The handle  http://hdl.handle.net/1887/35468 holds various files of this Leiden University dissertation.

**Author:** Groeneveld, Dafna Jordana  
**Title:** On the miscellaneous aspects of von Willebrand factor  
**Issue Date:** 2015-09-23
Part 2

Aspects of clearance of VWF
No evidence for a direct effect of von Willebrand factor’s ABH blood group antigens on von Willebrand factor clearance

Dafna Groeneveld¹, Tamara van Bekkum¹, Ka Lei Cheung¹, Richard Dirven¹, Giancarlo Castaman², Pieter Reitsma¹, Bart van Vlijmen¹ and Jeroen Eikenboom¹.

Published in Journal of Thrombosis and Haemostasis 2015; 13(4):592-600

¹Einthoven Laboratory for Experimental Vascular Medicine, Department of Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, The Netherlands, ²Center for Bleeding Disorders, Department of Heart and Vessels, Careggi University Hospital, Florence, Italy.
Abstract

**Background:** One of the major determinants of von Willebrand factor (VWF) plasma levels are the ABO blood groups, and individuals with blood group O have approximately 25% lower plasma levels. The exact mechanism behind this relationship remains unknown, although effects on clearance have been postulated. **Objectives:** Whether clearance of VWF is directly dependent on the presence of ABH antigens on VWF. **Methods:** Three type 3 von Willebrand disease (VWD) patients were infused with Haemate-P® and the relative loading of VWF with ABH antigens at different time points was measured. VWF-deficient mice were injected with purified plasma-derived human VWF obtained from donors with either blood group A, B or O. **Results:** In mice we found no difference in clearance rate between plasma derived A-, B- or O-VWF. Faster clearance of the blood group O VWF present in Haemate-P® infused in type 3 VWD patients would have resulted in a relative increase in the loading of VWF with A and B antigens over time. However, we observed a 2-fold decrease of A and B antigens in 2 out of 3 patients and stable loading in the third patient. **Conclusion:** There is no direct effect of ABH antigens on VWF in VWF clearance. We demonstrate that in a direct comparison within one individual O-VWF is not cleared faster compared to A- or B-VWF. Clearance differences in blood group O versus non-O individuals may therefore be related to the blood group status of the individual rather than the ABH antigen loading on VWF itself.

Introduction

When activated upon vascular damage, von Willebrand factor (VWF) supports platelet adhesion and activation and initiates platelet plug formation [1]. VWF is synthesized in endothelial cells and megakaryocytes and is stored in cell-specific organelles, respectively Weibel-Palade bodies (WPB) and α-granules [2,3]. Part of the synthesized VWF is secreted constitutively into the blood, where it has a half-life of about 12 hours [4].

The variation in plasma VWF levels is 66% genetically determined and the ABO blood group locus accounts for 30% of that variation [5]. VWF is decorated with ABO blood group sugars and those ABH antigens are attached to the N-linked oligosaccharide chains [6]. Several studies have shown that ABO blood groups are associated with VWF plasma levels [7,8]. Individuals with blood group O have approximately 25% lower VWF plasma levels. Individuals with the Bombay blood group, lacking expression of ABH antigens, even have lower VWF levels [9]. How ABO blood groups influence VWF levels exactly remains unknown.

The VWF levels correlate with the amounts of A and B antigens present on the VWF molecule [10,11]. The ABH antigens could have an effect on biosynthesis, secretion,
and clearance of VWF or a combination thereof. However, ABH antigens on VWF do not play a role in synthesis and secretion as the plasma VWF propeptide levels are independent of ABO blood group [12,13]. In endothelial cells derived from different blood group donors, the constitutive VWF secretion showed no difference, however those cells did not express ABH antigens on their surface nor on VWF [14]. Another study showed that the rise in VWF level after desmopressin treatment appears to be similar in patients with type 1 von Willebrand disease (VWD) with different blood groups, suggesting that secretion efficiency is not affected by blood group [15].

Nossent et al.[12] estimated, that the half-life of endogenous VWF in healthy controls with blood group O is almost 2 hours shorter compared with blood group non-O controls. In healthy individuals the VWF half-life after DDAVP administration was also shorter in blood group O individuals [16]. Furthermore, the ratio in plasma between VWF propeptide and mature VWF, which are released in equimolar amounts, is increased in individuals with blood group O compared with non-O individuals. This suggests faster clearance of mature VWF in blood group O individuals [12,13,17]. These observations clearly show that VWF plasma levels in individuals with blood group O are lower due to faster clearance of VWF. However, this supposition is not yet challenged in a direct comparison. All studies investigated the clearance of O-VWF in blood group O individuals. The question remains whether clearance differences are to be attributed to the blood group antigens on the VWF molecule itself or to the ABO blood group status of the individual that clears the VWF protein. The present study is the first investigating simultaneous VWF clearance of the different blood groups in a direct comparison.

**Methods**

**Mice**

VWF-deficient (VWF-/-) female mice on a C57BL/6J background (embryos originally obtained from Jackson Laboratories (Bar Harbor, ME, USA) were between 8 to 12 weeks old. Mice were housed under a 12-h light/dark cycle. Standard diet and drinking water were provided *ad libitum*. All procedures were approved by the animal welfare committee of the Leiden University (under registration #10037).

**Patients**

After written informed consent, VWF concentrate (Haemate-P®, CSL Behring, Marburg, Germany) was infused in three type 3 VWD patients (2 females and 1 male, all blood group O) in a dosage of 50 units FVIII per kg body weight. Blood samples were collected before administration of Haemate-P®, and at 10 min, 1, 2, 4, 6, 8, and 24 hours thereafter. The study was approved by the local ethical review committee of the Leiden University Medical Center.
VWF purification
Fresh frozen plasma from donors with blood groups A (n=3), B (n=3) or O (n=3) (San-quin, Leiden, The Netherlands), was cryoprecipitated and precipitates were dissolved in elution buffer (25mM TEA, 15mM sodium citrate, 150mM NaCl, pH 7.5). Plasma VWF was separated by gel filtration chromatography on a Sepharose 4B column (GE Healthcare, Little Chalfont, UK). Collected fractions were measured for VWF antigen (VWF:Ag) [18]. VWF purified from donors with blood group O, containing only the H-antigen, is indicated as O-VWF. VWF purified from A and B donors is indicated as A-VWF (A-antigen present) and B-VWF (B-antigen present).

Clearance of plasma derived human VWF in mice
Female VWF-/- mice were subcutaneously injected with 50 µl Temgesic (0.1 mg/kg) for analgesia one hour prior to experiments. Three different human blood donors were used for each blood group, and five mice were used per human blood donor, so a total of 15 mice were used for each blood group. Fortyfive mice were injected into the tail vein with 200 µl of 2U/ml purified plasma derived (pd)-VWF. At 1, 15, 30, 60, 120, 240 and 360 minutes after injection, 50 µL of citrated-blood (final concentration of 0.32%) was collected via tail transection and tails were subsequently cauterized. After centrifugation (2500g for 15min), plasma was collected and human VWF:Ag was measured [18].

Macrophage depletion in mice
Female VWF-/- mice (n=4 per group) were injected intravenously with either 200µl/mouse clodronate liposomes (gift of Roche Diagnostics GmbH, Mannheim, Germany) or saline (200µl/mouse) as a control. Two days after treatment, mice were injected via tail injection with 200 µl Haemate-P® (5U/ml VWF:Ag) and citrated-blood was collected at several time points for determination of plasma VWF:Ag levels and clearance rates. Macrophage depletion was confirmed by staining liver and spleen cryostat sections with an antibody to a specific mouse macrophage marker F4/80 (AbD Sero-tec, Oxford, UK).

Measurement of A, B and H antigen expression on VWF
The amount of loading on VWF with A and B antigens was quantified as described [11]. Immobilized anti-VWF IgG (A0082, DAKO, Glostrup, Denmark) was saturated with VWF from the sample. Next, immobilized VWF is saturated with murine monoclonal anti-A or anti-B antibody (Immu Clone, Immucor, Rodermark, Germany). The amount of bound anti-A or anti-B antibody is quantified using an HRP-labelled goat anti-mouse IgM (Nordic Immunological laboratories, Tilburg, The Netherlands). The peroxidase activity of a sample was normalized against the peroxidase activity obtained with an A1 or B reference plasma sample, and the results were expressed as normalized ratios (nA-ratio or nB-ratio) [11]. The amount of H antigens expressed on
VWF was measured similar to O'Donnell et al. [19]. Plates were coated with anti-VWF Ig (DAKO), washed and incubated for 2 hours with plasma samples (1:20, 1:40, 1:50 and 1:100 dilution). Plates were washed and incubated for 2 hours with Lectin from Ulex europaeus/UEA-I-HRP (1:150 dilution, AMS Biotechnology, Abingdon, UK) and peroxidase activity was measured. Blood group O plasma from a healthy control was used as standard curve and was assigned a value of 1 U/ml. To determine the percentage of H antigen expressed per unit VWF, the amount of H antigen detected was divided by the amount of VWF:Ag present in the ELISA well and multiplied by 100.

**Detection of A, B or H antigen on VWF using Western Blotting**

Plasma samples were reduced using 20 mM dithiothreitol, and separated by Novex 6% (A and B antigens) or Novex 4% (H-antigen) Tris-Glycine gel electrophoresis (Invitrogen, Carlsbad, CA, USA). Equal amounts of VWF protein were loaded. Gels were immunostained with polyclonal rabbit anti-human VWF antibody conjugated to HRP (DAKO). Duplicate lanes were immunostained with murine monoclonal anti-A or anti-B antibody (Immucor) (1:10 dilution) or with Lectin from Ulex europaeus/UEA-I-HRP (AMS Biotechnology) (5µg/ml). Blots for detection of A and B antigens were subsequently incubated with a HRP-labelled goat anti-mouse IgM (Nordic). Staining was visualized with Supersignal WestFemto (Thermo Scientific, Rockford, IL, USA).

**Pharmacokinetic data of infused VWD patients**

In a previous study [20], ten type 1 VWD, ten type 2A VWD and eight type 3 VWD patients received a single i.v. VWF concentrate infusion with a median of 79 IU/kg VWF:RCo (32 IU/kg FVIII:C). From that study we obtained the calculated half-lives of VWF:RCo and VWF:Ag [20]. Another six type 3 VWD patients received a single i.v. VWF concentrate infusion with a median of 45 (range 25-50) IU/kg FVIII:C. Plasma samples were drawn at different time points and assayed for VWF:Ag and VWF:RCo and half-lives were calculated.

**Data analysis and statistics**

Analysis of pharmacokinetic data was performed using GraphPad Prism (version 6.0, La Jolla, CA, USA). Clearance data from mice experiments were fitted to the two phase exponential decay equation \( Y=A\times e^{(-k_1\times t)}+B\times e^{(-k_2\times t)}+\text{Plateu} \) to obtain \( A, k_1, B, \) and \( k_2 \). These parameters were used to calculate mean residence time (MRT) employing the equation; \( \text{MRT}=(A/k_1^2+B/k_2^2)/(A/k_1+B/k_2) \) as previously described [21]. Clearance data from type 3 VWD patients were fitted to the one phase exponential decay equation \( Y=A\times e^{(-kt)} \) to obtain \( k \). Half-lives were calculated using the first order equation; \( t_{\frac{1}{2}}=\text{ln}2/k \). Quantification of western blots was performed using Fiji (Fiji Is Just Image, http://fiji.sc). Statistical analyses were performed by the Student’s unpaired t-test or One-Way ANOVA followed by Tukey’s multiple comparisons test.
Results

VWF clearance is not influenced by ABO blood group in mice

We first investigated whether sampling strategy has any influence on the clearance of VWF in VWF -/- mice. As shown in Figure 1A, no difference in decay curves of injected Haemate-P® was observed between mice that were sampled only once (MRT 2.2h±0.9) and mice that were sampled multiple times (MRT 2.0h±0.8). Further experiments were therefore done using the multiple sampling approach. Furthermore, the amount of injected Haemate-P® did not affect clearance rates (low dose VWF, MRT 2.3h±0.6 versus high dose VWF, MRT 2.5h±0.5) (Figure 1B).

As shown in Figure 1C no significant difference was observed in clearance rate between A-VWF (MRT 1.8h±0.3), B-VWF (MRT 1.4h±0.2) or O-VWF (MRT 1.9h±0.3).

Figure 1. Clearance of pd-VWF from blood groups A, B and O donors in VWF -/- mice. Panel A; In one group (single sampling, grey triangle) different mice (total n=12, n=3 per time point) were sampled at different time points after injection with 200μl Haemate-P® (5U/ml VWF:Ag); each mouse was sampled only once. In the second group (n=3, multiple sampling, black square) each mouse was sampled several times at different time points. Panel B; two different dosages (2.5U/ml and 50U/ml) of Haemate-P® were injected into VWF -/- mice (n=10 per dosage). Panel C; clearance profiles of human plasma derived-VWF from donors with either blood group A, B or O in VWF -/- mice (n= total of 15 per blood group, 5 per donor). Panel D; loading of VWF with A and B antigens in VWF -/- mice after injection with 200μl (10U/ml VWF:Ag) Haemate-P® [11]. Because VWF:Ag levels at the last two time points were below the threshold of the assay, loading was not measured for these time points. Data represent mean ±SD.
When clearance rates of A-VWF and B-VWF were combined into non O-VWF (MRT 1.6h±0.3) there was also no differences in clearance compared with O-VWF (MRT 1.9h±0.3, p=0.2). These results suggest that in mice VWF clearance rate is not influenced by ABH antigens present on VWF. In another approach, VWF-/- mice were injected with Haemate-P®, a pd-VWF concentrate obtained from a pool of donors including all ABO blood groups. Injection of Haemate-P®, and subsequent measurement of the changes over time in the loading of A and B antigens on VWF, facilitates the simultaneous investigation of differential clearance of A-, B- and O-VWF in a single test animal. In case ABH antigens influence clearance of VWF, the proportions of O-VWF, A-VWF and B-VWF in the circulating pool of VWF will change over time. Thus, the normalized ratio of A and B antigen on VWF (expressed as nA and nB) in the pool of VWF should also change over time. The loading with A and B antigen should increase when O-VWF is cleared faster. The loading of VWF with A- and B antigens however, was not significantly affected over time (p for linear trend 0.06 and 0.79 for A- and B-antigen respectively, Figure 1D) indicating no effect of ABH antigens on VWF clearance. To investigate whether Kupffer cells mask a possible ABO dependant uptake of VWF by other cells in the liver, we depleted macrophages in VWF -/- mice. Depletion of macrophages by clodronate liposomes lead to a significant decrease in clearance rate of VWF (MRT 3.1h ±0.83) compared with saline treated animals (MRT 2.1h ±0.36, p <0.05) (Figure 2A), confirming that Kupffer cells contribute to VWF clearance. However, after depletion of Kupffer cells there was still no ABO dependent clearance observed (Figure 2B-C).

**Blood group O VWF is not cleared faster in VWD patients**

Since our mouse model has its limitations due to possible differences in clearance mechanisms of VWF compared to humans, we also studied clearance of VWF in humans. As no blood group specific purified VWF is available for infusions in humans, we infused three type 3 VWD patients with Haemate-P® (Table 1 for patient characteristics) and measured the amount of A and B antigens present on VWF. This experimental set-up allows to measure simultaneous VWF clearance of the different blood...
groups in a direct comparison in one individual. As described in the previous paragraph, faster clearance of O-VWF would lead to increased normalized ratios of A and B antigen on VWF in time. However, the opposite was observed for two patients; a 2-fold decrease in the amount of A and B antigen loading on VWF during infusion. Interestingly, in the third patient the loading of both A and B antigens on VWF remained steady over time (Figure 3A). The amount of loading with A and B antigens was also confirmed using the non-saturating assay as described by O’Donnell et al [10] (data not shown).

In principle, endogenous anti-A and anti-B antibodies could shield the antigens on VWF to the antibodies used in the ELISA. However, we could not detect direct antibody binding by adding an anti IgM antibody to captured VWF from the samples (data not shown). Furthermore, Western-blotting confirmed the decrease in the amount of antigen loading on VWF at the latest time point for patient 2, while the amount of antigen loading on VWF for patient 3 remained the same (Figure 3B). The decreased loading therefore reflects a true decrease in VWF loaded with A and B antigens. Also the loading of H-antigens on VWF was declining in time and analysis of the Western blots showed that the density of the bands was also decreasing in time (Figure 3C, D, Table 2). A- and B-VWF are thus not cleared faster in our patients, leading to the reduced normalized ratios (Figure 3A, B) since the relative amount of O-VWF present in the circulation would then increase over time. These results suggest that ABH antigens are removed from the VWF molecule.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Time (min)</th>
<th>FVIII:C (IU/mL)</th>
<th>VWF:Ag (IU/mL)</th>
<th>VWF:RCo (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.08</td>
<td>&lt;0.04</td>
<td>&lt;0.20</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.85</td>
<td>2.14</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.73</td>
<td>1.61</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>0.70</td>
<td>0.60</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.11</td>
<td>&lt;0.04</td>
<td>&lt;0.20</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.17</td>
<td>3.20</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.17</td>
<td>3.20</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>1.11</td>
<td>2.65</td>
<td>2.01</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>1.04</td>
<td>2.45</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>1.01</td>
<td>2.18</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>0.99</td>
<td>1.90</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>1440</td>
<td>0.71</td>
<td>0.55</td>
<td>0.40</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>&lt;0.03</td>
<td>&lt;0.04</td>
<td>&lt;0.20</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.16</td>
<td>2.88</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.16</td>
<td>2.74</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.85</td>
<td>2.38</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>0.84</td>
<td>1.84</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>0.87</td>
<td>1.55</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>0.85</td>
<td>1.30</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>1440</td>
<td>0.70</td>
<td>0.18</td>
<td>&lt;0.20</td>
</tr>
</tbody>
</table>
Figure 3. Clearance of Haemate-P® in VWD type 3 patients. Panel A; loading of VWF with A (black bars) and B (grey bars) antigens at different time points. Data represent the mean loading from duplicate wells. Panel B; Western blot of plasma samples after reducing conditions from time points 10 min, 6 hours and 24 hours after infusion of patients 2 and 3. Blots were stained for VWF, A-antigen and B-antigen. Panel C; Western blot of plasma samples after reducing conditions from time points 10 min, 2 hours (for patient 3), 6 hours, 8 hours and 24 hours (for patient 2) after infusion. Blots were incubated with anti-VWF and UEA-I lectin. Due to the low VWF:Ag level, the latest time point for patient 3 was excluded. Equal amounts of VWF:Ag were loaded per lane (see also Table 2 for quantification of the western blots). Panel D; loading of VWF with H antigen at different time points for patient 2 (black bars) and 3 (grey bars). Due to limited availability of plasma from patient 1, this patient was omitted. Data represent mean±SD from 2 independent dilutions measured in duplicate.

<table>
<thead>
<tr>
<th>Time point (min)</th>
<th>Area</th>
<th>Percentage</th>
<th>VWF</th>
<th>H-antigen</th>
<th>VWF</th>
<th>H-antigen</th>
<th>Relative density*</th>
<th>Corrected density†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>10</td>
<td>25049806</td>
<td>20033434</td>
<td>25</td>
<td>30</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>20702492</td>
<td>17463898</td>
<td>20</td>
<td>26</td>
<td>0.83</td>
<td>0.87</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>23908271</td>
<td>15613522</td>
<td>24</td>
<td>24</td>
<td>0.95</td>
<td>0.78</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>1440</td>
<td>31636454</td>
<td>13187108</td>
<td>31</td>
<td>20</td>
<td>1.26</td>
<td>0.66</td>
<td>0.52</td>
</tr>
<tr>
<td>Patient 3</td>
<td>10</td>
<td>32375999</td>
<td>24766806</td>
<td>29</td>
<td>35</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>28513342</td>
<td>17474078</td>
<td>26</td>
<td>26</td>
<td>0.88</td>
<td>0.74</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>27722856</td>
<td>10558492</td>
<td>25</td>
<td>21</td>
<td>0.86</td>
<td>0.59</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>480</td>
<td>22476170</td>
<td>10691593</td>
<td>20</td>
<td>19</td>
<td>0.69</td>
<td>0.53</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* Time point 10 minutes is set as a standard with a relative density of 1 for both VWF and H-antigen band. Rest of bands are relative to t=10min.
† Corrected density was calculated by dividing the relative density of H-antigen band by the relative density of VWF band.
Clearance of Haemate-P® in blood group O vs non-O VWD patients

To investigate whether the ABO blood group status of the individual influences the clearance rather than the ABH antigen loading of the VWF protein per se, we studied the clearance of Haemate-P® in VWD patients with blood group O and non-O. The half-lives of both VWF:Ag and VWF:RCo were shorter in type 3 VWD patients with blood group O compared to patients with non-O (8.4h, 95%CI 3.9-12.9 vs 9.6h, 95%CI 3.2-16.0 for VWF:Ag and 5.8h, 95%CI 3.0-8.5 vs 8.6h, 95%CI 4.3-13.0 for VWF:RCo, Figure 4A), however this difference was not statistically significant, probably due to the small group size. The same difference was also observed in type 1 (10.9h, 95%CI 6.1-15.8 versus 16.1h, 95%CI 10.9-21.3 for VWF:Ag and 5.7h, 95%CI 3.9-7.5 versus 10.5h, 95%CI 5.3-15.6 for VWF:RCo) and type 2A VWD patients (6.9h, 95%CI 2.0-11.8 versus 11.0h, 95%CI 3.6-18.3 for VWF:Ag and 3.8h, 95%CI 0.7-6.9 versus 9.0h, 95%CI 4.8-13.1 for VWF:RCo) (Figure 4B,C). These preliminary results support our hypothesis that blood group O individuals clear VWF faster than blood group A or B individuals independent of the blood group status of VWF.

Discussion

In this study we investigated the assumed faster clearance of VWF from individuals with blood group O. Since we cannot measure possible differences in clearance rates of human VWF from different donors simultaneously in humans, we first investigated whether we could detect a clearance difference in VWF -/- mice. This mouse model has advantages since it allowed us to 1) measure simultaneous VWF clearance of different blood groups in a direct comparison and 2) measure clearance profiles of VWF puri-
fied from individuals with either blood group A, B or O. However, in VWF -/- mice we did not observe differences in the clearance of purified human A-, B- or O-VWF. We also did not observe the expected increase in the relative amount of A and B antigen loading on VWF in VWF -/- mice injected with Haemate-P®. In Haemate-P® infused type 3 VWD patients we could also not observe the expected increase.

Mice have genes homologous to the human A-, B- and H-transferase genes. However, the enzymatic activity is much lower than the human homologues [22]. The expression of the genes is limited to a few tissues [23]. Clearance of VWF in mice might therefore not be dependent on the ABH antigens present on VWF, either because the VWF clearance system is different than in humans or because mice might not recognize human ABH antigens on VWF. This could explain why we did not observe a faster clearance of O-VWF. However, although the enzymatic activity is lower, mice do express the transferase genes. It is therefore more likely that the VWF clearance system in mice is able to recognize human ABH antigens present on VWF. This is supported by results from a study of Wolofsky et al. who observed increased phagocytosis of infected human blood group O erythrocytes compared to infected blood group A and B erythrocytes in mice [24].

Mouse ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) does not cleave human VWF very efficiently [25], so whether proteolysis has an influence on ABO dependent VWF clearance could not be answered. Increased susceptibility to ADAMTS13 proteolysis of O-VWF has been postulated as a mechanism for increased clearance of O-VWF. However, basal VWF levels are similar in normal and ADAMTS13 -/- mice [26]. Additionally, wild type VWF and the p.Ser-1618Pro VWF variant, which results in more susceptibility to ADAMTS13 cleavage, had similar clearance rates in a rat model [27]. It is therefore unlikely that (potential) increased ADAMTS13 susceptibility of O-VWF would lead to an accelerated clearance.

Given the limitations of the mouse model, we also investigated the assumed faster clearance of O-VWF in patients with type 3 VWD. By infusion of Haemate-P®, we were able to study clearance of VWF loaded with A, B and H antigens simultaneously in one individual, excluding any variability between subjects and variation over time. To our surprise, loading of VWF with A and B antigens decreased over time. This decrease could not be explained by simple shielding of antigens in vivo. Faster removal of A- and B-VWF compared to O-VWF would lead to a decreased ratio, although this would be contradictory to the assumed faster clearance of O-VWF. However, the amount of H-antigen present on VWF also decreased during clearance. The decrease in A, B and H-antigen loading indicates that either the A, B and H-antigens are removed from the VWF molecule or heavily glycosylated VWF is cleared faster, leading to the reduced amounts of loading. All three patients have blood group O, and whether interaction between endogenous Anti-A and Anti-B antibodies and the antigens on VWF could influence the clearance of VWF, by subsequent clearance of the whole complex or by removing ABH antigens on VWF, is as yet unknown. However, we could not detect direct
antibody binding to antigens on VWF in our plasma samples, nor could we demonstrate antibody binding to ABH antigens on VWF by incubating VWF deficient plasma from different blood group donors with Haemate-P®. The ideal experimental set-up to tackle this problem would be infusing a VWD type 3 patient with blood group AB, thereby excluding possible antibody-antigen interaction, however this type of patient is very rare [28], and was not available.

In 2 infused patients we observed a 2-fold decrease in A and B antigen loading, but in the other patient we did not observe such a strong decrease in antigen loading. We have no reasonable explanation for the discrepancy between patients 1 and 2 versus patient 3. There is no difference in the amount of A- and B-antibodies present, as antibody titers between the three patients are comparable, nor have the patients received different batches of Haemate-P®.

Taking together the results, we could not demonstrate the assumed faster clearance of O-VWF. It could be that the association between ABO blood group and plasma VWF levels is not just via an effect on VWF clearance but could include individual differences, for example receptor mediated uptake of VWF [29]. Mannucci et al. [30], found that their recombinant VWF product, which lacks the ABH antigens, had a similar pharmacokinetic profile compared with human pd-VWF in patients with VWD. This findings support our results that ABH antigens on VWF itself do not affect clearance.

Nevertheless, there is overwhelming evidence suggesting increased VWF clearance in individuals with blood group O [12,13,16,17]. However, all those studies investigated the clearance of O-VWF in a blood group O individual and A-VWF in a blood group A individual. Since we demonstrated that within one individual O-VWF is not cleared faster compared to A- or B-VWF, we postulate that the ABO blood group dependent differences in VWF clearance depend on the ABO blood group status of the individual and not on the ABH antigens on VWF per se. Since VWF is the carrier protein of factor VIII (FVIII) and clearance of FVIII is largely dependent on VWF, clearance of (infused) FVIII will most likely also be dependent of the blood group status of the individual. This hypothesis is supported by our preliminary results of a shorter half-life in blood group O individuals, independent of the ABO blood group of the circulating VWF. However more pharmacokinetic data is required to substantiate this assumption.

**Authorship Contributions**

D.J. Groeneveld designed the study, performed the research and data analysis and wrote the manuscript; T. van Bekkum., K. Cheung, and R.J. Dirven performed part of the research and reviewed the manuscript; G. Castaman performed part of the research and reviewed the manuscript. P.H. Reitsma discussed and interpreted the data, and reviewed the manuscript; B. van Vlijmen designed the study, performed part of the research, interpreted the data and reviewed the manuscript; J. Eikenboom designed the study, interpreted the data, and contributed to writing and reviewing of the manuscript. All authors approved the final version of the manuscript.
Acknowledgements
CSL Behring bv (Breda, The Netherlands) provided supplies of Haemate-P® to conduct the study.

Disclosure of Conflicts of Interest
The authors declare no conflict of interest.
References


