Up-regulation of platelet activation in hemophilia A

Esther R. van Bladel,[1] Mark Roest,[2] Philip G. de Groot,[2] and Roger E.G. Schutgens[3]

[1]Department of Clinical Chemistry and Hematology/Van Creveldlaboratory, University Medical Center Utrecht, Utrecht; [2]Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht; and [3]Van Creveldkliniek/Department of Hematology, University Medical Center Utrecht, Utrecht, the Netherlands

ABSTRACT

Background
Platelets are an underappreciated factor in the classification of the bleeding tendency of patients with hemophilia. In this cross-sectional study, we investigated platelet activation status and responsiveness in relation to residual factor VIII activity and, within the group with severe hemophilia (<1% residual factor VIII activity), to annual factor VIII consumption.

Design and Methods
Twenty-one patients with mild-moderate hemophilia A, 13 with severe hemophilia A and 21 healthy controls were studied. The basal level of platelet activation and platelet responsiveness to activation and inhibition were determined by the measurement of platelet P-selectin expression and soluble platelet activation markers.

Results
Patients with severe hemophilia A had a higher percentage of activated platelets at baseline (15.9%) when compared to patients with mild-moderate hemophilia A (8.2%, P=0.014) and controls (6.4%, P<0.001). Both patients with mild-moderate hemophilia A and those with severe hemophilia A had higher levels of the soluble platelet activation markers platelet factor 4 (1.4 and 1.8 pg/10^6 platelets), CXCL7 (65.8 and 48.2 pg/10^6 platelets) and RANTES (12.8 and 9.5 pg/10^6 platelets), compared to controls (platelet factor 4: 0.3 pg/10^6 platelets, P<0.001 and <0.001; CXCL7 20.0 pg/10^6 platelets, P<0.001 and <0.001; RANTES 4.5 pg/10^6 platelets, P<0.001 and =0.003, respectively). In support of these observations, we found clinical evidence that higher platelet P-selectin expression correlates with lower factor VIII consumption in patients with severe hemophilia (Spearman's r -0.65, P=0.043).

Conclusions
This study indicates that platelets from patients with severe hemophilia A are in a pre-activated state and that this pre-activated state is associated with factor VIII consumption.

Key words: hemophilia A, platelets, activation, up-regulation, FVIII consumption.

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Correspondence:
Esther R. van Bladel, Department of Clinical Chemistry and Hematology/Van Creveldlaboratory, University Medical Center Utrecht, Heidelberglaan 100 (G03.550), 3584 CX Utrecht, The Netherlands.
E-mail: e.bladel@umcutrecht.nl

Introduction

Hemophilia A is an X-linked disorder of coagulation factor VIII (FVIII). Patients with hemophilia suffer from a bleeding tendency of various severity. Currently, the bleeding severity is roughly classified by the residual FVIII activity of the patient. In general, patients with lower FVIII activity levels have a more severe clinical phenotype than patients with higher FVIII activity.[1] Accordingly, hemophilia A is classified into mild (FVIII activity 6-40%), moderate (FVIII activity 1-5%) and severe (FVIII activity <1%).[2,3] Although this classification based on residual FVIII activity is associated with clinical phenotype, it is not a precise predictor of the bleeding pattern in individual patients. Within the group of patients classified as having severe hemophilia A, up to 10% of patients have a mild clinical phenotype,[4,5] indicating that residual FVIII activity level is not the sole determinant of clinical phenotype.

Differences in bleeding phenotype within the hemophilia A classifications are not completely understood. Prothrombotic factors, FVIII half-life, genotype and fibrinolytic activity did not correlate with bleeding patterns in individual patients.[6,7] No information is available on the contribution of platelets to the clinical phenotype in hemophilia. In normal hemostasis, platelets and the coagulation system interact with each other to form a plug at the site of vascular damage.[8] Platelets are the most important source of negatively charged phospholipids. We hypothesized that the reduced coagulation caused by the absence of FVIII could be (partly) compensated for by increased platelet reactivity. We, therefore, investigated platelet activation and platelet responsiveness to different agonists in patients with hemophilia in relation to the patients' residual FVIII activity.

Design and Methods

Patients and setting

During regular visits at the Van Creveldkliniek (VCK), University Medical Center Utrecht, blood was collected from patients with severe (<1% FVIII activity), moderate (1-5% FVIII activity) and mild (6-40% FVIII activity) hemophilia A. The blood was drawn from the antecubital vein through 20-gauge needles into vacuum citrate tubes. Data on residual FVIII activity, inhibitor status, annual FVIII consumption and blood group were collected from the patients' medical files.

All participants of the current study were aged 18 years or older. Subjects were excluded when taking non-steroidal anti-inflammatory drugs, which are known to influence platelet function. Healthy, male volunteers of similar age served as controls. During a period of 1 year, between February 2009 and February 2010, 35 patients with hemophilia and 21 healthy controls were included in this study. One patient was excluded, because blood was erroneously stored on ice after drawing, leaving 34 patients with hemophilia for analysis. Based on their residual FVIII activity levels, patients were divided into those having mild-moderate hemophilia (n=21) and those with severe hemophilia (n=13). All patients gave written informed consent to participation in the study, which was approved by the Medical Ethics Committees of the University Medical Center Utrecht and performed in accordance with the Declaration of Helsinki.

Materials

R-phycoerythrin-labeled antibodies for fluorescence activated cell sorting (FACS) analyses, raised against human P-selectin (#555524), were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies against human platelet factor 4 (PF4) (MAB7951, AF795), human chemokine (C-X-C motif) ligand 7 (CXCL7) (MAB393, BAF393), human chemokine (C-C motif) ligand 5 (RANTES) (MAB278, AB-278-NA) and human soluble P-selectin (DY137 Duoset) were all purchased from R&D Systems (Abingdon, UK). Rabbit anti-von Willebrand factor (vWF) propeptide and rabbit anti-vWF propeptide/biotin were prepared as described by Borchiellini *et al.*[9] Polyclonal rabbit anti-human vWF antibodies (A0082), rabbit anti-goat horseradish peroxidase (HRP) (P0449) and streptavidin-poly-HRP (P0397) were purchased from DAKO (Glusdorp, Denmark).

For platelet responsiveness assays, adenosine diphosphate (ADP) was purchased from Roche (Almere, The Netherlands), iloprost (Ilomedine) from Bayer Schering Pharma AG (Berlin, Germany) and cross-linked collagen-related peptide (CRP) was a generous gift from R. Farndale (Cambridge, UK).

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from BDH (Poole, UK). Sodium chloride (NaCl), hydrogen peroxidase, Tween-20 and bovine serum albumin (BSA) were purchased from Sigma (Zwijndrecht, The Netherlands). Magnesium sulphate ($MgSO_4$) and potassium chloride (KCl) were purchased from Riedel (Seelze, Germany), formaldehyde and orthophenyldiamine from Calbiochem (Merck, Darmstadt, Germany). Sulfuric acid (H_2SO_4) was purchased from Mallinckrodt Baker Inc. (Deventer, The Netherlands), Amplex UltraRed reagent from Invitrogen (Breda, The Netherlands) and SuperSignal ELISA Pico chemiluminescent substrate from Thermo Scientific (Rockford, IL, USA).

Platelet activation and responsiveness

Platelet responsiveness to agonists was determined with concentration series of ADP and CRP while platelet responsiveness to inhibitors was measured with concentration series of iloprost in platelets activated with a suboptimal dose of ADP. Serial dilutions of ADP (500 μM, 50 μM, 5 μM, 500 nM, 50 nM and 5 nM) were prepared in 50 μL HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, pH 7.4, filtered through a 0.22 μm filter) with 2 μL phycoerythrin-labeled mouse anti-human P-selectin antibodies. Similarly, serial dilutions of CRP (2.5 μg/mL, 250 ng/mL, 25 ng/mL, 2.5 ng/mL and 250 pg/mL) were prepared in 50 μL HBS with 2 μL phycoerythrin-labeled mouse anti-human P-selectin antibodies. Furthermore, a serial dilution of iloprost (250 ng/mL, 25 ng/mL, 2.5 ng/mL, 250 pg/mL, 25 pg/mL and 2.5 pg/mL) was prepared in 50 μL HBS with 5 μM ADP and 2 μL phycoerythrin-labeled mouse anti-human P-selectin antibodies. A negative control sample, containing only 50 μL HBS with 2 μL phycoerythrin-labeled mouse anti-human P-selectin antibodies, was prepared to determine the basal level of platelet activation.

The platelet activation test was initiated by addition of 5 μL fresh, citrate anticoagulated, whole blood to each sample of the serial dilutions. After 20 min of incubation, the samples were fixed with 500 μL 0.2% formyl saline (0.2% formaldehyde in 0.9% NaCl, filtered through a 0.22 μm filter) and kept at 4°C until analyses. All samples were analyzed on a FACSCalibur flow cytometer from BD Biosciences (Franklin Lakes, NJ, USA) within 1 day after processing. Single platelets were gated based on forward and side scatter properties. The mean fluorescence intensity (MFI) in the platelet gate was measured by FACS analysis. Platelets were defined as positive for P-selectin expression when the MFI exceeded that of the isotype control. One individual performed all the assays.

Enzyme-linked immunosorbet assays

Citrated whole blood was centrifuged twice at 2000xg for 10 min at room temperature and the resultant plasma was collected and frozen at -80°C for evaluation. Plasma levels of soluble platelet activation markers PF4, CXCL7, soluble P-selectin and RANTES, and of vWF propeptide and vWF antigen (vWF:Ag) were measured by enzyme-linked immunosorbent assay (ELISA). Plasma samples from patients and controls were mixed randomly for all ELISA measurements. Each antigen was measured on a separate Nunc maxisorb ELISA plate (Thermofischer Scientific, Roskilde, Denmark). Capture antibodies, monoclonal mouse anti-human PF4 (1 μg/mL), purified monoclonal mouse anti-human CXCL7 (1 μg/mL), mouse anti-human P-selectin (1 μg/mL), purified mouse monoclonal anti-human RANTES (500 ng/mL), rabbit anti-human vWF propeptide (5 μg/mL) and polyclonal rabbit-anti-human vWF (5 μg/mL) were coated on different plates overnight. Unbound antibodies were washed with five steps of phosphate-buffered saline (PBS)/0.5% Tween.

Plasma samples were diluted 1/75 for PF4 and CXCL7, 1/10 for soluble P-selectin, 1/4 for RANTES, 1/20 for vWF propeptide and in 1/20, 1/40, 1/80 and 1/160 dilutions for vWF:Ag measurements, and then added in duplicate to the plate with the corresponding capture antibody. Each plate contained two calibration curves (duplex) consisting of dilution ranges of a standard serum sample with known PF4, CXCL7, soluble P-selectin and RANTES concentrations or a dilution range of a normal pooled plasma sample with known vWF propeptide and vWF:Ag concentrations. Dilutions were made in PBS/1% BSA for PF4, CXCL7, soluble P-selectin and RANTES measurements, in PBS/0.05% Tween/3.8% EDTA for vWF propeptide and in PBS/0.05% Tween/3% BSA for vWF:Ag measurements.

For PF4 and CXCL7 measurements unbound antigens were removed by five washing steps with PBS/0.5% Tween. Detection antibodies, polyclonal goat anti-human PF4 (0.05 μg/mL) and biotinylated goat anti-human NAP-2 (50 ng/mL) were added to the corresponding plates. After five washing steps with PBS/0.5% Tween detection antibodies, rabbit anti-goat-HRP (2 μL in 13 mL) and streptavidin-poly-HRP (2 μL in 13 mL), were added for 1 hour to bind the biotin on the detection antibody. After five other washing steps, Amplex Ultrared reagent was added and fluorescence intensity was measured (emission: 490 nm; excitation: 520 nm), following incubation for 75 min, with a Fluostar Galaxy fluorimeter from BMG Labtech GmbH (Offenburg, Germany).

For soluble P-selectin, RANTES and vWF propeptide measurements, unbound antigens were removed by five washing steps with PBS/0.5% Tween. Detection antibodies, mouse anti-human P-selectin (0.01 μg/mL), purified goat anti-human RANTES (1 μg/mL) and rabbit anti-propeptide/biotin (2.5 μg/mL), were added to the corresponding plates. After five washing steps with PBS/0.5% Tween, streptavidin-poly-HRP was added to the soluble P-selectin plate and the vWF propeptide plate, and rabbit anti-goat HRP was added to the RANTES plate for 1 h to bind the detection antibody. After another five washing steps, SuperSignal ELISA Pico chemiluminescent substrate was added and luminescence was measured (emission: 490 nm) after 60 min of incubation, with a SpectraMax L microplate reader from Molecular Devices Inc. (Silicon Vally, CA, USA).

Three washing steps with PBS/0.5% Tween were performed to remove unbound antigens for the vWF:Ag measurements. Polyclonal rabbit-anti-human vWF/HRP secondary antibodies (1.1 mg/mL) were added to the plates and incubated for 1 h. After another three washing steps, activated orthophenyldiamine reagent was added to stain the wells. After 3 min of incubation, the staining reaction was stopped with H_2SO_4, after which the absorption was measured (absorption at 490 nm), with a VersaMax tunable microplate reader from Molecular Devices Inc. (Silicon Vally, CA, USA).

Statistical analysis

Initial quantification of FACS data was performed using BD CellQuest Pro software, version 6.0 (BD Biosciences, Franklin Lakes, NJ, USA). To determine platelet responsiveness, the concentration generating a response halfway between the baseline and maximum MFI of P-selectin-expressing platelets (EC_{50}) was calculated using GraphPad Prism version 4.00 (GraphPad Software, San Diago, California, USA). The maximal effect of stimulation or inhibition is represented by the platelet response to the highest used (ant-)agonist concentration, in terms of both percentage of P-selectin expressing platelets and the MFI of P-selectin expressing platelets.

A statistical analysis was performed with SPSS version 15.0.1 for Windows (SPSS Inc., Chicago, IL, USA). Data are expressed as median and interquartile range (IQR) and figures show individual values and group medians unless indicated otherwise. Comparisons between two groups were performed by Mann-Whitney U testing. *P* values lower than 0.05 were considered to be statistically significant.

Results

Baseline characteristics

Of the 34 patients with hemophilia analyzed, one with moderate hemophilia and nine (69%) with severe hemophilia received regular prophylaxis with FVIII concentrates. Within the group of patients with severe hemophilia, three (23%) had active inhibitors during the period of this study and four (30.8%) had a hepatitis C infection at the time of inclusion into the study. Hemophilic arthropathy was present in 12 (92.3%) patients with severe hemophilia and in nine (42.9%) with mild-moderate hemophilia. The baseline characteristics of the study population are shown in Table 1. The characteristics of the individual patients are shown in Table 2.

Basal level of platelet activation

The percentage of platelets expressing P-selectin on the platelet membrane was increased in patients with severe hemophilia [15.9% (IQR 10.3-21.1%)] when compared to that in patients with mild-moderate hemophilia [8.2% (IQR 4.8-14.5%)] (P=0.014) and in healthy controls [6.4% (IQR 4.7-7.6%)] (P<0.001) (Figure 1A).

Similarly, platelets from patients with severe hemophilia showed greater P-selectin expression [MFI 6.5 (IQR 4.8-9.1)] than platelets from either patients with mild-moderate hemophilia [MFI 4.2 (IQR 3.3-5.9)] (P=0.010) or healthy controls [MFI 3.8 (IQR 3.1-4.1)] (P<0.001) (Figure 1B).

When correlating the mean annual FVIII consumption of the patients with severe hemophilia to the percentage of platelets expressing P-selectin on the platelet membrane, we found that a higher percentage of P-selectin expression was correlated with lower FVIII consumption (Spearman's r -0.65) (P=0.043) (Figure 1C). Correcting for the patients' weight did not influence this correlation (corrected Spearman's r -0.63) (P=0.048).

To study the effect of FVIII infusion itself on platelet activation, we measured P-selectin expression in five patients with severe hemophilia before and 15 and 60 min after a bolus infusion of FVIII to peak levels of 1.0 U/L. No differences were found (*data not shown*).

Table 1. Baseline characteristics of the study population.

	Healthy control (n=21)	Mild/moderate Hemophilia A (n=21)	Severe hemophilia A (n=13)	*P* mild-moderate *versus* control	*P* severe *versus* control
Male, n (%)	21 (100)	21 (100)	13 (100)	1.000	1.000
Median age, years (IQR)	37.0 (28.5-50.5)	40.0 (25.5-60.0)	39.0 (28.0-54.5)	1.000	0.696
Treatment, n (%)					
Prophylaxis	NA	1 (4.8)	9 (69.2)	NA	NA
On demand	NA	20 (95.2)	4 (30.8)	NA	NA
Inhibitors, n (%)	NA	0 (0)	3 (23.1)	NA	NA
Infection, n (%)					
Chronic hepatitis	0 (0)	0 (0)	4 (30.8)	NA	NA
Human immunodeficiency virus	0 (0)	0 (0)	0 (0)	NA	NA
Hemophilic arthropathy, n (%)	NA	9 (42.9)	12 (92.3)	NA	NA
Median platelet count, $\times 10^9$/L (IQR)	238 (202-282)	226 (197-284)	208 (160-287)	0.642	0.330
Median MPV, fL (IQR)	8.4 (6.8-8.9)	8.3 (7.3-8.5)	7.5 (7.1-8.6)	0.764	0.517
Blood group O, n (%)	9 (42.9)	11 (52.4)	6 (46.2)	0.542	0.853

IQR: indicates interquartile range, MPV: mean platelet volume, NA: not applicable.

Table 2. Individual patients' characteristics.

Residual FVIII activity (%)	Inhibitor status (BU)	Mutation	Time between last major bleed and inclusion (days)	Mean annual FVIII consumption (IU)	Blood group
0	0.0	1619delC mutation in exon 11	>90	165000	O
0	0.0	2946insA mutation in exon 14	>90	287000	O
0	0.0	Ala200Pro mutation in exon 5	0-180	316000	A
0	0.0	Type II inversion	>90	132500	O
0	0.0	Type II inversion	>90	92000	A
0	0.0	Type II inversion	>90	527250	A
0	0.1	Unknown	>90	169750	O
0	0.1	2946insA mutation in exon 14	>90	260000	O
0	0.1	Ala1920THR mutation in exon 17	>90	166750	O
0	0.1	Type I inversion + Asp1241Gln mutation in exon 14	0-180	166250	A
0	12.0	Type I inversion variant + Asp1241Gln mutation in exon 14	± 60	0	AB
0	23.2	Deletion of exons 1 to 22	>90	0	A
0	34.5	Type II inversion	>90	0	A
2	0.0	Unknown	>90	188000	O
2	0.1	Unknown	>90	36750	O
2	0.1	c.6506>A(p.Arg92150His) mutation in exon 23	>90	88250	AB
3	0.0	Unknown	>90	3000	O
3	0.0	Unknown	>90	31000	O
3	0.0	Unknown	>90	250	A
3	0.0	Unknown	>90	10500	A
3	0.0	CGC>TGC;Arg1689Cys mutation in exon 14	>90	27000	A
3	0.0	Val483Gly missense mutation in exon 10	>90	1500	B
3	0.1	c.5338C>A(p.Pro1761Gln) mutation in exon 15	0-180	73000	O
4	0.0	Unknown	>90	2000	O
4	0.1	Unknown	>90	3250	O
5	0.0	Val483Gly missense mutation in exon 10	>90	18500	O
5	0.1	Unknown	>90	10375	A
5	0.1	Unknown	>90	13875	A
5	ND	558 C>A (ASP167 GLU) mutation in exon 4	>90	0	O
8	0.1	Tyr656Cys mutation in exon 13	>60	61500	B
8	0.2	Unknown	0	56000	A
9	0.1	Unknown	>90	12875	A
17	0.0	Unknown	>90	1500	O
23	0.0	Unknown	>90	5750	O

ND indicates not determined.

Soluble platelet activation markers

Plasma concentrations of soluble platelet activation markers in normal healthy controls were: PF4 0.3 pg/10^6 platelets (IQR 0.0-0.6); CXCL7 20.0 pg/10^6 platelets (IQR 17.7-32.5); and RANTES 4.5 pg/10^6 platelets (IQR 3.1-6.7). The plasma concentrations of these soluble platelet activation markers were significantly higher in patients with severe hemophilia [PF4 1.8 pg/10^6 platelets (IQR 1.4-2.6); CXCL7 48.2 pg/10^6 platelets (IQR 35.6-103.7); and RANTES 9.5 pg/10^6 platelets (IQR 5.8-16.1)] (P<0.001, P<0.001 and P=0.003, respectively, compared to values in normal controls) and in patients with mild-moderate hemophilia [PF4 1.4 pg/10^6 platelets (IQR 0.9-2.1); CXCL7 65.8 pg/10^6 platelets (IQR 40.2-80.5); and RANTES 12.8 pg/10^6 platelets (IQR 9.5-21.2)] (P<0.001, P<0.001 and P<0.001, respectively, compared to values in normal controls). No significant differences in these markers were found between patients with mild-moderate and severe hemophilia (Figure 2A-C).

Plasma concentrations of soluble P-selectin did not dif-

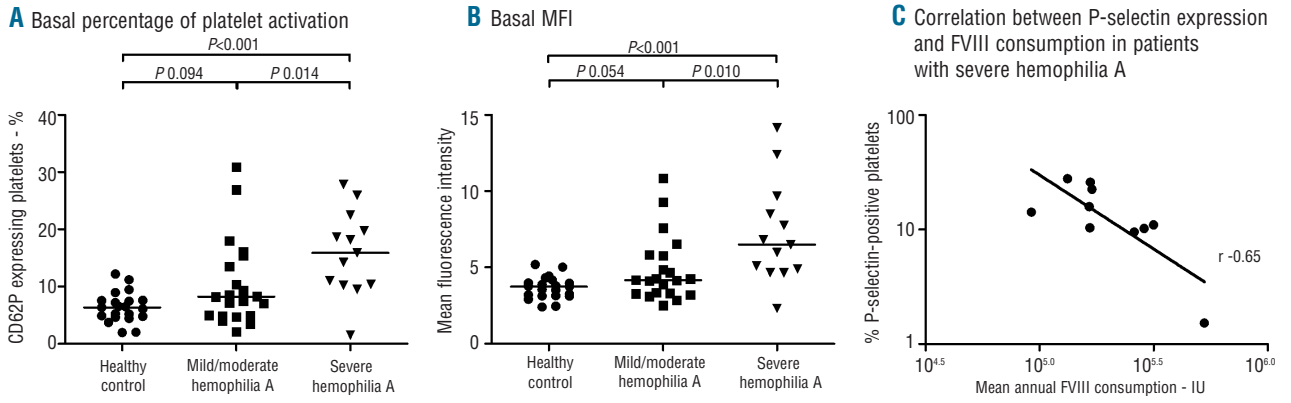

Figure 1. Baseline P-selectin expression. Citrated fresh whole blood was incubated for 20 min with phycoerythrin-labeled mouse anti-human P-selectin antibodies, and then fixed with 0.2% formyl saline. (A) Percentage of P-selectin-expressing platelets and (B) mean fluorescence intensity of all platelets were determined by FACS analysis. (C) Correlation between mean annual FVIII consumption (calculated over a 4-year period) and percentage of P-selectin-expressing platelets, r shows Spearman's rank correlation coefficient.

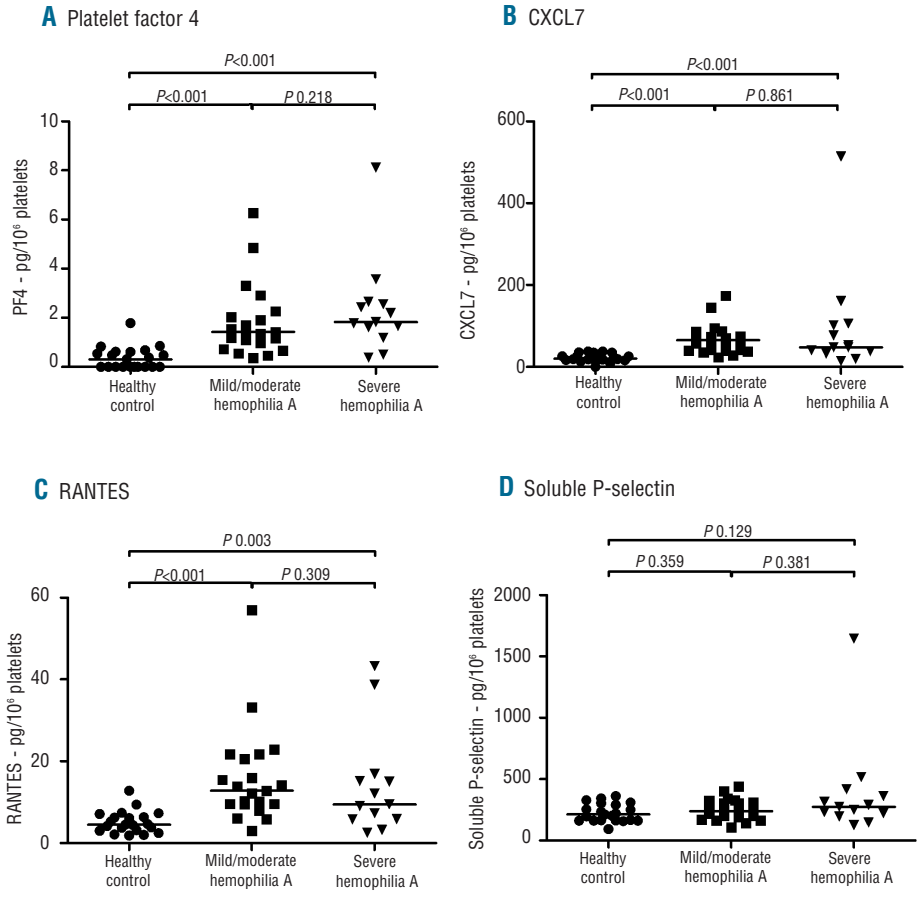

Figure 2. Plasma concentrations of soluble platelet activation markers. Citrated whole blood was centrifuged twice at 2000xg for 10 min at room temperature, and the resultant plasma was collected and frozen at -80°C for evaluation. Plasma levels of (A) platelet factor 4 (PF4), (B) human chemokine (C-X-C motif) ligand 7 (CXCL7), (C) RANTES and (D) soluble P-selectin were measured by enzyme-linked immunosorbent assay, and are expressed per 10^6 platelets.

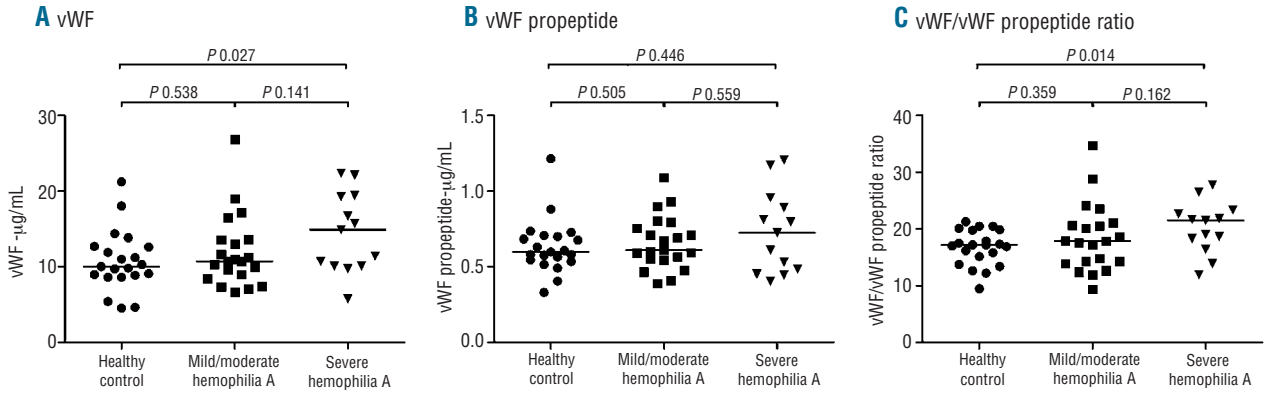

Figure 3. Plasma concentrations of vWF. Citrated whole blood was centrifuged twice at 2000xg for 10 min at room temperature, and the resultant plasma was collected and frozen at -80°C for evaluation. Plasma levels (in µg/mL) of (A) vWF antigen (vWF:Ag) and (B) vWF propeptide were measured by enzyme-linked immunosorbent assay. The vWF:Ag /vWF propeptide ratio was determined (C).

fer between patients with hemophilia and healthy controls (Figure 2D).

von Willebrand factor

Patients with severe hemophilia had higher vWF plasma levels [14.9 µg/mL (IQR 10.1-19.3)] than those in healthy controls [10.0 µg/mL (IQR 8.7-12.7)] (P=0.027), whereas patients with mild-moderate hemophilia had similar levels of vWF [10.7 µg/mL (IQR 8.7-13.6)] (P=0.538) as healthy controls (Figure 3). We observed similar vWF propeptide levels in patients with severe hemophilia [0.7 µg/mL (IQR 0.5-0.9)] compared to those with mild-moderate hemophilia [0.6 µg/mL (IQR 0.5-0.8)] (P=0.559) and healthy controls [0.6 µg/mL (IQR 0.5-0.7)] (P=0.446). The vWF/vWF propeptide ratio was higher in patients with severe hemophilia [21.5 (IQR 17.4-23.0)] (P=0.014), but not in those with mild-moderate hemophilia [17.9 (IQR 14.1-20.9)] (P=0.359) compared to in healthy controls [17.2 (IQR 14.4-19.8)] (Figure 3).

Platelet responsiveness

Platelet responsiveness to ADP stimulation, represented by the EC_{50}, of patients with severe hemophilia [EC_{50} 0.9 µM (IQR 0.4-0.9)] and mild-moderate hemophilia [EC_{50} 0.7 µM (IQR 0.5-0.8)] did not differ from that of healthy controls [EC_{50} 0.6 µM (IQR 0.5-0.7)] (P=0.184 and P=0.110 for patients with severe and mild-moderate hemophilia, respectively). The percentages of activated platelets after maximal ADP stimulation in all patients with hemophilia were comparable to those in healthy controls. Patients with severe hemophilia had higher MFI after maximal stimulation with ADP [MFI 112.1 (IQR 84.5-134.8)] compared to patients with mild-moderate hemophilia [MFI 92.1 (IQR 75.5-107.7)] (P=0.063) and healthy controls [MFI 80.8 (IQR 74.7-100.6)] (P=0.017) (Figure 4A-C).

The platelet responsiveness to CRP showed marked variability and was equivalent in patients with severe hemophilia [EC_{50} 69.2 ng/mL (IQR 24.4-276.4)] to that in patients with mild-moderate hemophilia [EC_{50} 47.6 ng/mL (IQR 21.8-280.0)] (P=0.710) and in healthy controls [EC_{50} 65.9 ng/mL (IQR 26.8-250.7)] (P=0.986). When maximally stimulated with CRP, both patients with severe hemophilia and those with mild-moderate hemophilia showed lower percentages of P-selectin-expressing platelets [95.0% (IQR 89.9-95.9) and 94.7% (IQR 92.4-97.3), respectively] with a lower amount of P-selectin on the surface of each platelet [MFI 280.1 (IQR 252.8-302.6) and 278.7 (IQR 191.1-319.0), respectively] compared to that on platelets from healthy controls [96.3% (IQR 95.3-97.6)] (P=0.007 and 0.014, respectively); [MFI 307.1 (IQR 283.1-341.8)] (P=0.058 and 0.076, respectively) (Figure 4D-F).

Platelets of patients with either mild-moderate or severe hemophilia were more sensitive to inhibition by iloprost [EC_{50} 1.8 ng/mL (IQR 0.3-6.5) and 1.0 ng/mL (IQR, 0.3-3.9), respectively] when compared to those of healthy controls [EC_{50} 8.3 ng/mL (IQR 6.0-11.1)] (P<0.001 and P<0.001, respectively). However, the maximal inhibition by iloprost of platelets from patients with mild-moderate hemophilia [26.0% (IQR 21.4-34.3) and MFI: 25.2 (IQR 22.7-29.1)] and from patients with severe hemophilia [34.0% (IQR 27.9-42.3) and MFI 34.0 (IQR 27.9-42.3)] was comparable to that of platelets from healthy controls [28.3% (IQR 25.0-36.5)] (P=0.141 and P=0.184, respectively) [MFI 25.6 (IQR, 24.4-28.8)] (P=0.521 and P=0.228, respectively) (Figure 4G-I).

Discussion

We have shown that patients with hemophilia have a higher basal level of activated platelets in their circulation, have a stronger platelet response to ADP stimulation and have platelets that are more sensitive to inhibition with the prostacyclin analog iloprost than those of healthy controls. Furthermore, patients with hemophilia had higher plasma concentrations of the platelet activation markers PF4, CXCL7 and RANTES. vWF concentrations were raised in patients with severe hemophilia A. Among the patients with severe hemophilia, platelet activation correlated with FVIII consumption. These data indicate that platelet activation is up-regulated in the presence of a deficiency in secondary hemostasis, reducing the risk of bleeding complications.

Comparing baseline levels of platelet activation in patients with hemophilia with those of healthy controls, we found that both platelet P-selectin expression and plasma concentrations of the soluble platelet activation markers PF4, CXCL7 and RANTES are increased in patients with hemophilia. Theoretically, this increased platelet activation

could partially compensate for a deficiency in FVIII by providing more negatively charged surface to enhance the constrained coagulation cascade in hemophiliacs. In line with this theory one would expect platelet activation to show the greatest increase in the more severely affected patients. In our study hemophilia severity was classified based on residual FVIII activity. Comparing platelet activation in patients with mild-moderate and severe hemophilia, we did indeed find higher baseline platelet P-selectin expression in the more severely affected patients (residual FVIII<1%). However, no significant differences were found between patients with mild-moderate and severe hemophilia with regards to the soluble platelet activation markers PF4, CXCL7 and RANTES.

In an additional analysis within the group of patients with severe hemophilia, we classified hemophilia severity according to annual FVIII consumption. Again, platelet P-selectin expression was found to correlate with clinical phenotype. However, to fully characterize clinical phenotype, in future studies the phenotype should not be scored solely on the amount of FVIII consumed, but also on the age at the time of the first joint bleed, joint bleeding frequency and joint damage. To exclude FVIII infusion as a cause of up-regulation of platelet P-selectin expression, we measured P-selection expression in five patients with severe hemophilia before and 15 and 60 min after the infusion. FVIII infusion had no effect on the basal MFI of P-selectin on platelets.

Platelet responsiveness was triggered by increasing concentrations of ADP and CRP and activation was measured by P-selectin expression. The platelet responsiveness to both ADP and CRP did not differ between patients with hemophilia and controls. However, the maximal response to stimulation was different in patients with hemophilia. Upon maximal stimulation with ADP, an agonist which by itself can only initiate partial platelet activation, the platelets of patients with hemophilia expressed more P-selectin on the cell surface than did the platelets of healthy controls. However, upon maximal stimulation with CRP a smaller percentage of platelets became activated in patients with hemophilia. Whether this lower response to CRP can lead to an aggravation of the bleeding phenotype in individual patients remains to be determined in future studies.

Platelets from patients with hemophilia were more responsive to inhibition by iloprost than were platelets from controls. This was shown by the low EC_{50} of iloprost in patients with hemophilia after suboptimal stimulation by ADP. In patients with hemophilia, whom we have shown to have higher baseline platelet activation and a higher maximal response to stimulation by ADP, this increased inhibitory mechanism could have developed to

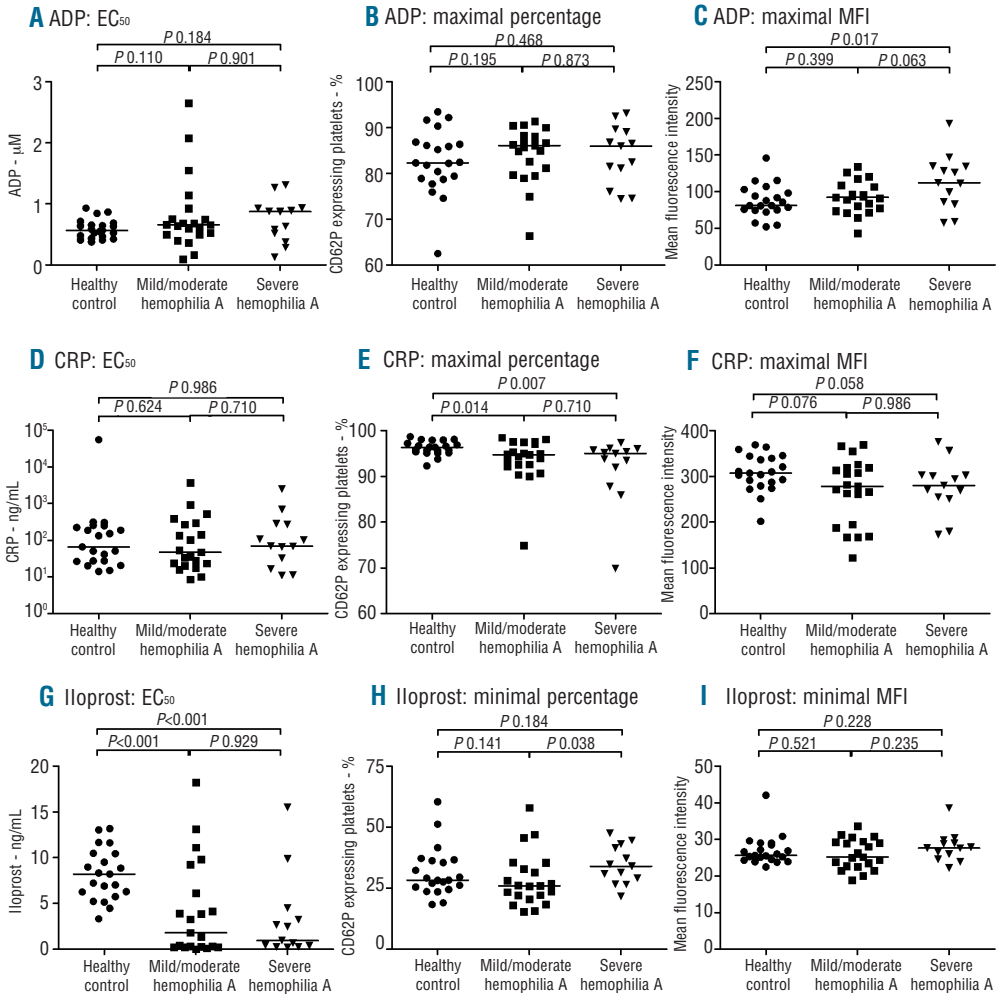

Figure 4. Platelet responsiveness. Citrated fresh whole blood was stimulated with concentration series of ADP and CRP, and incubated for 20 min. For all concentrations, the percentage of platelets expressing P-selectin and the mean fluorescence intensity (MFI) of these platelets were measured. The ADP and CRP concentrations generating a half maximal mean fluorescence intensity of P-selectin expressing platelets were determined (A, D). The platelet response to the highest used ADP concentration of 500 µM and the highest used CRP concentration of 2500 ng/mL, represented by both the percentage of P-selectin expressing platelets (B, E) and the MFI of P-selectin-expressing platelets (C, F), were determined. To determine platelet responsiveness to inhibition, platelets were inhibited with a concentration series of iloprost in the presence of 5 µM ADP. After 20 min of incubation, the samples were fixed with 0.2% formyl saline. The iloprost concentrations generating the half maximal inhibition on mean fluorescence intensity of P-selectin-expressing platelets were determined (G). The platelet response to the highest used iloprost concentration of 250 ng/mL, represented by both the percentage of P-selectin-expressing platelets (H) and the MFI of P-selectin-expressing platelets (I), were determined.

prevent platelet activation in the absence of vascular injury.

To our knowledge, this study provides the first evidence that in patients, deficiency in secondary hemostasis is associated with increased platelet activation and responsiveness. A platelet responsiveness test was used that covers the complete range of the platelet response, from the (ant-)agonist concentration yielding no response, to concentrations yielding the maximal platelet response to the used (ant-)agonist. This total range of (ant-)agonist concentrations allowed for analysis of the concentration yielding a half maximal response together with the maximal effect of different (ant-)agonists, providing new parameters of platelet responsiveness. The platelet responsiveness was measured for established (ant-)agonists representing established hemostatic pathways *in vivo*.

Our findings of increased vWF concentration in patients with severe hemophilia support previous observations of Vlot *et al.*[10] and Fijnvandraat *et al.*[11] who also found higher vWF:Ag levels in patients with severe hemophilia. Increased vWF:Ag levels due to infection and stress in hemophiliacs have been suggested as a rationale for this finding. The results did not change when blood group analysis was included (*data not shown*). The absence of a relationship between vWF propeptide and hemophilia A suggests that the rise in vWF plasma concentration reflects chronic endothelial cell activation rather than acute infection or stress. Chronic inflammation in joints or chronic hepatitis C may be an explanation for this observation. The origin of the increased vWF in the plasma of patients with severe hemophilia may be secretion by platelets as well as endothelial cells. This chronic rise in vWF plasma concentration could be an additional factor of the primary hemostatic system compensating for the deficiency in secondary hemostasis present in patients with hemophilia.

For this study, patients were selected during regular visits at the Van Creveldkliniek. The Van Creveldkliniek is the largest hemophilia clinic in The Netherlands, treating approximately 800 patients with hemophilia. Patients are treated according to bleeding phenotype, resulting in individually tailored prophylaxis regimens in the patients with severe hemophilia or on demand treatment in the patients with a milder phenotype. The populations of the current study are representative of any other hemophilia populations in western countries, in which treatment is tailored to bleeding phenotype.

The present study has potential limitations that should be addressed. First, to ensure sufficient power for a reliable comparison, patients with mild and moderate hemophilia were grouped together. Second, our study is a cross-sectional analysis and clinical phenotype in the patients with severe hemophilia was based only on retrospectively examined annual FVIII consumption. Third, in this study we decided to include only adult hemophiliacs. Studying a younger population would probably have given additional insight into the origin, innate or acquired, of up-regulation of platelet activation and responsiveness. Last, blood sampling and platelet activation and reactivity measurements were performed at a single time point for each patient. Although repeated measurements were not performed in patients, we do not expect large variability in results over time. Repeated measurements in healthy donors show equivalent results over time (*data not shown*).

In summary, this study reveals a higher basal level of platelet activation, shown by higher P-selectin expression on the platelet surface and higher plasma concentrations of soluble platelet activation markers, and an increased platelet response to stimulation by ADP and to inhibition by iloprost in patients with hemophilia. Platelet P-selectin expression was inversely correlated with FVIII consumption in patients with severe hemophilia. Furthermore a chronic increase in vWF plasma concentration is present in patients with severe hemophilia. This study introduces a novel insight into hemostasis in patients with hemophilia A, in whom platelets are in a pre-activated state, and this pre-activated state is associated with the patients' FVIII consumption.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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