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ORIGINAL ARTICLE

Protein C or Protein S deficiency associates with paradoxically impaired platelet-dependent thrombus and fibrin formation under flow

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Abstract

Background: Low plasma levels of protein C or protein S are associated with venous thromboembolism rather than myocardial infarction. The high coagulant activity in patients with thrombophilia with a (familial) defect in protein C or S is explained by defective protein C activation, involving thrombomodulin and protein S. This causes increased plasmatic thrombin generation.

Objective: Assess the role of platelets in the thrombus- and fibrin-forming potential in patients with familial protein C or protein S deficiency under high-shear flow conditions.

Patients/Methods: Whole blood from 23 patients and 15 control subjects was perfused over six glycoprotein VI–dependent microspot surfaces. By real-time multicolor microscopic imaging, kinetics of platelet thrombus and fibrin formation were characterized in 49 parameters.

Results and Conclusion: Whole-blood flow perfusion over collagen, collagen-like peptide, and fibrin surfaces with low or high GPVI dependency indicated an unexpected impairment of platelet activation, thrombus phenotype, and fibrin formation but unchanged platelet adhesion, observed in patients with protein C deficiency and to a lesser extent protein S deficiency, when compared to controls. The defect extended from diminished phosphatidylserine exposure and thrombus contraction to delayed and suppressed fibrin formation. The mechanism was thrombomodulin independent, and may involve negative platelet priming by plasma components.

Bibian M. E. Tullemans, Cristiana Bulato, Gina Perrella, Paolo Simioni, and Johan W. M. Heemskerk contributed equally to this work.

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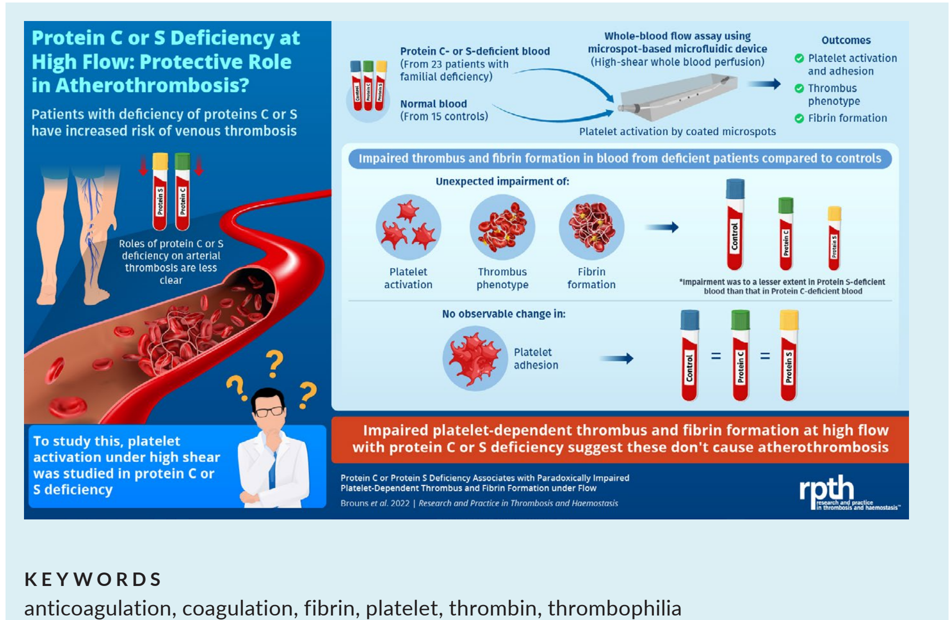


KEYWORDS
anticoagulation, coagulation, fibrin, platelet, thrombin, thrombophilia

Essentials

- Patients with protein C or S deficiency are at increased risk of venous thrombosis.
- Using a whole-blood flow assay, we measured thrombus formation for 23 patients with protein C or S deficiency.
- At arterial flow, we found paradoxically reduced platelet activation and blood clotting.
- This reduction was more prominent for patients with deficiency of protein C than of protein S.

1 | INTRODUCTION

Low plasma levels of the natural anticoagulants protein C or protein S are strongly associated with venous thromboembolism, and appear to be a common cause of familial thrombophilia.[1-5] However, atherothrombotic complications are less frequently seen in such patients. An early case report points to arterial thrombosis in some patients with protein C or protein S deficiency,[6] while the ARIC cohort study concluded that low protein C can be a risk factor for ischemic stroke but not for coronary heart disease.[7] This was confirmed by a systematic review indicating that hypercoagulability is a stronger risk factor for stroke than for myocardial infarction.[8]

The apparent absence of a link to myocardial infarction and hence atherothrombosis may suggest that the protein C and S anticoagulants are stronger negative regulators of blood clotting in the venous than in the arterial (cardio)vasculature. For arterial thrombus formation, the shear-dependent activation of platelets and subsequent coagulation are crucial.[9] However, to what extent these processes are altered in patients with thrombophilia is essentially unknown. An incidental report pointed to a supporting role of protein C in high-shear flow-dependent platelet adhesion.[10] However, in mice, the platelet-expressed protein S was found to negatively regulate fibrin-thrombus clotting in large veins but not in large arteries.[11]

The protein C pathway, unraveled in human and mouse plasmatic systems, provides a feedback loop in which thrombin downregulates its own generation.[5,12] In this pathway, locally formed thrombin binds to endothelial-expressed thrombomodulin (TM), which complex activates protein C on the endothelial protein C receptor.[13] Protein S herein acts as a cofactor, supporting the role of activated protein C (APC) to inactivate the coagulation process.[14,15] Through the inactivation of factors Va and VIIIa, APC thus halts ongoing thrombin generation.[16] This implies impaired anticoagulant activity—and hence increased coagulant activity—in patients with a deficiency in protein C or protein S. Accordingly, the plasma from such patients shows a reduced TM-dependent conversion of protein C into APC, and an impaired ability of APC to suppress thrombin generation and hence the clotting process.[17]

Glycoprotein VI (GPVI, binding collagen) and glycoprotein Ib-V-IX (GPIb, binding von Willebrand factor [VWF]) are essential platelet receptors triggering the processes of thrombus and fibrin formation at arterial wall-shear rate on collagen and tissue factor (TF) surfaces.[18,19] Herein, the signaling collagen receptor GPVI induces integrin $\alpha IIb\beta 3$ activation, granule secretion, and surface exposure of the procoagulant phospholipid phosphatidylserine.[20,21] The key role of GPVI in arterial thrombus formation was confirmed by a reduction of platelet aggregates, absence of phosphatidylserine exposure, and impaired fibrin clot formation with blood from patients with homozygous GPVI deficiency.[22]

Recently, we developed a microspot-based microfluidic system, in which the kinetics of platelet adhesion, platelet activation, thrombus structure, and fibrin clot formation can be monitored simultaneously

during high-shear whole-blood perfusion.[23] This multiparameter generating flow assay using a collagen and TF surface proved to be sensitive to the anticoagulant actions of TM and APC. In addition, it detected a procoagulant phenotype in the blood from patients carrying the prothrombotic factor V–Leiden mutation,[24] that is, a mutation leading to a factor Va form that cannot be inactivated by APC.[23]

In this article, we applied this multiparameter microfluidic test to evaluate the blood from patients with impaired anticoagulant activity due to a congenital deficiency in protein C or protein S. The patients were recruited from one of the largest cohorts of congenital thrombophilia subjects worldwide.[5] Our results point to a paradoxical impairment of the GPVI-dependent platelet-thrombus buildup and fibrin formation under shear that was most prominent in cases of protein C deficiency.

2 | MATERIALS AND METHODS

2.1 | Materials

Cross-linked triple-helical collagen-related peptides were used form the sources described before,[23] that is, triple-helical H-GPC(GPO)$_3$GFOGER(GPO)$_3$GPC-NH$_2$, in short GFOGER-(GPO)$_n$, a peptide binding to GPVI and integrin $\alpha 2\beta 1$[25]; and H-GPC(GPP)$_5$GPRGQOGVMGFO(GPP)$_5$GPC-NH$_2$, collagen type III–derived VWF–binding peptide (VWF-BP) VWF-III.[26] Collagen-I Horm derived from equine tendon (collagen-I) was obtained from Nycomed (Hoofddorp, The Netherlands). Human collagen-III (C4407) came from Sigma-Aldrich (Zwijndrecht, The Netherlands). The selective spleen tyrosine kinase (Syk) inhibitor PRT-060318, 2-((1R,2S)-2-aminocyclohexylamino)-4-(m-tolylamino)pyrimidine-5-carboxamide (Syk-IN), was from Bio-Connect (Huissen, The Netherlands); human TM from American Diagnostica (Pfungstadt, Germany); human APC from Innovative Research (Novi, MI, USA); recombinant human TF (Innovin) from Siemens (Erlangen, Germany); human fibrinogen from Enzyme Research Laboratories (Swansea, UK); human α-thrombin from Stago (Saint Ouen-l'Aumone, France); human factor XIIIa from Zedira (Darmstadt, Germany). Bovine serum albumin was obtained from Sigma-Aldrich; *D*-phenylalanyl-*L*-prolyl-*L*-arginine chloromethyl ketone from Calbiochem (San Diego, CA, USA); 3,3′dihexyloxa carbocyanine iodide (DiOC$_6$) from AnaSpec (Fremont, CA, USA); Alexa Fluor (AF)568-labeled annexin A5 from Life Technology (Carlsbad, CA, USA); and AF647-labeled human fibrinogen from Molecular Probes (Eugene, OR, USA). Other materials were from sources described before.[27]

2.2 | Preparation of microspot coatings

Glass coverslips (24 × 60 mm, Thermo-Fisher, Breda, The Netherlands) containing microspots were freshly prepared, as described before.[23,28] Coating concentrations were optimized to achieve maximal platelet adhesion. Microspots (all 2.0 µl) consisted of the following components (Table 2): for microspots I and II, collagen-I (50 µg/ml) without or with TF (500 pM), respectively; and for microspots III and IV, GFOGER-(GPO)$_n$ (250 µg/ml) + VWF-BP (100 µg/ml) without or with TF (500 pM), respectively. Furthermore, for microspot V, collagen-III (100 µg/ml); for microspot VI, fibrin (prepared from fibrinogen [1 mg/ml, 0.5 µl] plus thrombin [20 nM, 1 µl]), and after 30 min added factor XIIIa (0.7 µg/ml)[28]; for microspot VII, collagen-I (50 µg/ml) ± TM (0.7 µg/ml). For experiments with microspots V to VII, TF was present in the recalcified blood, as described elsewhere.[23]

2.3 | Blood drawing from patients and control subjects

Blood samples were obtained after medical ethical permission and full informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Padova University Hospital (protocol code 4303/AO/17; July 28, 2017). Patients with known deficiency of plasma protein C (n = 12) or protein S (n = 11) were enrolled among the cohort of patients previously referred to the Thrombotic Unit of Padova University Hospital for thrombophilia screening and identified as thrombophilia carriers.[5] Inclusion criteria were age >18 years, documented protein C or protein S deficiency, availability to come to our center the day of the study. Exclusion criteria were antiplatelet or anticoagulant medication in the previous 4 weeks, surgery or acute infection in the previous 3 months, or cancer. The criteria used for the classification of protein C and protein S defects were in line with the current literature, and reduced levels of anticoagulant factors (activity and/or antigen) were confirmed in two consecutive determinations and in at least one first-degree relative.[29] Healthy control subjects (n = 15) were included on the same days as patients among volunteering nonfamily companions of patients or volunteers among the Thrombotic Unit staff. The control group consisted of subjects without a history of cardiovascular, autoimmune, and acute diseases and not undergoing antithrombotic, antibiotic, or hormonal therapy. Characteristics of patients (families) and of blood hematologic variables are indicated in Table 1. Particularly, two patients among the protein C deficiency group (patients 10 and 12) and two patients among the protein S deficiency group (patients 20 and 21) had previous deep vein thrombosis. Patients and controls were recruited fasting during the same weeks (October 7–11, 2019) in the morning. Blood was drawn by venipuncture directly into BD Vacutainer citrate tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing 0.109 M (3.2%) sodium citrate and processed within 4 h.

2.4 | Operational procedures of whole-blood flow measurements by microfluidics

Citrated blood (1000 µl) was recalcified and perfused through a microfluidic chamber using two syringes on pulse-free pumps, connected to a *y*-shaped mixing tubing. The *y*-tube and shape of

TABLE 1 Characteristics of patients and blood variables of patients and control subjects

Subject	Deficiency	Age	Sex	Platelet count[a], 10^9/L	Mean platelet volume, fL	WBC, 10^9/L	RBC, 10^9/L	Hema tocrit, %	Medication
C01	None	28	8M	*148*	*10.8*	5.3	4.17	0.38	None
|	|	|	|	|	|	|	|	|	|
C15	None	64	7F	260	8.4	8.3	5.44	0.47	None
Pt01	Protein C	36	F	238	7.8	7.5	3.94	36.6	None
Pt02	Protein C	49	F	297	8.3	*10.7*	4.31	35.2	None
Pt03	protein C	25	F	163	8.8	7.7	3.90	36.4	None
Pt04	Protein C	51	F	159	7.2	4.7	3.81	35.8	None
Pt05	Protein C	82	F	190	*6.0*	4.0	3.62	35.3	None
Pt06 (Fa1)	Protein C	53	M	210	7.2	5.1	4.62	40.4	None
Pt07 (Fa1)	Protein C	23	F	223	8.2	5.3	3.86	*34.5*	None
Pt08 (Fa1)	Protein C	18	F	207	7.5	6.9	4.32	37.2	None
Pt09	Protein C	31	F	210	8.6	5.1	4.75	44.5	None
Pt10[b]	Protein C	67	M	248	n.d.	6.4	4.54	40.6	None
Pt11 (Fa2)	Protein C	20	F	233	7.9	5.1	4.46	42.6	None
Pt12 (Fa2)	Protein C	54	M	232	7.4	4.0	4.60	44.1	None
Pt13 (Fa3)	Protein S	65	M	158	8.6	5.8	4.99	44.1	None
Pt14 (Fa3)	Protein S	37	F	221	8.4	6.3	4.52	*32.9*	None
Pt15 (Fa4)	Protein S	68	F	191	7.3	5.6	4.30	42.0	None
Pt16 (Fa4)	Protein S	73	F	*137*	7.9	5.4	4.52	42.3	None
Pt17	Protein S	49	F	203	7.8	4.3	*3.35*	*33.4*	None
Pt18	Protein S	51	F	250	8.0	5.1	4.11	*25.8*	None
Pt19	Protein S	62	F	245	6.8	6.5	3.71	36.1	None
Pt20[b]	Protein S	30	M	210	9.4	4.9	5.06	36.5	None
Pt21	protein S	29	F	287	8.1	6.6	4.01	42.3	None
Pt22	protein S	28	F	217	*10.4*	6.1	4.47	42.6	None
Pt23	protein S	55	F	174	*10.4*	5.8	4.47	46.6	None

Note: Gray blocks indicate relatives of four families (Fa1-Fa4).

[a]Normal ranges[40]: platelet count 150–400 × 10^9/L; mean platelet volume 7.0–9.5 fl; white blood cell count (WBC) 4.0–10.0 1 × 0^9/L; red blood cell count (RBC) 4.4–5.8 × 10^9/L (M) and 3.6–5.3 × 10^9/L (F); hematocrit 0.41–0.51% (M), 0.35–0.47% (F). Values outside normal ranges are indicated in *italics*.

[b]Indicates patients with previous (>6 months) venous thromboembolism.

the chamber inlet allowed full mixing by pressure injection of the recalcification medium (32 mM $MgCl_2$ and 63 mM $CaCl_2$ in N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid buffer, pH 7.45) into the blood, as detailed before.[23] Coinfusion was at a volume ratio of 10 (blood) to 1 (recalcification medium), resulting in a final wall shear rate of 1000 s^{-1}. For flow runs over microspots V to VII, TF (10 pM) was added to the recalcification medium. Immediately before the start of perfusion, samples of blood were labeled with $DiOC_6$, (0.5 μg/ml, final concentration [f.c.]), AF647-fibrinogen (8.5 μg/ml, f.c.), and AF568-annexin A5 (4.0 μg/ml, f.c.). The probe $DiOC_6$ is routinely used to label (adhered) platelets in whole-blood microfluidics by our and other laboratories.[18,28,30,31] Of note, erythrocytes in the blood were not stained by $DiOC_6$, and adhered transiently only if the microchannels were partly occluded (Figure S1). Erythrocytes were excluded upon image analysis. Leukocytes did not adhere to the platelet-fibrin thrombi under the present high-shear flow conditions with control or patient blood.

2.5 | Recording of time-dependent platelet-fibrin thrombus formation

During blood flow, at time points 0, 2, 4, 6, 8, and 10 min, overlay brightfield and fluorescence microscopic images were acquired from indicated microspots. An inverted EVOS fluorescence microscope (Life Technology, Ledeberg, Belgium) was used, equipped with brightfield illumination, three light-emitting diode cubes (excitation wavelengths: green fluorescent protein 470 nm, red fluorescent protein 531 nm, and Cy5 626 nm), and an Olympus 60× oil-immersion objective with high *z*-axis resolution (UPLSAPO60, numerical

TABLE 2 Explanation of microspots (M) and parameters (P) used to quantify thrombus and fibrin formation under flow

Microspot composition		Triggers	Ref.
I.	Collagen-I (VWF)[a]	GPIb, GPVI, α2β1	23
II.	Collagen-I (VWF)[a] + TF	GPIb, GPVI, α2β1, FVIIa	23
III.	GFOGER-$(GPO)_n$ + VWF-BP	GPIb, GPVI, α2β1	23
IV.	GFOGER-$(GPO)_n$ + VWF-BP + TF	GPIb, GPVI, α2β1, FVIIa	23
V.	Collagen-III (VWF)[a] + TF	GPIb, GPVI, α2β1	28
VI.	Fibrin + FXIIIa (VWF)[a] + TF	GPIb, GPVI, αIIbβ3	28
VII.	Collagen-I (VWF)[a] ± TM	GPIb, GPVI, α2β1 ± APC	23

P	Time (min)	Image type	Description	Unit
Platelet parameters				
P1	0–10	$DiOC_6$	Platelet adhesion	%SAC
P2	0–10	AF568-annexin A5	Platelet PtdS exposure	%SAC
Thrombus parameters				
P3	0–10	Brightfield	Thrombus coverage	%SAC
P4	0–10	Brightfield	Thrombus morphology score	0–5
P5	0–10	Brightfield	Thrombus aggregation score	0–3
P6	0–10	Brightfield	Thrombus contraction score	0–3
Fibrin parameters				
P7	0–10	AF647-fibrin(ogen)	Fibrin deposition	%SAC
P8	0–10	Brightfield	Fibrin score	0–3
P9	0–15	AF647-fibrin	Shorter time to fibrin	15 – *t* min

Note: Indicated are (most active) triggers regarding platelet receptors and coagulation activation. Numbering of parameters is according to appearance in heatmap.

Abbreviations: $DiOC_6$, 3,3′dihexyloxa carbocyanine iodide; FVIIa, factor VIIa; GPIb, glycoprotein Ib; GPVI, glycoprotein VI; PtdS, phosphatidylserine, SAC, surface-area coverage; TF, tissue factor; TM, thrombomodulin; VWF, von Willebrand factor; VWF-BP, von Willebrand factor–binding peptide.

[a]Binding VWF from plasma. Details on scoring, similarly as in Herfs et al.[40]

aperture 1.35).[23] Images were collected as monochromatic at 8 bit (1360 ×1024 pixels, resolution 0.108 μm/pixel). Flow runs were checked for the absence of platelet aggregates or clots not caused by a microspot.

2.6 | Standard image analysis and delineation of thrombus parameters

The collected microscopic images were analyzed using semi-automated scripts, written in the open-access program Fiji/ImageJ. Separate scripts were developed for images of brightfield light and for each fluorescent label, as described.[23] The scripts operated by (i) an optimized fast Fourier transformation to reduce background noise, (ii) filtering of image-wide structures, (iii) vertical and horizontal dilate and erode steps to remove noise and enhance relevant structures, (iv) overlays to verify binary mask images, and (v) back-loops to reset thresholds if the analysis was incorrect. The combination of brightfield and fluorescence images resulted in a total of nine parameters per microspot and time point (Table 2), that is, platelet parameters P1-2; thrombus parameters P3-6, of which thrombus scores (P4-5) were given in comparison to preselected reference images; and fibrin parameters (P7-9). Image analysts were blinded to the experimental condition.

For precise determination of fibrin formation, brightfield videos were recorded to obtain a series of subtraction images, directly showing the growth of fibrin fibers. These were compared with simultaneously recorded images of AF647-fibrino(gen) fluorescence, after which the latter were thresholded to not include the lower fluorescence of platelet-fibrinogen aggregates.[32] This resulted in an offset to detect only the higher AF647 fluorescence of formed fibrin fibers, which was also checked against reference images from AF647 platelet aggregates formed under noncoagulant conditions (see Figure S1).

2.7 | Data handling, heatmaps, and statistics

Prism 8 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Flow runs were performed in duplicate or triplicate, and parameters of thrombus formation (P1-9) were averaged to obtain value per microspot, time point, and donor. Mean parameter values between groups were compared per blood sample using a paired Student's *t* test. For the representation of heatmaps in R, raw values

per parameter across indicated microspots were univariate scaled from 0 to 10.[33] For subtraction heatmaps, a standard filter was applied for changes outside the range of mean ± standard deviation (SD) was to indicate relevant effects.

Numerical data of interventions are presented as means with standard error of the mean. Data from individual patients were compared to normal ranges established in the cohort of control subjects (set as mean ± SD), as described before.[23] Statistical comparison between groups was using a Mann-Whitey U-test for numerical or continuous variables. Values of $p < 0.05$ were considered to be significant.

3 | RESULTS

3.1 | Impaired collagen/TF-induced thrombus and fibrin formation under flow with blood from patients deficient in protein C and protein S

To determine how protein C– and protein S–based thrombophilia influenced the platelet and coagulation properties under flow, we collected blood samples from 12 patients with a previously established protein C deficiency (patients 01–12, 9 women, 3 men) and 11 patients with a known protein S deficiency (patients 13–23, 9 women, 2 men). Patients and 15 healthy control subjects were recruited in the same experimental week (see Methods).

Characteristics of the included patients, of which four were related, and the blood variables are indicated in Table 1. For the majority of patients, counts of platelets and white and red blood cells were within normal ranges, with few exceptions slightly outside of the normal range. In all patients, the activity levels of protein C (21%-64%) or protein S (15%-59%) were singly subnormal (Figure 1). No abnormalities were seen in the antithrombin levels. Two of the patients, one with protein C deficiency and one with protein S deficiency, had experienced a previous (>6 months) venous thromboembolism (Table 1).

For all blood samples, freshly taken from patients and control subjects, we assessed thrombus formation ability using a validated flow chamber test, by perfusing the blood under continuous recalcification at a shear rate of 1000 s^{-1} over a series of microspots. On collagen-I microspots, thrombi formed over time triggered by glycoprotein VI and TF (Table 2). As a standard procedure,[23] platelet adhesion was quantified by preadding to the blood the label $DiOC_6$, while platelet activation (phosphatidylserine expression) was detected by added AF568-annexin A5. Real-time fluorescence microscopy of the two labels provided time-dependent information of the platelet parameters P1-2 (Figure 2). Brightfield images were collected simultaneously, which provided information on the thrombus phenotype (thrombus coverage, morphology, platelet aggregation and contraction), together forming the thrombus parameters P3-6. Addition of AF647-fibrinogen to the blood, under flow accumulating into the thrombi as AF647-fibrin, provided information on the clotting process, quantified as the fibrin parameters P7-9. The AF647 fluorescence images were thresholded to limit the slowly accumulating

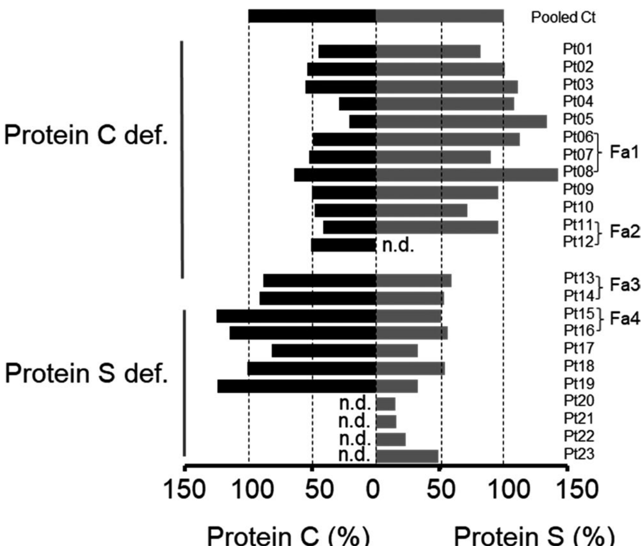


FIGURE 1 Characterization of anticoagulant factors of patients with deficiency in protein C (Pt01-12) or protein S (Pt13-23). Indicated are plasma activities of protein C and protein S per patient, compared to normal pooled plasma from control subjects (Ct). Families are indicated by accolades (Fa1-4). Additional coagulation parameters (mean ± standard deviation) of plasmas from patients with protein C (protein S) deficiency: prothrombin time–international normalized ratio 1.1 ± 0.1 (1.1 ± 0.1), antithrombin activity 102 ± 5% (101 ± 10%), von Willebrand factor antigen 112 ± 36% (132 ± 29%). n.d., not determined

fluorescence of AF647-fibrin.[32] By collecting multicolor image information at 6 time points for up to 10 min, we obtained 49 parameter values per microspot and flow run.

As required, flow perfusion of the blood from control subjects over collagen-I plus TF resulted over time in an accumulating platelet adhesion and phosphatidylserine exposure, followed by platelet aggregate buildup, contraction of the thrombi, and subsequent fibrin formation (Data S1). With blood samples from the patients who deficient in protein C or protein S, we noticed surprising differences. At end-term, the thrombi obtained with blood from many of these patients remained relatively small with a less contracted appearance, a low platelet phosphatidylserine exposure, and a retarded fibrin formation (Figure 2). The reduction in phosphatidylserine exposure was more obvious for protein C deficiency than for protein S deficiency, when compared to control subjects (Data S1; see also below).

Stringent analysis of all parameter values and presentation in a subtraction heatmap provided detailed insight into the differences in these processes over time per patient, when compared to the mean values of the controls (Figure 3). For microspots of collagen-I plus TF, we found for several patients a small reduction in phosphatidylserine exposure (parameter P2), but no time-dependent changes in platelet adhesion (parameter P1) (Figure 3, left part). Markedly, already at early time points (2–8 min) the thrombus parameters P3-6 were strongly decreased with the blood from 10 of 12 patients with protein C deficiency, and the blood from 6 out of 11 patients with protein S deficiency. For all of those patients, the reduction in

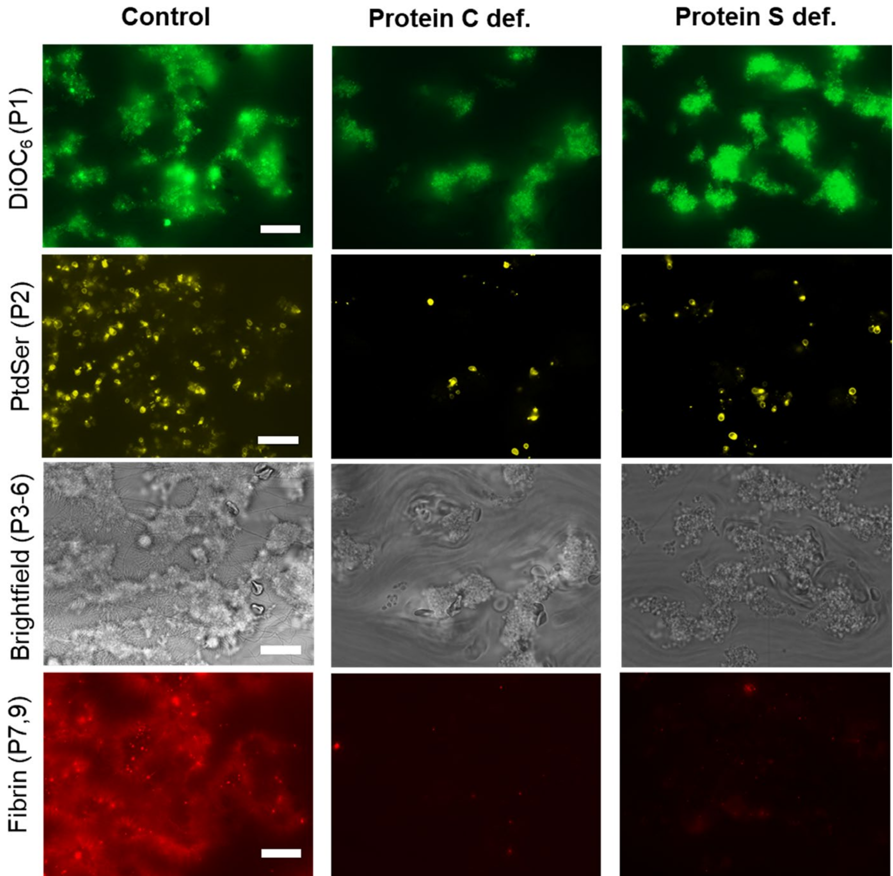


FIGURE 2 Imaging of whole-blood thrombus-fibrin formation under flow on collagen-I. Blood samples from day-controls (Ct1-12; $n = 12$) and patients with known deficiency in protein C (Pt01-12) or protein S (Pt13-23), collected on citrate, were used for assessment of thrombus-fibrin formation at a shear-rate of 1000 s^{-1}. The microfluidic flow chambers were coated with different microspots, coded by numbering I to VII (Table 2). Before perfusion under recalcification, blood samples were prelabeled with 3,3′dihexyloxa carbocyanine iodide ($DiOC_6$; detecting platelets), AF568-annexin A5 (detecting platelet PtdSer exposure) and AF647-fibrinogen (detecting accumulating fibrin). Overlay microscopic images, taken at time points 0, 2, 4, 6, 8, and 10 min, were analyzed for thrombus parameters (P1-9). Shown are representative 6-min images of flow runs over microspot II. (collagen-I + tissue factor, TF) from a control subject (Ct11), a patient with protein C deficiency (Pt07), and a patient with protein S deficiency (Pt18). Bars = 20 μm

thrombus parameters was followed by a delay and reduction in fibrin parameters P7-9.

Examination of the thrombus formation data after whole-blood flow over microspots of collagen-I only indicated a similar set of changes in the patient groups. In particular, for 7 of the patients deficient in protein C and 6 of the patients deficient in protein S, the same thrombus parameters were reduced on the collagen-I only surface (Figure 3, middle part). Hence, in, the majority of patients the impairments in thrombus formation appeared to be independent of triggering of the TF/factor VIIa pathway.

3.2 | TM-insensitive fibrin formation under flow with blood from patients with protein C or protein S deficiency

In blood samples from the majority of patients, with three exceptions (patients 01, 13, and 18), the co-coating of TM with collagen (ΔTM) did not retard the formation of fibrin, in contrast to the TM-dependent delay of fibrin formation of control blood samples (Figure 3, right part of green-black boxes). The difference in time to fibrin formation for each patient group in comparison to the control group was highly significant ($p < 0.001$). This confirmed impaired TM-induced protein C activation in the majority of patients, a difference that is in line with reported TM effects on thrombin generation measurements.[23,34] Accordingly, we concluded that the reduced levels of protein C or protein S in the patients' samples annulled the ability of TM to modulate the clotting under flow, thus confirming a functional impairment of the protein C pathway for most of the patients.

3.3 | Impaired thrombus and fibrin formation over high GPVI-stimulating surfaces under flow with patient blood

Additional image analysis of the flow data on collagen-I + TF, comparing the two patient groups with the control group, indicated that platelet deposition (P1) was unaltered in cases of protein C or

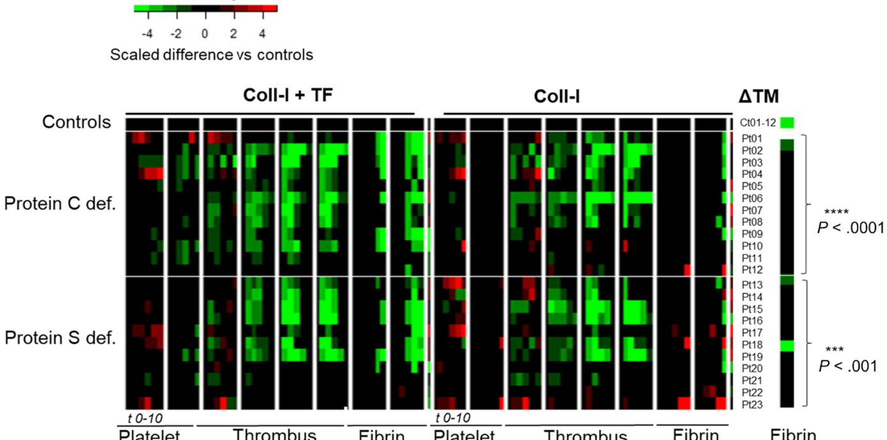


FIGURE 3 Reduced parameters of thrombus-fibrin formation on collagen-I and tissue factor (TF) spots in blood from patients with a protein C or protein S deficiency. Prelabeled blood samples from indicated subjects were flowed over microspots, coded as in Table 1: I, collagen-I; II, collagen-I + TF; and VII, collagen-I ± thrombomodulin™. Image parameters (P1-9) were obtained from time points 0, 2, 4, 6, 8, and 10 min. *Platelet parameters*: adhesion (P1, 3,3′dihexyloxa carbocyanine iodide [$DiOC_6$] surface-area coverage) and phosphatidylserine (PtdSer) exposure (P2, AF568-annexin A5 coverage); *thrombus parameters*: coverage (P3, brightfield, thrombus coverage), morphology score (P4), aggregation score (P5), and contraction score (P6); and fibrin parameters: fibrin deposition (P7, AF647-fibrin coverage), fibrin score (P8) and shorter time-to-fibrin (P9, 15 – *t* min). Data from duplicate flow runs were averaged. Values of each parameter over time were univariate scaled 0 to 10 across surfaces, and a subtraction was made for each individual patient in comparison to means of the control subjects. Subtraction heatmap is shown of relevant differences of P1-9 (0–10 min) per indicated patient. Differences were filtered for medium effect size (outside mean ± standard deviation [SD]) compared to means of controls. Green color: decrease; red color: increase. Right column: univariate scaled effect of TM co-coating (ΔTM) on microspots for fibrin formation (P7, 4–8 min) compared to controls (*P*, 1-way analysis of variance [ANOVA]). Green color: decrease below range of controls (outside mean ± SD)

protein S deficiency (Figure 4A). In contrast, phosphatidylserine expression (P2), as a marker of platelet procoagulant activity was substantially reduced in the protein C–deficient group but insignificantly changed in the protein S–deficient group (Figure 4B). Regarding fibrin deposition (P7), the analysis showed significant reductions between 6 and 10 min for either patient group, although the overall reduction was greater in cases of protein C deficiency (Figure 4C).

To obtain more information on a possibly altered role of the platelet collagen receptor GPVI, we also perfused blood samples from the patients over two microspots with a highly GPVI-stimulating surface, that is, the peptide GFOGER-$(GPO)_n$.[25] To allow shear-dependent platelet adhesion, the GFOGER-$(GPO)_n$ coating was combined with VWF-BP (binding von Willebrand factor), while the downstream microspot was postcoated with TF (Table 2). After analysis of the multicolor images collected over time, again 49 parameter values were obtained per microspot. A subtraction heatmap was again composed of the scaled parameters. This did not show patient-dependent changes in the platelet parameter P1 on GFOGER-$(GPO)_n$ with or without TF (Figure S2). However, the thrombus parameters P3-6 on either microspot were decreased at early time points with blood from 9 of 12 patients with protein C deficiency, and a lower fraction of 5 of 11 patients with protein S deficiency. For the patient blood samples showing a reduction in thrombus parameters, this was almost always followed by a later reduction and delay in fibrin parameters P7-9.

Groupwise comparison of the raw parameter data confirmed the heatmap-based analysis. For the highly GPVI-dependent surface of GFOGER-$(GPO)_n$ plus TF, no differences were seen in platelet adhesion (Figure 4D), whereas phosphatidylserine exposure was reduced in the protein C–deficient group but not in the protein S–deficient group (Figure 4E). In the presence of TF, fibrin formation was substantially delayed and reduced again in the protein C–deficient group only (Figure 4F). No significant difference was obtained for the group of patients deficient in protein S. Taking these findings together, we noticed a consistent reduction in phosphatidylserine exposure and fibrin formation at both collagen-I and GFOGER-$(GPO)_n$ microspots in the case of protein C deficiency, with a lesser reduction in part of the patients with protein S deficiency.

3.4 | Impaired thrombus formation at low GPVI-stimulating surfaces under flow with patient blood

Using blood samples from a subgroup of 8 protein C and 6 patients deficient in protein S, we evaluated the thrombus formation for two additional surfaces with low GPVI dependency, that is, collagen-III or fibrin formed in the presence of factor XIIIa.[28] Microscopic images were again analyzed and the obtained parameter values univariately scaled. The subtraction heatmap versus control blood samples indicated for the majority of patients deficient in protein C and half of the patients deficient in protein S a limited effect on platelet

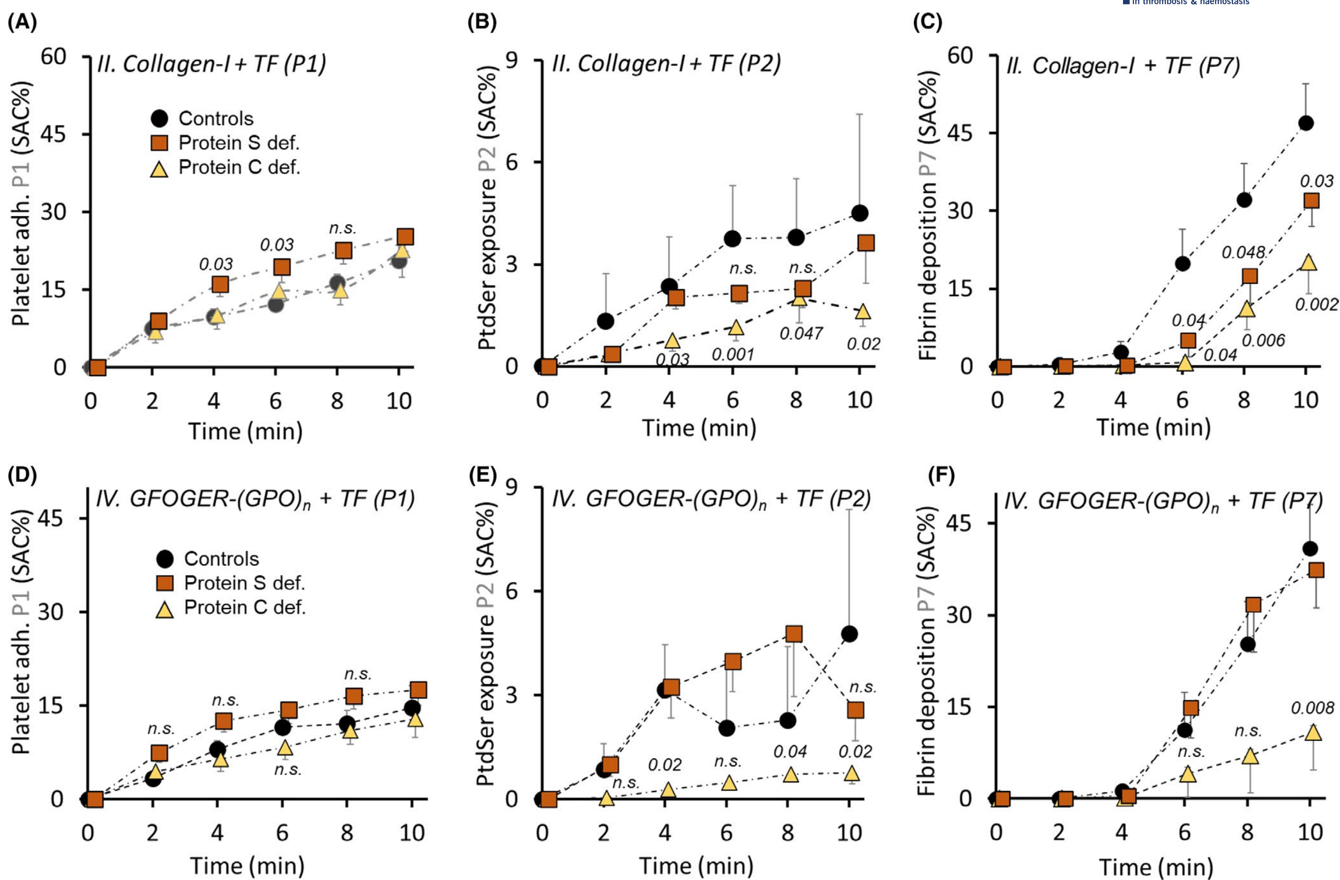


FIGURE 4 Time-dependent effect on platelet adhesion and fibrin formation on GPVI-dependent microspots and tissue factor (TF) in protein C or protein S deficiency. Prelabeled blood from controls or patients was flowed over collagen-I + TF (II.) (A-C) or GFO-GER-(GPO)$_n$ + TF (IV) (D-F); as for Figure 3. Images taken at $t = 0$–10 min were analyzed for platelet adhesion (P1), phosphatidylserine exposure (P2), and fibrin formation (P7). Shown is per subject group (controls, protein C or protein S deficiency): time dependency of platelet adhesion (A, D), phosphatidylserine exposure (B, E), and fibrin formation (C, F). Italic numbers, *p* values based on normal distribution (mean ± SE, *P* vs. controls, 1-way ANOVA)

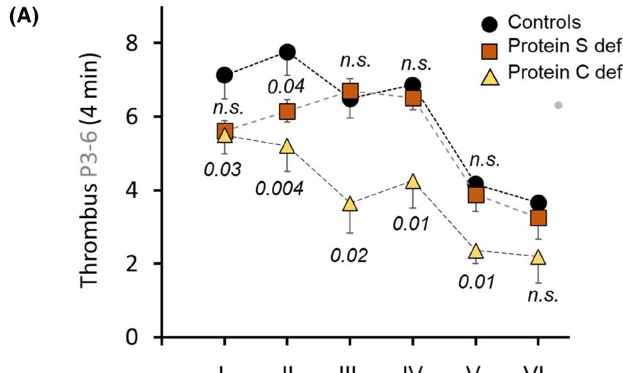
adhesion but a substantial reduction over time of the thrombus parameters (P3-6) (Figure S3). When evaluated per patient with protein S deficiency, the impairment of thrombus formation was consistent across the various surfaces, for example, with reduced thrombus parameters for patients 13, 15, 18, and 19 (Figures S2 and S3).

3.5 | Altered thrombus characteristics across surfaces under flow with patient blood

Finally, we focused on all thrombus characteristics related to the extent of platelet activation, that is, thrombus coverage, and morphology, aggregation size, and contraction scores (parameters P3-6), as these provided a signature of platelet activation in the thrombus-forming process before the start of overt coagulation.[23] When evaluating the means of these four parameters over time for the six surfaces (collagen-I, GFOGER-[GPO]$_n$ with[out] TF collagen-III and fibrin) at an early time point of 4 min, values were significantly lower in the protein C group than in the control group, regardless of the surface type but with an exception of fibrin/factor XIIIa (Figure 5A). For the protein S group, mean values were less affected and reduced only for the surface collagen-I plus TF. Statistical comparison of the mean values for other time points indicated that the lowering in case of protein C deficiency was maintained for up to 6 min (Figure 5B). Interestingly, for the weakly platelet-activating surface fibrin/factor XIIIa,[28] significance for the protein C–deficient group was only approached at 10 min (Figure 5B). For the protein S group, regression analysis indicated a correlation between the protein S activity level and the mean of thrombus parameters (4 min) for collagen-I ($p = 0.04$) and collagen-I + TF ($p = 0.05$) but not for the other surfaces. Taken together, these findings indicate that, upon flow over a variety of GPVI-dependent surfaces, the thrombus parameters related to platelet activation and fibrin clot formation are most consistently impaired with blood from the patients with protein C deficiency.

4 | DISCUSSION

In this article, we provide new findings that in blood from patients with isolated protein C deficiency, and to a lesser extent with protein S deficiency, the shear-dependent formation of thrombi and



(B)

	Protein C def.					Protein S def.				
	2 min	4 min	6 min	8 min	10 min	2 min	4 min	6 min	8 min	10 min
I. Collagen-I	0.004	0.030	0.077	0.136	0.603	0.056	0.070	0.344	0.364	0.331
II. Collagen-I +TF	0.003	0.004	0.037	0.192	0.367	0.015	0.042	0.183	0.649	0.129
III. GFOGER-(GPO)n	0.003	0.017	0.002	0.091	0.118	0.684	0.977	0.980	0.378	0.131
IV. GFOGER-(GPO)n + TF	0.021	0.013	0.033	0.122	0.528	0.260	0.445	0.620	0.226	0.142
V. Collagen-III (TF)	0.004	0.010	0.024	0.034	0.011	0.401	0.747	0.416	0.694	0.461
VI. Fibrin + FXIIIa (TF)	0.147	0.211	0.161	0.129	0.061	0.803	0.762	0.766	0.557	0.380

FIGURE 5 Time-dependent reduction in thrombus buildup and contraction across microspots in protein C or protein S deficiency. Prelabeled blood samples from indicated subjects were flowed over microspots, coded as in Table 1: I, collagen-I; II, collagen-I + tissue factor (TF); III, GFOGER-$(GPO)_n$ + von Willebrand factor–binding peptide (VWF-BP); IV, GFOGER-$(GPO)_n$ + VWF-BP + TF; V. collagen-III; VI. fibrin + factor XIIIa. In flow runs over microspots V.-VI., TF was added to the blood. Thrombus formation parameters P1-9 over time (0–10 min) were univariate scaled (0–10) across surfaces, as for Figure 3. Investigated numbers of subjects (controls, protein C deficiency, and protein S deficiency) were for I-IV: n = 12, 12, and 11; and for V-VI: n = 7, 8, and 6, respectively. A, Thrombus parameters (means of P3-6) per subject groups at 4 min, indicated for microspots I.-VI. B, Time-dependency of changes in thrombus parameters in protein C or protein S deficiency versus controls (means of P3-6) per microspot. Indicated are heatmapped p values versus controls at 2, 4, 6, 8, and 10 min. Blue = negative sign of change; yellow = positive sign of change. Mean ± standard error, P versus controls, 1-way analysis of variance. n.s., not significant

fibrin is delayed and impaired. Moreover, we detected the same reductions at six collagen-like surfaces with a high or low GPVI dependency of platelet activation. In the flow measurements where coagulation was not blocked, the same impairments were seen in the presence and absence of TF, triggering the extrinsic coagulation pathway. Collectively, the results suggest a GPVI-dependent but TF-independent defect in arterial thrombus-fibrin formation for the majority of patients with thrombophilia and protein C deficiency, and for part of the patients with protein S deficiency. The slightly enhanced defects in the presence of TF may point to a diminished formation of the protease APC, which is formed in a protein S–dependent way. It furthermore appeared that the molecular mechanism is TM independent, as no TM effects on coagulation inactivation were detected in almost all patient samples; a finding that was expected from earlier data.[23]

For the study, we collected blood from 12 patients with protein C deficiency and 11 patients with protein S deficiency. The overrepresentation of 18 female patients versus 5 male patients is expected in such cohorts.[17] Prior evaluation of the patient plasmas did not reveal abnormal levels of VWF or antithrombin (the latter was a patient inclusion criterion). None of the patients or control subjects was taking anticoagulant or antiplatelet medication at least 4 weeks before donating the blood sample, hence excluding a medication-related effect. For the majority of patients and controls, blood cell counts were within normal ranges. Sex- or age-related differences are not expected to affect the outcome of the whole-blood flow tests, as noted earlier.[33] Accordingly, the observed changes in thrombus formation are unlikely to be explained by these confounding factors.

In the positive interactions between blood flow, platelet activation, and thrombin generation, GPVI is known to play an important role as a collagen and fibrin receptor.[28,32,35,36] The GPVI-induced processes involve platelet phosphatidylserine exposure—evoked by collagen and thrombin—as well as the ensuing fibrin clot formation. With blood from the majority of patients deficient in protein C and part of the patients deficient in protein S, we observed a consistent reduction in both phosphatidylserine exposure and fibrin formation on various collagen-like microspots. From earlier work, it is known that a reduced phosphatidylserine expression indeed leads to lower thrombin generation and delayed fibrin clot formation.[19] Hence, the low phosphatidylserine exposure observed in flow runs with patient blood reflects an impairment in GPVI- and thrombin-dependent platelet activation processes.

One explanation for our findings might be a negative priming of the patients' platelets, for example, by past exposure of the circulating platelets to traces of thrombin.[9] The phenomenon of platelet desensitization or exhaustion, leading to a dysfunctional response to agonists, has been described for patients with acute ischemic stroke or cancer.[37,38] However, if such priming is relevant in the present

cases of protein C or protein S deficiency—that is, in patients with thrombophilia without recent thrombotic symptoms—is unclear. At present, we hypothesize that the reduced thrombus formation involves a TM-independent negative priming of platelets related to certain plasma components. Further research is needed to disclose the precise mechanism and to determine whether the defect lies in the blood plasma or the platelets. Of note, a similar loss-of-thrombus function was not observed in patients carrying factor V Leiden,[23] thus suggesting that the defect is not linked to factor Va inactivation.

So far, few conflicting studies have reported on the role of protein C in arterial thrombus formation. In one publication, it was demonstrated that immobilized protein C or APC can activate platelets via the GPIb-V-IX complex and apolipoprotein E receptor-2 receptors.[10] However, no coagulant effects were observed. Differently from our data, an early paper stipulated that APC can inhibit thrombus formation under flow.[39] Limitations of our study, although providing multiparameter data of platelet activation under flow, are that the blood volume and the flow protocol did not allow simultaneous measurements of other platelet function tests. These will need to be performed in the future.

In summary, the present data point to a paradoxical positive role of protein C—and to a lesser extent protein S—on platelet and coagulation activation under flow, likely through a GPVI- and coagulation-dependent mechanism. The observed impaired thrombus formation at high, arterial shear rate might explain why the patients with thrombophilia may be more frequently exposed to venous thromboembolism than to myocardial infarction.

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RELATIONSHIP DISCLOSURE

JWMH is a cofounder and shareholder of FlowChamber. The other authors declare no relevant conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: SLNB, JPvG, PS, and JWMH; methodology and analysis: SLNB, BMET, and GP; patient contacting and clinical laboratory testing: CB; post hoc plasma analysis: RvO and JPvG; resources: EC, LS, MEJK, PEJvdM, PS, and JWMH; data curation: BMET, GP, CB, EC, and HMHS; writing, drafting, and editing: EC, LS, PS, and JWMH; supervision: EC, MEJK, PEJvdM, PS, and JWMH.

DATA AVAILABILITY STATEMENT

All primary data are included in Data S1.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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