ORIGINAL ARTICLE

Microfluidic assay of hemophilic blood clotting: distinct deficits in platelet and fibrin deposition at low factor levels

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Summary. *Background:* Coagulation factor deficiencies create a range of bleeding phenotypes. Microfluidic devices offer controlled hemodynamics and defined procoagulant triggers for measurement of clotting under flow. *Objectives:* We tested a flow assay of contact pathway-triggered clotting to quantify platelet and fibrin deposition distal of dysfunctional thrombin production. Microfluidic metrics were then compared with PTT or % factor activity assays. *Methods:* Whole blood (WB) treated with low level corn trypsin inhibitor (4 μg mL^{-1}) from nine healthy donors and 27 patients (deficient in factor [F] VIII, 19 patients; FIX, one patient; FXI, one patient; VWF, six patients) was perfused over fibrillar collagen at wall shear rate = 100 s^{-1}. *Results:* Using healthy WB, platelets deposited within 30 s, while fibrin appeared within 6 min. Compared with healthy controls, WB from patients displayed a 50% reduction in platelet deposition only at < 1% factor activity. In contrast, striking defects in fibrin deposition occurred for patients with < 13% factor activity (or PTT > 40 s). Full occlusion of the 60-μm high channel was completely absent over the 15-min test in patients with < 1% factor activity, while an intermediate defect was present in patients with > 1% factor. *Conclusion:* Spontaneous bleeding in patients with < 1% factor activity may be linked to deficits in both platelet and fibrin deposition, a risk known to be mitigated when factor levels are raised to > 1% activity (PTT of ~40–60 s), a level that does not necessarily rescue fibrin formation under flow.

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Introduction

Microfluidic platforms for the assessment of patient-specific hemostatic function are finding new and diverse applications [1–3]. Microfluidic devices are typically casted in poly(dimethylsiloxane) (PDMS), utilize pressure-driven flows, and are designed to perform multiple clotting tests in parallel with relatively low volumes of blood (< 1 mL) [4]. The main advantages of these systems are control of the hemodynamic environment and the composition of the exposed procoagulant protein surface. For example, by flowing whole blood over a surface of collagen type 1 and lipidated tissue factor (TF), Colace *et al.* [5,6] demonstrated robust activation of the extrinsic coagulation system, resulting in rapid platelet adhesion and fibrin deposition. With sufficient TF on a surface, platelet and fibrin deposition on collagen/TF were insensitive to function-blocking antibodies against factors VIII and IX (FVIII and FIX) of the intrinsic coagulation pathway in a model of acquired hemophilia [7]. Importantly, the expression of TF varies in human tissues [8,9]. High TF levels may compensate for impaired FVIII or FIX function in some, but not all, vasculature locations. In the joints, for example, where bleeding in hemophilia is common, TF expression is low [8,9].

In hemophilia, the clinical severity of disease is defined by the activity of the residual critical clotting factor, although the bleeding phenotype can vary widely even among patients with identical mutations [10–13]. This suggests that quantitative assessment of coagulation factor levels or activities alone is not fully predictive of bleeding risk. Traditional tests such as the partial thromboplastin time (PTT) use platelet-free plasma (PFP) to obtain a clotting time in the presence of intrinsic activators such as kaolin or silica beads. While PTT accurately evaluates the severity of the factor deficiency in plasma, it does not always provide reliable predictions of bleeding

risk and the use of more global assays of whole blood function under flow have been proposed [14]. During bleeding episodes experienced by hemophiliacs, deficits in thrombin production may produce deficits in platelet activation/deposition, fibrin polymerization or platelet-dependent fibrin deposition. The relative priority of such dysfunctional pathways, distal of FVIIIa/IXa formation, is not fully resolved in hemophilia.

With the goal of assessing intrinsic coagulation in a low tissue factor environment under flow, we developed a model of hemostasis triggered by collagen (no exogenous TF was added). In this system, whole blood lightly anticoagulated with a low dose of the activated factor XII (FXIIa) inhibitor, corn trypsin inhibitor (CTI), was perfused over a type 1 collagen surface at a venous shear rate for 20 min. Platelet and fibrin accumulation were measured in real time using fluorescently conjugated monoclonal antibodies. Healthy donors as well as hemophilic or von Willebrand disease patients were studied and the assay outputs were compared with clinical laboratory test results such as residual coagulation factor activity level and PTT. We observed that platelet deposition in the microfluidic injury model correlated well with PTT. We found that fibrin generation under flow was switch-like and occurred only for residual factor levels $\geq 13\%$, a result that contrasts with the continuous range of PTT values. The quantity of fibrin generated was not correlated with factor activity (or PTT) above the critical value. These results illustrate that the local generation of thrombin plays a critical role in the deposition of platelets (in the absence of TF) and that a threshold level of thrombin is required for fibrin production to proceed under flow. In regions of the body where low TF conditions are expected, the attenuation of thrombin generation potential has an impact on both platelet accumulation and fibrin generation.

Materials and methods

Blood collection and preparation

Blood was drawn from healthy donors ($n = 9$) or patients ($n = 27$) attending a routine visit at the Hemophilia Program of the Hospital of the University of Pennsylvania under Internal Review Board approval of the University of Pennsylvania. All patient subjects were chronic patients in a comprehensive bleeding disorders center and had in most cases extensive duration of follow-up that enabled a very good assessment of bleeding phenotype. All subjects' bleeding phenotype was felt to be consistent with their hemostatic diagnosis. No correlation of bleeding phenotype and plasma platelet/fibrinogen levels was attempted. Healthy donors were self-reported as free of any oral medications for at least 10 days. Information regarding patient sex, age, diagnosed bleeding disorder, recent therapy and HIV/HCV viral status was collected. Table 1 presents the demographics of the patient population. In addition, laboratory values, which included platelet count, residual coagulation factor activity and partial thromboplastin time (PTT), were collected when available. The residual coagulation factor of interest was FVIII in Hemophilia A and VWD diagnoses, FIX in Hemophilia B diagnoses and Factor XI (FXI) in Hemophilia C diagnoses. The PTT test assesses the function of the intrinsic coagulation pathways by measuring the time to clot formation in platelet-free plasma (PFP) in the presence of a contact activator. PTT values (normal reference range, 20.8–34.4 s) were negatively correlated with residual coagulation factor activity levels, as expected (Fig. 1A).

Blood was drawn into 4 µg mL^{-1} corn trypsin inhibitor (CTI; Haematologic Technologies, Essex Junction, VT, USA), a low level that is a quantity sufficient to prevent visible clotting in the test tube for ~30 min but not sufficient to overcome contact activation at the model collagen injury site. Blood samples were treated with 0.125 µg mL^{-1} fluorescently conjugated anti-CD41a antibody (clone VI-PL2; Becton Dickson, Franklin Lakes, NJ, USA; 0.125 µg mL^{-1} final concentration) to label platelets and fluorescently conjugated anti-fibrin antibody (T2G1; gift from the Mortimer Poncz Laboratory, Children's Hospital of Philadelphia; 0.5 µg mL^{-1} final concentration) to label fibrin. Perfusion of whole blood samples was started within 10–15 min of venipuncture.

Microfluidic hemostasis model

The microfluidic device used in this study consisted of eight channels fed by individual inlets and perfused by withdrawal into a syringe pump (PHD 2000; Harvard Apparatus, Holliston, MA, USA) through a single outlet [15]. The device was casted in poly(dimethylsiloxane) (Sylgard; Ellsworth Adhesives, Germantown, WI, USA) as previously described [16]. A 250-µm wide strip of collagen type 1 (Chronopar; Chronolog, Havertown, PA, USA) was localized to the region where the eight channels run parallel using a microfluidic patterning technique (Fig. 1B). This technique resulted in a 250 µm × 250 µm collagen patch in each channel. The device was blocked with 0.5% bovine serum albumin in HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) prior to perfusion with whole blood. Blood samples were perfused at a local wall shear rate of 100 s^{-1} (1 µL min^{-1} per channel), conditions that are relevant to the hemodynamic environment of the venous circulation. In some experiments the local wall shear rate was increased to 1000 s^{-1} to mimic the conditions of the arterial circulation. The microfluidic devices were mounted on an inverted microscope (IX81; Olympus, Center Valley, PA, USA) and the collagen patches were imaged by a CCD at 30-s intervals (Hamamatsu, Bridgewater, NJ, USA). The total perfusion time was 20 min. Representative traces for platelet adhesion and fibrin generation for healthy donor blood and severe hemophilic blood are shown in Fig. 1(C,D, respectively).

Table 1 Twenty-seven patients were assessed in the microfluidic hemostasis model. Patients were organized first by diagnosis and then by % residual critical factor activity within the diagnosis category. The PTT and platelet count were available for all but two patients. Patients also reported when they last received therapy. Those who received therapy in < 48 h are indicated

Patient	Diagnosis	Residual coagulation factor (%)	PTT (s)	Platelets (10^3 µL^{-1})	Recent therapy	Ristocetin cofactor (%)
1	Severe hemophilia A	93	34.9	243	Y	–
2	Severe hemophilia A	63	31.2	247	Y	–
3	Severe hemophilia A	31	38.6	260	Y	–
4	Severe hemophilia A	32	32.9	148	Y	–
5	Severe hemophilia A	19	35.6	229	Y	–
6	Severe hemophilia A	16	32.9	243	Y	–
7	Severe hemophilia A	6	46.6	189	Y	–
8	Severe hemophilia A	1	51.3	235	N	–
9	Severe hemophilia A	< 1	64.9	279	Y	–
10	Severe hemophilia A	< 1	84.6	225	N	–
11	Severe hemophilia A	< 1	88.8	210	N	–
12	Severe hemophilia A	< 1	90.8	104	N	–
13	Severe hemophilia A	< 1	73.9	435	N	–
14	Moderate hemophilia A	5	66.1	135	N	–
15	Moderate hemophilia A	2	62	–	N	–
16	Moderate hemophilia A	2	67.3	79	N	–
17	Mild hemophilia A	13	51.8	153	N	–
18	Mild hemophilia A	5	59	207	N	–
19	Mild hemophilia A	20	52.2	142	N	–
20	Severe hemophilia C	2	55.9	230	N	–
21	Severe hemophilia B	< 1	62.1	97	N	–
22	VWD (Type 1)	159	31.7	132	Y	127
23	VWD (Type 1)	126	31	215	N	110
24	VWD (Type 1)	93	24.3	–	N	57
25	VWD (Type 1)	33	–	197	N	16
26	VWD (Type 2A)	32	–	195	N	5
27	VWD (Type 3)	< 1	67	177	N	< 1

Flow cytometric analysis of platelet activation

Whole blood (4 µg mL^{-1}) CTI was separated into eight separate 100 µL aliquots. One-microliter samples were drawn at predefined time-points (0–35 min) from a single aliquot and added to a well containing 30 nM Apixaban (Selleck Chemicals, Houston, TX, USA) and 100 µM PPACK (Haematologic Technologies) in 100 µL of HBS. An aliquot was briefly mixed before the sample was drawn and discarded immediately after. To measure platelet activation, 20 µg mL^{-1} fluorescent fibrinogen (Life Technologies, Grand Island, NY, USA), 1:100 fluorescently labeled anti-P-selectin (Becton Dickson), and 1:100 fluorescently labeled Annexin V (Life Technologies) were added to the diluted whole blood. Samples were incubated for 10 min with the fluorescent labels before reading with the flow cytometer (Accuri C6; Becton Dickson). Positive controls were activated with 5 nM convulxin (Centerchem, Norwalk, CT, USA).

Statistical analysis

Total platelet and fibrin fluorescence, which are proportional to platelet and fibrin mass, respectively, were recorded at the 15-min time-point or at the first frame that indicated full channel occlusion. Fifteen minutes was chosen, as opposed to the full perfusion time of 20 min,

because most samples reached an endpoint (i.e. steady state growth or full occlusion) in < 15 min. These values represent the mean of eight individual clotting events performed in parallel for each donor. Interdonor variability was calculated as the ratio of the standard deviation to the mean of the device averages for all donors multiplied by 100 (% coefficient of variation). Intradonor variability was calculated using the same formula considering the eight individual channel readings for a single donor; that value was then averaged over all donors. Platelet growth rate was calculated assuming no fluorescence at the first time-point. Fibrin growth rate was calculated using the first frame at which fibrin fluorescence was > 100 fluorescence units (an arbitrary value). The final time-point for both rates was either full channel occlusion, a non-occlusive steady state platelet aggregate, or 15 min, depending upon which event occurred first. The time to fibrin initiation was extrapolated from the fibrin growth rate line at a value of 100 fibrin fluorescence units. Statistical significance was assessed using the two-tailed Student's *t*-test.

Results

Low CTI prevents platelet activation under resting conditions

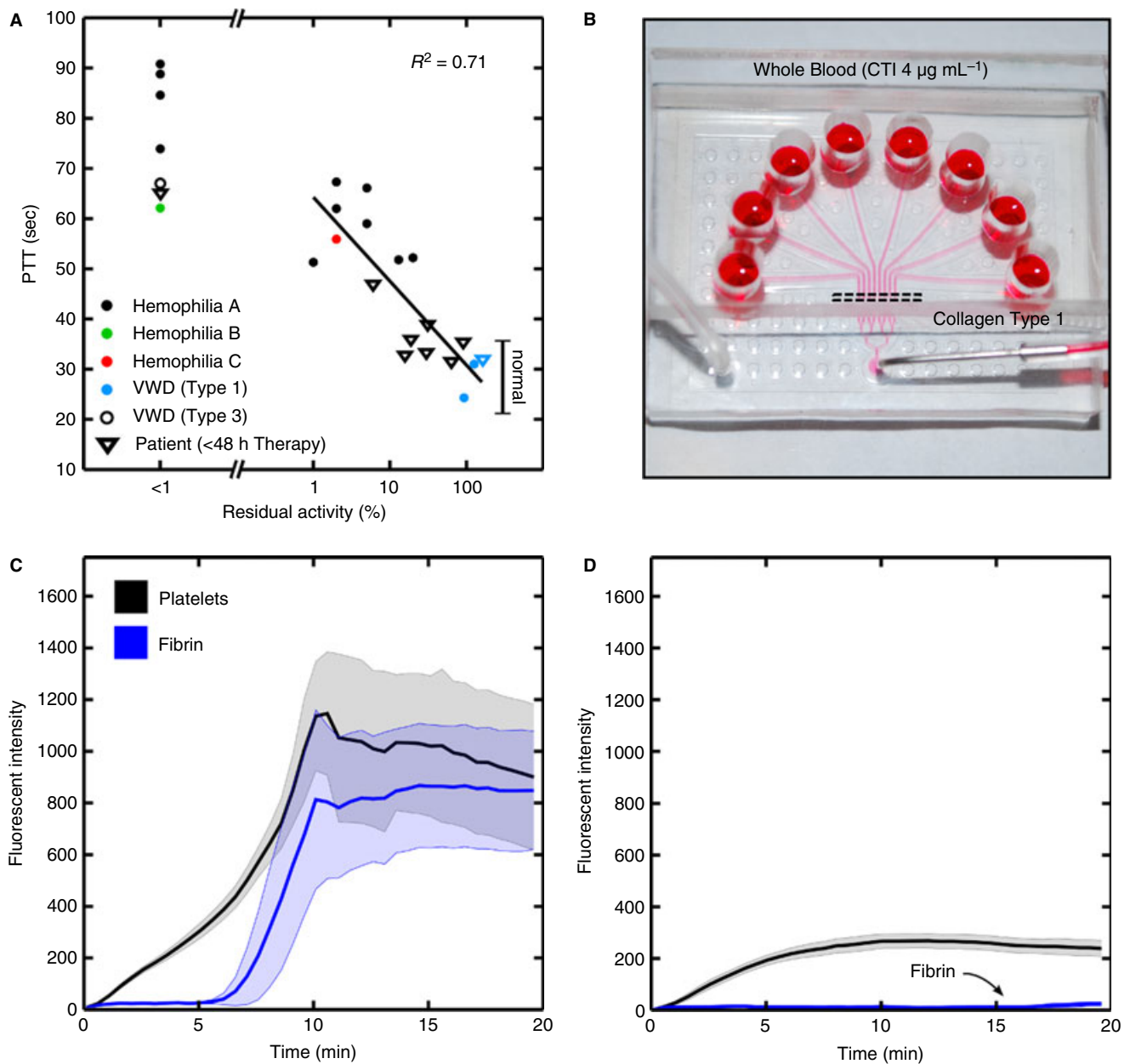Platelet activation in healthy donor whole blood (4 µg mL^{-1} CTI) aliquots incubated for up to 35 min

Fig. 1. (A) Patient PTT values were negatively correlated with residual coagulation factor activity values > 1%. (B) Whole blood samples were perfused simultaneously through eight separate channels in the microfluidic assay. A stripe of collagen type 1 was patterned in the region where the channels run parallel. Platelet accumulation and fibrin deposition were monitored in real time at the collagen patch. (C) Raw platelet and fibrin fluorescent intensity data at the collagen patch for a healthy donor and for a patient (D) with severe hemophilia A.

was assessed using flow cytometry. No increase in healthy donor $\alpha_{IIb}\beta_3$ activation (as measured by fluorescent fibrinogen binding), P-Selectin expression or PS exposure was observed over the 35-min time period (Figure S1). Without exogenous activation (i.e. collagen), low-dose CTI at 4 μg mL^{-1} provides for anticoagulation for at least 30 min in blood from healthy donors. Even longer prolongation of clotting time, often in excess of an hour or more, was observed in low-dose CTI-treated blood obtained from patients.

Critical factor deficiencies resulted in reduced platelet adhesion and fibrin generation

Representative images depicting overlays of platelet and fibrin fluorescence for healthy donors and severe hemophilia patients are presented in Fig. 2. Acceleration of FXIIa formation on collagen-adherent platelets is the essential event that triggers thrombin generation in this collagen assay lacking exogenously added TF [17]. In this assay, high-dose CTI (40 μg mL^{-1}) treatment of healthy
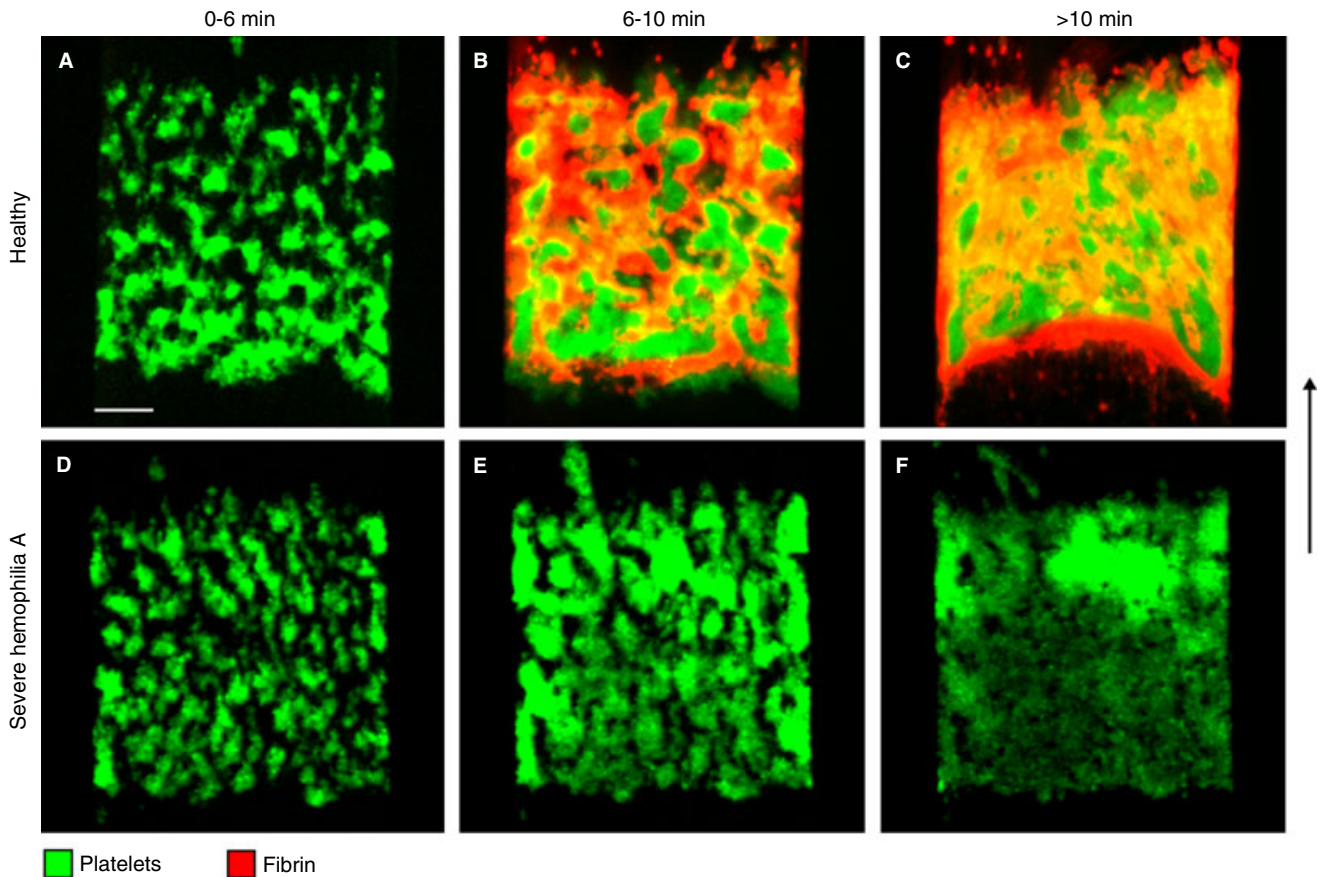
Fig. 2. Representative images depicting overlays of platelet (green) and fibrin (red) fluorescence intensity are presented for healthy donors (A–C) and patients (D–F). For all donors, platelet adhesion preceded fibrin deposition (A, D). Initiation of fibrin generation was observed for healthy donors in approximately 6.5 min, while only patients with residual factor activities > 13% deposited fibrin (B, E). Thrombi formed by healthy donors always led to full occlusion of the microfluidic channel (C) by 15 min. Patient experiments resulted in one of three outcomes, full channel occlusion, continued growth or steady state. A steady state thrombus, which appears washed out, is pictured (F). The scale bar indicates 50 μm.

WB prevents significant fibrin formation at 15 min in the absence of added TF (Figure S2) [5,18]. Fully consistent with XIIa being the most proximal trigger of thrombin production in the assay, WB from a patient with < 2% FXI activity produced absolutely no detectable fibrin after 15 min of WB perfusion (Fig. 3, red circle). The platelet-derived components that activate FXII to FXIIa are not fully delineated in this assay, while collagen type 1 has been reported to be an activator of XII [19].

Total accumulation of platelets and fibrin on the collagen after 15 min of whole blood perfusion are presented in Fig. 3(A,B, respectively). Patient data were split into three categories based on % residual coagulation factor activity levels (inverted triangles indicate recent therapy within 48 h prior to blood draw). Average platelet deposition was not significantly different from the healthy population in the > 13% or 1–6% critical factor groups, while a 50% reduction in total platelet deposition was noted in patients with < 1% residual factor activity ($P < 0.001$). With respect to fibrin generation, we observed that residual factor activities below 13% were not sufficient to

generate fibrin under flow conditions, with the exception of a single donor (out of 14 patients) who generated a relatively low but detectable level of fibrin (possibly a result of low TF function). Average fibrin deposition for patients in the ≥ 13% activity group displayed a wide range around a mean value that was not statistically different from healthy controls. These data are summarized in Table 2. Interdonor and intradonor variability in platelet and fibrin deposition are presented in Table S1.

Figure 3(C) illustrates that total platelet accumulation was not correlated with residual activity when % factor activity was > 1%. However, a defect in platelet deposition was seen at < 1% activity levels. In Fig. 3(D), fibrin deposition was similar to that of healthy controls when factor activity levels were ≥ 13%. However, at < 13% activity a striking defect in fibrin deposition occurred under flow. Total platelet accumulation or fibrin deposition at 15 min was negatively correlated with PTT values ($R^2 = 0.42$, Fig. 3E). When PTT was > 40 s, fibrin deposition under flow was abolished in all but two samples. For PTT between 20 and 40 s (or % residual factor
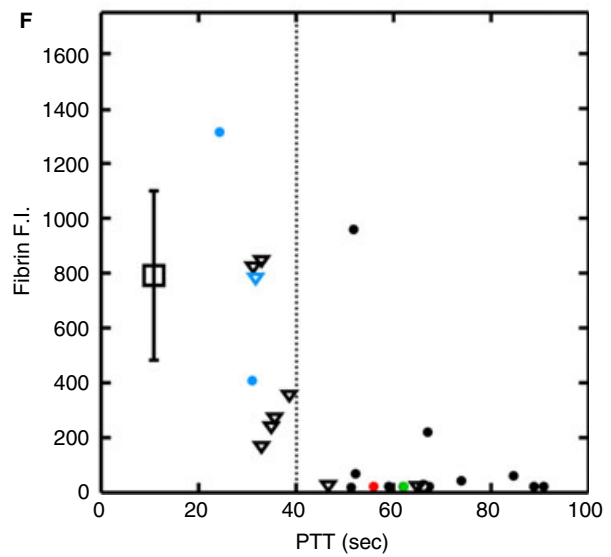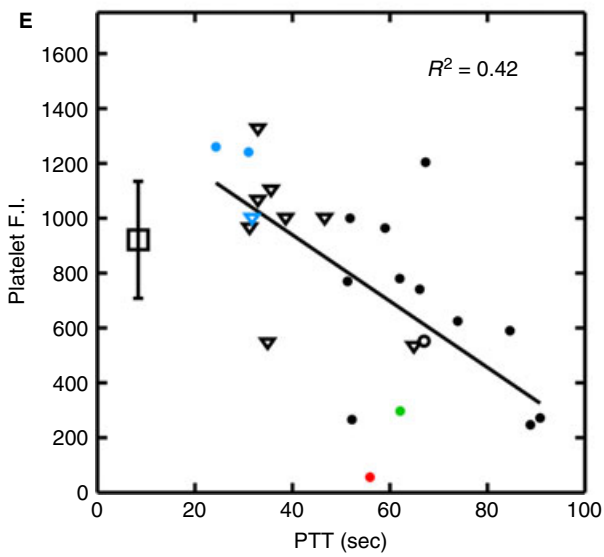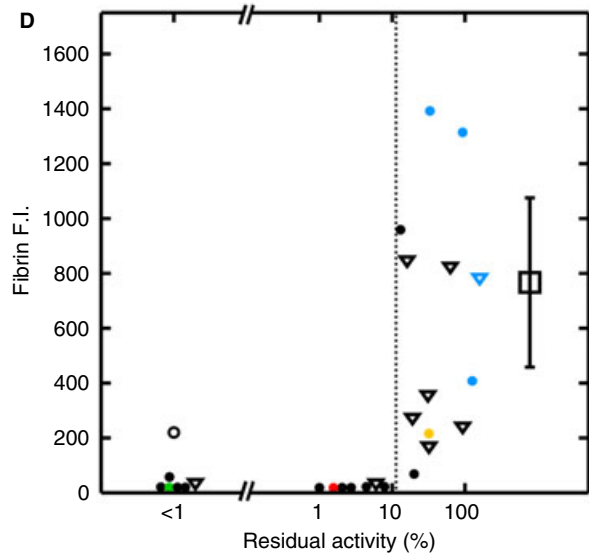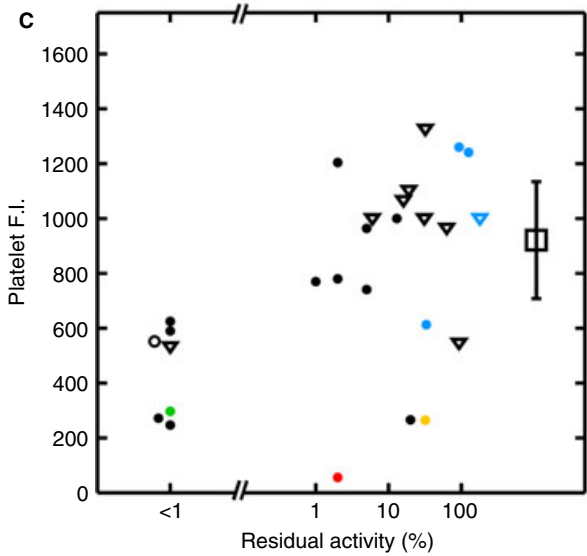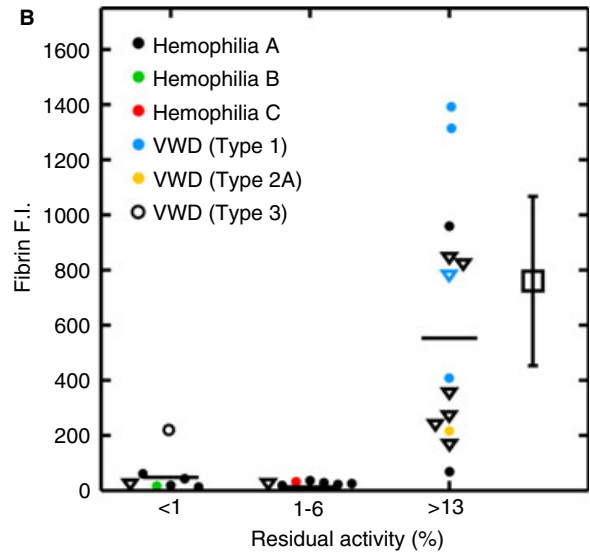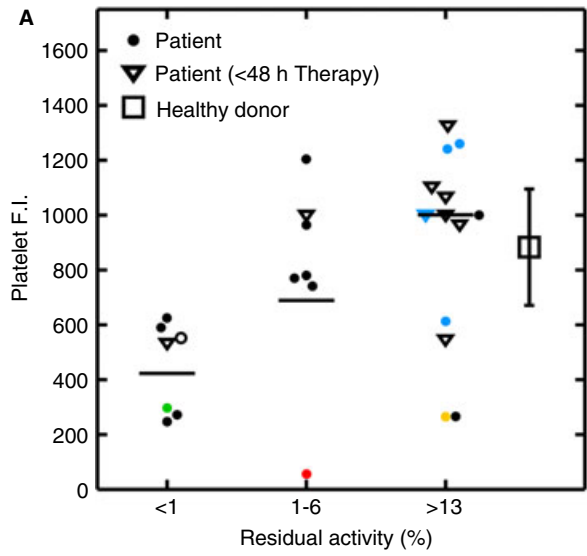
Fig. 3. Platelet (A) and fibrin (B) fluorescence intensities were organized into three groups based on residual coagulation factor activity. Patient samples with < 1% activity displayed a 50% reduction in total platelet accumulation ($P < 0.001$), while all but one patient with < 13% activity had a complete defect in fibrin production. (C) Platelet accumulation was not correlated with residual coagulation factor activity of > 1%. (D) Strong switch-like behavior around 13% residual coagulation factor activity (dotted line) was observed for fibrin deposition. (E) An inverse relationship was detected between platelet accumulation and PTT, while switch-like behavior was seen for fibrin (F) with respect to PTT around 40 s (dotted line). Note: neither residual coagulation factor activity nor PTT were obtained for healthy donors. The location of platelet and fibrin accumulation data for those donors is arbitrary.

Table 2 Patient samples were organized according to % residual factor activity and separated into four groups: severe (< 1%), moderate (1–5%), mild (6–50%) or normal levels (> 50%). Clinical assays were not performed on healthy donors. Average platelet and fibrin fluorescent intensity are reported for all donors. These values were recorded upon the first event to occur among full channel occlusion, steady state growth or 15 min. The number of experiments that reached occlusion is also reported

		Clinical assays		Microfluidic assay		
	Patient (y/n)	Average activity (%)	Average PTT(s)	Average platelet F.I.	Average fibrin F.I.	Number occluded
Severe (*n* = 7)	y	< 1%	76	450	53	0/7
Moderate (*n* = 6)	y	2.8	60	752	13	1/6
Mild (*n* = 9)	y	22	42	849	478	6/9
Normal level (*n* = 5)	y	107	31	1000	484	4/5
Healthy (*n* = 9)	n	–	–	882	760	9/9

> 13%) in patients, fibrin production varied widely; however, for PTT > 40 s (or % residual factor), little fibrin was deposited (Fig. 3F).

In addition to a deficiency in FVIII, three of six patients diagnosed with VWD displayed a defect in the ristocetin cofactor activity assay. Results from the ristocetin cofactor assay were only available for these patients and showed a modest but positive correlation with total platelet adhesion and platelet accumulation rate ($R^2 = 0.47$ and 0.42, respectively). However, the association between the ristocetin cofactor assay and residual FVIII activity was very strongly positive, $R^2 = 0.75$, as expected. At venous shear rates VWF function is not required because collagen can capture flowing platelets under these conditions. With this in mind the relationship between platelet adhesion and FVIII activity in hemophilic patients points to FVIII activity as the dominate variable, as opposed to VWF function.

Consideration of platelet and fibrin accumulation rates

Platelet and fibrin accumulation were also analyzed with respect to growth rate. We observed significant reductions in platelet growth rate as % factor activity decreased (Fig. 4A). We calculated a 60% reduction in the < 1% factor activity group ($P < 0.0001$), a 40% reduction for 1–6% ($P < 0.01$) and a slight 25% reduction for the ≥ 13% group ($P < 0.05$) relative to the healthy controls. As no fibrin was detected in all but one of the experiments with ≤ 6% residual activity, fibrin growth rates were only available for the ≥ 13% group. We detected a 40% reduction in fibrin growth rate in the patient population as compared with the healthy controls ($P < 0.01$, Fig. 4B). Interdonor and intradonor variability for platelet and fibrin growth rates are presented in Table S1.

At < 1% coagulation factor activity levels, the platelet growth rate was substantially less than that observed for healthy controls (Fig. 4C). For % activity > 1%, no significant correlation between residual activity and fibrin growth rate was observed (Fig. 4D). A negative correlation ($R^2 = 0.48$) between platelet growth rate and PTT values was detected (Fig. 4E). Furthermore, no relationship between fibrin growth rate and PTT was observed (Fig. 4D) for PTT < 40 s. When PTT was > 40 s, the fibrin growth rate under flow was typically extremely low in all but two cases.

At early times in the assay (0–2.5 min), the platelet growth rate was not strongly correlated with PTT, indicating that this stage of platelet deposition was dominated by collagen-triggered signaling, with a lesser influence of thrombin production (Figure S3A). Interestingly, at this early time between 0 and 2.5 min, overall platelet adhesion for all patients with prolonged PTT (between PTT = 40–100 s) was 60% of healthy controls ($P < 0.001$), indicating at least a partial role for thrombin at this time-point. By 15 min, the inverse correlation between platelet growth rate and PTT was much stronger ($R^2 = 0.48$), indicating that thrombin and fibrin production at later times helped to drive net platelet deposition (Figure S3B).

Full channel occlusion and fibrin initiation time

The overall time of the experiment was assessed, defined as the first to occur among three possible events: (i) reaching a steady state, non-occlusive aggregate size (Fig. 2F), (ii) reaching a fully occlusive aggregate (Fig. 2C), or (iii) 15 min. All three of these outcomes were observed in the patient population, while all healthy controls reached full occlusion (Fig. 5A). In the < 1%
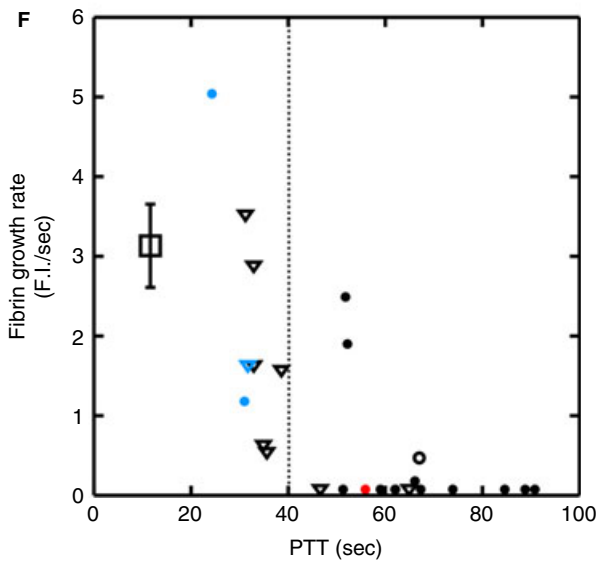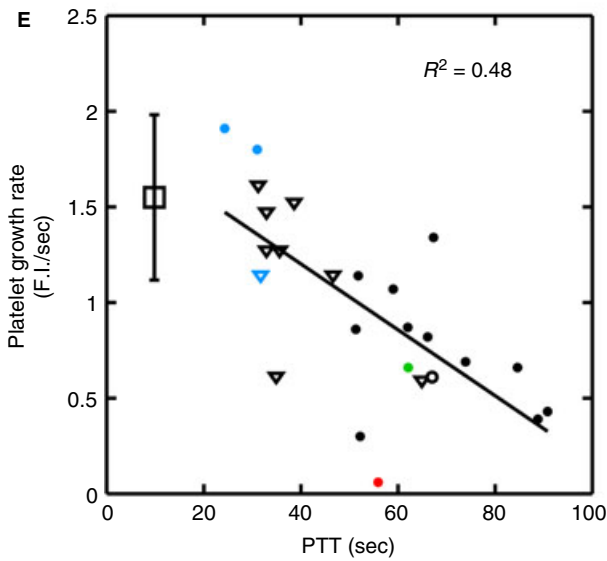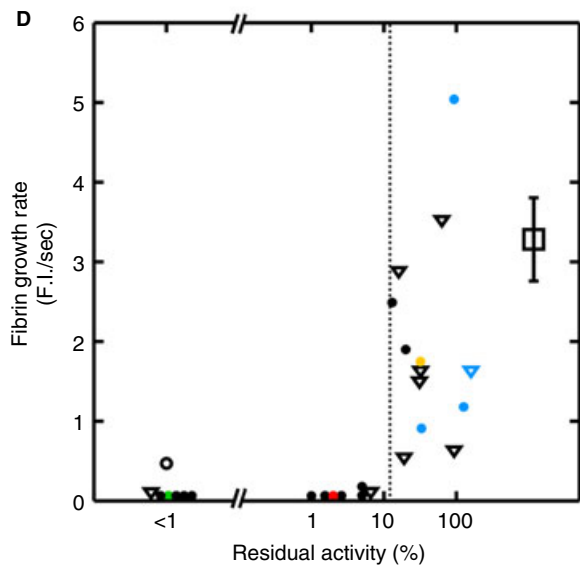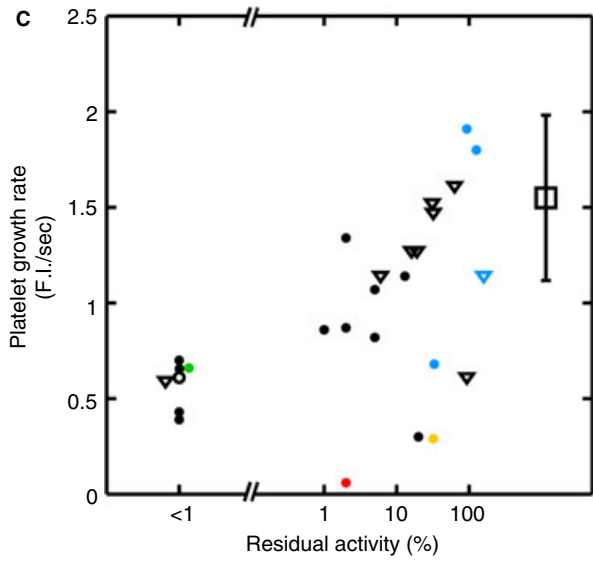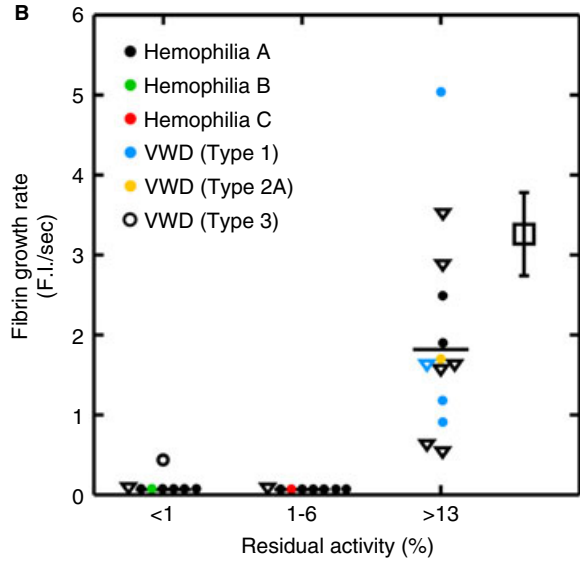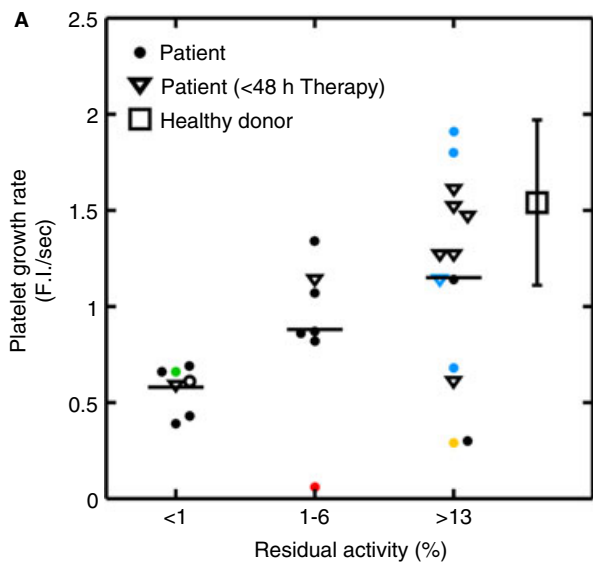
residual factor activity group, steady state, non-occlusive thrombi were reached in three cases (average time of 10 ± 0.5 min), while steady state had not been achieved by 15 min in the rest (iv). We observed significant embolus from steady state, non-occlusive thrombi in this patient population and a washed out appearance that was morphologically distinct from clots generated from healthy donors, indicative of clot instability (Fig. 2F). In the 1–6% group, full occlusion was reached in two cases (average time of 15 ± 0.25 min) while steady state had not been reached in the rest (v). Finally, in the $\geq 13\%$ residual activity group, nine experiments proceeded to full channel occlusion with an average time of 13 ± 2.0 min, which was 30% longer than healthy donor occlusion times (10 ± 1.5 min, $P < 0.001$). The rest (iv) had not reached a steady state size or full occlusion. These data are also summarized in Table 2.

Fibrin initiation time was extrapolated from the fibrin growth rate curve for all healthy donors and patients who generated fibrin before one of the three experimental endpoints was reached. In healthy controls we observed an average initiation time of 6.5 ± 1 min and in patients a time of 10 ± 2.5 min, a statistically significant increase ($P < 0.001$, Fig. 5B). Interdonor and intradonor variations for channel occlusion times as well as fibrin initiation times are presented in Table S1. For blood with < 13% residual coagulation factor activity, occlusion and fibrin initiation was not typically observed within the 15-min test (Fig. 5C,D). Similarly, occlusion and fibrin initiation was not typically observed when PTT > 40 s (Fig. 5E,F).

The overall platelet deposition, platelet growth rate and occlusion time were not correlated with platelet count (Figure S4). Furthermore, no statistical difference was present between patients with less than or greater than 150 000 platelets per microliter in any of these measured variables (data not shown).

Arterial shear rates potentiate platelet deposition but not fibrin deposition

In order to assess the role of arterial shear rate in platelet and fibrin deposition during perfusion of low CTI-treated whole blood over collagen, simultaneous experiments were conducted at 100 and 1000 s^{-1} in two separate microfluidic devices. Samples were collected from five healthy donors and a single patient diagnosed with severe

hemophilia A (Figure S5). The arterial shear rate environment resulted in a 33% reduction in time to full channel occlusion in the healthy population ($P < 0.02$). A 9-fold increase in platelet accumulation was noted at 1000 s^{-1} ($P < 0.05$) for the patient sample, but full channel occlusion was not achieved (Figure S6). Fibrin deposition measured at full channel occlusion time was reduced 4-fold at arterial shear in healthy donors ($P = 0.05$), probably due to the washout of thrombin and the reduced time for fibrin formation. Fibrin deposition was not observed in the patient sample (Figure S6) at either local wall shear rate.

Discussion

Using a microfluidic model of hemostasis, we have described a novel assay of platelet and coagulation function under venous flow conditions of low CTI-treated whole blood clotting on collagen. This technique measured platelet and fibrin deposition onto a patterned collagen type 1 surface using lightly anticoagulated whole blood under venous flow conditions. We characterized the assay using healthy human donors and demonstrated that fibrin generation was catalyzed at the collagen-localized platelet mass in 6.5 min (for our healthy population). With whole blood from healthy donors, visual signs of clotting took > 30 min to appear in the test tube and platelet activation markers of thrombin production were not detected after 35 min of whole blood incubation *ex vivo*. These results indicated that thrombin generation was enhanced at the microfluidic injury site, possibly due to platelet activation [17,20] as well as collagen activation of the contact pathway [19].

We analyzed the blood of 27 patients of the Hemophilia Program of the Hospital of the University of Pennsylvania who had been previously diagnosed with a bleeding disorder. We observed a significant 2-fold reduction in platelet adhesion between healthy controls and patients with < 1% residual critical factor activity, while patients with 1–6% and $\geq 13\%$ showed no defect. This result persisted for the < 1% group when platelet growth rate was calculated in order to normalize for the variety of endpoints observed in this study (15 min total perfusion, full channel occlusion, steady state, and non-occlusive thrombus before 15 min). Platelet growth rate revealed a significant defect in both the 1–6% and > 13% residual factor activity groups, indicating some intermediate
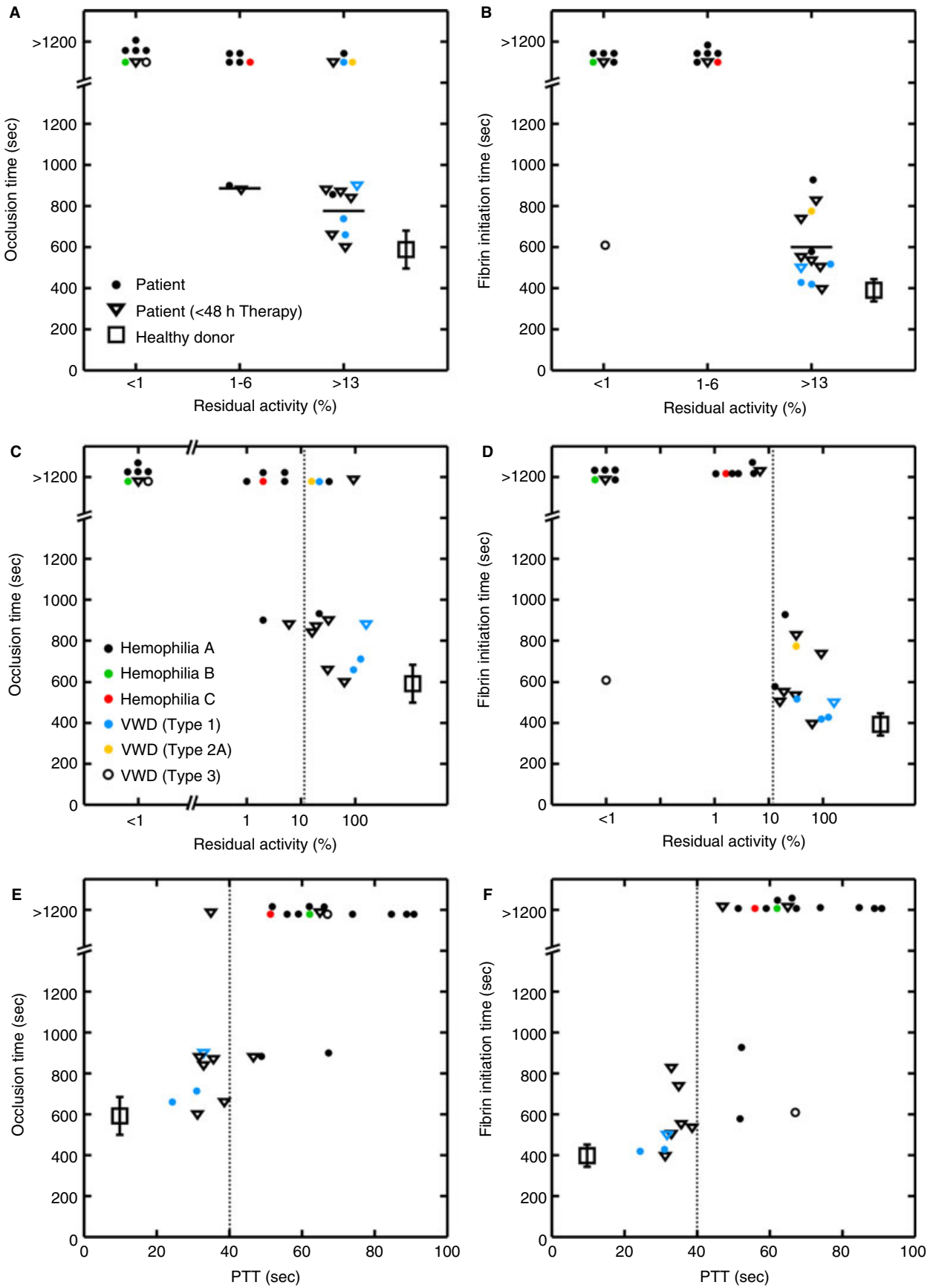
Fig. 5. Time to full channel occlusion and time to fibrin initiation were organized into three groups based on residual coagulation factor activity. (A) Patient samples with < 1% activity failed to fully occlude the channels by 900 s and did not do so by 1200 s. Two of seven patient samples reached full occlusion in the 1–6% activity group while 9 of 13 did so in the > 13% group. (B) For all but one sample with < 13% residual activity, a complete defect in fibrin initiation time is shown. Samples with > 13% activity displayed an average 70% increase in fibrin initiation time ($P < 0.001$). (C) Full channel occlusion was not correlated with residual coagulation factor activity > 1%. (D) Strong switch-like behavior around 13% residual coagulation factor activity (dotted line) was observed for fibrin initiation time. (E) Switch-like behavior was present for both full channel occlusion time (E) and fibrin initiation time (F) with respect to PTT around 40 s (dotted line). Note: neither residual coagulation factor activity nor PTT were obtained for healthy donors. The location of platelet and fibrin accumulation data for those donors is arbitrary.

deficiency in thrombin generation potential for these groups. Taken together, these results indicate that intrinsically generated thrombin enhances platelet deposition in this assay of platelet adhesion to collagen in the absence of exogenously added TF.

Fibrin deposition revealed a more striking result as all but one donor with < 13% of factor activity levels displayed a complete defect. Fibrin deposition in the ≥ 13% group was not significantly different from healthy controls. Fibrin accumulation and fibrin growth displayed switch-like behavior, which has been reported before for surfaces of collagen and TF [6,7]. In the case of the extrinsic pathway, a critical level of TF was required to overcome the effects of flow-mediated washout of activated coagulation factors and fibrin monomer, as well as endogenous inhibitors, such as TFPI [6]. We found that a minimum level of 13% residual FVIII activity (in hemophilia A patients) was required to overcome these effects in our microfluidic assay. Our group has previously demonstrated the importance of thrombin flux and washout in a model of fibrin deposition under flow using purified proteins [21]. Interestingly, our results indicate that an intermediate level of thrombin generation may exist for the 1–6% residual coagulation factor activity group that is capable of enhancing platelet deposition but not of generating fibrin.

Severe hemophilia is associated with < 1% of normal coagulation factor activity. Based upon the findings with microfluidic tests, the bleeding in patients with < 1% factor activity may be linked to deficits in both platelet and fibrin deposition. During treatment or in patients with moderate to mild hemophilia, the spontaneous bleeding risk is mitigated when factor levels are raised to > 1% activity [22]. Here we demonstrated that the ability to generate low levels of thrombin under flow at 1–6% of normal FVIII may potentiate platelet deposition and aggregate stability to maintain hemostasis via PAR1/ PAR4 signaling [16]. However, a > 1% activity level (PTT of ~40–60 s) does not necessarily rescue fibrin formation under flow. Recently our group has demonstrated that the polymerization of fibrin into a growing thrombus provides stability against increasing shear rates [7]. In patients with severe hemophilia A we observed a complete defect in the ability to achieve full microfluidic channel occlusion. In fact three of four samples produced steady state clots that appeared to embolize after the initial collagen-driven platelet deposition phase. These

results suggest the need for further evaluation of the maintenance of elevated trough levels to reduce the variation of bleeding phenotypes observed in patients on prophylactic therapy [23].

In this study we set out to investigate the role of thrombin generated via the contact pathway in thrombus growth in a microfluidic assay of vessel injury under flow conditions in a low TF environment. We observed that whole blood flow over exposed collagen type 1 could support platelet aggregation and fibrin generation in a manner influenced by the activation of intrinsic coagulation. Defects in the function of FVIII, FIX or FXI led to measurable reductions in overall accumulation of platelets and fibrin, as well as in their growth rates. Changes in the microfluidic platelet function in patient samples could be predicted by the PTT test, highlighting an important role for thrombin in aggregate growth and stability. Intrinsic coagulation was enhanced at the microfluidic injury site, occurring in just 6.5 min for healthy donors, as compared to > 30 min in the test tube. The generation of measurable quantities of fibrin in patient samples was switch-like with respect to residual coagulation factor activity as well as PTT, suggesting a requirement for threshold levels of thrombin generation. In the future, whole blood microfluidic assays to assess platelet and coagulation function may replace static assays as they capture more details of the hemostatic mechanism.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Flow cytometry on whole blood samples incubated in low CTI (4 µg mL^{-1}), PPACK or PPACK and 5 nM Convulxin for up to 35 min was performed at 5-min intervals.

Fig. S2. Whole blood from a single donor (and from the same blood draw) was perfused over collagen type 1 in separate microfluidic devices simultaneously ($n = 8$, per condition).

Fig. S3. Platelet growth rate was calculated for all patients and healthy donors between the start of the experiment and 2.5 min.

Fig. S4. Total platelet accumulation, platelet growth rate and occlusion time are plotted against platelet count.

Fig. S5. Platelet and fibrin kinetic traces for healthy donors and a single severe hemophilia A patient ($n = 8$, < 1% residual F VIII activity) for two different shear rate experiments performed simultaneously.

Fig. S6. Representative images of platelets (green) and fibrin (red) are presented for a healthy donor (A–C) and a patient (severe hemophilia A [< 1% FVIII activity], D–F) at a shear rate of 1000 s^{-1}.

Table S1. Intradonor and interdonor variability in the outputs listed was calculated as described in the Materials and methods section.

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