Original Article

Blood group antigen A on von Willebrand factor is more protective against ADAMTS13 cleavage than antigens B and H

M. HAYAKAWA,[*] S. KATO,[*] T. MATSUI,[†] K. SAKAI,[*] Y. FUJIMURA,[‡] and M. MATSUMOTO[*]

[*]*Department of Blood Transfusion Medicine, Nara Medical University, Nara, Japan;* [†]*Clinical Laboratory Medicine, Graduate School of Health Sciences, Fujita Health University School of Health Sciences, Toyoake, Japan; and* [‡]*Japanese Red Cross Kinki Block Blood Center, Ibaraki, Japan*

Running Title: Blood group A inhibits VWF cleavage by ADAMTS13

Correspondence: Masanori Matsumoto, Department of Blood Transfusion Medicine, Nara Medical University, Shijyo-cho 840, Kashihara city, Nara 634-8522, Japan
Tel: +81 744 22 3051
Fax:+81 744 29 0771
E-mail: mmatsumo@naramed-u.ac.jp

Abstract: 247 words
Main text: 2972 words
Figure / Table: 4 / 1
Reference: 32
Supporting information: 1

Essentials

- ABO(H) blood group may influence the susceptibility of von Willebrand facor (VWF) to ADAMTS13.
- We have developed a quantitative ELISA of VWF-degradation product (VWF-DP) by ADAMTS13.
- Production of VWF-DP after exposure of high shear stress was validated in VWF multimer analysis.
- Blood group antigen A on VWF is more protective against ADAMTS13 cleavage than antigens B and H.

Summary. *Background:* ADAMTS13 specifically cleaves the peptide bond between Y1605 and M1606 within the von Willebrand factor (VWF)-A2 domain.
Objective: VWF contains ABO(H) blood group antigens, which may influence the susceptibility of VWF to ADAMTS13.
Methods: Using a unique monoclonal antibody recognizing the Y1605 residue, we have developed a sandwich ELISA to quantitatively analyze the generation of a VWF-degradation product (VWF-DP) by ADAMTS13.
Results: Production of VWF-DP after exposure to four different degrees of high shear stress was validated in comparison to the reduction in high molecular weight multimers using VWF multimer analysis. In analysis of plasma from 259 healthy individuals, plasma levels of VWF antigen (VWF:Ag) were significantly lower in blood group O than in the other groups, and were significantly correlated with plasma VWF-DP levels. The ratio between VWF-DP and VWF:Ag was significantly higher in blood group O than in blood groups A and AB. The ratio in blood group B was also significantly higher than those in A and AB, but did not differ from blood group O. Finally, to examine whether or not ABO(H) blood group antigens contributed to VWF cleavage, 82 plasma samples were exposed to high shear stress using a cone-plate shear stress inducer. The difference in the VWF-DP/VWF:Ag ratio before and after high shear stress in blood group O was significantly greater than those in groups A and AB.
Conclusion: These results indicate that blood group antigen A on VWF was more protective against ADAMTS13 cleavage than antigens B and H.

Key words: ABO(H) blood group, ADAMTS13, Shear stress, von Willebrand factor, multimer analysis

Introduction

von Willebrand factor (VWF) is a large, multimeric plasma glycoprotein that plays an essential role in regulating hemostasis and thrombosis [1]. Each VWF molecule can bind to several platelets, which then aggregate to form a platelet plug under high shear stress conditions. VWF is exclusively synthesized in and secreted from vascular endothelial cells as unusually large VWF multimers (UL-VWFMs) [2]. Larger VWFMs bind more actively to platelets under high shear stress [3]. In this condition, VWF is specifically cleaved by ADAMTS13 within the peptide bond between Y1605 and M1606 of the A2 domain [4]. Cleavage by ADAMTS13 down-regulates the function of VWF. Regulation of the molecular size of VWF is important for proper hemostasis, because circulation of UL-VWFM due to ADAMTS13 deficiency results in thrombotic thrombocytopenic purpura (TTP), and a lack of high molecular weight VWFMs (HMW-VWFMs) results in bleeding disorders such as type 2A von Willebrand disease (VWD).

A 250-kDa mature VWF subunit consists of 2050 amino acid residues and contains 13 N-linked carbohydrate moieties that express ABO(H) blood group antigens [5]. There are two N-linked carbohydrate moieties within the VWF-A2 domain [6]. Therefore, ABO(H) blood group antigens might affect the cleavage of VWF by ADAMTS13 [6, 7]. The antigens of the ABO blood system consist of complex carbohydrate molecules known as the A, B, and H determinants [8]. The A and B alleles of the ABO locus encode A and B glycosyltransferase activities; these modify the terminal sugar moiety of the precursor H antigen to produce the A or B determinant by addition of α-N-acetylgalactosamine (GalNAc) or α-D-galactose, respectively. Individuals with blood group O lack the transferase enzymes and have the basic H structure with a solitary terminal fructose moiety.

It is well established that plasma levels of VWF antigen (VWF:Ag) vary among individuals with different ABO(H) blood groups, in the rank order O<A<B<AB [9]. In people with blood groups A and B, plasma VWF:Ag levels of homozygotes (AA or BB) are higher than those of heterozygotes (AO or BO) [10]. In 2003, Bowen conducted a VWF multimer analysis and predicted that the rate of VWF proteolysis by ADAMTS13 was greatest in individuals with blood group O, and decreased among those with other groups in the following order: O≥B>A≥AB [11]. Recently, Rauch et al [12] used a novel enzyme-linked immunosorbent assay (ELISA) to demonstrate greater VWF proteolysis in group O than non-group O individuals, but they did not characterize the epitope of the monoclonal antibody (mAb) they used.

We previously succeeded in developing a unique mAb termed N10 that recognizes the

Y1605 residue exposed by ADAMTS13 cleavage [13], and here we describe the quantitative analysis of VWF proteolysis by ADAMTS13 with a sandwich ELISA using N10. Our previous study showed that N10 did not react with intact VWF, but rather with the monomeric and dimeric N-terminal VWF polypeptides (residues 764–1605) [13]. In this study, we validated the results of the novel ELISA using plasma exposed to very high shear stress conditions. We also found that blood group antigen A on VWF was more protective against ADAMTS13 cleavage than antigens B and H.

Methods

Sample collection

Citrated plasma samples obtained from 259 normal healthy volunteers were analyzed (Table 1). This study was conducted with the approval of the ethics committee of Nara Medical University. Written informed consent was obtained from all participants.

Reagents

The anti-ADAMTS13 monoclonal antibody A10 (IgG1-κ) [14] was used for inhibition of ADAMTS13 activity. A10-IgG completely inhibited ADAMTS13 activity at a final concentration of 50 ug/mL in the static assay [14]. Blood group A trisaccharide and blood group B trisaccharide were purchased from Dextra Laboratories, Ltd. (Reading, United Kingdom).

Quantitative ELISA to assess VWF proteolysis by ADAMTS13

The quantitative sandwich ELISA used to estimate the amount of plasma VWF-degradation product (VWF-DP) generated by ADAMTS13 was performed as follows. A 96-well microplate was coated with the primary mAb N10 (IgG, 10 μg/mL) [13]. Plasma samples were diluted 20-fold and applied to the coated plates. VWF-DP was detected by anti-VWF mAb, which recognized the N-terminal region of VWF. A value of 100% was defined as the amount of VWF-DP in pooled plasma from normal healthy volunteers.

Measurements

Plasma levels of VWF antigen (VWF:Ag) were measured by sandwich ELISA using rabbit anti-VWF polyclonal antibodies (DakoCytomation, Copenhagen, Denmark) [15]. Plasma levels of VWF collagen binding activity (VWF:CB) were analyzed by VWF:CB ELISA (PROGEN Biotechnik GmbH, Heidelberg, Germany).

Generation of high shear stress

1) Syringe method

To examine the effect of various degrees of high shear stress applied to plasma, we created an original instrument (Fig. S1) in which two 1-mL syringes (Terumo Corporation, Tokyo, Japan) were connected with an 18-gauge injection needle with an inner diameter of 0.47 mm (Terumo Corporation). Every second over a 6-minute period (360 times), plasma was transferred back and forth between the syringes to generate high shear stress.

Shear stress (τ) $\tau = 4\mu V/r$

μ: viscosity, V: average flow velocity, r: vessel inner diameter.

Assuming that plasma viscosity (μ) was 2.0 mPa·s [16], V was 634.5 mm/second for 108 dyne/cm^2 of shear stress.

Plasma volume (PV) to be replaced in one second = $V \cdot \pi r^2$

The PVs in this instrument were 440, 660, 880, and 1100 μL for 108, 162, 216, and 270 dyne/cm^2 of shear stress, respectively.

2) Cone-plate method

This was performed using a cone-plate shear stress inducer (Toray Medical, Tokyo, Japan), as previously described [17].

VWF multimer analysis

VWF multimer analysis was performed according to the method of Ruggeri and Zimmerman [18], with some modifications [19, 20]. Multimers were classified as HMW-VWFMs if they corresponded to bands >10 in the VWF multimer analysis [21]. The blots were scanned and subjected to densitometric analysis using ImageJ (National Institute of Health, Bethesda, USA). The HMW-VWFM ratio was calculated as the density of HMW-VWFMs relative to total VWFMs.

Statistical analysis

The values are expressed as the mean (minimum-maximum) because the distribution was not normal. Comparisons between the four blood groups were calculated using the Kruskal-Wallis H test. Significant differences between the four groups were further analyzed by the Mann-Whitney U test. Correlations between two variables were tested by Spearman rank correlation. A p value less than 0.05 was considered to be significant.

Results

Effects of various degrees of high shear stress in citrated plasma

Using the syringe method, various degrees of high shear stress (108, 162, 216, and 270 dyne/cm^2) were applied to normal citrate plasma samples obtained from one healthy volunteer (blood group AB). The experiments were repeated five times on different occasions, each time under three different conditions: citrated plasma alone [without ethylenediaminetetraacetic acid (EDTA) and A10-IgG] or citrated plasma with either EDTA (final concentration 5 mM) or A10-IgG (final concentration 50 μg/mL).

In citrated plasma alone, HMW-VWFMs clearly decreased with increasing shear rates, as shown in Fig. 1. In contrast, HMW-VWFMs were almost unchanged in the presence of EDTA or A10 (Fig. 1). These results were confirmed by densitometric analysis of HMW-VWFM ratios as shown in Fig. 2A, 2B, and 2C. HMW-VWFM ratios in plasma with EDTA or A10 did not correlate significantly with the degree of shear stress (0 to 270 dyne/cm^2). In contrast, in citrated plasma alone, the ratio decreased significantly with increasing shear stress (p<0.001, Fig. 2A).

ELISA quantification of VWF-DP generated under various high shear stress conditions

To validate our new ELISA method for the quantification of VWF-DP, we measured the plasma levels of VWF-DP using samples obtained from the abovementioned syringe-based experiments. A strong positive correlation ($r = 0.92$, $p < 0.001$) was found in citrated plasma alone between the plasma levels of VWF-DP and shear stress (Fig. 2D). On the other hand, VWF-DP was not generated in plasma with either EDTA or A10-IgG (Fig. 2E and 2F). ADAMTS13 cleavage of VWF was enhanced by high shear stress. Therefore, these results strongly suggest that the generation of VWF-DP is mainly dependent on the cleavage of VWF by ADAMTS13.

Differences in VWF:Ag and VWF-DP among the four ABO blood groups

Plasma levels of VWF:Ag were significantly different among the four groups (Fig. 3A). The levels in blood group O were significantly lower than those in groups A, AB, and B. The rank order of VWF:Ag plasma levels was AB≥B>A>O, which is consistent with the results of a previous report [9]. Plasma levels of VWF-DP also differed significantly among the four groups (Fig. 3B): those in blood group O were significantly lower than those in groups A, AB, and B. Notably, there was a significant relationship between the plasma levels of VWF:Ag and VWF-DP (Fig. S2). Thus, we analyzed the ratio of VWF-DP to VWF:Ag (VWF-DP/VWF:Ag ratio). This ratio differed significantly among the

four blood groups. Specifically, the VWF-DP/VWF:Ag ratio in blood group O was significantly higher than those in blood groups A and AB (Fig. 3C). The ratio in blood group B was also significantly higher than those in groups A and AB. Interestingly, the ratio in blood group B did not differ significantly from that in group O. Our novel ELISA clearly indicated that the degree of VWF proteolysis in normal individuals occurred in this rank order: O=B>AB=A.

Effects of high shear stress on VWF-DP in the four ABO blood groups

Plasma samples from 82 healthy individuals with different blood groups (Table S1) were exposed to high shear stress generated by a cone-plate shear stress inducer. These individuals were a subset of the abovementioned 259 volunteers, and the samples were freshly prepared for this experiment. VWF-DP was measured both before and after exposure to high shear stress. In the 82 individuals, plasma levels of VWF:Ag in blood group O were significantly lower than those in other blood groups (Fig. 4A). There were no differences in VWF-DP before and after high shear stress exposure (Fig. 4B). We then analyzed the ratio of the differences in VWF-DP and VWF:Ag before high shear stress (ΔVWF-DP/VWF:Ag). The VWF-DP/VWF:Ag ratio was significantly increased after exposure to high shear stress in all blood groups (Fig. S3). The ΔVWF-DP/VWF:Ag ratio varied significantly among the four blood groups (Fig. 4C). This difference ratio was significantly higher in blood group O than in groups A and AB. It was also significantly higher in blood group B than in group AB, but was similar in blood groups B and O. The rank order of the difference ratios was O≥B>AB=A, which was identical to the rank order of the VWF-DP/VWF:Ag ratios, as shown in Fig. 3C. These results indicate that blood group antigen A was more protective against ADAMTS13 cleavage than blood group antigens B and H.

Effect of blood group antigens A and B on ADAMTS13 cleavage

To analyze the effect of blood group antigens A and B on ADAMTS13 cleavage, we added blood group A or B trisaccharide (1.36 μM each) to blood group O plasma (O-plasma). Each plasma sample was exposed to a high shear stress of 270 dyne/cm^2 three times using the syringe method. Differences in VWF-DP before and after shear stress were not significantly different between O-plasma with blood group A trisaccharide, O-plasma with blood group B trisaccharide, and O-plasma alone (Fig. S4).

Discussion

In this study, we measured VWF-DP cleaved by ADAMTS13 using a novel ELISA based on monoclonal antibody N10, which specifically recognizes the Y1605 residue exposed by ADAMTS13 cleavage and does not react with the VWF-A2 domain before this cleavage [13]. In 2016, Rauch et al [12] reported a similar ELISA using a different, commercially available monoclonal antibody, but they did not report that antibody's precise epitope. We identified the exact epitope of N10 in a previous report [13]. In this study, we validated the quantification of reduced VWF-DP levels caused by high shear stress created with a syringe method (Fig. 1). In general, it is difficult to detect the cleavage products of multimeric VWF due to their heterogeneous lengths. However, this assay showed good correlation with the reduction of HMW-VWFMs after exposure to high shear stress.

This study confirmed previous findings that plasma VWF levels correlated with the amounts of A and B antigens on the VWF molecule [22, 23]. The ABO blood group carbohydrates linked to the plasma VWF molecule may influence its clearance from plasma, as may VWF biosynthesis by and secretion from vascular endothelial cells [24]. A recent study indicated that neither synthesis nor secretion of the VWF molecule was affected by ABO blood groups [25]. Therefore, plasma VWF levels were mainly controlled by the clearance of VWF from plasma. It is reported that plasma VWF:Ag levels are influenced by clearance via hepatocytes [26] or macrophages [27] in an ABO(H)-dependent manner, but this theory remains unaddressed and thus far no corresponding receptors have been identified [28]. The susceptibility of VWF to proteolysis by ADAMTS13 is another mechanism of VWF clearance [29]. Thus, plasma VWF levels are assumed to be partially regulated by ADAMTS13 cleavage, which is in turn influenced by ABO determinants [29].

It is highly conceivable that the ABO(H)-bearing carbohydrate moieties on the VWF molecule affect its susceptibility to ADAMTS13 cleavage [30]. In this study, VWF in individuals with blood group O was most sensitive to this cleavage, consistent with previously published results [11]. Furthermore, our ELISA using the mAb N10 also clearly indicated that VWF in individuals with blood group B was more sensitive than in those with blood group A. Bowen [11] already reported that the carbohydrate of blood group A was more protective than that of group B. Our study confirmed this observation using a novel and specific method. In fact, the difference between blood groups A and B correspond to the sugar chain structure on the protein or glycolipid. The blood group A antigen contains a terminal α-1,3-linked GalNAc, whereas the blood group B antigen has an α-1,3-linked galactose, suggesting that the *N*-acetyl group of GalNAc in the blood group A structure plays a key role in ADAMTS13 proteolysis via steric hindrance or

charge effects.

We examined the effect of blood group A trisaccharide (GalNAc-a-1,3-[Fuc-a-1,2] Gal) or B trisaccharide (Gal-a-1,3[Fuc-a-1,2]Gal) on ADAMTS13 activity in blood group O plasma (Fig. S5). There was no difference in the amount of VWF-DP induced by shear stress in the presence or absence of the trisaccharide, indicating that the free A or B trisaccharide moiety by itself has no effect on the proteolytic activity of ADAMTS13. In addition, the blood group H antigen on VWF in blood group O plasma was converted to the blood group A or B antigen by glycosyltransferase A or B. The converted blood groups A and B showed no difference in VWF-DP production following exposure to high shear stress using a syringe method (data not shown). The amount of blood group antigen A converted from blood group O might be insufficient to affect ADAMTS13 proteolysis of VWF. Moreover, these results might be affected by antibodies against the antigens of blood groups A and B in blood group O plasma. However, in the experiments involving shear stress, results were greatly affected by the properties of the solution, such as velocity. Therefore, we created shear stress in plasma using the syringe method in order to maintain consistency. We are now trying alternative methods to confirm the effect of the blood group A antigen.

This study had several limitations. First, the amount of VWF-DP was not quantified using a specific standard for measurement of degraded VWF by ADAMTS13. VWF-DP exists even in the plasma of healthy individuals, but its amount is extremely small relative to the amount of intact VWF. It was difficult for us to detect VWF-DP in a dose-dependent manner without using the ELISA method. Therefore, we quantified VWF-DP under four degrees of high shear stress (Fig. 2D), and demonstrated a relationship between VWF-DP and VWF cleavage by ADAMTS13. A second limitation is that we could not analyze the effects of high shear stress using a cone-plate inducer in all 259 samples due to the relatively large plasma volume necessary for this assay. Therefore, for this experiment we collected fresh samples from only 82 subjects. The third limitation is that we used two different methods to generate shear stress. The syringe method can induce much higher shear stress than the cone-plate method. While shear stress is generated via oscillatory motions using the syringe method, it is generated continuously with the cone-plate method. However, the degradation of VWF by ADAMTS13 due to high shear stress was observed with both methods.

ELISA for quantitative analysis of VWF-DP might be useful for the diagnosis of type 2A VWD and acquired von Willebrand syndrome (AVWS) induced by nonphysiologic high shear stress. Some cases of type 2A VWD are caused by a mutation in the VWF A2 domain that increases susceptibility to ADAMTS13 cleavage. Excessively high shear

stress in the blood stream, leading to AVWS, may be caused by the use of mechanical circulatory support systems as well as by various cardiovascular diseases, such as aortic stenosis and congenital structural disease. Heyde's syndrome is a well-known disease characterized by calcific aortic valve stenosis and massive gastrointestinal bleeding with no identified cause [31]. High shear stress arising from passage through a narrowed aortic valve enhances the proteolysis of VWF by ADAMTS13 and causes AVWS [32]. To date, these types of type 2A VWD and shear-induced AVWS are diagnosed by VWF multimer analysis, which identifies a lack of HMW-VWFMs. However, VWF multimer analysis is time consuming and difficult to perform in ordinary hospitals. The ELISA method used to measure VWF-DP in this study can be completed within 4 hours and is sensitive to VWF cleavage by ADAMTS13. Moreover, quantitative results might predict the severity of type 2A VWD and shear-induced AVWS.

In conclusion, we developed a novel ELISA technique that uses a monoclonal antibody targeting the VWF-Y1605 residue to quantitatively analyze VWF-DP cleaved by ADAMTS13. This system showed that blood group antigen A on VWF was more protective against ADAMTS13 cleavage than antigens B and H.

Addendum

M.H. performed experiments, analyzed data, and wrote the paper. S. K. performed experiments and prepared the materials. T.M. interpreted the results, and commented on the manuscript. K. S. performed research. Y. F. designed the research and wrote the manuscript. M.M. designed and directed the study and wrote the manuscript.

Acknowledgments

This work was supported by research grants from the Ministry of Health, Labour, and Welfare of Japan and from the Japan Agency for Medical Research and Development.

Disclosure of Conflict of Interests

K.S., Y.F., and M.M. are inventors of monoclonal antibody N10. The other authors have no conflict of interest.

Supporting Information

Additional supporting information added in the Supporting Information section.

Table S1 Characteristics of subjects whose plasma samples were exposed to high-shear stress

Figure S1. Syringe-based technique to generate high shear stress
We developed an original instrument to analyze the effects of various degrees of high shear stress. Two 1-mL syringes were connected with an 18-gauge injection needle. Plasma was transferred between syringes every 1 second to generate high shear stress.

Figure S2. Changes of VWF collagen binding activity
We analyzed VWF collagen binding activity (VWF:CB) using VWF:CB ELISA in the same plasma as Figure 2. First, we confirmed the change of VWF:Ag after high shear stress. VWF:Ag did not significantly change with shear stress in citrated plasma alone or in EDTA (n=5 for each shear stress) (A, B). Both VWF:CB and VWF:CB/VWF:Ag decreased with increasing shear stress in citrated plasma alone (C and E, $p<0.001$). In contrast, in citrated plasma with EDTA, VWF:CB decreased with shear stress (D, $p=0.016$) but VWF:CB/VWF:Ag did not (F). These results indicated that the HMW-VWFM ratio corresponded to the VWF-CB/VWF:Ag ratio.

Figure S3. Correlation between VWF-DP and VWF:Ag
Plasma levels of VWF degradation product (VWF-DP) significantly correlated with VWF antigen (VWF:Ag) in 259 healthy individuals ($r = 0.736$, $p<0.0001$).

Figure S4. Differences in the VWF-DP/VWF:Ag ratio before and after high shear stress
The ratio of the VWF degradation product (VWF-DP) to VWF antigen significantly increased after exposure to high shear stress (108 dyne/cm^2) in all four blood groups (**$p<0.01$). Differences were analyzed by the Wilcoxon t-test.

Figure S5. Effect of blood group antigens A and B against ADAMTS13 cleavage
To analyze the effect of blood group antigens A and B against ADAMTS13 cleavage, we added blood group A trisaccharide (1.36 μM) or blood group B trisaccharide (1.36 μM) to plasma from blood group O individuals (O-plasma). The syringe method was used to expose plasma samples to high shear stress (270 dyne/cm^2); this was repeated three times per sample. Differences in VWF-DP before and after high shear stress were not significantly different between the O-plasma with blood group with A trisaccharide, O-plasma with blood group with B trisaccharide, and O-plasma alone (control). Comparisons between the three groups were made using the Kruskal-Wallis H test. N.S.:

no significant difference.

References

1 Sadler JE. Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem*. 1998; **67**: 395-424.

2 Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, Schade AJ, McIntire LV, Fujikawa K, Lopez JA. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002; **100**: 4033-9.

3 Matsumoto M, Kawaguchi S, Ishizashi H, Yagi H, Iida J, Sakaki T, Fujimura Y. Platelets treated with ticlopidine are less reactive to unusually large von Willebrand factor multimers than are those treated with aspirin under high shear stress. *Pathophysiol Haemost Thromb*. 2005; **34**: 35-40.

4 Zheng XL. Structure-function and regulation of ADAMTS-13 protease. *J Thromb Haemost*. 2013; **11 Suppl 1**: 11-23.

5 Matsui T, Fujimura Y, Nishida S, Titani K. Human plasma alpha 2-macroglobulin and von Willebrand factor possess covalently linked ABO(H) blood group antigens in subjects with corresponding ABO phenotype. *Blood*. 1993; **82**: 663-8.

6 Chion A, O'Sullivan JM, Drakeford C, Bergsson G, Dalton N, Aguila S, Ward S, Fallon PG, Brophy TM, Preston RJ, Brady L, Sheils O, Laffan M, McKinnon TA, O'Donnell JS. N-linked glycans within the A2 domain of von Willebrand factor modulate macrophage-mediated clearance. *Blood*. 2016; **128**: 1959-68.

7 Preston RJ, Rawley O, Gleeson EM, O'Donnell JS. Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities. *Blood*. 2013; **121**: 3801-10.

8 Lowe JB. The blood group-specific human glycosyltransferases. *Baillieres Clin Haematol*. 1993; **6**: 465-92.

9 Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ, Jr., Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*. 1987; **69**: 1691-5.

10 Shima M, Fujimura Y, Nishiyama T, Tsujiuchi T, Narita N, Matsui T, Titani K, Katayama M, Yamamoto F, Yoshioka A. ABO blood group genotype and plasma von Willebrand factor in normal individuals. *Vox Sang*. 1995; **68**: 236-40.

11 Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *J Thromb Haemost*. 2003; **1**: 33-40.

12 Rauch A, Caron C, Vincent F, Jeanpierre E, Ternisien C, Boisseau P, Zawadzki C, Fressinaud E, Borel-Derlon A, Hermoire S, Paris C, Lavenu-Bombled C, Veyradier A, Ung A, Vincentelli A, van Belle E, Lenting PJ, Goudemand J, Susen S. A novel ELISA-based diagnosis of acquired von Willebrand disease with increased VWF proteolysis. *Thromb Haemost*. 2016; **115**: 950-9.

13 Kato S, Matsumoto M, Matsuyama T, Isonishi A, Hiura H, Fujimura Y. Novel monoclonal antibody-based enzyme immunoassay for determining plasma levels of ADAMTS13 activity. *Transfusion*. 2006; **46**: 1444-52.

14 Uemura M, Tatsumi K, Matsumoto M, Fujimoto M, Matsuyama T, Ishikawa M, Iwamoto TA, Mori T, Wanaka A, Fukui H, Fujimura Y. Localization of ADAMTS13 to the stellate cells of human liver. *Blood*. 2005; **106**: 922-4.

15 Bartlett A, Dormandy KM, Hawkey CM, Stableforth P, Voller A. Factor-VIII-related antigen: measurement by enzyme immunoassay. *Br Med J*. 1976; **1**: 994-6.

16 van Breugel HF, de Groot PG, Heethaar RM, Sixma JJ. Role of plasma viscosity in platelet adhesion. *Blood*. 1992; **80**: 953-9.

17 Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, Anbo H, Kawai Y, Watanabe K, Itagaki I. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest*. 1991; **87**: 1234-40.

18 Ruggeri ZM, Zimmerman TS. Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *J Clin Invest*. 1980; **65**: 1318-25.

19 Warren CM, Krzesinski PR, Greaser ML. Vertical agarose gel electrophoresis and electroblotting of high-molecular-weight proteins. *Electrophoresis*. 2003; **24**: 1695-702.

20 Budde U, Schneppenheim R, Plendl H, Dent J, Ruggeri ZM, Zimmerman TS. Luminographic detection of von Willebrand factor multimers in agarose gels and on nitrocellulose membranes. *Thromb Haemost*. 1990; **63**: 312-5.

21 Budde U, Drewke E, Mainusch K, Schneppenheim R. Laboratory diagnosis of congenital von Willebrand disease. *Semin Thromb Hemost*. 2002; **28**: 173-90.

22 O'Donnell J, Boulton FE, Manning RA, Laffan MA. Amount of H antigen expressed on circulating von Willebrand factor is modified by ABO blood group genotype and is a major determinant of plasma von Willebrand factor antigen levels. *Arterioscler Thromb Vasc Biol*. 2002; **22**: 335-41.

23 Morelli VM, de Visser MC, van Tilburg NH, Vos HL, Eikenboom JC, Rosendaal FR, Bertina RM. ABO blood group genotypes, plasma von Willebrand factor levels and loading of von Willebrand factor with A and B antigens. *Thromb Haemost*. 2007; **97**: 534-41.

24 Groeneveld DJ, van Bekkum T, Cheung KL, Dirven RJ, Castaman G, Reitsma PH, van Vlijmen B, Eikenboom J. No evidence for a direct effect of von Willebrand factor's ABH blood group antigens on von Willebrand factor clearance. *J Thromb Haemost*. 2015; **13**: 592-600.

25 Nossent AY, V VANM, NH VANT, Rosendaal FR, Bertina RM, JA VANM, Eikenboom HC. von Willebrand factor and its propeptide: the influence of secretion and clearance on protein levels and the risk of venous thrombosis. *J Thromb Haemost*. 2006; **4**: 2556-62.

26 Lenting PJ, Westein E, Terraube V, Ribba AS, Huizinga EG, Meyer D, de Groot PG, Denis CV. An experimental model to study the in vivo survival of von Willebrand factor. Basic aspects and application to the R1205H mutation. *J Biol Chem*. 2004; **279**: 12102-9.

27 van Schooten CJ, Shahbazi S, Groot E, Oortwijn BD, van den Berg HM, Denis CV, Lenting PJ. Macrophages contribute to the cellular uptake of von Willebrand factor and factor VIII in vivo. *Blood*. 2008; **112**: 1704-12.

28 Lenting PJ, Christophe OD, Denis CV. von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. *Blood*. 2015; **125**: 2019-28.

29 Gallinaro L, Cattini MG, Sztukowska M, Padrini R, Sartorello F, Pontara E, Bertomoro A, Daidone V, Pagnan A, Casonato A. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood.* 2008; **111**: 3540-5.

30 Federici AB, Elder JH, De Marco L, Ruggeri ZM, Zimmerman TS. Carbohydrate moiety of von Willebrand factor is not necessary for maintaining multimeric structure and ristocetin cofactor activity but protects from proteolytic degradation. *J Clin Invest.* 1984; **74**: 2049-55.

31 Heyde EC. Gastrointestinal bleeding in aortic stenosis. *N Engl J Med.* 1958; **259**: 196.

32 Vincentelli A, Susen S, Le Tourneau T, Six I, Fabre O, Juthier F, Bauters A, Decoene C, Goudemand J, Prat A, Jude B. Acquired von Willebrand syndrome in aortic stenosis. *N Engl J Med.* 2003; **349**: 343-9.

Table 1 Subject characteristics

blood group	A	AB	B	O
number	87	45	52	75
age (median)	23	23	23	23
age (range)	18-33	20-40	18-33	19-35
male/female	56/31	30/15	34/18	48/27

Figure legends

Figure 1. VWF multimer analysis of citrated plasma with or without EDTA or A10 after exposure to high shear stress

Citrated plasma samples were subjected to four degrees of high shear stress (108, 162, 216, and 270 dyne/cm^2) using a syringe method; for each shear stress level the experiment was repeated five times. VWF multimer analysis showed that the amount of high molecular weight VWF multimers (HMW-VWFMs) in citrated plasma without EDTA or A10 (citrated plasma alone) gradually decreased with increasing shear stress. In contrast, there were no apparent changes in HMW-VWFMs in citrated plasma with EDTA or A10 (inhibitory anti-ADAMTS13 monoclonal antibody).

Figure 2. Effects of high shear stress on HMW-VWF and VWF-DP

The HMW-VWFM ratio was calculated as the density of HMW-VWFMs relative to total VWFMs. In citrated plasma alone, the HMW-VWFM ratio decreased with increasing shear stress (n=5 in each shear stress) (A, $p<0.001$), and the VWF-DP/VWF:Ag ratio increased with increasing shear stress (n=5 in each shear stress) (D, $p<0.01$). In citrated plasma with either EDTA or A10, the HMW-VWFM ratio (B, E) and VWF-DP/VWF:Ag ratio (C, F) did not significantly change with shear stress. A significant relationship between the HMW-VWFM ratio and the VWF-DP/VWF:Ag ratio was found only in citrated plasma alone (n=25)(G, $p<0.001$). The relationship was not found in citrated plasma with neither EDTA nor A10 (H, I).

Figure 3. Comparison of VWF:Ag and VWD-DP in ABO(H) blood groups

A total of 259 healthy individuals (blood group A, n = 87; AB, n = 45; B, n = 52; O, n = 75) were analyzed. (A) Plasma levels of VWF antigen (VWF:Ag) in blood group O were significantly lower those than in the other groups. Those in blood group A were significantly higher than those in groups AB and B. The rank order of VWF:Ag plasma levels was AB≥B>A>O. (B) Plasma levels of VWF degradation products (VWF-DP) in blood group O were also significantly lower than those in other groups. (C) The ratio between VWF-DP and VWF:Ag was calculated in each group. The ratio in blood group O was significantly higher than those in groups A and AB. The ratio in blood group B was significantly higher than those in groups A and AB. The rank order of the ratios of VWF-DP/VWF:Ag was O=B>AB=A.

The values are expressed as mean (range). *$p<0.05$, **$p<0.01$.

Figure 4. Differences before and after high shear stress in ABO blood groups

Eighty-two plasma samples (blood group A, n = 24; AB, n = 18; B, n = 20; O, n = 20) were exposed to high shear stress generated by a cone-plate shear stress inducer. (A) Plasma levels of VWF antigen (VWF:Ag) in blood group O were significantly lower than those in other groups. (B) Differences in VWF degradation product (VWF-DP) before and after high shear stress did not differ among the four blood groups. (C) The ratio of the differences in VWF-DP and VWF:Ag (i.e., ΔVWF-DP/ΔVWF:Ag) in blood group O was significantly higher than those in groups A and AB. The difference ratio in blood group B was significantly higher than that in group AB. The rank order of the difference ratio was O≥B>AB=A. N.S.: no significant difference. * $p<0.05$, ** $p<0.01$.