-215433A
Oseltamivir phosphate	Selleckchem	S2597

#### Antibodies

Target antigen	Vendor or Source	Catalog #
FITC-rat anti-mouse GPIIb $\alpha$ mAb (clone XiaG5)	Emfret Analytics	M040-1
FITC-rat anti-mouse GPVI mAb (clone JAQ1)	Emfret Analytics	M011-1
FITC-rat anti-mouse integrin $\alpha$ <sub>Ib</sub> $\beta$ <sub>3</sub> mAb (clone Leo.H4)	Emfret Analytics	M021-1
FITC-rat IgG negative control	Emfret Analytics	P190-1
FITC-rat anti-mouse CD41 mAb (clone MWReg30)	BD Pharmingen	553848
FITC-rat IgG negative control	BD Pharmingen	553924
Neu1 (clone H-300)	Santa Cruz	SC-32936
Purified rat anti-mouse GPIIb $\alpha$ (clones XiaG5/XiaG7)	Emfret Analytics	M040-0/M042-0
Purified rat anti-mouse integrin $\alpha$ Ib (clone 386627)	R&D	MAB 4118
Purified rat anti-mouse integrin $\beta$ 3 (clone Luc.A5)	Emfret Analytics	M030-0
Purified rat anti-mouse GPVI (clone JAQ1)	Emfret Analytics	M011-0
Alex fluor 488 goat anti-rabbit secondary antibody	Invitrogen	A11008

### Methods

**Recombinant mVWF/p.V1316M.** Baby hamster kidney cells were transfected with pNUT-mouse VWF and pNUT-mouse VWF/p.V1316M encoding mouse VWF and mouse VWF/p.V1316M, as previously described.<sup>1</sup> Serum-free-conditioned medium was collected, and VWF antigen levels were quantified, taking normal pooled human plasma as a reference (100%).

**Desialylation by VWF.** Citrated plasma from WT or 2B mice was mixed 9:1 (v/v) with citrated platelet-rich plasma from WT mice. Washed platelets (prepared as previously described)<sup>2</sup> were incubated in the presence of WT or 2B recombinant murine VWF

(p.V1316M). All samples were incubated for 1 hour at 37°C without stirring.

**RCA pull-down.** Washed platelet (500  $\mu$ L at  $3.10^8$ /mL) were lysed (NaCl 0.15 M, HEPES 5 mM, NP40 1%, octylglucopyranoside 0.1%, SDS 0.1%, EDTA 1 mM, cantharidine 10  $\mu$ M, 5  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin) and centrifuged 14 000g during 20 min. Biotinylated-RCA (5  $\mu$ g/mL) was added to the supernatant and incubated during 1h at 4°C while rotating. Then, samples were incubated for 1h with streptavidin magnetic beads at room temperature while rotating. The beads were then washed and proteins were eluted with SDS sample buffer.

**Western-blot.** Samples were separated by 4-12% SDS-PAGE, and immunoblotted with anti-GPIIb $\alpha$  antibodies (mix of clones XiaG5/XiaG7, 1/100), GPVI (1/100),  $\alpha$ IIb (1/1000),  $\beta$ 3 (1/200). Immunoreactive bands were visualized using Enhanced Chemiluminescence Detection Reagents (Pierce). Images of the chemiluminescent signal were captured using G:BOX Chemi XT16 Image Systems and quantified using Gene Tools version 4.0.0.0 (Syngene).

**In vivo sialidase inhibitors.** The neuraminidase inhibitors N-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA, 0.1 mg/g total body weight) and oseltamivir phosphate (0.025 mg/g total body weight) were respectively injected intraperitoneally and intravenously into 2B and WT mice. An injection of Hank's balanced salt solution was used as a control. After 6, 24, 48, 96, and 168 h (*i.e.* up to 7 days), the mice were bled, and the platelet count and RCA binding were measured.

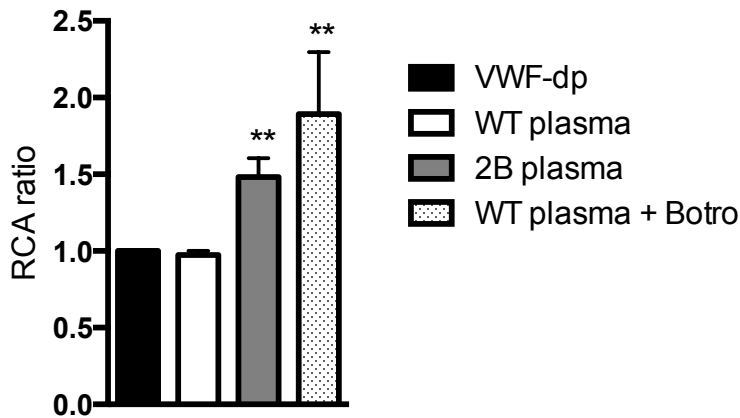
**In vivo desialylation.** WT mice received an intravenous injection of *C. perfringens*  $\alpha$ 2-3,6,8 neuraminidase. The platelet count and RCA binding were measured after 1 hour.

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### Supplemental data

**Supplemental Figure 1:** Histogram of RCA lectin binding on WT mouse platelets in the presence of VWF-deficient plasma (KO VWF), WT plasma, 2B plasma or WT plasma in the presence of botrocetin (5  $\mu\text{g}/\text{mL}$ ). The fold increase in each experiment was calculated with the VWF-deficient plasma as set as 1. (Mean  $\pm$  SD, n=3, One-way Anova followed by Dunnett's test. \*\*p<0.01).



**Supplemental Figure 2:** Histogram of MFI of MALII/streptavidin (left) and RCA (right) lectin binding on WT mouse platelets treated or not with neuraminidase (0.05 U/mL).

