Investigation of the Endothelial Protein C Pathway Response and its Influence on the Thrombotic Risk in Carriers of the Factor V Leiden Mutation

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* Equal contributions as first author

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Schwarz, N, Yadegari, H. Potentials of Endothelial Colony-Forming Cells: Applications in Hemostasis and Thrombosis Disorders, from Unveiling Disease Pathophysiology to Cell Therapy. *Hamostaseologie* 2023; 43:325-337. doi: 10.1055/a-2101-5936

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Best Abstract Award

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List of Abbreviations

α2-AP	α2-antiplasmin
AT	Antithrombin
APC	Activated protein C
AUC	Area under the curve
Cell-ELISA	Cell-based enzyme-linked immunosorbent assays
ECFC	Endothelial colony–forming cell
EPCR	Endothelial protein C receptor
F1+2	Prothrombin fragment 1+2
FII	Prothrombin
FIX	Coagulation factor IX
FIXa	Activated coagulation factor IX
FU	Fluorescence units
FV	Coagulation factor V
FVa	Activated coagulation factor V
FVIIa	Activated coagulation factor FVII
FVIII	Coagulation factor VIII
FVIIIa	Activated coagulation factor VIII
FX	Coagulation factor X
FXa	Activated coagulation factor X
FXI	Coagulation factor XI
FXIa	Activated coagulation factor XIa
FXII	Coagulation factor XII
FXIIa	Activated coagulation factor XIIa
FXIIIa	Activated coagulation factor XIIIa
FVL	Factor V Leiden
OECA	Oligonucleotide-based enzyme capture assay
PAI-1	Plasminogen activator inhibitor-1
PAP	Plasmin-α2-antiplasmin complex
PC	Protein C

PS	Protein S
rFVIIa	Recombinant activated factor VII
sFM	Soluble fibrin monomer
SHAPE	Stimulated hemostasis activity pattern evaluation
t-PA	Tissue-type plasminogen activator
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complex
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
u-PA	Urokinase-type plasminogen activator
VTE	Venous thromboembolism
VWF	Von-Willebrand-factor

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Abstract

Venous thrombosis and its potentially life-threatening complication pulmonary embolism are caused by the formation of an intravascular blood clot. Though there is a myriad of potential etiologies for this condition, the factor V Leiden mutation (FVL) is its most common genetic risk factor in populations of Caucasian descent. FVL affects the anticoagulant protein C (PC) system, in which PC is activated as a result of coagulation activation in an interplay between factors in the circulation and endothelial receptors.

However, under comparable environmental conditions, the clinical expressivity of the thrombotic phenotype is highly variable between FVL carriers. Recently, in vivo human studies showed that the formation of activated PC (APC) in response to coagulation activation induced by recombinant activated factor VII is increased in FVL carriers without a history of thrombosis (asymptomatic) compared to FVL carriers with a history of thrombosis (symptomatic). Starting from this observation, this thesis focuses on the question of which factors modulate the thrombotic risk in FVL carriers via either direct or indirect interaction with the mutation. Following the hypothesis that differences in the PC system may contribute to this phenomenon, symptomatic and asymptomatic FVL carriers were examined for differences in pathways affecting the PC system, in terms of 1.) thrombin formation, 2.) APC formation, and 3.) fibrinolysis.

Experimental strategies included the measurement of in vitro and in vivo thrombin generation in FVL carriers and other thrombophilia patients, as described in **chapter 2.1**. Alterations in the fibrinolytic system following recombinant FVIIa-induced coagulation activation in symptomatic and asymptomatic FVL carriers were addressed in **chapter 2.2** using the same in vivo model. Furthermore, in order to study APC formation in detail, an ex vivo model of the PC pathway using patient-derived endothelial cells and autologous plasma was established (**chapter 2.3**) and applied to symptomatic and asymptomatic FVL carriers (**chapter 2.4**).

While analyses of in vitro thrombin generation and potential influencing factors did not show differences between FVL carriers with and without a history of thrombosis, or between FVL carriers and FVL non-carriers, changes in the fibrinolytic system after in vivo stimulation of thrombin and APC formation showed specific differences between symptomatic and asymptomatic FVL carriers. The ex vivo model of the PC system revealed that the APC response to thrombin formation was significantly more

pronounced in asymptomatic than symptomatic FVL carriers, although no differences in the expression of the PC system-relevant membrane proteins thrombomodulin and endothelial PC receptor, or APC inactivation kinetics were observed. In subsequent crossover experiments, endothelial cells were identified as the causative factor of a more pronounced APC response in asymptomatic FVL carriers.

Taken together, these results affirm the hypothesis that components of the PC system modulate the thrombotic risk in FVL carriers and indicate the involvement of endothelial features. Further studies are warranted to study the endothelial influence on APC formation in FVL in more detail. A more general achievement of the presented research is the successful establishment of a personalized ex vivo model that can be applied to study the functionality of the anticoagulant PC system in individual patients, including potentially in clinical diagnostics in the future.

1 Introduction

1.1 The hemostatic system

Blood is constantly circulating, maintaining the nutrient and oxygen supply of every cell and organ in the body. In the case of vessel wall injury, excessive bleeding is limited by the formation of a physiological blood clot. This process of clot formation and subsequent lysis, i.e. hemostasis, is very tightly regulated and relies on various proenzymes and inhibitors (synthesized predominantly in the liver and released into the bloodstream), bone-marrow-derived cells in circulation, and the endothelium (the cellular lining of blood vessels). In the following sections, the three main elements of hemostasis, namely coagulation activation, regulation, and fibrinolysis, are further described.

1.1.1. Coagulation activation and clot formation

When a vessel is damaged, blood becomes exposed to the subendothelial matrix and subendothelial cells. In response, coagulation is activated in different ways.

Platelet adhesion to the exposed subendothelial matrix, referred to as primary hemostasis, is a critical initial step in the immediate arrest of bleeding.[1] This process is initiated when platelets come into contact with exposed collagen from the subendothelial matrix and become activated, which results in the release of soluble agonists and the upregulation of adhesion receptors,[2] and it is further augmented when exposed collagen captures circulating Von-Willebrand-factor (VWF), a glycoprotein and multimer composed of repeating subunits. In response to collagen binding, VWF unfolds and exposes its a1 domain, which is a binding site for platelets.[3] The adhesion of platelets to VWF induces further platelet activation,[2] causing the platelets to aggregate and adhere to the vessel wall, which initiates the primary closure of the vascular damage via an unstable platelet clot.[4]

Plasmatic coagulation activation, referred to as secondary hemostasis, is divided into two initial pathways which were both described in 1964.[5,6] The extrinsic pathway is initiated by the cellular presentation of tissue factor (TF) to the circulation. TF instantaneously forms a complex with activated coagulation factor VII (FVIIa), as factor

VII in its activated form is constantly present in low levels in the plasma in order to ensure an immediate coagulation activation response.[7] The FVIIa-TF complex then activates coagulation factor X (FX), forming activated FX (FXa).

An alternative pathway, known as the intrinsic or contact pathway, relies on the exposure of coagulation factor XII (FXII) to subendothelial collagen which is thereby activated. Activated FXII (FXIIa) acts as a catalyst for activation of coagulation factor XI (FXI), followed by activation of coagulation factor IX (FIX) via enzyme activity of activated FXI (FXIa). Together with activated coagulation factor VIII (FVIIIa), activated FIX (FIXa) proceeds to catalyze the activation of FX. At that point, both pathways converge into the common pathway, where thrombin, the key enzyme of the coagulation cascade, is formed. While the extrinsic pathway is considered physiologically relevant in the initiation of coagulation, the intrinsic pathway is more involved in the amplification and propagation of the coagulation cascade via positive feedback loops once it has been initiated.[8,9]

Prothrombin (FII) activation requires the formation of the prothrombinase complex, composed of FXa as the catalyzing enzyme and activated coagulation factor V (FVa) as a co-factor. This complex is formed on a negatively charged phospholipid surface and FII is activated.[10] Subsequently, thrombin acts as an activator of coagulation factor V (FV) and coagulation factor VIII (FVIII), thereby amplifying the coagulation cascade in a positive feedback loop.[11] This process is a crucial contributor to clot formation.

Fibrin and aggregated platelets are both key components of a stable blood clot. While platelet aggregation happens early in primary hemostasis as described above, the formation of fibrin is the last step of the secondary coagulation cascade.

The fibrin clot formation process starts with thrombin cleaving fibrinogen to form soluble fibrin monomers.[12] The final step in this process is fibrin cross-linking via thrombin-activated coagulation factor XIII (FXIIIa), which is a transglutaminase that circulates in human blood as a heterodimer and catalyzes the polymerization of fibrin monomers resulting in fibrin mesh formation.[13] In combination with aggregated platelets bound to the subendothelial matrix, a clot is formed at the site of injury, thereby closing the damaged vessel wall and stopping the bleeding. **(Figure 1)**

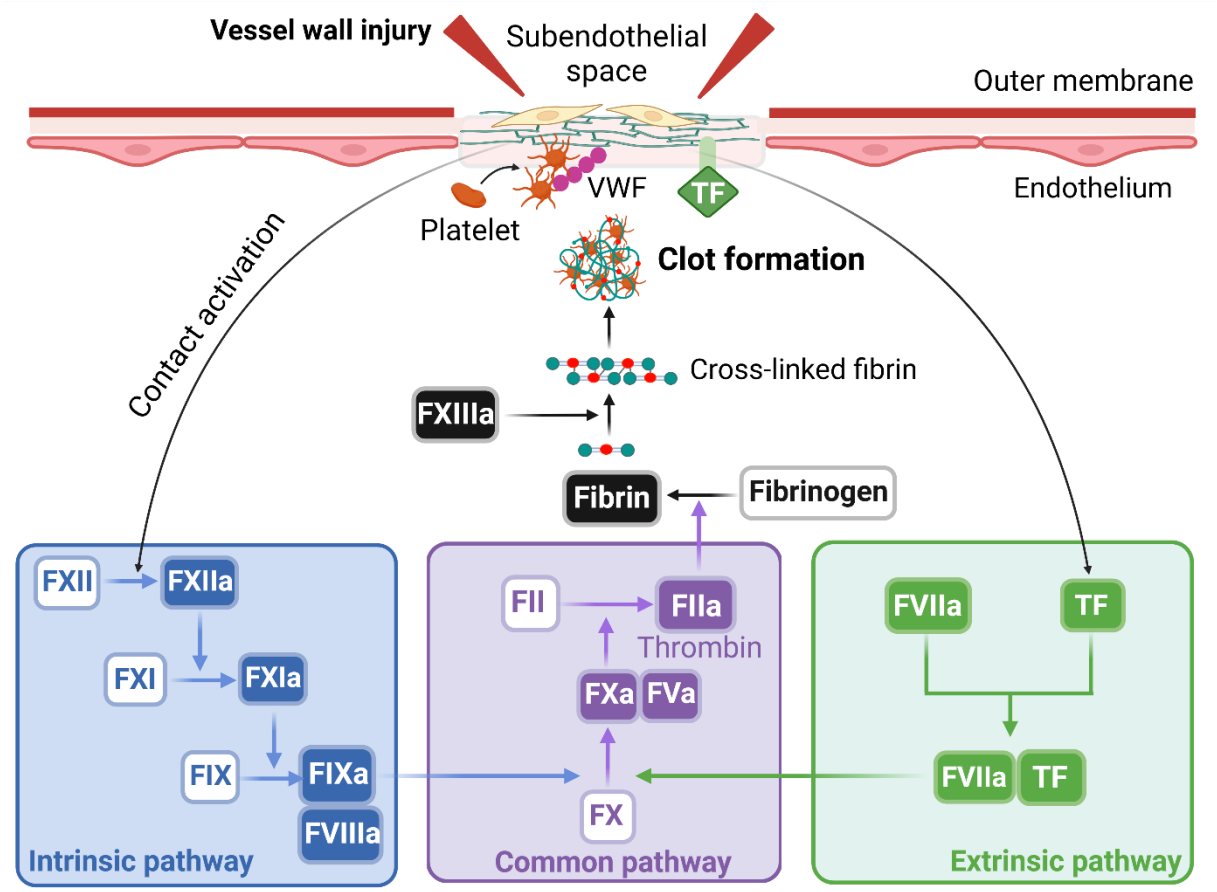

Figure 1. Coagulation activation and clot formation in vessel wall injury. When a blood vessel is damaged, blood is exposed to the subendothelial space. In a primary reaction, platelets are activated upon contact with collagen and begin to aggregate mediated by VWF polymers anchored to the subendothelial matrix, resulting in the formation of a platelet plug. Secondarily, the coagulation cascade is activated in the plasma. Upon contact with the subendothelial matrix, FXII is activated, which initiates the intrinsic pathway. FXIIa is a catalyst for FXI activation and FXIa activates FIX, which acts in combination with FVIIIa as an activator for FX. The extrinsic pathway is initiated via subendothelial TF forming a FX-activating complex with FVIIa. In the common pathway, FXa together with FVa activates FII. Thrombin (FIIa) cleaves fibrinogen to form fibrin monomers, which are cross-linked by FXIIIa. Together with aggregated platelets, the fibrin mesh forms a stable clot that closes the vessel wall injury and stops the bleeding.

1.1.2 Regulation of coagulation

Coagulation activation is a dynamic and self-amplifying process. Therefore, without regulation, a clot would excessively grow and eventually form a thrombus, i.e., a pathophysiological clot occluding the vessel lumen leading to thrombosis.[14]

To counterbalance coagulation activation and mitigate excessive clot formation, there exist natural inhibitors such as serpins, a superfamily of proteins inhibiting serine protease activity.[15] One example is antithrombin (AT), a direct inhibitor of thrombin and other proteases, which is constantly present in the circulation and immediately counteracts coagulation activation.[16] AT activity is greatly enhanced by the

glycosaminoglycans heparin, dermatan sulfate, and heparan sulfate,[17] which bind to AT and thereby induce a conformational change.[18] Heparan sulfate represents 50 % to 90 % of the overall glycosaminoglycans in the body and is mainly expressed by endothelial cells.[19,20] In addition to heparin sulfate, endothelial cells produce nitric oxide,[21] prostacyclin,[22] and ecto-ADPase,[23] which interfere with activation pathways in platelets, in order to prevent excessive platelet aggregation and clot formation.

Tissue factor pathway inhibitor (TFPI) is the major regulator of TF-induced blood coagulation. TFPI binds to the TF-FVIIa complex inhibiting its ability to activate FX and also providing a direct inhibitory effect on FXa activity.[24] It is primarily produced by endothelial cells and is either released into the bloodstream or bound to the endothelial surface.[25]

The protein C (PC) pathway, which is described in more detail in **chapter 1.3**, is initiated when PC binds to the endothelial protein C receptor (EPCR) and becomes activated via a complex formed between thrombin and the endothelial receptor thrombomodulin (TM).[26,27] Activated PC (APC) is then released into circulation and acts as an anticoagulant by proteolytic inactivation of FVa and FVIIIa.[28,29] As a cofactor, protein S (PS) enhances the proteolytic activity of APC.[30,31] (**Figure 2**)

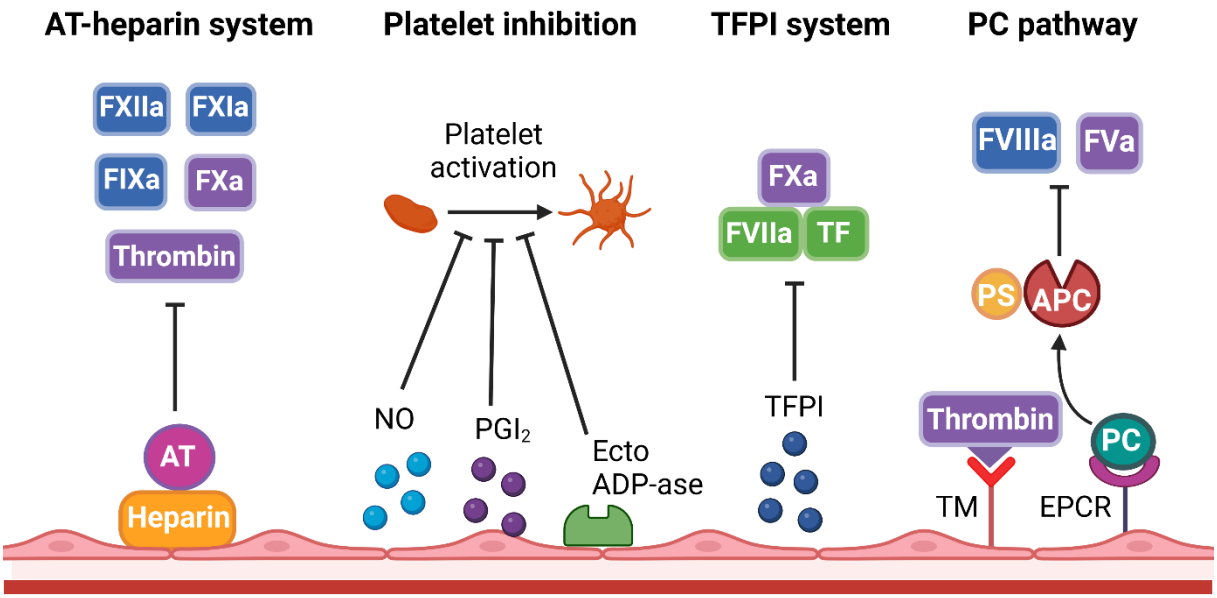

Figure 2. Regulation of coagulation. As a counterbalance mechanism, various endothelial cellular responses have an inhibitory effect on coagulation activation. Platelet activation is inhibited by the expression of nitric oxide, prostacyclin, or ecto-ADPase, whereas heparan sulfate serves as a co-factor for AT inhibitory activity on thrombin and other coagulation factors. TFPI is an inhibitor of the extrinsic coagulation activation released or presented by various cells. The PC pathway inhibits coagulation by activation of PC bound to EPCR via the thrombin-TM complex, which is an inhibitor of FVIIIa and FVa. NO, nitric oxide; PGI_2, prostacyclin.

1.1.3 Fibrinolysis

After the vessel injury is closed and bleeding is stopped, the clot is degraded to maintain the blood flow in a process called fibrinolysis. The central enzyme of fibrinolysis is plasmin, which cleaves fibrin molecules.[32] Plasmin activation and its enzymatic activity are regulated by activating and inhibiting mechanisms.

Fibrinolysis is initiated when plasminogen is converted to plasmin by tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA) released from endothelial cells.[33] Plasminogen activator inhibitor-1 (PAI-1), which is also expressed by the endothelium, is an inhibitor of both plasminogen activators and thereby downregulates fibrinolysis.[34] Thrombin-activatable fibrinolysis inhibitor (TAFI) is activated during coagulation activation by thrombin. Via cleavage of fibrin residues that bind plasminogen and plasminogen activators and thereby propagate fibrinolysis, TAFI interrupts this positive feedback mechanism and inhibits fibrinolysis.[35] Plasmin activity is directly downregulated by complex formation with α2-antiplasmin (α2-AP).[36] (**Figure 3**)

Overall, these mechanisms ensure that fibrinolysis and clot degradation occur in a controlled and regulated manner.

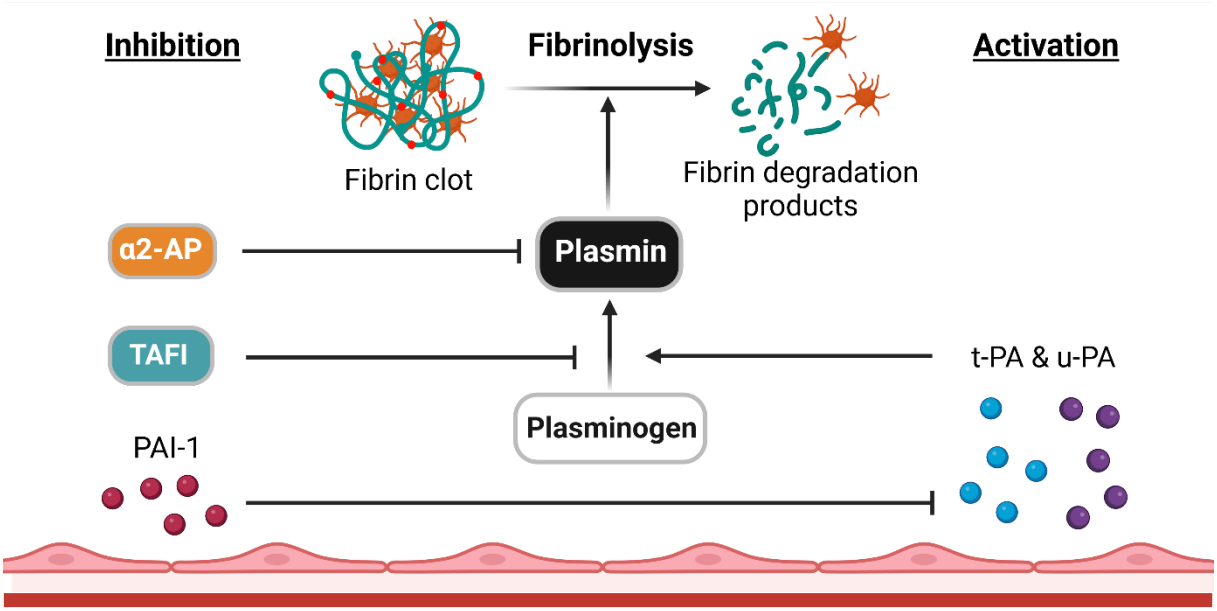

Figure 3. Activation and inhibition of fibrinolysis. After a fibrin clot is formed and bleeding is stopped, the clot is degraded in a controlled manner via fibrinolysis. Released from endothelial cells, t-PA and u-PA are activators of plasminogen that initiate plasmin formation. They are inhibited by PAI-1 which is also released from the endothelium. TAFI cleaves fibrin residues and thereby inhibits a positive feedback mechanism, in which plasminogen is further activated. α2-AP directly binds plasmin and inhibits its proteolytic activity.

1.2 Hereditary thrombophilia

Hemostasis is a delicate equilibrium between pro- and anticoagulant factors. Imbalances due to altered levels or dysfunction of coagulation factors and inhibitors eventually result in an increased bleeding or thrombosis risk. The term thrombophilia is used in a broad sense to characterize populations, disorders, situations, etc. in which there is an increased predisposition for thrombosis.[37] Per the general definition, thrombosis can occur in an artery, a vein, within the microvasculature. A possible life-threatening complication of deep venous thrombosis is known as pulmonary embolism, in which a piece of the thrombus (i.e. an embolus) breaks off the vessel wall and travels via the right heart into the pulmonary circulation, where it then occludes a pulmonary artery. Deep venous thrombosis, pulmonary embolism, or a combination thereof are collectively termed venous thromboembolism (VTE).[38]

While acquired thrombophilia often occurs later in life due to autoimmune response, malignancy, or environmental factors, hereditary thrombophilia characterizes a genetic predisposition for increased risk of venous thrombosis due to the “classical” thrombophilic risk factors as described below.

1.2.1 Discovery and epidemiology of genetic risk factors

Early discoveries of hereditary thrombotic risk factors include deficiencies of anticoagulant proteins. In 1965, reduced levels of AT were shown to be associated with recurrent thrombosis in a family.[39] Deficiencies of PC and PS, both contributing to the anticoagulant PC pathway, were described as causes of inherited thrombophilia in the 1980s.[40,41] The relative thrombotic risk is calculated to be about 3 to 11-fold in PC deficiency, 2 to 19-fold in AT deficiency, and 2 to 26-fold in PS deficiency.[42,43] Fortunately, these conditions are quite rare and only represent a minor fraction of inherited thrombophilia cases.[44] In 1996, genetic analysis of FII revealed a G to A transition at position 20210 (FII 20210G>A) that results in increased mRNA processing, and due to increased FII levels, is linked to VTE risk in carriers of the mutation.[45,46] FII 20210G>A was found to be the second most prevalent form of hereditary thrombophilia occurring in 1 to 4 % of the overall population[47,48] and it yields a 3 to 5-fold VTE risk as compared to the general population.[45,49] (**Table 1**)

A breakthrough was achieved when Dahlbäck and colleagues described the concept of APC resistance in 1993. They found a poor anticoagulant response of the

activated partial thromboplastin clotting time to exogenously added APC in VTE-affected families.[50] One year later, a mutation resulting in the replacement of arginine 506 with glutamine in the FV protein (and thereby the loss of an APC cleavage site) was described by Bertina and colleagues from the Dutch city of Leiden simultaneously with three other groups.[51–54] The defect was henceforth referred to as FV Leiden (FVL) and it was shown to be the most prevalent inherited thrombophilic risk factor, found in 20 to 25% of patients with VTE and 50% of patients with hereditary thrombophilia.[55,56] FVL occurs in 3 to 7 % of the white population and causes a 7-fold thrombotic risk in heterozygotes.[57,56] (**Table 1**)

Table 1. Mechanism, prevalence, and relative thrombotic risk of heterozygous genetic defects of inherited thrombophilias.

Thrombophilia type	Mechanism	Prevalence (%)	Relative risk (-fold)	Genetic defect
FVL	APC resistance	3 - 7	4 - 7	1691G>A
FII 20210G>A	Increased FII levels	1 - 4	3 - 5	20210 G>A
PC deficiency	Reduced APC formation	0.2	3 - 11	Various
PS deficiency	Reduced APC formation	0.03 - 0.13	2 - 26	Various
AT deficiency	Impaired inhibition of e.g., thrombin and FXa	0.02	2 - 19	Various

1.2.2 Factor V Leiden

FV is an important cofactor of the coagulation cascade. While 20 - 25% of total FV is stored in platelets, it exists mainly in the circulation at a concentration of approximately 21 nmol/L.[58] The FV molecule is a single-chain glycoprotein of 330 kDa and possesses an A1-A2-B-A3-C1-C2 architecture.[59] FV has little intrinsic procoagulant activity before its activation through proteolytic cleavage by thrombin or FXa.[60] Upon activation, the B-domain is released and a hetero-dimer is formed that consists of a 105 kDa heavy chain (A1-A2 domains) and a 71/74 kDa light chain (A3-C1-C2 domains) that are non-covalently associated in a calcium-dependent manner.[61] (**Figure 4**) In its activated form, as described above, FVa serves as a cofactor of FXa in the prothrombinase complex that catalyzes the conversion of FII into thrombin.[60]

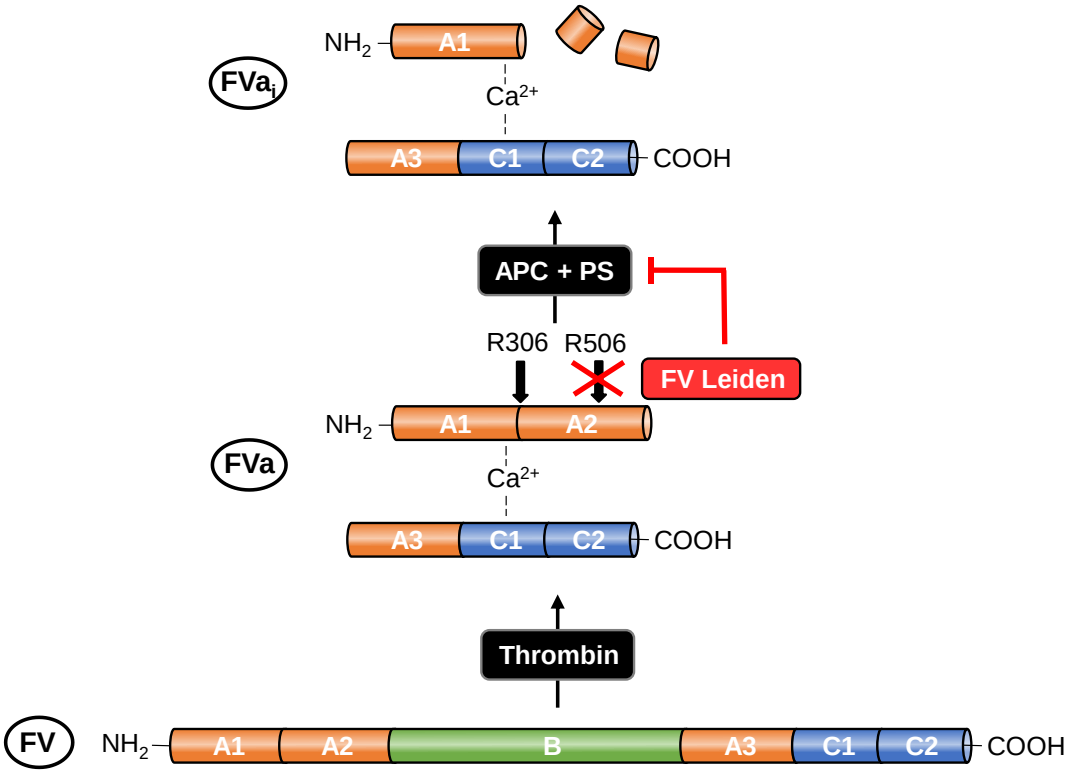

Figure 4. Schematic representation of the activated and inactivated forms of FV. FV consists of A1, A2, B, A3, C1, and C2 domains, and has little procoagulant activity until it is activated through proteolytic cleavage by thrombin (or FXa). During activation, the B domain is lost and a hetero-dimer is formed in a calcium-dependent manner. Once activated on the endothelial surface, APC in complex with PS inactivates FVa via cleavage at arginine 306 and 506 resulting in loss of the A2 domain from the hetero-dimer. The FVL mutation causes a substitution of a glutamine for an arginine at position 506, which impairs the inactivation of FVa by APC and thereby induces a shift towards procoagulant activity resulting in a prothrombotic phenotype of FVL carriers. Modified after Segers et al, 2007.[62]

The procoagulant activity of FVa is downregulated by APC in a complex with its cofactor PS, which proteolytically cleaves the A2 domain at arginine 306 and arginine 506.[29] The FVL mutation is associated with hypercoagulability due to the loss of the arginine 506 cleavage site, causing APC resistance of FVa. (**Figure 4**) APC resistance is defined as a poor anticoagulant response in plasma after the addition of APC.[50] Based on this principle, it can be diagnosed by clotting-based tests, e.g. the measurement of the prolongation of the activated partial thromboplastin time when APC is added to plasma.[63] To confirm the diagnosis of FVL, the underlying single nucleotide polymorphism is confirmed via genetic testing.[64,65]

Although carriers share the same genetic background, the clinical phenotype of FVL is highly variable. Some carriers develop severe unprovoked and recurrent VTE at a young age, while others remain asymptomatic throughout their entire life.[64,66] Coinheritances of other common thrombophilic genetic risk factors[67] or the FV HR2

haplotype[68] with the FVL mutation were found to be associated with an additionally increased thrombotic risk. However, 80 % of symptomatic FVL carriers are not affected by such clustering of known genetic thrombophilic defects.[67] Overall, reasons for the variability in expressivity of the FVL mutation remain poorly understood.

1.2.3 Thrombotic risk assessment strategies

Thrombophilia screening, i.e., testing for FVL and other thrombophilic risk factors is performed in symptomatic patients who experienced a thrombotic event or in their asymptomatic relatives since a family history of VTE is associated with an increased thrombotic risk.[69,70] For the prevention and management of VTE, there are several anticoagulant medications available. However, the bleeding risk associated with continuous anticoagulant treatment must be weighed against the therapeutic benefit of preventing (recurrent) thrombosis in VTE-prone individuals.[71] Therefore, besides the clinical evaluation of further risk factors,[72] laboratory tests have been proposed to detect plasmatic hypercoagulability and contribute to an individualized thrombotic risk assessment.

Coagulation factor levels, and, as a part of thrombophilia testing, inhibitor plasma levels are examined quantitatively in immunoassay-based systems or functionally by evaluation of clotting times.[73,74] In contrast to these tests, which only allow for an assessment of the pro- and anticoagulant potential of an individual, the measurement of activation markers of coagulation aims at identifying an actual hypercoagulable state.

Such activation markers include the thrombin-antithrombin complex (TAT) or prothrombin fragment 1+2 (F1+2), which is generated by the cleavage of prothrombin into thrombin.[75,76] In addition to these, in vitro thrombin generation in response to TF, which reflects the extrinsic pathway of the plasmatic coagulation cascade, can be measured.[77] Increased fibrinolytic activity is detected by measurement of plasmin-α2-antiplasmin complex (PAP), soluble fibrin monomer (sFM), or fibrin degradation products, such as D-dimer, with the latter also indicating previous coagulation activation and clot formation.[78,79]

While results from current routine testing might provide valuable insights into single aspects of hemostasis, depicting an overall picture of hemostasis and thrombosis risk remains challenging. Furthermore, crucial variables such as the endothelial

anticoagulation response are not reflected by routine coagulation assays, and many mechanisms contributing to VTE risk are not fully understood.

1.3 The protein C pathway

The endothelial PC pathway serves as a major anticoagulant, anti-inflammatory, and anti-apoptotic system. Its physiological impact is significant, as demonstrated for example by lethal thrombotic complications in infants with severe homozygous PC deficiency and the significantly increased risk of VTE in PC-deficient adults.[40,80] Moreover, the FVL mutation and PS deficiency, which are both linked to the functionality of the PC pathway, are also associated with increased thrombotic risk.[44] The central components of the PC pathway and its influence on coagulation and other mechanisms are described as follows.

1.3.1 Central components

PC, the key protein of the PC pathway and zymogen of the serine protease APC, circulates in plasma at levels around 70 nmol/L.[80] Proteolytic activation of PC occurs on the surface of the endothelial cell and relies on coagulation activation. Thrombin binds to the endothelial receptor TM and the thrombin-TM-complex activates the pro-enzyme. This process is enhanced by EPCR, which binds and localizes PC on the endothelial surface near the activating complex.[81,82] Once formed and released into circulation, APC has a half-life of 15 to 20 minutes which is mainly determined by the protease inhibitors protein C inhibitor, α1-antitrypsin, α2-macroglobulin, and α2-AP.[83,84] In the circulation, the proteolytic activity of APC is augmented by its co-factor, PS.[85] Bound to EPCR on the endothelial surface, APC acts by cleavage and thereby activation of protease-activated receptor 1 (PAR-1) across the endothelial membrane.[86,87] (**Figure 5**). Overall, the activation and progression of the PC pathway rely on both, circulating and endothelial-derived factors.

1.3.2 Mechanisms

The anticoagulant properties of APC are mainly based on the inactivation of FVa and FVIIIa preventing further thrombin formation and propagation of coagulation activation.[84] Moreover, the complex formation of thrombin with TM blocks the

procoagulant activity of the enzyme and promotes its clearance by plasma protease inhibitors and internalization of the complex via the endothelial membrane.[88,89] The formation of APC is linked to the amount of thrombin bound to TM, while simultaneously, thrombin formation is downregulated. By directly counteracting coagulation activation and, subsequently, the formation of APC, the PC pathway is unique amongst all anticoagulation pathways.

Another function of APC with a direct impact on hemostasis is the stimulation of fibrinolysis. PAI-1, the most important inhibitor of plasmin activators t-PA and u-PA, is inactivated when it forms a complex with free APC.[90] In addition, the downregulating effect on thrombin formation results in reduced formation of the fibrinolysis inhibitor TAFI.[91] Overall, APC formation indirectly causes a shift towards increased fibrin clot degradation.

Besides exerting anticoagulant and pro-fibrinolytic effects as a circulating enzyme, a proportion of APC remains bound on the endothelial surface where it influences intracellular signaling via PAR-1. PARs are a subfamily of G-protein-coupled receptors which were initially described as thrombin receptors on platelets and endothelial cells associated with pro-inflammatory signaling and vascular leakage.[92,93] Like some other G-protein coupled receptors, PAR-1 is capable of biased downstream signaling which occurs either via G proteins or via β-arrestin-2, dependent on cleavage site and cell type.[94] The formation of a complex between PAR-1, EPCR, and APC leads to cleavage at arginine 46 within PAR-1 and results in β-arrestin-2-biased signaling.[86,87]

The exact mechanisms behind the inhibition of inflammation and cytoprotection mediated by APC on the endothelial cell are not fully understood. One potential direct anti-inflammatory pathway is the inhibition of nuclear factor kappa-light-chain-enhancer, a major transcription factor for several inflammatory cytokines, by β-arrestin-2[95] whereas the downregulation of the coagulation cascade by circulating APC also indirectly inhibits inflammation.[96,97] Furthermore, APC-activated β-arrestin-2-biased signaling has cytoprotective effects by suppression of pro-apoptotic transcription factor p53[98,99] and activation of endothelial-barrier-stabilizing Rac1.[100,101] (**Figure 5**)

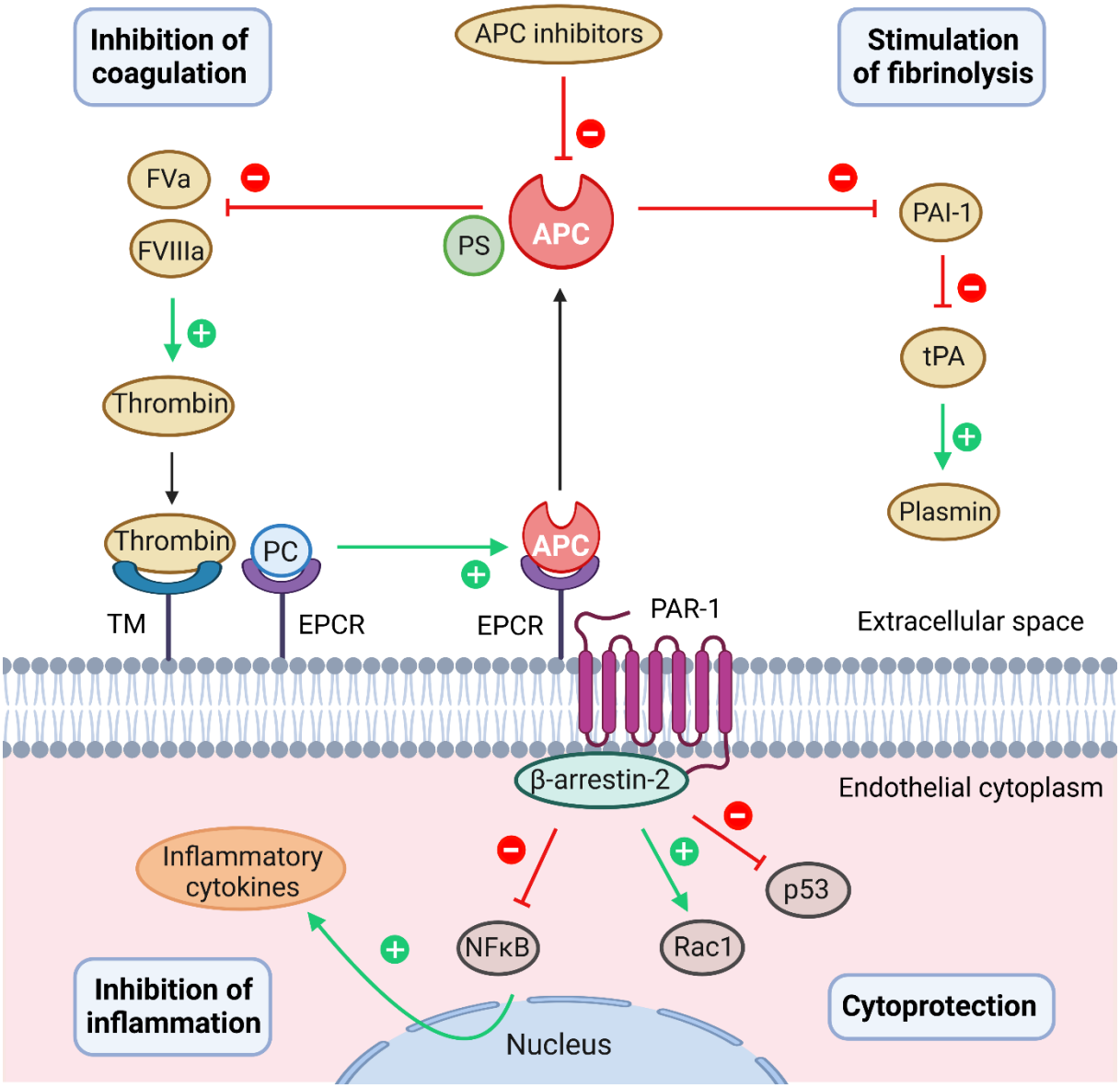

Figure 5. Activation and biological functions of the PC pathway. PC is converted to APC on the phospholipid-rich surface of endothelial cells by the thrombin-TM complex. EPCR enhances PC activation. Together with its co-factor PS, APC blocks prothrombinase formation and activation of coagulation by inactivating FVa and FVIIIa. APC stimulates fibrinolysis directly by forming a complex with PAI-1 or indirectly by preventing thrombin-induced activation of TAFI. In complex with EPCR and PAR-1, APC exerts anti-inflammatory activity by suppressing the nuclear factor kappa-light-chain enhancer (NFκB) and thereby the secretion of cytokines. Moreover, the complex promotes cytoprotection by suppression of pro-apoptotic p53 and activation of endothelial-barrier-stabilizing Rac1.

Taken together, APC is a potent modulator of various pathological processes. While the anticoagulant functions of the PC pathway and their association with classical hereditary thrombophilias are well-described, the overall interplay of its downstream mechanisms, the influence on endothelial cell biology variations, and the influence of such on the FVL thrombophilia phenotype remain unknown.

1.4 Research objectives

Hereditary thrombophilia risk factors are mutations associated with an increased prevalence of VTE. The most common classical risk factor in populations of Caucasian descent is FVL. Inactivation of mutant FVa by APC is impaired which results in reduced efficacy of the anticoagulant PC pathway. While the single point mutation can be easily diagnosed, its clinical expressivity is highly variable between FVL carriers, ranging from a life-long asymptomatic course to VTE at an early age. This raises the research question, of which factors modulate the thrombotic risk in FVL carriers.

The endothelial PC pathway is a major anticoagulant system counterbalancing thrombin formation. Its clinical impact is demonstrated by an increased thrombotic risk in carriers of PC deficiency, PS deficiency, and FVL. Recently, a human in vivo model of stimulated hemostasis activity pattern evaluation (SHAPE) has been described which combines standardized activation of extrinsic coagulation by a low dose of recombinant FVIIa with subsequent monitoring of coagulation and anticoagulation biomarkers.[102] Using this model, higher thrombin and APC formation rates were observed in response to coagulation activation in FVL carriers compared to non-carriers.[102] Moreover, after stimulation, higher APC levels were measured in asymptomatic FVL carriers than in FVL carriers with a history of VTE.[103]

Due to these observations, which provided the basis for this thesis, I hypothesized, that differences in the endothelial PC pathway response influence the expressivity of the thrombotic phenotype in FVL. Following this hypothesis, symptomatic and asymptomatic FVL carriers were systematically examined for differences in pathways connected to the PC system, i.e., 1.) thrombin formation, 2.) APC formation, and 3.) fibrinolysis. In addition to the primary aim of identifying potential differences in these pathways, various secondary aims were pursued in the studies contributing to this cumulative thesis.

As described in **chapter 2.1**, the “thrombin formation” hypothesis was investigated by comparative analysis of in vitro and in vivo thrombin formation in relation to the APC levels observed in plasma after in vivo coagulation activation by rFVIIa. The secondary aim of these studies was to investigate whether the extent of the APC response to thrombin formation was associated with an increased risk of thrombosis in thrombophilia other than FVL. The study population therefore included not only FVL

carriers and healthy controls but also patients with familial thrombophilia, in whom no established risk factors were found.

The fibrinolytic system and the PC system are interconnected, and alterations in fibrinolysis have been proposed as a thrombotic risk factor. However, studies on the association of various fibrinolysis markers with the clinical risk of VTE have yielded inconclusive results.[104–107] **Chapter 2.2** of this thesis addresses the interaction between the APC response and fibrinolysis in FVL carriers using the SHAPE in vivo model. By comparison of coagulation activation-induced changes in fibrinolysis biomarkers between healthy individuals, and thrombophilia patients with and without a history of VTE, the interplay between the PC pathway and fibrinolytic mechanisms and their influence on the thrombotic phenotype was examined. As a secondary aim, they were also studied in patients with other types of thrombophilia.

For further insights into the PC pathway on a personalized level, in vitro studies with a focus on the endothelial APC response were performed. Here, endothelial colony-forming cell (ECFC) cultures isolated from peripheral blood were applied. These endothelial progenitor cells intrinsically contribute to the formation of new blood vessels and the reconstitution of injured endothelium.[108] After differentiation in culture, ECFCs highly resemble mature vascular endothelial cells phenotypically and functionally[109] and are therefore representative of an individual's endothelial properties. In **chapter 2.3**, the establishment of an ex vivo model combining both plasma and autologous ECFCs is described. By TF-initiated coagulation activation, the patient-specific thrombin and endothelial anticoagulant APC response was monitored in an in vitro setting. To demonstrate the functionality of the assay, the APC response was modeled in healthy individuals, FVL carriers, and, as a secondary aim of these studies, under induced inflammation.

Using the established ex vivo model, the endothelial APC response was further examined in FVL carriers with and without a history of thrombosis in **chapter 2.4**. In addition, other endothelial and plasmatic variables were compared to identify potential differences between symptomatic and asymptomatic FVL carriers in order to identify factors influencing the FVL thrombophilia phenotype.

Overall, this thesis aimed for further characterization of the interplay between the PC pathway and coagulant and fibrinolytic mechanisms and its potential influence on the thrombotic risk in FVL carriers. Using different personalized models of hemostasis, comparisons between individuals with and without a history of VTE were made to

provide insights into mechanisms contributing to and protecting from the development of thrombosis. Ultimately, the overall aim of this research was to improve the understanding of the variable expressivity of FVL and as a long-term objective, individualized thrombotic risk assessment and management.

2. Results

2.1 Impaired in vivo activated protein C response rates indicate a thrombophilic phenotype in inherited thrombophilia

This chapter is published as:

Reda, S*, **Schwarz, N***, Müller, J, Oldenburg, J, Pötzsch, B#, Rühl, H.# Impaired in vivo activated protein C response rates indicate a thrombophilic phenotype in inherited thrombophilia. *Haematologica*. 2022 (5):1197-1200. doi: 10.3324/haematol.2021.280573

* SR and NS contributed equally as co-first authors

# BP and HR contributed equally as co-senior author

The publication is listed in Appendix A.

My contributions

Data collection, figure preparation, and draft of the manuscript.

Summary

Thrombophilia is an abnormality of blood coagulation that increases the risk of thrombosis. Known risk factors for inherited thrombophilia including FVL and FII 20210G>A only partly explain the estimated heritability of VTE of about 40 - 60 % observed in family-based studies.[110] Consideration of laboratory evaluation of in vitro thrombin generation has been proposed for the identification of thrombotic risk based on elevated values in families with unexplained thrombophilia.[111] Moreover, human in vivo studies of induced coagulation activation demonstrated that APC response rates were higher in asymptomatic than symptomatic FVL carriers.[103] However, it remains unclear whether increased in vitro thrombin generation indeed reflects the situation in vivo and whether an altered APC response correlates with thrombotic risk in other thrombophilia patients.

In a study population of 30 healthy individuals and 51 patients with a history of VTE, thereof 28 with known hereditary risk factors (FVL or FII 2021G>A), and 23 unrelated subjects with unexplained familial VTE, in vivo coagulation activation was induced by administration of 15 µg/kg rFVIIa, and the subsequent changes of hemostasis biomarkers were monitored over 8 hours (SHAPE approach). APC was measured using an oligonucleotide-based enzyme capture assay (OECA) and other biomarkers were evaluated using commercially available assays. In vitro thrombin generation was assessed using the calibrated automated thrombogram assay. The coagulation activation-induced changes of biomarkers over time were expressed as area under the curve (AUC) and groups were compared using either the unpaired Student t-test or the two-sided Mann-Whitney test. Correlations were measured by the determination of Pearson's correlation coefficient.

Addressing the question of whether in vitro thrombin generation reflects the in vivo situation, in vitro and in vivo thrombin formation were comparatively analyzed. In vitro, thrombin formation kinetics were higher in the familial thrombophilia cohort than in FVL or FII 20210G>A carriers and healthy controls, as indicated by an elevated endogenous thrombin potential. In vivo, the indirect markers of thrombin formation F1+2 and TAT behaved differently between groups. Resting and stimulated plasma levels of F1+2 were increased in the FVL and FII 20210G>A carriers. In contrast, there was no difference between the cohort with unexplained familial thrombophilia and healthy controls. TAT levels were higher in both FVL and FII 20210G>A carriers and patients with unexplained familial thrombophilia compared to healthy controls after infusion of rFVIIa. Although the AUC of the in vivo thrombin generation parameters F1+2 and TAT correlated with each other in healthy controls and patients with a history of VTE, they did not correlate with in vitro thrombin generation. Furthermore, in vitro thrombin formation parameters did not differ between FVL carriers with and without a history of VTE.

To investigate the reactivity of the PC system to thrombin formation, plasma levels of APC were measured. After infusion of rFVIIa, APC increased significantly in all cohorts. Changes in APC did not differ in FVL and FII 20210G>A carriers but were higher compared to healthy controls. In contrast to thrombin formation rates, the APC response was significantly lower in the cohort with unexplained familial thrombophilia than in the FVL/FII 20210G>A cohort and did not differ from healthy controls.

These results suggest that different factors determine the outcome of in vitro and in vivo thrombin generation measurement. Since the APC response is a direct marker of the APC formation capacity of the endothelium, the disproportionately low APC response in relation to the thrombin formation rate indicated an impaired endothelial APC-generating activity in the cohort with unexplained familial thrombophilia. This is in agreement with previous observations of 1) opposite changes of thrombin and PC activation markers in patients with abnormalities of the PC pathway,[112] 2) a higher APC response to in vivo coagulation activation in asymptomatic FVL carriers than in those with prior VTE,[103] and 3) results in the present study demonstrating that thrombin and APC formation rates correlated with each other in both cohorts of patients with VTE but not in healthy controls. In conclusion, these data show that an impaired APC response to increased thrombin formation indicates a thrombophilic phenotype in inherited thrombophilia patients.

2.2 Fibrinolysis biomarker, thrombin, and APC level alterations after coagulation activation depend on type of thrombophilia and clinical phenotype

This chapter is published as:

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* SR and NS contributed equally as co-first authors

# BP and HR contributed equally as co-senior authors

The publication is listed in Appendix B.

My contributions

Data collection, figure preparation, and draft of the manuscript.

Summary

Coagulation and fibrinolysis are highly interconnected; thus, alterations in the fibrinolytic system have been proposed as thrombophilic risk factors. While plasminogen deficiency and increased α2-AP levels appear not to be associated with thrombotic risk,[113,104,105] studies on the association between plasma levels of TAFI and PAI-1, and thrombotic risk have yielded inconclusive results.[107,114–117] Previously, a human endotoxemia model has been used for studies on the interaction between the coagulation and fibrinolytic systems in vivo.[118,119] However, it has been shown that the endotoxin-induced fibrinolytic response is mainly driven by inflammatory mediators rather than by coagulation activation.[120,121] In this study, biomarker changes in the fibrinolytic system were examined using an in vivo model of coagulation activation (SHAPE approach) to identify potential variables influencing the thrombotic risk in thrombophilia patients, including FVL carriers.

The study population included 38 asymptomatic carriers of thrombophilic risk factors, 56 thrombophilic patients with a history of venous thromboembolism, thereof 19 FVL carriers each, and 35 healthy controls. In vivo coagulation activation was induced by administration of 15 µg/kg rFVIIa and the subsequent increase of hemostasis biomarkers was monitored over 8 hours (SHAPE approach). APC was measured using the OECA, and other biomarkers were evaluated using commercially available assays. The coagulation activation-induced changes of biomarkers over time were expressed as AUC and groups were compared using either the two-sided Mann-Whitney test (two groups), the Friedman test followed by pairwise comparison using the Nemenyi procedure, or the Kruskall-Wallis test followed by pairwise comparison using the Dunn procedure (three groups). Correlations were measured by the determination of Pearson’s correlation coefficient.

Prior to administration of rFVIIa, PAI-1 was approximately 3-fold higher in both symptomatic and asymptomatic patients with thrombophilia compared to healthy controls. Moreover, in the cohort with asymptomatic thrombophilia compared to the controls, APC was 1.8-fold higher while sFM was 1.5-fold lower. D-dimer levels in the VTE+ cohort were 1.4-fold higher than in the control group. Plasma levels of the other studied parameters did not exceed normal values and did not differ significantly between cohorts. In all cohorts PAP increased and PAI-1 decreased significantly over time in response to coagulation activation. While TAT increased temporarily in all cohorts and decreased to normal levels after 8 hours, changes in PAP and PAI-1 did

not reverse during the observation period. The AUC of PAP or TAT as a measure of plasmin or thrombin formation was greater in the overall VTE cohort than in healthy controls and PAP and TAT correlated with each other. As evidenced by the respective AUCs, asymptomatic FVL carriers in the cohort of asymptomatic patients with thrombophilia showed lower PAP and PAI-1 levels as compared to carriers with a history of thrombosis while APC formation was higher. In contrast, no difference in any parameter was observed between FII 20210G>A carriers with and without a history of thrombosis or compared to patients with familial thrombophilia without FVL or FII 20210G>A indicating that this phenomenon is related to the FVL phenotype.

The subgroup analysis of rFVIIa-induced thrombin activation, APC response, and fibrinolysis biomarker changes revealed an association between decreased PAP and PAI-1 formation and increased APC formation rates in asymptomatic FVL carriers. However, this fibrinolytic response profile did not apply to other thrombophilia patients. Moreover, these data demonstrate that low-grade thrombin formation induces an anticoagulant and a long-lasting fibrinolytic response and that the SHAPE approach is a useful tool to assess the fibrinolytic response in vivo. In conclusion, the data show that the fibrinolytic response differs in thrombophilia patients, depending on underlying thrombophilic risk factors, and might help to explain the variable clinical expressivity of FVL.

2.3 Ex vivo modeling of the PC (Protein C) Pathway Using Endothelial Cells and Plasma: A Personalized Approach

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* BP and HR contributed equally as co-senior authors

The publication is listed in Appendix C.

My contributions

Conceptualization, data collection, data analysis, figure preparation, and draft of the manuscript.

Summary

The PC pathway is critically involved in the regulation of coagulation, as well as in anti-inflammatory and cytoprotective signaling.[122] APC, the key enzyme of this pathway, is generated on the surface of endothelial cells and downregulates thrombin formation through the inactivation of FVa and FVIIIa. However, the activity of the PC pathway shows a high degree of interindividual variability depending on the strength of the pro- and anticoagulant drivers operating within the plasma and on the APC formation potential of the endothelium.[84] In vivo studies showed that the APC response differed between FVL carriers and non-carriers and between asymptomatic and symptomatic FVL carriers.[102] Therefore, it was hypothesized that differences in APC generation by the endothelium may cause these discrepancies in APC response rates. Further investigation of this hypothesis required an ex vivo model allowing for analysis of both the extent of thrombin generation and the ability of the endothelium to generate APC in a patient-specific manner.

ECFCs were isolated from heparinized peripheral blood obtained from FVL carriers (n=6) and healthy controls (n=5) and surface marker expression was measured by flow cytometry. Confluent monolayers of endothelial cells were overlaid with either buffer containing PC and thrombin, or citrated, defibrinated plasma. For measurements in plasma, thrombin formation was initiated by the addition of 1 pmol/L TF. Thrombin and APC formation rates were measured over time in supernatant samples using the respective OECA. To induce downregulation of TM expression, the cell culture medium was supplemented with 1 ng/ml interleukin 1β, and ECFCs were stimulated for 24 hours. In vivo APC and thrombin formation were monitored in study participants after infusion of rFVIIa (15 µg/kg) over 2 hours (SHAPE approach). Ex vivo APC and thrombin formation over time were expressed as AUC and groups were compared using the two-sided Mann-Whitney test.

It was demonstrated, that ECFCs can be routinely isolated from FVL carriers and that they do not vary from ECFCs obtained from non-carriers in terms of morphological appearance and surface marker expression as measured by flow cytometry. ECFC-

dependent APC formation, as performed in a purified system and the plasma matrix, was comparable to the APC formation capacity of human umbilical vein endothelial cells. Following inflammatory stimulation with interleukin 1β, surface expression of TM was significantly reduced and the median peak APC concentration on ECFCs with autologous plasma was significantly lower than without cytokine stimulation. The model was applied to study the kinetics of thrombin and APC formation in FVL carriers (n=6) compared to non-carriers (n=5) ex vivo. When ECFCs were combined with autologous plasma and coagulation activation was induced, APC formation rates were significantly higher in FVL carriers compared to non-carriers as evidenced by a higher ratio between the AUC of APC generation to the AUC of thrombin generation. Furthermore, by direct comparison of the ex vivo APC formation with in vivo data obtained from the same individuals, it was shown that the ex vivo response of endothelial cells resembles the in vivo situation.

By reflecting an impaired APC response caused by cytokine-induced downregulation of TM expression, the general ability of the model to simulate procoagulant endothelial cell dysfunction was demonstrated. Moreover, these data confirm and extend previous in vivo findings in FVL carriers. Therefore, this autologous system represents significant progress in the investigation of the PC pathway on a personalized level. In further studies, this ex vivo approach can be used to investigate unexplained differences in APC generation between symptomatic and asymptomatic FVL carriers without the need for exposition to in vivo coagulation activation and, in a broader sense, allows for examinations of endothelial differences and their influence on the APC response.

2.4 The Endothelium Modulates the Prothrombotic Phenotype of Factor V Leiden: Evidence from an Ex Vivo Model

This chapter is submitted as follows:

Schwarz, N, Müller, J, McRae, HL, Reda, S, Pezeshkpoor, B, Oldenburg, J, Pötzsch, B*, Rühl, H.* The Endothelium Contributes to the Prothrombotic Phenotype of Factor V Leiden: Evidence from an Ex Vivo Model. *bioRxiv* preprint doi: 10.1101/2023.11.15.567299

* BP and HR contributed equally as co-senior authors

The publication is listed in Appendix D.

My contributions

Conceptualization, data collection, data analysis, figure preparation, and draft of the manuscript.

Summary

The clinical expressivity of the FVL mutation is highly variable, ranging from life-long absence of VTE to early and recurrent thrombosis in carriers. This interpatient variability challenges our understanding of the interplay between genetic predisposition and molecular interactions contributing to the development of VTE. The PC pathway, which becomes impaired by FVL, is a unique anticoagulant mechanism, where the formation of APC as an anticoagulant is directly linked to thrombin formation. In vivo studies have shown that APC formation in response to thrombin formation is increased in asymptomatic FVL carriers compared to carriers with a history of thrombosis.[103] Potential explanations of this observation include alterations of plasmatic and/or endothelial components of the PC system that might modulate thrombin and APC formation rates. Among the potential plasmatic variables are increased levels of coagulation factors,[123] and other factors that cause APC resistance,[124] or affect APC inactivation kinetics,[125–127] all of which have been associated with increased thrombotic risk. Moreover, endothelial variables include, in particular, EPCR and TM, since downregulation in these markers or variants in their encoding genes have been suggested as thrombotic risk factors.[128–131] In this study, plasmatic and endothelial variables that might modulate the APC response in FVL carriers were addressed using the ex vivo model of the PC pathway and other methods. By comparisons between symptomatic and asymptomatic carriers, potential underlying mechanisms of the variable expressivity of the FVL phenotype were examined.

ECFCs and citrated plasma were obtained from FVL carriers with and without previous VTE and healthy controls (n=7 each). APC and thrombin formation were measured in ECFC-overlaid plasma using the previously described ex vivo model. Plasma levels of coagulation factors and inhibitors were measured using a coagulation analyzer and corresponding reagents. For measurement of APC inhibition in plasma over time, human APC (5 ng/mL) was added to citrated plasma, the mixture was incubated at 37°C under agitation (300 rpm) and quenched samples taken over time were measured using the APC-OECA. APC resistance was measured in a prothrombinase-based assay. In brief, FV was fully activated in plasma and the diluted mixture was incubated with or without APC at 37°C for 20 minutes. After stopping the reaction by the addition of an APC-inhibiting aptamer, FXa, FII, and a thrombin-specific fluorogenic substrate were added and the formation of thrombin as reflected by fluorescence units (FU) was monitored over 10 minutes using a fluorescence plate reader. The residual prothrombinase activity was determined by calculation of the APC sensitivity ratio (FU without APC / FU with APC). Surface expression of EPCR and TM was measured on ECFCs using cell-based enzyme-linked immunosorbent assays (cell-ELISAs). The Kruskall-Wallis test followed by pairwise comparison using the Dunn procedure or the Mann-Whitney test was used to compare datasets.

The APC response (AUC APC/AUC thrombin) was significantly higher in asymptomatic FVL carriers compared to those with a history of VTE and the healthy control cohorts which confirms previously obtained results from in vivo studies. Coagulation factors and inhibitors, inhibition kinetics of exogenously added APC, and APC sensitivity of the prothrombinase in plasma were found not to be different. Additionally, endothelial TM and EPCR expression on ECFCs, as determined by cell-ELISA, did not differ between the cohorts of symptomatic and asymptomatic FVL carriers.

In order to examine the influence of plasmatic and endothelial variation on the APC response separately, cross-over experiments with plasma and ECFCs from asymptomatic and symptomatic FVL carriers and healthy controls were performed. When plasma obtained from FVL carriers was overlaid on healthy control ECFCs, the APC response was not different between symptomatic and asymptomatic FVL carriers. In contrast, the APC response in normal plasma on ECFCs from asymptomatic FVL carriers remained significantly higher than on ECFCs from FVL carriers with a history of VTE.

In accordance with results from previous in vivo experiments, APC response rates to thrombin formation were higher in asymptomatic FVL carriers compared to carriers with a history of VTE.

Overall, the findings of this study confirm previous in vivo evidence that the extent of APC formation in response to thrombin modulates the thrombotic risk in FVL carriers. Although the causative factors remain yet to be identified, the observations on FVL ECFCs combined with healthy control plasma suggest, that this increased APC response is driven by the endothelium. Further studies are warranted to elucidate yet unknown endothelial mechanisms that might modulate the clinical expressivity of FVL.

3. Discussion and Conclusions

Although the FVL mutation is found in 20 to 25% of patients with VTE and in 3 to 7 % of the overall population of Caucasian descent,[55–57] little is known about the underlying reasons for its variable expressivity. In the common understanding of how FVL affects thrombotic risk, thrombogenicity is driven by the prolonged half-life of FVa, resulting in increased prothrombinase complex activity. Accordingly, the thrombotic risk is higher in homozygous FVL carriers, who have higher levels of APC-resistant FVa, than in heterozygous carriers.[56] However, under comparable environmental conditions, some carriers develop severe thrombosis at a young age, while others remain asymptomatic for life. Previous in vivo studies have shown that APC response to thrombin formation is higher in asymptomatic FVL carriers compared to carriers with a history of VTE leading to the hypothesis that the PC pathway may influence the thrombotic phenotype in these hereditary thrombophilia patients.[102,103] In this thesis, the influence of the endothelial PC pathway response on the thrombotic risk of FVL carriers was investigated along with thrombin formation rates, the fibrinolytic response, and other variables.

Besides the well-established forms of inherited thrombophilia, a vast number of additional risk factors have been suggested; interfering with either coagulation factor levels,[132–134] anticoagulatory responses,[135,130,129] or fibrinolytic potential.[107,117,136] However, previous studies have reported conflicting results, and the clinical relevance of these additional hereditary risk factors remains controversial.

While patient data is crucial for the confirmation of potentially pathological variables, biological models are helpful tools for the identification and targeted investigation of mechanisms and disease states. Accordingly, several models for FVL and the PC pathway have been described and studied.

The thrombin generation assay is a global dynamic assay that simultaneously measures the generation of thrombin initiated by the TF/FVIIa-dependent extrinsic pathway and its inhibition in plasma. When APC or TM and PC are added to FVL plasma, the APC-resistant phenotype is demonstrated via increased thrombin formation and mutation carriers can be distinguished from non-carriers using this method.[137–140] However, studies evaluating the thrombogenic potential of FVL in

asymptomatic carriers and patients with a history of thrombosis have yielded conflicting results.[139,140]

Human umbilical vein endothelial cells have been extensively used as an in vitro model to define several molecular and cellular features of the PC pathway, including the functions of TM,[141] EPCR,[142] and PAR signaling.[86,143] These primary cells are proliferative, display key features of mature endothelial cells, and are commercially available. While these features make human umbilical vein endothelial cells a convenient model of the endothelium, they lack a disease-specific state and are not suitable for studying the cellular functionality of the PC pathway on an individualized basis.

Transgenic mice carrying FVL[144,145] or knockouts of PC,[146] TM,[147,148] EPCR,[149,150] or other variants of such[151] were introduced as in vivo models, and their thrombotic phenotype was studied. Results obtained from FVL mice with different genetic backgrounds suggest that there are one or more variable loci that exert a profound modifying influence on the FVL thrombotic phenotype.[144] Heterozygous TM or EPCR knockout mice maintain a normal phenotype,[147–150] while the knock-in of certain variants in TM leads to mild thrombotic phenotypes.[148,152] Sood et al[151] described a transgenic mouse model in which TM and EPCR variants were experimentally validated as modulators of pregnancy success in FVL-carrying mothers. Nevertheless, results from this study do not provide insight into the thrombosis risk in the offspring carrying FVL along with either TM or EPCR variants. Although in vivo mouse models can be genetically manipulated and investigated systemically, there are significant differences between mice and humans such as life span, metabolic rate, and anatomical differences of the vein system which limit the translatability of results.

In **chapters 2.1** and **2.2**, a human in vivo model of coagulation activation was applied in FVL carriers and changes in hemostatic biomarkers were monitored. This method induced only slight activation of the coagulation cascade and therefore, the participants were safe from experiencing an actual thrombotic event. However, it allowed for the estimation and comparison of the coagulation and fibrinolytic response between different cohorts in a highly physiological context.

In **chapter 2.3** it was shown that ECFCs can be obtained from FVL carriers and, in combination with autologous plasma, the ex vivo model represents significant progress in the personalized investigation of the PC pathway. Furthermore, the increased APC response rates in asymptomatic FVL carriers, as observed using this model in **chapter**

2.4, demonstrated the ability of the autologous approach to analyze disease-specific phenotypic differences of the PC pathway and confirmed earlier results obtained in vivo.

The FVL mutation results in an APC-resistant phenotype and, in theory, increased thrombin formation and an increased thrombotic risk. However, there are several modulators for coagulation activation and fibrinolysis, which induce variability in the expressivity of FVL. The above-described models were therefore used for the evaluation of the highly complex and interactive response of the PC pathway to coagulation activation and fibrinolysis in FVL carriers.

The studies described in **chapter 2.1** confirm that in vivo thrombin formation in the resting state is slightly increased in FVL carriers, as demonstrated by increased baseline values of F1+2. However, in the case of coagulation activation, the formation of APC in response to thrombin generation is disproportionately higher compared to those in healthy individuals. These data indicate a potential protective mechanism against excessive thrombin formation mediated by the PC pathway. Besides its anticoagulant function, APC is a proposed regulator for fibrinolysis. On the one hand, the downregulation of thrombin results in lower levels of activated TAFI, which is an inhibitor of fibrinolysis.[153,91] On the other hand, APC binds to PAI-1 and thereby blocks its inhibiting effect on plasminogen activators.[90,154] In **chapter 2.2**, it is shown that in vivo APC formation in response to coagulation activation is lower while PAI-1 levels are higher in symptomatic versus asymptomatic FVL carriers. This confirms the proposed fibrinolytic effect of APC and suggests that an increased fibrinolytic response as mediated by an increased APC response in asymptomatic FVL carriers might be protective against the development of VTE.

For further evaluation of the mechanism behind the increased PC pathway response, an ex vivo model of the PC pathway was established, as described in **chapter 2.3**, which allowed for confirmation of the increased APC formation via FVL patient-derived ECFCs in autologous plasma. When ECFCs obtained from symptomatic and asymptomatic FVL carriers were combined with healthy control plasma, as described in **chapter 2.4**, the increased APC response in asymptomatic carriers persisted. In contrast, when healthy control ECFCs were combined with plasma from FVL carriers, there was no difference in APC formation between symptomatic and asymptomatic carriers. Accordingly, investigation of potential influencing factors in plasma revealed no differences in coagulation factors and

inhibitors, APC inhibition kinetics, or APC resistance between the two cohorts. These results strongly indicate that the endothelium is a modulator for APC formation and potentially determines the thrombotic risk in FVL carriers.

TM and EPCR mediate the formation of APC on the endothelial surface.[81,128] Although results from cell-ELISAs in **chapter 2.4** did not indicate any difference in TM and EPCR expression on ECFCs obtained from symptomatic and asymptomatic FVL carriers in a resting state, the localization of receptors across the endothelial membrane can change upon coagulation activation. Both TM and EPCR can be internalized by the cell in response to stimulation and ligand binding,[88,155–157] which makes the receptors unavailable for further ligand binding and promotion of APC formation. Therefore, different internalization rates could be a possible explanation for the variable APC response observed between symptomatic and asymptomatic FVL carriers.

Variants of receptor-coding genes can alter the function, stability, or binding affinity of the receptor to their ligands. Accordingly, polymorphisms in the TM- and EPCR-coding genes (*THBD* and *PROCR*) have been postulated to affect APC formation and thrombotic risk. Several studies have proposed a reduced thrombotic risk associated with the *THBD* c.1418C>T or *PROCR* 4678 G>C polymorphism, either via functional increase of APC formation or reduced receptor shedding.[158–161] In contrast, the *PROCR* 4600 A>G variant was associated with lower PC activation, increased EPCR shedding, and higher VTE risk.[162,163] However, while the association between increased thrombotic risk and the *PROCR* 4600 A>G variant was confirmed by larger trials and meta-analyses,[129,131,164] these studies did not find an association between the *THBD* c.1418C>T[130,165–167] or the *PROCR* 4678 G>C polymorphism[162,131] and reduced risk of VTE. As described in **chapter 2.4**, the APC response in symptomatic and asymptomatic FVL carriers, who were also carriers of the aforementioned *THBD* or *PROCR* variants, fell within the range of the APC response in their respective cohorts. Therefore, these do not explain our observed differences in the APC response. However, a potential effect of *THBD* or *PROCR* variants on the APC response cannot be excluded and would require further examination in study populations of accordingly selected patients.

Besides TM and EPCR, PARs are additional endothelial receptors that contribute to the functions of the PC pathway. The discovery of bidirectional PAR signaling opened a wide and multidisciplinary field of research. The highly complex and dynamic PAR

signaling cascade regulates endothelial cell apoptosis, exocytosis, barrier function, inflammatory cytokine production, and transcriptional activation.[168,169] Although the activation of PARs by either thrombin or APC is well characterized, little is known to date about its feedback on the coagulation system. Since PAR-1 in particular is known to closely interact with APC and EPCR on the endothelial surface, it could potentially influence the formation and release of the enzyme, which warrants further investigations.

To date, although increased APC response has been described in FVL, this phenomenon has not been observed in FII20210G>A carriers or thrombophilia patients without known risk factors as described in **chapter 2.2**. Recent studies demonstrated that human endothelial cells express and produce several coagulation factors including FII and FV and can generate thrombin without the exogenous addition of any proteins or phospholipids.[170,171] Besides circulating coagulation proteins, endothelial cell-derived coagulation factors may influence local thrombin and APC formation and have an impact on the thrombotic phenotype. It would also be interesting to investigate, whether endothelial cell-derived FV is resistant to APC as has been established in plasmatic FV in FVL carriers.

In conclusion, the findings of this thesis demonstrate differences between symptomatic and asymptomatic FVL carriers related to the endothelial PC pathway response and confirm the central hypothesis of this thesis that components of the PC system modulate the thrombotic risk in FVL. These data provide evidence that higher APC levels in response to coagulation activation reduce coagulation and increase fibrinolysis in asymptomatic FVL carriers, thereby providing a protective effect from the development of VTE, (**Figure 6**) which at least partially explains the variable clinical expressivity of the thrombophilic mutation. Furthermore, the established and successfully applied ECFC-based ex vivo model is presented as a tool for functional assessment of the PC pathway with respect to clinically relevant endpoints on an individualized level.

Although the underlying mechanism remains undefined, these data strongly suggest that endothelial variables are a major driver of variability in the APC response in symptomatic and asymptomatic FVL carriers, and studying the endothelial physiology in this population is essential for characterizing this phenomenon. Furthermore, the proposed mechanism of how APC levels affect the thrombotic risk in FVL carriers (**Figure 6**) should be evaluated in future studies, and studies are warranted to examine

the role of the endothelium in other thrombophilias. The autologous ECFC model described in this thesis has great utility for future studies in this area of research as well as potential application in clinical diagnostics.

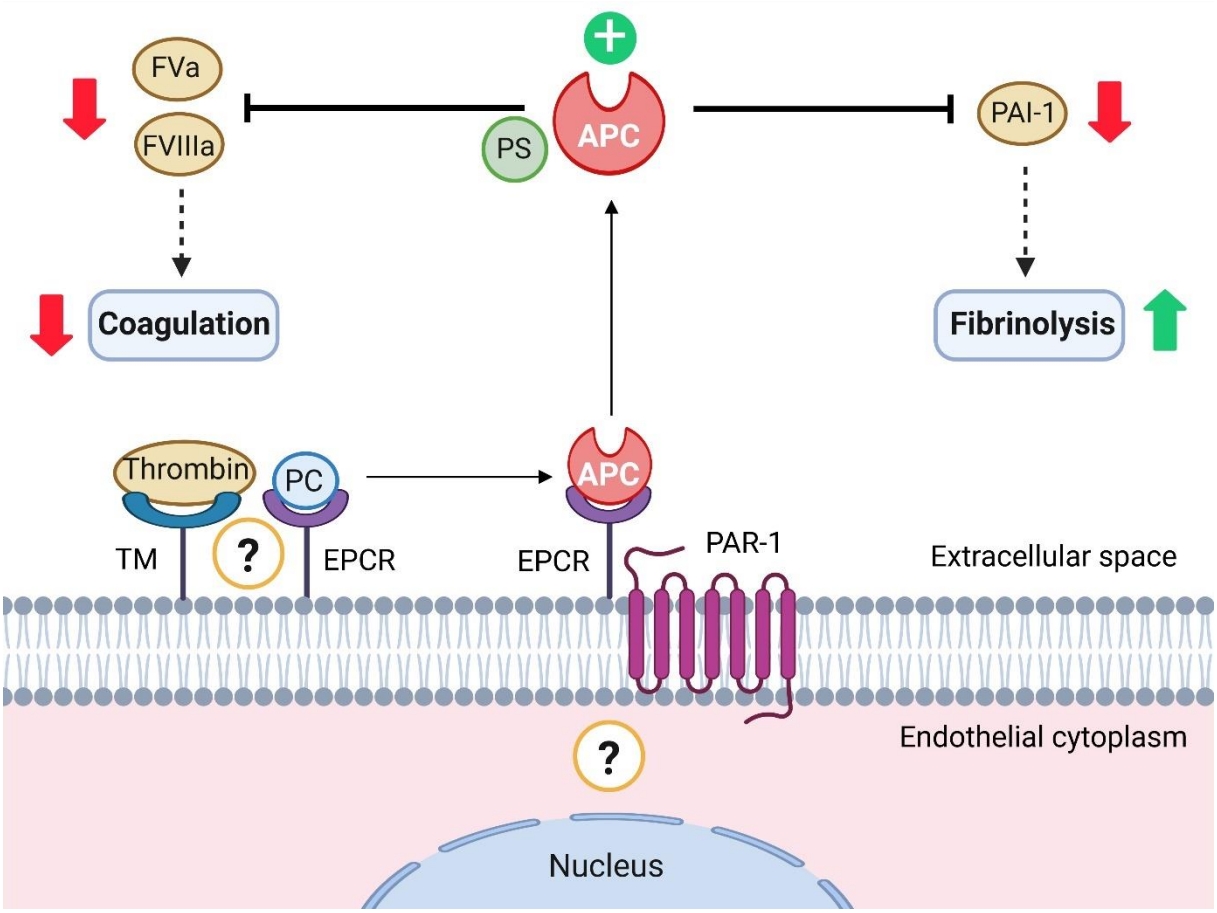

Figure 6. The proposed mechanism on how the PC pathway response affects the thrombotic risk in FVL carriers. The formation of APC in response to coagulation activation is higher in asymptomatic FVL carriers compared to carriers with a history of VTE. On the one hand, this likely yields a stronger inhibition of FVa and FVIIIa which results in reduced coagulation activation. On the other hand, by inactivation of PAI-1, the fibrinolytic response is potentially increased. Overall, reduced coagulation activation and increased fibrinolysis may lead to a reduced thrombotic risk. While the exact mechanism behind the increased APC response remains unknown, it is most likely driven by the endothelium. Potential candidates include TM and EPCR since their PC-activating properties underlie the internalization in response to coagulation activation or genetic variables. Furthermore, endothelial cell-derived coagulation factors or PAR-signaling could potentially influence the PC pathway response.

4. References

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5. Appendix

5.1 Appendix A

Publication and supplement to chapter 2.1

Impaired *in vivo* activated protein C response rates indicate a thrombophilic phenotype in inherited thrombophilia

Venous thromboembolism (VTE) is a multifactorial disease. Hereditary risk factors include the common mutations factor V Leiden (FVL) and prothrombin (FII) 20210G>A, with a prevalence of 3–15% among whites, as well as deficiencies of the coagulation inhibitors antithrombin (AT), protein C (PC), and protein S.[1] In the recent past, novel risk loci have been found by genome-wide association studies.[2,3] However, their consideration in addition to the classical thrombophilic defects results in an estimated heritability of VTE of only 15%, in contrast to 40–60% heritability observed in family-based studies.[4] In order to identify further unknown genetic thrombophilic defects, consideration of the laboratory phenotype of increased thrombin formation in addition to the clinical phenotype of VTE has been proposed, based on the observation of elevated *in vitro* thrombin generation parameters in families with unexplained thrombophilia and in carriers of genetic variations in hemostasis-related genes other than FVL and FII 20210G>A.[5]

It remains unclear, however, if increased *in vitro* thrombin formation rates indeed reflect increased *in vivo* thrombin formation. In order to investigate this, we comparatively analyzed *in vitro* and *in vivo* thrombin formation in a cohort of healthy individuals and in thrombophilic patients. *In vivo* coagulation activation was induced by low-dose recombinant activated factor VII (rFVIIa). Subsequent hemostasis biomarker-monitoring included measurement of activated PC (APC) as a measure of the endothelial-dependent anticoagulant response. Recently, using this stimulated hemostasis activity pattern evaluation (SHAPE) approach, we were able to show increased *in vivo* thrombin generation rates and a comparable APC response in FVL and FII 20210G>A carriers.[6,7] Moreover, we found that APC response rates correlated with the thrombotic risk in FVL carriers.[7]

The study population consisted of 30 healthy individuals and 51 patients with a history of VTE, thereof 28 FVL or FII 20210G>A carriers (FVL/FII 20210G>A cohort), and 23 unrelated subjects with unexplained familial VTE (FH cohort). A diagram of patient recruitment and selection criteria is shown in Figure 1, along with a description of study procedures. Blood samples were drawn before

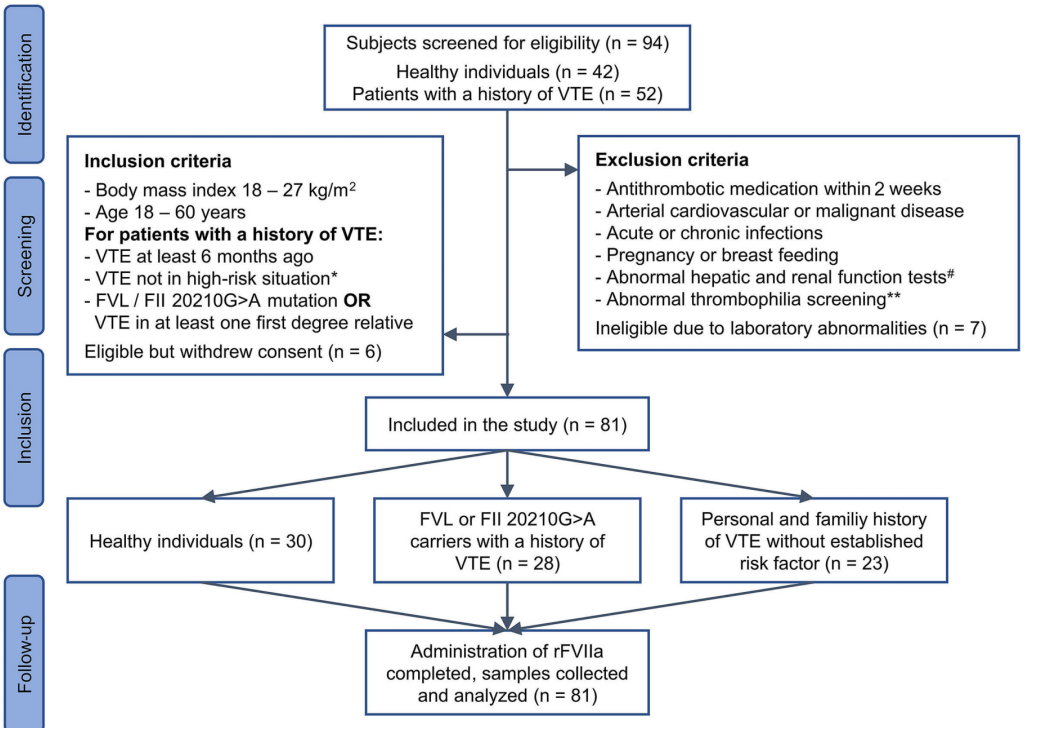

Figure 1. Eligibility criteria and study procedures. Healthy individuals were recruited from blood donors. Patients with a history of venous thromboembolism (VTE) were recruited from the thrombophilia outpatient clinic of our hospital. The study proposal was approved by the Ethics Committee of the Medical Faculty of the University Bonn (reference number 016/16). Written informed consent was received prior to participation. All finally included study participants (n=81) received morning administration of 15 µg/kg recombinant activated factor VII (rFVIIa) as single intravenous bolus injection after overnight fast. Blood samples were drawn immediately before and 10 minutes (min), 30 min, 1, 2, 3, 5, and 8 hours after administration, each from a new venipuncture. After discarding the first 2 mL, blood was drawn into citrate tubes (10.5 mmol/L, Sarstedt, Nümbrecht, DE). Citrate tubes were supplemented with aprotinin (10 µmol/L) and bivalirudin (250 µg/mL) for activated protein C (APC) measurement. Plasma samples were obtained by centrifugation (2,600 x g, 10 min) within 30 min and stored at less than -70°C until assayed. All finally included study participants completed rFVIIa administration and follow-up blood sampling. All collected samples were analyzed. *Surgery, trauma, immobilization, pregnancy, and puerperium. #Transaminases, γ-glutamyl transferase, urea, creatinine in serum. **Decreased plasma levels of antithrombin, protein C, protein S, anti-cardiolipin and anti-β2 glycoprotein I immunoglobulin G (IgG) and IgM, functional lupus anticoagulants (activated partial thromboplastin time, dilute Russell viper venom time), and factor V Leiden (FVL) and prothrombin (FII) 20210G>A mutation (except for inclusion into the FVL/FII 20210G>A cohort).

Table 1. **Baseline characteristics and rFVIIa-induced biomarker changes.**

	Healthy controls, N = 30	VTE, FVL or FII 20210G>A, N = 28*	*P*	VTE, family history of VTE, no RF, N = 23	*P*
Age, years (range)	35 (21-60)	41 (18-60)	-	38 (20-53)	-
Sex (male/female)	12 / 18	12 / 16	-	9 / 14	-
BMI, kg/m^2 (range)	23 (18-27)	24 (18-27)	-	24 (19-27)	-
DVT / PE / both, n	-	15 / 3 / 10	-	10 / 6 / 7	-
Fibrinogen, g/L	252 (221-284)	262 (250-309)	-	267 (256-331)	-
FII, %	103 (98-116)	124 (115-135)	**10^{-5}**	114 (103-120)	-
Factor XI, %	102 (90-115)	101 (95-107)	-	115 (100-127)	**0.044**
Antithrombin, %	107 (100-111)	98 (93-104)	-	100 (98-106)	-
sTM, ng/mL	1.62 (1.30-2.15)	1.61 (1.47-2.19)	-	1.62 (1.19-1.86)	-
sEPCR, ng/mL	45.6 (26.0-81.6)	57.0 (35.4-91.0)	-	72.5 (46,4-108.0)	-
PC, %	106 (97-118)	112 (103-122)	-	105 (97-116)	-
F1+2, nmol/L	0.16 (0.12-0.21)	0.25 (0.17-0.30)	**0.002**	0.15 (0.12-0.20)	-
AUC, nmol·h/L	0.29 (0.16-0.45)	0.34 (0.23-0.49)	-	0.42 (0.19-0.73)	-
TAT, ng/mL	<21.2 (<21.3-<21.3)	<21.3 (<21.3-29.7)	-	<21.3 (<21.3-24.7)	-
AUC, pmol·h/L	35.9 (0.81-109.5)	123.9 (45.1-188.2)	**0.008**	141.6 (12.8-332.3)	**0.021**
APC, pmol/L	0.68 (0.40-1.11)	1.13 (0.75-1.43)	**0.022**	0.79 (0.39-1.11)	-
AUC, pmol·h/L	6.55 (5.22-8.82)	15.1 (10.7-22.7)	**$<10^{-4}$**	9.46 (5.50-14.55)	-

Age and body mass index (BMI) are shown as mean (range), all other variables as median (interquartile range). The area under the curve (AUC) quantifies changes of prothrombin activation fragment F1+2 (F1+2), thrombin-antithrombin complex (TAT), and activated protein C (APC) over 8 hours after intravenous injection of recombinant activated factor VII (rFVIIa). *P* describes significant (<0.05) differences to healthy controls. *P* was calculated using the unpaired Student *t*-test (prothrombin, FII; protein C, PC) or the Mann-Whitney test (all other parameters) and corrected for multiple testing using the Bonferroni method. DVT: deep vein thrombosis; FVL: factor V Leiden; PE: pulmonary embolism; sEPCR: soluble endothelial PC receptor; sTM: soluble thrombomodulin; VTE: venous thromboembolism. *14 heterozygous FII 20210G>A carriers, 1 homozygous and 13 heterozygous FVL carriers, thereof 2 with HR2 haplotype..

and during 8 hours after administration of 15 µg/kg rFVIIa. No adverse events were observed. APC was measured using an oligonucleotide-based enzyme capture assay (OECA).[8] The thrombin biomarkers prothrombin activation fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT), and other hemostasis parameters were determined using commercially available assays. *In vitro* thrombin generation was assessed before rFVIIa administration, using the calibrated automated thrombogram (CAT) assay (Thrombinoscope, Maastricht, NL). Table 1 lists demographic features and measurement results of hemostasis parameters in the three cohorts at baseline, and rFVIIa-induced changes of F1+2, TAT, and APC over time, expressed as area under the curve (AUC). Hemostasis parameters at baseline were comparable in FVL and FII 20210G>A carriers (*Online Supplementary Table S1*).

In vitro thrombin formation kinetics were higher in the FH cohort than in FVL/FII 20210G>A carriers and healthy controls, indicated by an elevated endogenous thrombin potential (ETP) (Figure 2A), Additionally, peak thrombin concentration was increased compared with FVL/FII 20210G>A carriers, whereas lag time and time-to-peak did not differ significantly (*Online Supplementary Figure S1A* to *C*). The difference in the ETP was more pronounced at 1 pmol/L tissue factor (TF) concentration. This could be explained by higher FXI levels in the FH cohort, which have been shown to affect *in vitro* thrombin generation at a greater extent at lower TF concentrations.[9] In the resting state, plasma levels of F1+2 were slightly increased in the FVL/FII 20210G>A cohort, giving additional evidence of increased thrombin formation.

After infusion of rFVIIa, plasma levels of F1+2 (Figure 2B) and TAT (Figure 2C) increased significantly in all three cohorts (peak *vs.* baseline values, Wilcoxon signed-rank test $P<0.05$ after Bonferroni correction). F1+2 increased in every participant, indicating that rFVIIa activates the clotting cascade, resulting in thrombin formation. Every FVL/FII 20210G>A carrier showed an increase of F1+2 and TAT, whereas four subjects in the FH group and seven healthy controls showed an isolated increase of F1+2. This absence of a TAT increase could indicate a comparably lower thrombin formation rate. The most probable explanation of this discrepancy is the longer F1+2 half-life of approximately 2 hours in comparison to the TAT half-life of 44 minutes,[10] making F1+2 a more sensitive thrombin generation marker. The *in vivo* thrombin generation parameters F1+2 AUC and TAT AUC correlated with each other in healthy controls and patients with a history of VTE (Figure 2D). However, they did not correlate with *in vitro* thrombin generation (representatively shown for ETP and TAT AUC, *Online Supplementary Figure S1D* and *E*), suggesting that different factors determine and interfere with the outcome in both distinct and complex methodological approaches. In addition, compared with FVL and FII 20210G>A carriers, a more heterogenous risk profile can be expected in the FH cohort.

If the endothelium is intact, the thrombin formation capacity is effectively controlled by APC formation. The extent to which thrombin formation induces an increase in APC might therefore indicate the functionality of the APC-generating pathway in an individual patient and, moreover, modulate the thrombotic potential of increased thrombin formation rates. In order to investigate the reactivity of the PC system to thrombin formation we measured plasma levels of APC. After infusion of rFVIIa, APC increased significantly in all cohorts (Wilcoxon signed-rank test, $P<0.05$ after Bonferroni correction). Changes in APC (and thrombin biomarkers) did not differ in FVL and FII 20210G>A carriers (*Online Supplementary Figure S2*). In contrast to thrombin formation rates the APC response was significantly lower in the FH cohort than in the FVL/FII 20210G>A cohort and

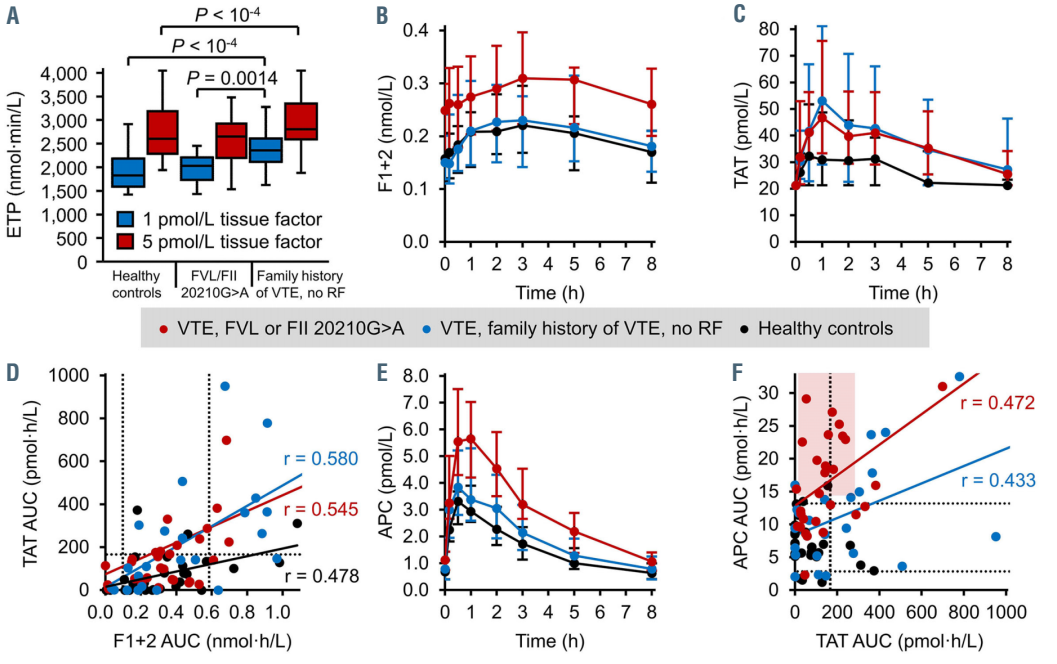

Figure 2. *In vitro* thrombin generation and *in vivo* thrombin-activated protein C response to rFVIIa. *In vitro* thrombin generation was measured by the calibrated automated thrombogram (CAT, Thrombinoscope, Maastricht, NL) in healthy controls (n=30) and in patients with venous thromboembolism (VTE) with factor V Leiden (FVL) or prothrombin (FII) 20210G>A mutation (n=28), or a family history of VTE without an established risk factor (RF, n=23). Plasma levels of prothrombin activation fragment 1+2 (F1+2), thrombin-antithrombin complex (TAT), and activated protein C (APC) were measured in the same population before (t=0) and after intravenous injection of 15 µg/kg recombinant activated factor VII (rFVIIa). (A) Endogenous thrombin potential (ETP) measured by CAT, presented as median and interquartile range (IQR, boxes), 1.5-fold IQR (whiskers), and outliers (circles). *P*-values <0.05 (Mann-Whitney test) are shown. (B) F1+2 and (C) TAT in plasma (median, IQR). (D) Area under the F1+2 generation curve (F1+2 AUC) in comparison to TAT AUC. Dotted lines indicate 90th percentiles of F1+2 AUC and TAT AUC, and 10th percentile of F1+2 AUC in healthy controls. (E) APC in plasma (median, IQR). (F) TAT AUC in comparison to APC AUC. Dotted lines indicate 90th percentiles of TAT AUC and APC AUC, and 10th percentile of APC AUC in healthy controls. The red area highlights the absence of a thrombin-related increase of APC in patients with unexplained familial thrombophilia (blue symbols). r: Pearson's correlation coefficient.

did not differ from healthy controls (Figure 2E). As the APC response is a direct marker of the APC formation capacity of the endothelium, the disproportionately low APC response in relation to the thrombin formation rate indicates an impaired endothelial APC-generating activity in the FH cohort. This relative APC deficiency after coagulation activation would consecutively result in increased thrombin formation. Several data support this conclusion: i) previously, reciprocal and opposite changes of indirect thrombin and PC activation markers were observed in patients with abnormalities of the PC pathway in a basal state;[11] ii) in a previous study, asymptomatic FVL carriers showed a higher APC response in the SHAPE approach than those with prior VTE;[7] iii) in the present study, thrombin and APC formation rates (TAT AUC and APC AUC) correlated with each other in both FVL/FII 20210G>A carriers and patients with unexplained familial thrombosis, but not in healthy controls (Figure 2F). With seven subjects (25%) in the FVL/FII 20210G>A cohort and six subjects (26%) in the FH cohort, both TAT AUC and APC AUC lay above the 90th percentiles of the healthy controls in similar rates of patients. However, only two individuals (9%) in the FH cohort showed a disproportionately high APC formation rate, as evidenced by an APC AUC (slightly) above and TAT AUC within the 90th percentiles of the healthy controls. In the FVL/FII 20210G>A cohort such a pattern was observed more often (29%), and more distinctively (Figure 2F). Thrombomodulin (TM) and endothelial PC receptor (EPCR) are two main factors that determine the APC formation capacity of the endothelium and variants in both genes have been suggested as thrombotic risk factors.[12,13] In order to assess interindividual variations in TM and EPCR, we measured plasma levels of soluble EPCR and TM but did not find significant differences between cohorts.

Potential sources of bias or imprecision include the size of the study population, the precision of rFVIIa dosing and times of blood draw, and laboratory analysis. In order to account for these issues, sample size, rFVIIa dosage and blood sampling times were chosen in orientation to previous pharmacokinetic studies on rFVIIa, yielding expected pharmacokinetic results (*Online Supplementary Figure S1F*).[14] The OECA for APC measurement has been extensively assessed.[8] Except for sECPR and sTM the other assays were covered by accreditation with the national accreditation body and were performed according to ISO standards. Moreover, the age and sex distribution, and the body mass index were similar in the different subgroups, ruling out a potential confounding effect of these variables. Finally, one might argue that instead of assessing a genetic hypercoagulable state in patients with unexplained familial thrombosis, an effect of the previous VTE may have been measured, as we did not include asymptomatic family members.

In conclusion, the data indicate that a dysbalanced

APC response characterized by increased thrombin formation rates and simultaneously decreased APC formation rates contributes to the increased thrombotic risk of patients with familial thrombosis. Further studies are now warranted to elucidate the pathophysiological and genetic basis of the described phenotype. Moreover, the data show that the SHAPE procedure is a useful tool to measure the functionality of the PC pathway, which is helpful to investigate prothrombotic mechanisms in patients with thrombophilia without an established risk factor.

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Contributions: HR, JM, and BP designed the experiments. SR, NS, and HR collected the data, SR and HR analyzed the data. SR, NS, JM, JO, BP, and HR drafted and edited the manuscript.

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Data-sharing statement: the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All authors have complete and on-going access to the study data.

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SUPPLEMENTARY APPENDIX

Impaired *in vivo* activated protein C response rates indicate a thrombophilic phenotype in inherited thrombophilia

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Supplementary data to

Impaired *in vivo* activated protein C response rates indicate a thrombophilic phenotype in inherited thrombophilia

SUPPLEMENTARY TABLES

Table S1. Comparison of baseline hemostasis parameters between FVL and FII 20210G>A carriers

	FVL, n = 14	FII 20210G>A, n = 14	*P*
Fibrinogen, g/L	250 (244;267)	279 (260;345)	**0.005**
FII, %	122 (108;135)	125 (121;134)	-
Factor XI, %	100 (95;101)	105 (96,114)	-
Antithrombin, %	97 (92;102)	99 (94;108)	-
sTM, ng/mL	1.49 (1.43;1.80)	1.83 (1.60;2.32)	-
sEPCR, ng/mL	35.2 (15.4;57.9)	76.1 (36.8;94.8)	-
Protein C, %	112 (110;120)	110 (98;126)	-
Thrombin, pmol/L	<0.46 (<0.46;0.64)	<0.46 (<0.46;0.87)	-
F1+2, nmol/L	0.21 (0.13;0.33)	0.27 (0.21;0.29)	-
TAT, ng/mL	<21.3 (<21.3;<21.3)	28.1 (<21.3;38.7)	-
APC, pmol/L	1.32 (1.04;1.63)	0.85 (0.50;1.15)	**0.019**

P describes significant (< 0.05) differences between factor V Leiden (FVL) and prothrombin (FII) 20210G>A carriers with a history of venous thromboembolism and was calculated using the unpaired Student t-test (FII, protein C) or the Mann-Whitney test (all other parameters). APC, activated protein C; F1+2, prothrombin activation fragment 1+2; sEPCR, soluble endothelial PC receptor; sTM, soluble thrombomodulin; TAT, thrombin-antithrombin complex.

SUPPLEMENTARY FIGURES

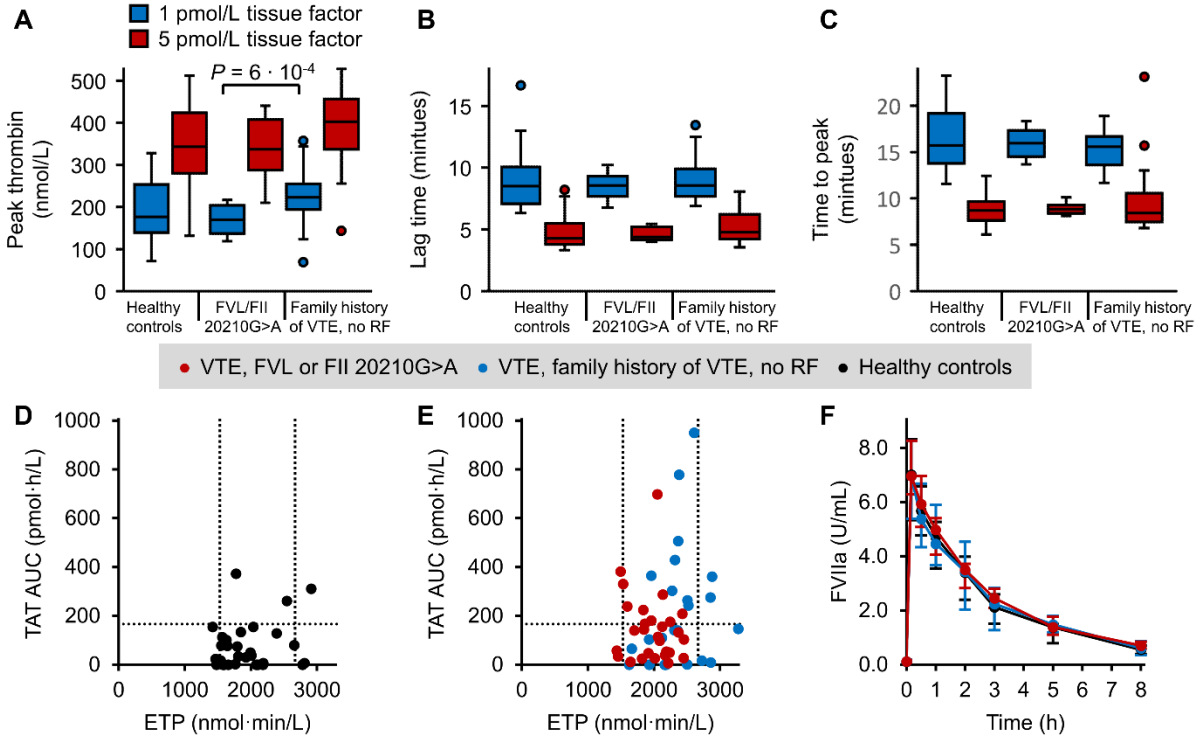

Figure S1. *In vitro* thrombin generation and kinetics of rFVIIa in plasma. *In vitro* thrombin generation was measured by the calibrated automated thrombogram (CAT) in healthy controls (n = 30) and in patients with venous thromboembolism (VTE) with factor V Leiden (FVL) or prothrombin (FII) 20210G>A mutation (n = 28), or a family history of VTE without an established risk factor (RF, n = 23). Plasma levels of thrombin-antithrombin complex (TAT) were measured in the same population before (t = 0) and after i.v. injection of 15 µg/kg recombinant activated factor VII (rFVIIa). (**A**) Peak thrombin concentration, (**B**) lag time and (**C**) time to peak measured by CAT, presented as median and interquartile range (IQR, boxes), 1.5 fold IQR (whiskers), and outlyers (circles). *P* values < 0.05 (Mann-Whitney test) are shown. (**D**) Endogenous thrombin potential (ETP, 1 pmol/L tissue factor) in comparison to the area under the curve (AUC) of TAT formation in healthy controls and (**E**) patients with VTE. Dotted lines indicate 90th percentiles of ETP and TAT AUC, and 10th percentile of ETP in healthy controls. (**F**) Activated factor VII (FVIIa) in plasma (median, IQR).

Figure S2

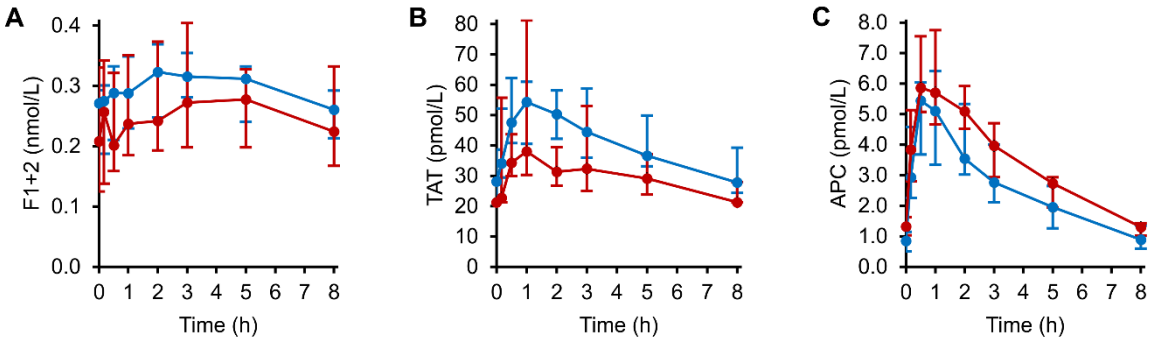

Figure S2. rFVIIa-induced thrombin/APC response in FVL and FII 20210G>A carriers. Plasma levels of (**A**) prothrombin activation fragment 1+2 (F1+2), (**B**) thrombin-antithrombin complex (TAT), and (**C**) activated protein C (APC)were measured before (t = 0) and after i.v. injection of 15 µg/kg recombinant activated factor VII (rFVIIa) in factor V Leiden carriers (FVL, n=14, red symbols) or FII 20210G>A carriers (n = 15, blue symbols).

5.2 Appendix B

Publication and supplement to chapter 2.2

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ORIGINAL ARTICLE

Fibrinolysis biomarker, thrombin, and activated protein C level alterations after coagulation activation depend on type of thrombophilia and clinical phenotype

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Abstract

Background: Recently, we have shown alterations in the anticoagulant response to recombinant activated factor VII (rFVIIa)–induced coagulation activation in patients with thrombophilia.

Objectives: This study aimed to extend this *in vivo* model to fibrinolysis biomarkers.

Methods: This interventional *in vivo* study included 56 patients with thrombophilia and previous venous thromboembolism (VTE+), 38 without VTE (VTE−), and 35 healthy controls. Plasma levels of D-dimer, plasmin–α2-antiplasmin (PAP) complex, and plasminogen activator inhibitor-1 (PAI-1) were monitored for over 8 hours after rFVIIa infusion (15 μg/kg) along with thrombin markers and activated protein C (APC).

Results: Throughout cohorts, median PAP increased by 40% to 52% ($P < 3.9 \times 10^{-10}$) and PAI-1 decreased by 59% to 79% ($P < 3.5 \times 10^{-8}$). In contrast to thrombin–antithrombin (TAT) complex, which also increased temporarily (44% to 115%, $P < 3.6 \times 10^{-6}$), changes in PAP and PAI-1 did not reverse during the observation period. The area under the measurement-time curves (AUCs) of PAP and TAT, which are measures of plasmin and thrombin formation, respectively, were each greater in the VTE+ cohort than in healthy controls (median PAP-AUC = 0.48 vs 0.27 ng·h/L [$P = .003$], TAT-AUC = 0.12 vs 0.03 nmol·h/L [$P = 2.5 \times 10^{-4}$]) and were correlated with one another ($r = 0.554$). As evidenced by the respective AUCs, asymptomatic factor (F)V Leiden carriers showed less PAP formation (0.22 vs 0.41 ng·h/L, $P = 9 \times 10^{-4}$), more pronounced PAI-1 decline (0.10 vs 0.18 ng·h/L, $P = .01$), and increased APC formation (28.7 vs 15.4 pmol·h/L, $P = .02$) than those within the VTE+ group ($n = 19$ each).

Conclusion: rFVIIa-induced thrombin formation is associated with fibrinolysis parameter changes outlasting the concomitant anticoagulant response. Both correlate with thrombosis history in FV Leiden and might help explain its variable clinical expressivity.

Sara Reda and Nadine Schwarz contributed equally to this study.

Bernd Pötzsch and Heiko Rühl are joint senior authors.

KEYWORDS
fibrinolysis, plasminogen activator inhibitor 1, protein C, thrombin, thrombophilia

Essentials

- Disorders in the breakdown of blood clots (fibrinolysis) may increase the risk of thrombosis.
- We studied proteins involved in fibrinolysis in blood taken after stimulation of clot formation.
- Protein levels in blood changed over time and did not fully normalize after 8 hours.
- These changes depended among others on the study participants' history of thrombosis.

1 | INTRODUCTION

Coagulation and fibrinolysis are highly interconnected, with thrombin formation playing a central role in both processes. Following coagulation activation through the activated factor VII (FVIIa)-tissue factor pathway, thrombin is generated and promotes fibrin formation and, via activation of FXIII, crosslinking of fibrin [1,2]. The pathophysiologic significance of thrombin is demonstrated in the setting of hereditary thrombophilia, caused by genetic variations that contribute to an increased risk of venous thromboembolism (VTE) by promoting thrombin formation directly or through interference with its regulation [3–5]. VTE, comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), is the third most common vascular disease following myocardial infarction and ischemic stroke, with an annual incidence of 1 to 2 per 1000 [5–7]. Factor (F)V Leiden and prothrombin (FII) 20210G>A have minor allele frequencies of approximately 5% and 2% to 3% and comprise the most common hereditary thrombophilias in the Caucasian population. Other thrombophilias include deficiencies of the coagulation inhibitors antithrombin (AT), protein C (PC), and protein S (PS) [5,8–11].

Alterations in the thrombin-mediated regulation of fibrinolytic activation have also been proposed as a form and mechanism of thrombophilia. Plasmin levels are directly regulated by complex formation with α2-antiplasmin, while plasminogen activation through tissue-type plasminogen activator (t-PA) is inhibited by plasminogen activator inhibitor-1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI) [12]. PAI-1 forms an inhibitory complex with t-PA, whereas TAFI, upon activation by either the thrombin–thrombomodulin complex, thrombin, or plasmin, inhibits fibrinolysis by removing carboxy-terminal lysine residues of partially degraded fibrin, resulting in decreased plasminogen binding and fibrinolysis [13,14]. While plasminogen deficiency and increased α2-antiplasmin levels appear not to be associated with thrombotic risk [15–18], studies on the association between plasma levels of TAFI and PAI-1 and thrombotic risk have yielded inconclusive results [19–23].

It is difficult to study the interactions between the coagulation and fibrinolytic systems *in vivo*. In the human endotoxemia model, activation of both coagulation and fibrinolysis is induced, but the increase of the fibrinolysis activation markers t-PA and plasmin–α2-antiplasmin complex (PAP) precedes the increase of the thrombin activation markers prothrombin fragment 1+2 (F1+2) and thrombin–antithrombin complex (TAT) [24–26]. Moreover, it has been shown that the endotoxin-induced fibrinolytic response is mainly driven by tumor necrosis factor and that the release of t-PA and PAI-1 during endotoxemia appears to be regulated independently of coagulation activation [26–29]. Recently, we have reported another approach to study coagulation activation *in vivo* using low-dose recombinant FVIIa (rFVIIa), which revealed increased thrombin generation rates in patients with thrombophilia [30–32]. Using this stimulated hemostasis activity pattern evaluation (SHAPE) approach, we were also able to show that a low APC response to rFVIIa-induced thrombin generation correlated with a history of VTE in FV Leiden carriers and unexplained familial thrombophilia [31,32]. The response to rFVIIa, including the effects on the fibrinolytic system, has been extensively studied in the context of hemophilia [33–38]. In most of these studies, utilized plasma concentrations of rFVIIa were several times higher than the median levels we observed in the SHAPE studies, of approximately 7 U/mL (4.7 nmol/L) [30–32]. However, Lisman et al. [33] showed that rFVIIa concentrations of this magnitude were sufficient to affect clot lysis time.

We therefore hypothesized that fibrinolytic changes following coagulation activation could also be assessed using the SHAPE approach and included fibrinolysis biomarkers in the spectrum of studied parameters. To assess whether, akin to the anticoagulant response to thrombin activation, fibrinolysis biomarker changes differed depending on type of thrombophilia and were clinically significant, patients with thrombophilia and without a history of VTE were comparatively studied against healthy controls.

2 | METHODS

This prospective study was carried out at the Institute of Experimental Hematology and Transfusion Medicine, Bonn, Germany, between July 2016 and October 2022. All cohorts were recruited in parallel. Sample collection was completed in February 2021. Analyses were performed continuously throughout the study period using frozen plasma samples. Measures for controlling inter- and intra-assay variability of oligonucleotide-based enzyme capture assays (OECAs) are described in section 2.3 titled "Laboratory analysis." Except for soluble fibrin monomer (sFM) and TAFI, assays were accredited by the German Accreditation Body (Deutsche Akkreditierungsstelle) and conducted

following ISO standards. Materials and devices used are listed in Supplementary Table S1.

2.1 | Study participant recruitment and eligibility criteria

Study participants were prospectively recruited. Patients with thrombophilia were recruited at our thrombophilia outpatient clinic. Healthy controls were blood donors. Figure 1 shows patient recruitment and describes inclusion and exclusion criteria. Initially, 146 subjects were recruited and further assessed for eligibility. All study participants were tested for FV Leiden and FII 20210G>A mutation using in-house methods as previously described [39,40]. Healthy volunteers were required to be noncarriers for participation in the study. Patients with thrombophilia were required to show no additional abnormalities in thrombophilia screening. Seventeen candidates were excluded from the study. One hundred twenty-nine subjects were included and received rFVIIa. No blood samples were lost after collection.

2.2 | rFVIIa administration

The subjects fasted overnight. In the following morning, blood samples were taken immediately before an intravenous bolus injection of 15-μg/kg rFVIIa was given. Thereafter, blood samples were drawn after 10 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 5 hours, and 8 hours. Twenty-one–gauge winged infusion sets and citrate tubes (10.5 mmol/L) were used, and a new antecubital vein puncture was performed for each blood draw. Citrate tubes containing 100 μmol/L argatroban were used for the thrombin–OECA. Citrate tubes containing 10 μmol/L aprotinin and 250 μg/mL bivalirudin were used for the APC–OECA. The first 2 mL of blood were not used for analysis. Whole blood was centrifuged (2600 × *g*, 10 minutes) within 30 minutes. All plasma samples were stored at <−70 °C before analysis.

2.3 | Laboratory analysis

Müller et al. [41,42] initially described the OECA for thrombin measurement and the OECA for APC measurement, which were used in various other studies [30–32,43–48]: In brief, bovine serum albumin–biotin-coated microtiter modules (10 μg/mL, 100 μL/well) were incubated overnight at 4 °C. After washing, 10 μg/mL streptavidin was added to the wells and incubated at room temperature for 1 hour. Then, plates were emptied. The aptamers for the thrombin–OECA (HD1-22) or for the APC–OECA (HS02-52G) were added to the wells. Thereafter, plates were washed, and plasma samples to be measured were placed in the wells. In case of the APC–OECA, citrated plasma was recalcified using 1 mol/L $CaCl_2$ (final concentration: 7.5 mmol/L), which has been shown to improve binding between aptamers and APC [49]. Plates were incubated and washed. To detect thrombin, the fluorogenic peptide substrate I-1560 was added to the wells. To detect APC, Pefafluor PCa was added. A plate fluorescence reader was used to measure changes in fluorescence over time. Two control samples, consisting of pooled normal plasma spiked with thrombin (136 and 13.6 pmol/L) or rAPC (91 and 9.1 pmol/L), and plasma-based calibrators covering a ½-log10 concentration range (0-272 pmol/L of thrombin or 0-182 pmol/L of rAPC) were processed simultaneously with the samples. Samples were always analyzed in triplicate. In all runs, aliquots of the same controls were used. Runs, in which a deviation of more than 10% was observed in at least 1 control, were repeated.

Plasma levels of FII, AT, plasminogen, α2-antiplasmin, PC, free PS, fibrinogen (Clauss method), and D-dimer were determined using a coagulation analyzer and corresponding reagents. FVIIa, F1+2, TAT, sFM, t-PA antigen, PAP, TAFI antigen, and PAI-1 antigen were measured using commercially available assays (Supplementary Table S2). The used t-PA and PAI-1 antigen assays detected both the free forms of the proteins and t-PA–PAI-1 complex [50].

2.4 | Data analysis

Median and IQR were used to describe data. The area under the curve (AUC) was used for longitudinal analysis of data, whereby an outcome (ie, hemostatic biomarker, *y*-axis) was measured repeatedly across time (*x*-axis) for each study participant and the area under the measurement-time curve represents a summary measure for an individual. The AUC was calculated using the formula $([(x_2 - x_1)\cdot(y_2 + y_1)]/2) + ([(x_3 - x_2)\cdot(y_3 + y_2)]/2) + \ldots + ([(x_n - x_{n-1})\cdot(y_n + y_{n-1})]/2) - y_1\cdot x_n$, with x_n being the latest timepoint with $y_n \geq y_1$ (ie, a plasma level not below baseline). Parameters that increased were corrected for baseline values (by subtracting $y_1\cdot x_n$) to isolate the effect of rFVIIa. Of note, the PAI-1 AUC was not corrected since PAI-1 decreased, and the TAFI-AUC was corrected for minimum values instead of baseline values. We did not account for within-subject variation when calculating AUCs and comparing AUCs between groups, since stimulation with rFVIIa was only performed once per individual. Analogous to the endogenous thrombin potential in *in vitro* thrombin generation [51], the AUC can be interpreted as the amount of the respective analyte that can be generated after a standardized coagulation activation. The Shapiro–Wilk test was applied to assess normality of data. For the comparison of values measured before and after administration of rFVIIa, the Friedman test was performed followed by multiple pairwise comparison using the Nemenyi procedure. To compare baseline values or the AUC between the 3 cohorts of healthy controls, patients with asymptomatic thrombophilia, and patients with thrombophilia and VTE, the Kruskal–Wallis test was used, followed by pairwise comparison using the Dunn procedure. The Bonferroni correction was used for multiple test correction. For comparisons between asymptomatic and symptomatic carriers of the FV Leiden mutation or the FII 20210G>A mutation, the Mann–Whitney test was used. *P* values of ≤.05 were considered statistically significant. The XLSTAT statistical and data analysis solution software (Addinsoft) was used for statistical analysis.

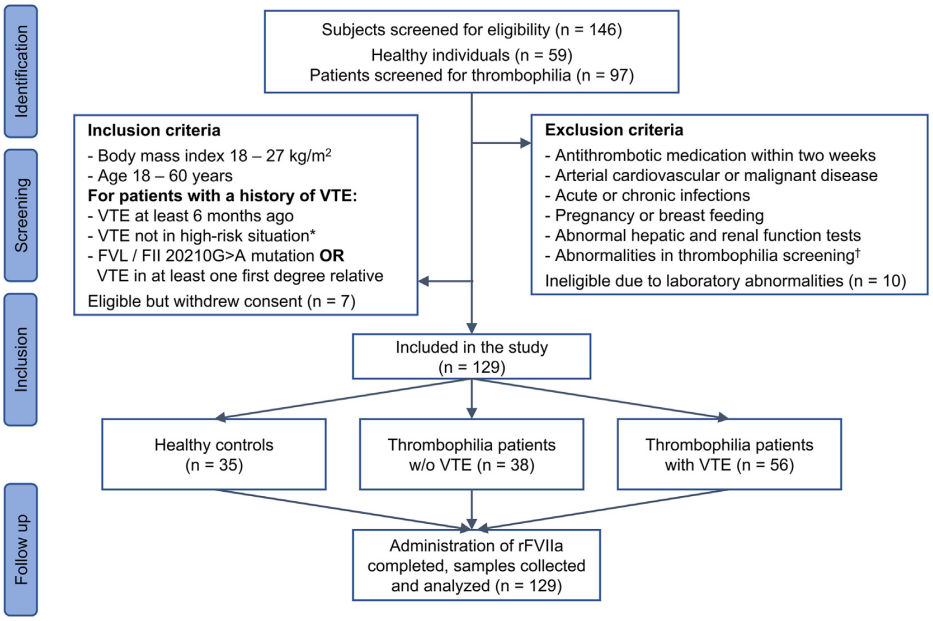

FIGURE 1 Eligibility criteria and study participant enrollment. *High-risk situations for VTE included pregnancy and puerperium, immobilization, trauma, and surgery. Renal and hepatic function tests included creatinine in serum, urea, γ-glutamyl transferase, and transaminases. †Abnormalities in a thrombophilia screening were defined as decreased plasma levels of protein C, free protein S, antithrombin, functional lupus anticoagulants (dilute Russell viper venom time, lupus anticoagulant–sensitive activated partial thromboplastin time), anticardiolipin IgG and IgM, and anti–β2-glycoprotein I IgG and IgM, and, only for healthy individuals to be included into the control group, FV Leiden and FII 20210G>A mutation. F, factor; Ig, immunoglobulin; VTE, venous thromboembolism.

3 | RESULTS

3.1 | Study population and hemostasis parameters at baseline

The final study population consisted of 35 healthy individuals, 38 asymptomatic carriers of FV Leiden or FII 20210G>A (19 each, VTE− cohort), and 56 patients with a history of at least 1 unprovoked VTE (VTE+ cohort). The basal characteristics of the study participants regarding age, sex (male/female), and body mass index (BMI) were similar (Table 1). Age (median: 35-41 years) and BMI (median: 22-24 kg/m^2) did not differ significantly between groups (Kruskal–Wallis test: P = .59 and P = .13, respectively).

Table 2 shows the results of hemostasis testing prior to the administration of rFVIIa. PAI-1 showed the greatest relative difference between the VTE+/− cohorts in comparison to the control group, with approximately 3-fold higher median levels ($P = 5 \times 10^{-4}$/$P = 2.1 \times 10^{-6}$). In the VTE− cohort, median APC levels were 1.8-fold higher (P = .009) and median sFM levels were 1.5-fold lower (P = .01) in comparison to healthy controls. Median D-dimer levels in the VTE+ cohort were 1.4-fold higher than in the control group (P = .005). Among the other parameters with statistically significant differences between cohorts (FII, plasminogen, α2-antiplasmin, fibrinogen), relative differences in median plasma levels in comparison to healthy controls ranged between 1.04 and 1.16, and measured values were within reference ranges in all cohorts. Plasma levels of free thrombin were below the limit of detection in most of the study participants with no differences between cohorts. Median plasma levels of the other studied parameters did not exceed normal values and did not differ significantly between cohorts.

3.2 | rFVIIa induces fibrinolysis parameter changes that are more pronounced in patients with thrombophilia and a history of VTE

Administration of rFVIIa was well tolerated by all subjects and no adverse events of any kind were encountered during the observation period. The pharmacokinetic profiles of rFVIIa were essentially identical in healthy controls and VTE− and VTE+ cohort with median peak levels of FVIIa activity of 6.91, 6.97, and 7.07 U/mL, respectively (Supplementary Figure S1A). Plasma level changes of hemostasis parameters after administration of rFVIIa are shown in Table 3. As shown in Figure 2A, D-dimer levels remained unchanged in the VTE− cohort and the control group, whereas they slightly but significantly increased in the VTE+ cohort. Among the other fibrinolysis parameters studied, plasma levels of sFM, plasminogen, and α2-antiplasmin did not change in comparison to baseline.

While t-PA antigen levels also remained unchanged after rFVIIa infusion (Supplementary Figure S1B), median plasma levels of PAP

TABLE 1 Characteristics of the study population.

Characteristics	Healthy controls, *n* = 35	Patients with thrombophilia	
		VTE−, *n* = 38	VTE+, *n* = 56
Age (y), median (range)	35 (21-60)	38 (18-60)	41 (20-57)
Sex (male/female), *n*	14 (40%)/21 (60%)	15 (39%)/23 (61%)	23 (41%)/33 (59%)
BMI (kg/m^2), median (range)	22 (18-27)	23 (18-27)	24 (19-27)
FV Leiden, *n*		19 (50%)[b]	19 (34%)[a]
FII 20210G>A, *n*		19 (50%)[c]	17 (30%)
Unexplained familial thrombophilia,[d] *n*			20 (36%)
History of DVT, *n*			26 (46%)
History of PE, *n*			12 (21%)
History of DVT and PE, *n*			18 (32%)

All study participants were of White, middle-European ethnicity.

BMI, body mass index; DVT, deep vein thrombosis; F, factor; PE, pulmonary embolism; VTE, venous thromboembolism.

[a]Thereof 1 homozygous and 2 with HR2 haplotype.

[b]Thereof 3 homozygous and 1 with HR2 haplotype.

[c]Thereof 1 homozygous.

[d]Family history of unprovoked VTE in at least 1 first-grade relative without an established thrombophilic risk factor.

TABLE 2 Comparison of baseline hemostasis parameters between cohorts.

	Healthy controls, *n* = 35	Patients with thrombophilia VTE−, *n* = 38		VTE+, *n* = 56	
Subjects	Plasma level	Plasma level	*P*	Plasma level	*P*
FII, %	103 (98-112)	113 (99-137)	.026	119 (107-127)	.002
F1+2 (nmol/L)	0.15 (0.12-0.21)	0.17 (0.14-0.19)	.456	0.20 (0.13-0.28)	.06
Thrombin (pmol/L)	<0.46 (<0.46 to 0.46)	<0.46 (<0.46 to 0.57)	.215	<0.46 (<0.46 to 0.60)	.05
AT, %	107 (98-110)	98 (93-106)	.059	99 (93-106)	.09
TAT (pmol/L)	<21.3 (<21.3 to <21.3)	<21.3 (<21.3 to 24.1)	.89	<21.3 (<21.3 to 26.0)	.54
Plasminogen, %	109 (101-114)	95 (84-103)	2×10^{-4}	102 (95-111)	.31
t-PA (ng/mL)	<1.71 (<1.71 to 3.10)	<1.71 (<1.71 to 1.99)	.596	<1.71 (<1.71 to 2.35)	.996
α2-antiplasmin, %	105 (98-113)	102 (98-109)	.60	112 (102-121)	.02
PAP (ng/mL)	136 (90-205)	133 (101-188)	.87	164 (123-230)	.17
TAFI, %	116 (95-143)	101 (81-122)	.20	121 (105-142)	.38
PC, %	107 (99-117)	104 (95-113)	.92	111 (102-128)	.45
APC (pmol/L)	0.63 (<0.39 to 0.99)	1.16 (0.72-1.39)	.009	0.90 (0.46-1.26)	.398
PAI-1 (ng/mL)	9.4 (6.0-15.5)	28.1 (11.5-34.5)	5×10^{-4}	26.0 (15.3-43.4)	2×10^{-6}
Fibrinogen (g/L)	2.58 (2.24-2.87)	2.33 (2.22-3.13)	.756	2.69 (2.52-3.31)	.08
sFM (μg/mL)	3.27 (2.40,4.30)	2.13 (1.75-3.23)	.01	3.33 (2.39-4.40)	.80
D-dimer (mg/L)	0.27 (0.19-0.34)	0.25 (0.19-0.35)	.876	0.38 (0.25-0.55)	.005

Data are presented as median and IQR. *P* values describe differences of values in asymptomatic carriers of thrombophilic risk factors (VTE−) and patients with thrombophilia and a history of VTE (VTE+) in comparison to healthy controls and were calculated using the Kruskal–Wallis test followed by pairwise comparison using the Dunn procedure. The Bonferroni method was used to correct for multiple testing and was performed for 3 comparisons.

APC, activated protein C; F, factor; PAI, plasminogen activator inhibitor; PAP, plasmin–α2-antiplasmin; PC, protein C; sFM, soluble fibrin monomer; TAT, thrombin–antithrombin; TAFI, thrombin-activatable fibrinolysis inhibitor; VTE, venous thromboembolism.

TABLE 3 Plasma level changes of hemostasis parameters after administration of rFVIIa.

	Healthy controls, *n* = 35		Patients with thrombophilia			
			VTE−, *n* = 38		VTE+, *n* = 56	
Subjects	Plasma level	Δ%	Plasma level	Δ%	Plasma level	Δ%
F1+2 (3 h), nmol/L	0.22 (0.17-0.30)	47	0.23 (0.20-0.30)	35	0.27 (0.21-0.36)	35
TAT (1 h), pmol/L	30.8 (21.3-44.5)	44	34.8 (26.9-51.0)	63	45.8 (29.9-75.6)	115
PAP (8 h), ng/mL	195 (148-237)	43	186 (124-256)	40	250 (187-342)	52
TAFI (0.5 h), %	101 (85-132)	−13	94 (78-109)[a]	−7	106 (97-135)[b]	−12
TAFI (8 h), %	124 (111-158)	23	117 (85-127)	24	139 (118-173)	31
APC (0.5 h), pmol/L	3.13 (2.46-3.67)	397	6.63 (5.24-8.12)	472	4.87 (3.53-6.08)	441
PAI-1 (8 h), ng/mL	3.9 (2.3-10.2)	−59	5.8 (2.6-9.8)	−79	6.7 (4.5-11.3)	−74
D-dimer (5 h), mg/L	0.29 (0.24-0.37)[c]	7	0.29 (0.23-0.41)[c]	16	0.44 (0.32-0.62)[b]	16

Δ% indicates the percentage by which plasma levels increased or decreased at the timepoints shown in brackets in comparison to baseline levels. The percentage by which TAFI increased between 0.5 and 8 hours after administration of rFVIIa is shown additionally. The Friedman test followed by pairwise comparison using the Nemenyi procedure was used to compare measurements at the 7 sampling timepoints to baseline values (or to the values measured at *t* = 0.5 hours in case of TAFI, respectively). The Bonferroni method was applied to correct for multiple testing and was performed for 7 comparisons.

APC, activated protein C; F, factor; PAI, plasminogen activator inhibitor; PAP, plasmin–α2-antiplasmin; TAFI, thrombin-activatable fibrinolysis inhibitor; TAT, thrombin–antithrombin; VTE, venous thromboembolism.

Corrected *P* values were as follows: [a]<.005; [b]<.05; [c]not significant (>.05); and <.0005 for all other comparisons.

increased in all cohorts (Table 3 and Figure 2B). The area under the PAP generation curve (PAP-AUC) in the VTE+ group, but not in the VTE− group, was greater than that in healthy controls, with 0.48 (0.25-0.81), respectively, 0.25 (0.25-0.53) vs 0.27 (0.09-0.41) ng·h/L (P = .003). TAFI antigen levels initially decreased and then increased within normal ranges in all cohorts, by 23% ($P = 4.4 \times 10^{-11}$) in healthy controls, 24% ($P = 1.9 \times 10^{-7}$) in the VTE− cohort, and 31% (P = .005) in the VTE+ cohort with no statistically significant

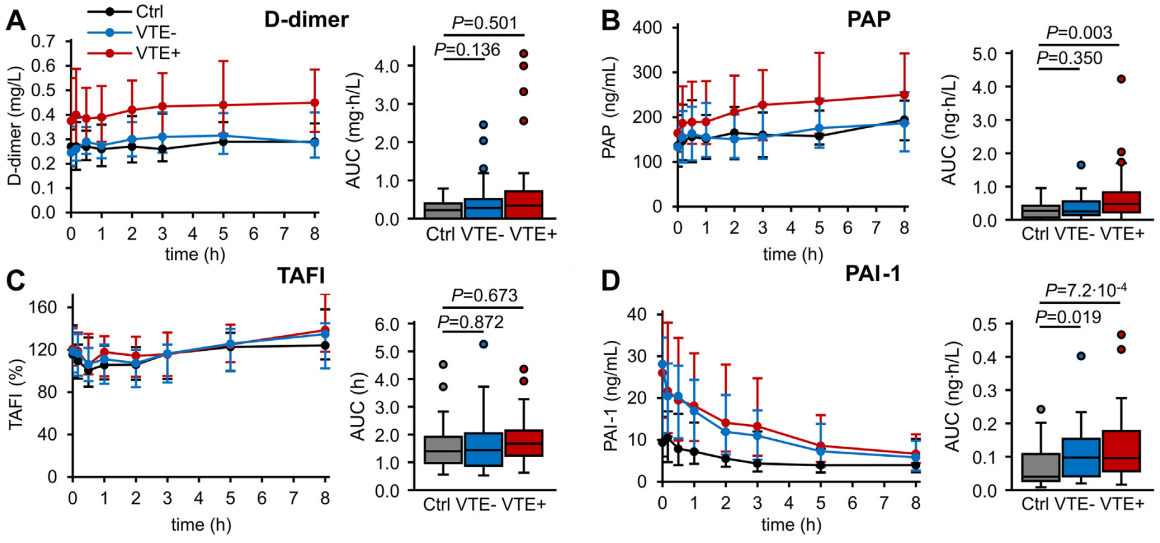

FIGURE 2 Fibrinolysis biomarker changes in response to *in vivo* coagulation activation by rFVIIa. Plasma levels of D-dimer (A), PAP (B), TAFI antigen (C), and PAI-1 antigen (D) were measured before (*t* = 0) and after intravenous injection of 15 μg/kg rFVIIa in healthy individuals (Ctrl, *n* = 35 [black]), asymptomatic carriers of FV Leiden or FII 20210G>A (VTE−, *n* = 38 [blue]), and patients with thrombophilia and a history of VTE (VTE+, *n* = 56 [red]). Plasma levels are shown as median and IQR. The AUCs are shown as box plots indicating quartiles and median of the data, the whiskers extending up to 1.5 times the IQR from the box, and circles showing outlying values. The AUCs were compared using the Kruskal–Wallis test followed by pairwise comparison using the Dunn procedure. The Bonferroni method was used to correct for multiple testing and was performed for 3 comparisons. AUC, area under the curve; Ctrl, control; F, factor; PAI, plasminogen activator inhibitor; PAP, plasmin–α2-antiplasmin; TAFI, thrombin-activatable fibrinolysis inhibitor; VTE, venous thromboembolism.

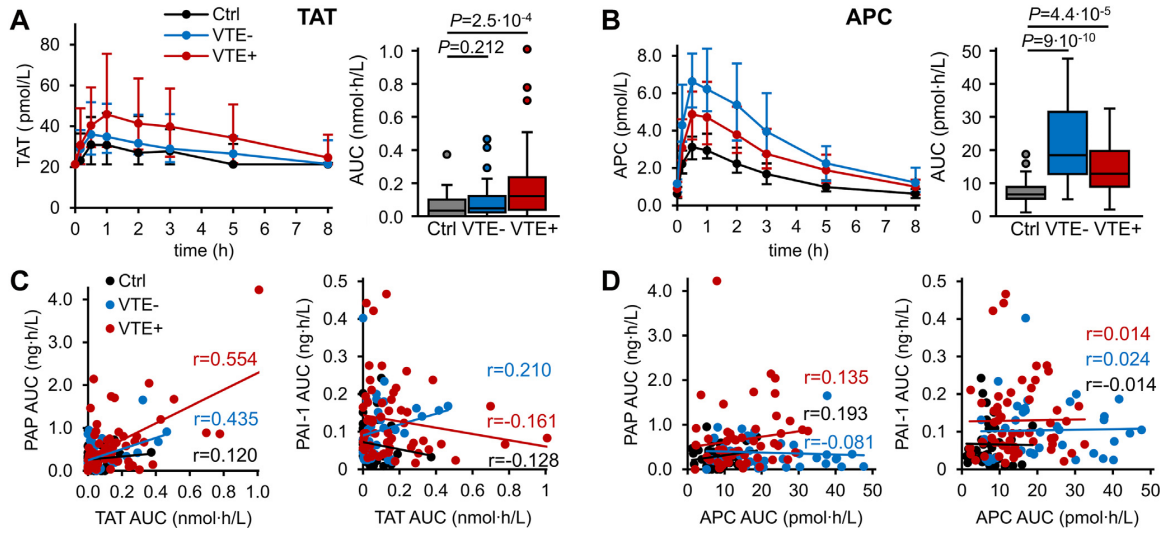

FIGURE 3 Association of fibrinolysis biomarker changes with thrombin–APC response to rFVIIa. Plasma levels of TAT (A), APC (B), PAP, and PAI-1 antigen were measured before (t = 0) and after intravenous injection of 15 μg/kg rFVIIa in healthy individuals (Ctrl, n = 35 [black]), asymptomatic carriers of FV Leiden or FII 20210G>A (VTE−, n = 38 [blue]), and patients with thrombophilia and a history of VTE (VTE+, n = 56 [red]). The AUCs are shown as box plots indicating quartiles and median of the data, the whiskers extending up to 1.5 times the IQR from the box, and circles showing outlying values. The AUCs were compared using the Kruskal–Wallis test followed by pairwise comparison using the Dunn procedure. The Bonferroni method was used to correct for multiple testing and was performed for 3 comparisons. The correlation between TAT-AUC (C) and APC-AUC (D) with PAP-AUC and PAI-1 AUC were analyzed by calculating the Pearson correlation coefficient (r). AUC, area under the curve; APC, activated protein C; F, factor; PAI, plasminogen activator inhibitor; PAP, plasmin–α2-antiplasmin; TAT, thrombin–antithrombin.

differences in the AUC between cohorts (Table 3 and Figure 2C). PAI-1 antigen levels decreased in all 3 cohorts. Eight hours after application of rFVIIa, PAI-1 concentrations below the baseline level of healthy controls were observed in the VTE− and VTE+ cohorts (Tables 2 and 3). The PAI-1 AUC was greater in the VTE− group (0.10, 0.04-0.15 ng·h/L, P = .02) and the VTE+ group (0.10, 0.06-018 ng·h/L, $P = 7.2 \times 10^{-4}$) than in healthy controls (0.04, 0.03-0.09 ng·h/L; Figure 2D).

3.3 | Fibrinolysis biomarker changes correlate with preceding rFVIIa-induced thrombin formation but not with the anticoagulant APC response

Plasma levels of free thrombin did not change statistically significantly after rFVIIa infusion (Supplementary Figure S1C), whereas F1+2 (Table 3, Supplementary Figure S1D) and TAT (Table 3 and Figure 3A) increased in all 3 cohorts. Compared with baseline values, median F1+2 and TAT levels increased by 47% ($P < 10^{-13}$) and 44% ($P = 3.6 \times 10^{-6}$) in healthy controls, by 35% ($P < 10^{-13}$) and 63% (6.3×10^{-11}) in the VTE− cohort and by 35% and 115% ($P = 10^{-13}$ each) in the VTE+ cohort, respectively. The area under the F1+2 generation curve did not differ between cohorts. In the VTE+ cohort, but not in the VTE− cohort, the area under the TAT generation curve (TAT-AUC) was greater than in healthy controls, with 0.12 (0.04-0.23), respectively, 0.05 (0.02-012) vs 0.03 (0.00-0.09) nmol·h/L ($P = 2.5 \times 10^{-4}$). As shown in Table 3 and Figure 3B, median plasma levels of APC increased in all 3 cohorts, in comparison to baseline by 397% in healthy controls, 472% in the VTE− cohort, and 441% in the VTE+ cohort ($P < 10^{-13}$ each). The APC-AUC was significantly greater in the VTE− cohort (18.5, 13.5-30.3 pmol·h/L, $P = 9.0 \times 10^{-10}$) and the VTE+ cohort (12.9, 9.3-19.6 pmol·h/L, $P = 4.4 \times 10^{-5}$) than in the control group (6.6, 5.4-8.8 pmol·h/L).

In the VTE+ and VTE− cohorts, TAT-AUC and PAP-AUC correlated moderately with one another (r = 0.554 and r = 0.435, respectively), whereas they did not correlate in healthy controls (Figure 3C). TAT-AUC and PAI-1 AUC neither correlated (Figure 3C), nor did APC-AUC and PAP-AUC or APC-AUC and PAI-1 AUC (Figure 3D).

3.4 | Fibrinolysis biomarker changes are associated with history of thrombosis only in FV Leiden carriers

Asymptomatic and symptomatic carriers of FV Leiden and FII 20210G>A were compared with respect to changes in PAP, PAI-1, TAT, and APC (Figure 4). The PAP-AUC and the PAI-1 AUC were smaller in asymptomatic FV Leiden carriers in comparison to those with a history of VTE, with 0.22 (0.06-0.32) ng·h/L vs 0.41 (0.21-0.69) ng·h/L ($P = 9.2 \times 10^{-4}$) and 0.10 (0.05-0.13) ng·h/L vs 0.18 (0.11-0.27) ng·h/L (P = .01), respectively. The TAT-AUC did not differ, and the

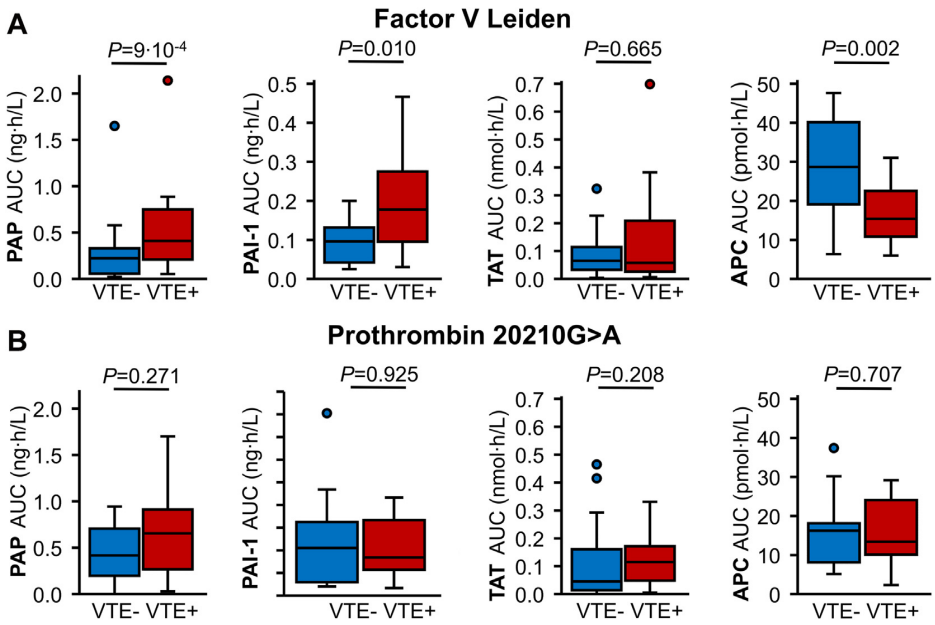

FIGURE 4 Comparison of fibrinolysis biomarker changes and thrombin–APC response in symptomatic and asymptomatic carriers of thrombophilia. Plasma levels of PAP, PAI-1, TAT, and APC were monitored for 8 hours after intravenous injection of 15 μg/kg rFVIIa in (A) asymptomatic (VTE−, blue) and symptomatic (VTE+, red) FV Leiden carriers (n = 19 each) and (B) asymptomatic (n = 19) and symptomatic (n = 17) FII 20210G>A carriers. The AUCs of plasma levels are shown as box plots indicating quartiles and median of the data, the whiskers extending up to 1.5 times the IQR from the box, and circles showing outlying values. The AUCs were compared using the Mann–Whitney test. APC, activated protein C; AUC, area under the curve; PAI, plasminogen activator inhibitor; PAP, plasmin–α2-antiplasmin; TAT, thrombin–antithrombin; VTE, venous thromboembolism.

APC-AUC was greater, with 28.7 (19.8-39.0) pmol·h/L vs 15.4 (11.0-21.2) pmol·h/L (P = .002; Figure 4A). The AUC of PAP, PAI-1, TAT, and APC did not differ significantly in asymptomatic FII 20210G>A carriers in comparison to VTE+ FII 20210G>A carriers (Figure 4B). In the absence of a corresponding cohort of asymptomatic individuals, changes of the abovementioned parameters in patients with unexplained familial thrombophilia were compared with FV Leiden and FII 20210G>A in the VTE+ group. The PAP-AUC did not differ between these 3 subgroups (Figure 5A), the PAI-1 AUC was greater in FV Leiden carriers than in the other 2 groups (Figure 5B), and the TAT-AUC did not differ between groups (Figure 5C). The APC-AUC was smaller in patients with unexplained familial thrombophilia than in VTE+ carriers of FV Leiden and FII 20210G>A (Figure 5D).

4 | DISCUSSION

Given that the coagulation and fibrinolytic systems are highly interrelated, we decided to study fibrinolysis biomarkers using a previously established human model of low-grade coagulation activation by rFVIIa [30–32]. The aim of these studies was to identify potential differences between patients with or without a history of VTE and with different types of thrombophilia. Subgroup analysis of rFVIIa-induced thrombin activation, APC response, and fibrinolysis biomarker changes in FV Leiden carriers, FII20210G>A carriers, and patients with unexplained familial thrombophilia revealed an association of decreased PAP and PAI-1 formation rates as well as increased APC and the absence of prior VTE only in FV Leiden carriers. This fibrinolytic response profile to coagulation activation was not observed in FII 20210G>A carriers or patients with unexplained familial thrombophilia. An association between increased APC response rates and the absence of prior VTE in FV Leiden carriers has been reported previously [31]. One might speculate if this phenomenon in combination with a characteristic fibrinolytic response to coagulation activation could suggest that an antifibrinolytic mechanism modulates thrombotic risk in FV Leiden carriers.

An antifibrinolytic effect of FV Leiden has been described before and suggested to be caused by increased TAFI activity due to increased thrombin formation [52,53]. TAFI levels initially decreased by 7% to 13% within 30 to 120 minutes through all cohorts, and then reverted to slightly above baseline levels during the observation period. As the thrombin–TM complex is involved in TAFI activation by thrombin [54,55], the initial slight decrease in TAFI levels might be explained by a fraction of TAFI being bound to the thrombin–TM complex on the cell surface and thereby removed from the circulation. However, since we only measured the total amount of TAFI, including both latent and activated forms, this remains speculative. Our results do, therefore, also not allow conclusions on potential

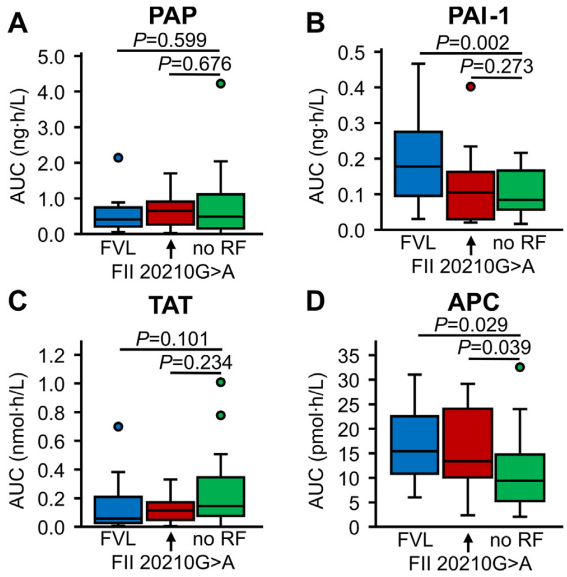

FIGURE 5 Comparison of fibrinolysis biomarker changes and thrombin–APC response in patients with thrombophilia and a history of VTE. Plasma levels of (A) PAP, (B) PAI-1, (C) TAT, and (D) APC were monitored for 8 hours after intravenous injection of 15 μg/kg rFVIIa in patients with a history of VTE, thereof 19 FV Leiden carriers (blue), 17 FII 20210G>A carriers (red), and 20 individuals with familial thrombophilia, in whom no thrombophilic risk factor was found (green). The AUCs of plasma levels are shown as box plots indicating quartiles and median of the data, the whiskers extending up to 1.5 times the IQR from the box, and circles showing outlying values. The AUCs were compared using the Kruskal–Wallis test followed by pairwise comparison using the Dunn procedure. The Bonferroni method was used to correct for multiple testing and was performed for 3 comparisons. APC, activated protein C; AUC, area under the curve; F, factor; PAI, plasminogen activator inhibitor; PAP, plasmin–α2-antiplasmin; TAT, thrombin–antithrombin; VTE, venous thromboembolism.

differences in the TAFI-mediated antifibrinolytic effect of FV Leiden in symptomatic and asymptomatic carriers. The test we used for TAFI measurement underestimates plasma levels in the case of the Thr325Ile polymorphism [56], which is associated with lower TAFI levels [57]. Since observed TAFI levels at baseline and after stimulation did not differ between cohorts, a genuine effect of this polymorphism or false low measurements of TAFI on the overall study results is unlikely.

In contrast to the small and transient decrease of TAFI, a sustained decrease of PAI-1 was observed through all cohorts. Starting from approximately 3-fold higher baseline levels consistent with those observed previously in a larger study population [58], the extent of this decline was significantly greater in patients with thrombophilia than in healthy controls. PAI-1 was measured using an assay that detects both free PAI-1 and PAI-1 complexed with t-PA, and thus, the observed decline in PAI-1 levels cannot be explained by ongoing complex formation following t-PA release. A potential explanation could be proteolysis of PAI-1 by thrombin or APC. F1+2 and TAT, indicating thrombin formation, increased in all cohorts and were associated with a distinctive increase of APC levels, which was significantly greater in patients with thrombophilia than in healthy controls. These data are consistent with our previous results [30–32]. *In vitro* experiments have shown that APC is able to directly neutralize PAI-1 [59], and increased APC generation and subsequent PAI-1 inactivation have been proposed to contribute to trauma-induced coagulopathy [60,61]. Yet, this concept remains controversial [62]. As thrombin and other serine proteases have also been shown to neutralize PAI-1 [63], it has conversely been suggested that increased APC generation might inhibit thrombin formation and thus impair inactivation of PAI-1 by thrombin [64]. Since neither the TAT-AUC nor APC-AUC correlated with the PAI-1 AUC in our study, probably due to differences in plasma half-lives, neither hypothesis can be clearly supported. However, our results indicate that even low-grade coagulation activation can level out baseline differences of PAI-1 and that higher baseline levels of PAI-1 do not prevent an increased fibrinolytic response to coagulation activation as observed in the VTE+ cohort. Taken together with the observed increase in PAP levels in all cohorts, these data indicate that in our *in vivo* model of coagulation activation, rFVIIa induces not only thrombin formation and an anticoagulant response but also a longer lasting fibrinolytic response.

We did not observe an increase of t-PA, most probably due to its shorter plasma half-life in comparison to PAP. In a previous *in vivo* study, in which a plasma half-life of PAP of 11 hours was determined, we were unable to calculate the plasma half-life of t-PA due to its complete clearance from the circulation within 15 minutes [65]. It has been shown *in vitro* that thrombin induces t-PA release from human endothelial cells [66,67]. However, in these studies, higher amounts of thrombin were used than those measured in the present study. Increases of F1+2 and TAT were observed in all cohorts, indicating thrombin formation, while no statistically significant increase in free thrombin levels was observed. The most likely explanation for this observation, which is consistent with our previous studies using this *in vivo* model [30–32], is the differences in the plasma half-life of the 3 thrombin markers. With approximately 2 hours, F1+2 has the longest half-life, followed by that of TAT of 44 minutes, while the catalytic half-life of thrombin is significantly shorter with less than 60 seconds [43,65]. Therefore, the thrombin burst induced by low-dose rFVIIa is too low to induce quantifiable plasma levels of thrombin but is sufficient enough to increase plasma levels of F1+2 and TAT.

Differences in baseline levels of hemostasis and fibrinolysis parameters could have modulated the response to rFVIIa-induced coagulation activation and were therefore compared between cohorts. Compared with PAI-1, other differences in baseline plasma levels between cohorts were minor. Prothrombin levels were higher in patients with thrombophilia (whether or not they experienced VTE) than in healthy controls, which can be attributed to the presence of FII 20210G>A carriers in these cohorts [10]. In line with our previous results, APC was higher in the VTE− cohort due to the presence of asymptomatic FV Leiden carriers [31]. Plasminogen levels were lower in asymptomatic thrombophilia carriers, and α2-antiplasmin levels were

higher in patients with VTE than in the control group. As both changes were within the respective references, it is unlikely that they could have affected the fibrinolytic response to rFVIIa-induced coagulation activation. Also, within reference ranges, sFM was lower in the asymptomatic VTE− cohort and D-dimer was higher in the VTE+ cohort, consistent with previous studies in which higher D-dimer in patients with VTE are attributed to an increased thrombotic risk [68].

There were potential sources of imprecision or bias in this study, including the error margin of laboratory tests, the adherence to blood draw times, the precision of dosing of rFVIIa, and the size of the study population. To control these potential issues, we chose cohort size, dosage of rFVIIa, and blood draw times based on previous pharmacokinetic studies on rFVIIa and obtained similar pharmacokinetic results [69,70]. The performance of the APC–OECA and the thrombin–OECA has been studied in detail previously [41,42]. Another potential source of bias is the analysis of data as AUCs, which could have exaggerated any differences. Although we did not detect a statistically significant difference in age or BMI, there are other confounding variables that may explain differences between groups, given that the study analyses were not adjusted for covariates. It cannot be ruled out that measurements in patients with familial thrombosis were affected by the prior VTE and not by yet unidentified genetic variation. Due to the aforementioned limitations, the study results should be considered hypothesis-generating.

5 | CONCLUSION

We have shown using an *in vivo* model that low-grade extrinsic coagulation activation induces both an anticoagulant and profibrinolytic endothelial response. The data obtained show that the SHAPE procedure is a useful tool to assess not only the functionality of the PC pathway but also the fibrinolytic properties of the endothelium, although the biological relevance of the induced changes remains yet to be determined. In this *in vivo* model, the fibrinolytic response notably lasts longer than the stages of coagulation activation and anticoagulant response. It remains an open question if this observation can be transferred to clinical situations of thrombotic risk, such as trauma or sepsis, in which thrombin formation rates are significantly higher. One might speculate that PAI-1 inactivation would then occur even more pronounced; however, other sources of PAI-1, such as activated platelets [71], might come into play. Moreover, the presence of tissue factor might not only promote thrombin formation [72] but also regulate plasminogen binding and activation [73], thereby possibly affecting the fibrinolytic response. Finally, our data show that the fibrinolytic response differs in patients with thrombophilia, depending on the underlying thrombophilic risk factors, and might modulate the thrombotic risk.

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ETHICS STATEMENT

The study proposal was approved by the Institutional Review Board and Ethics Committee of the Medical Faculty of the University Bonn (reference number: 016/16). Written informed consent was received prior to participation in compliance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

H.R., J.M., and B.P. conceived and designed the study. S.R., N.S., and H.R. performed the experiments and collected data. S.R. and H.R. analyzed the data. S.R., N.S., J.M., H.L.M., J.O., B.P., and H.R. drafted and edited the manuscript. All authors revised the manuscript, agreed with its content, and approved of submission.

RELATIONSHIP DISCLOSURE

B.P. and J.M. have a patent (DE102007063902B3) including the aptamer HS02-52G binding to APC. An assay for the quantification of APC levels in human plasma, based on this aptamer, has been licensed to ImmBioMed (Pfungstadt, Germany). B.P. and J.M. have a patent (DE102007041476) including the aptamer HD1-22 binding to thrombin. An assay for the quantification of thrombin levels in human plasma, based on this aptamer, has been licensed to ImmBioMed (Pfungstadt, Germany). J.O. has received research funding from Bayer, Biotest, CSL Behring, Octapharma, Pfizer, Swedish Orphan Biovitrum, and Takeda and consultancy, speakers bureau, honoraria, scientific advisory board, and travel expenses from Bayer, Biogen Idec, BioMarin, Biotest, Chugai Pharmaceutical Co Ltd, CSL Behring, Freeline, Grifols, LFB, Novo Nordisk, Octapharma, Pfizer, F. Hoffmann-La Roche AG, Sanofi, Spark Therapeutics, Swedish Orphan Biovitrum, and Takeda. The other authors have no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.rpth.2024.102351

Fibrinolysis biomarker, thrombin, and activated protein C level alterations after coagulation activation depend on type of thrombophilia and clinical phenotype

Supporting Information

Supplementary Tables

Table S1 Materials and devices

Article	Provider or manufacturer
21-gauge winged infusion set	Sarstedt, Nümbrecht, DE
Aprotinin	AppliChem, Darmstadt, DE
Argatroban	Mitsubishi Pharma, Düsseldorf, DE
BCS XP coagulation analyzer	Siemens Healthineers, Erlangen,
Biotinylated aptamer against activated protein C (HS02-52 G 3'-Bio)	Microsynth, Balgach, CH
Biotinylated aptamer against thrombin (HD1-22 3'-Bio)	Microsynth, Balgach, CH
Bivalirudin	The Medicines Company, Oxfordshire, UK
Bovine serum albumin-biotin	Thermo Fisher Scientific, Darmstadt, DE
Fluorogenic substrate for activated protein C (Pyroglu-Pro-Arg-AMC (Pefafluor PCa))	Pentapharm, Basel, CH
Fluorogenic substrate for thrombin (Boc-Asp(OBzl)-Pro-Arg-AMC (I-1560))	Bachem, Weil am Rhein, DE
Human α-thrombin	CellSystems, St. Katharinen, DE
Maxisorp Fluornunc microtiter modules	Nunc A/S, Roskilde, DK
Recombinant activated factor VII	Novo Nordisk, Bagsværd, DK
Recombinant activated protein C	Eli Lilly, Indianapolis, IN, US
Synergy 2 plate fluorescence reader	BioTek Instruments, Bad Friedrichshall, DE

Table S2 Commercially available assays

Parameter	Article	Provider or manufacturer
Activated factor VII	STACLOT VIIa-rTF	Stago, Asnières sur Seine, FR
anti-β2 glycoprotein I IgG	REAADS Anti-Beta2 Glycoprotein I IgG	Diapharma Group, West Chester, OH, US
anti-β2 glycoprotein I IgM	REAADS Anti-Beta2 Glycoprotein I IgM	Diapharma Group, West Chester, OH, US
anti-cardiolipin IgG and IgM	Aeskulisa Phospholipid Screen GM	Aesku diagnostics, Wendelsheim, DE
Dilute Russell viper venom time test	LA1, LA2	Siemens Healthineers, Erlangen, DE
Lupus anticoagulant	Actin FS, Actin FSL	Siemens Healthineers, Erlangen, DE
Plasminogen activator inhibitor-1 antigen	PAI-1 antigen ELISA	Technoclone, Vienna, AT
Plasmin-α2-antiplasmin complex	TECHNOZYM PAP complex ELISA	Technoclone, Vienna, AT
Prothrombin fragment 1+2	Enzygnost F1+2 (monoclonal)	Siemens Healthineers, Erlangen, DE
Soluble fibrin monomer	LIA sFM	Stago, Asnières sur Seine, FR
Thrombin-activatable fibrinolysis inhibitor antigen	ZYMUTEST (TOTAL) TAFI:Ag	Hyphen Biomed, Neuville-sur-Oise, FR
Thrombin-antithrombin complex	TAT micro	Siemens Healthineers, Erlangen, DE
Tissue-type plasminogen activator antigen	t-PA ELISA	Technoclone, Vienna, AT

Supplementary Figure

Figure S1

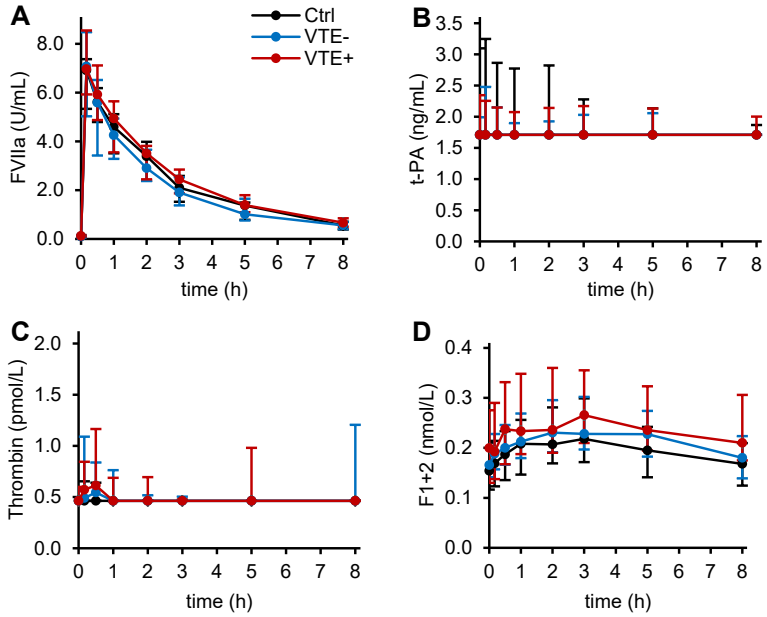

Figure S1. Hemostasis and fibrinolysis biomarker changes in response to *in vivo* coagulation activation. Plasma levels of **(A)** activated factor VII (FVIIa), **(B)** tissue-type plasminogen activator (t-PA) antigen, **(C)** free thrombin, and (**D**) prothrombin fragment 1+2 (F1+2) were measured before (t=0) and after IV injection of 15 µg/kg recombinant FVIIa in healthy controls (Ctrl, n=35, black), asymptomatic carriers of the thrombophilic risk factors factor V Leiden or prothrombin 20210G>A (VTE-, n=38, blue), and thrombophilic patients with a history of venous thromboembolism (VTE+, n=56, red). Plasma levels are shown as median and interquartile range.

5.3 Appendix C

Publication and supplement to chapter 2.3

Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Ex Vivo Modeling of the PC (Protein C) Pathway Using Endothelial Cells and Plasma: A Personalized Approach

Nadine Schwarz, Jens Müller, Hamideh Yadegari, Hannah L. McRae, Sara Reda, Nasim Shahidi Hamedani, Johannes Oldenburg, Bernd Pötzsch,* Heiko Rühl*

BACKGROUND: The endothelial cell–dependent PC (protein C) pathway is critically involved in the regulation of coagulation, anti-inflammatory, and cytoprotective signaling. Its reactivity shows high interindividual variability, and it contributes to prothrombotic disorders, such as the FVL (factor V Leiden) mutation.

METHODS: Endothelial colony–forming cells (ECFCs) were isolated from heparinized peripheral blood from healthy individuals and FVL carriers. Confluent monolayers of ECFCs were overlaid with plasma, and thrombin formation was initiated by addition of tissue factor (1 pmol/L). Subsequently, thrombin and APC (activated PC) formation rates were measured over time using oligonucleotide-based enzyme capture assays. To induce downregulation of TM (thrombomodulin) expression, ECFCs were stimulated with IL-1β (interleukin 1β). In vivo APC response rates were monitored in study participants after infusion of low-dose rFVIIa (recombinant activated factor VII).

RESULTS: The median peak APC concentration was 1.12 nmol/L in experiments with IL-1β stimulated ECFCs and 3.66 nmol/L without IL-1β. Although thrombin formation rates were comparable, APC formation rates were significantly higher in FVL carriers (n=6) compared to noncarriers (n=5) as evidenced by a higher ratio between the area under the curve of APC generation to the area under the curve of thrombin generation (median 0.090 versus 0.031, *P*=0.017). These ex vivo results were correlated with an increased APC response to rFVIIa-induced thrombin formation in FVL carriers in vivo.

CONCLUSIONS: Patient-specific ex vivo modeling of the PC pathway was achieved using blood-derived ECFCs. The correlation between in and ex vivo APC response rates confirms that the autologous PC model accurately depicts the in vivo situation.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: biomarkers ■ embolism ■ endothelium ■ protein C ■ thrombosis

The PC (protein C) pathway is an endothelial cell (EC)–dependent regulatory system that controls blood clotting and plays a central role in anti-inflammatory and cytoprotective cell signaling.[1] APC (activated PC), the key enzyme of this pathway, is generated from PC on the surface of ECs through an activation complex formed between thrombin and TM (thrombomodulin). This activation process is augmented when PC is bound to the EPCR (endothelial PC receptor).[2] Once formed, APC remains bound to EPCR or is released into the circulation. EPCR-bound APC induces cytoprotective cell signaling through canonical cleavage of PAR (protease-activatable receptors) 1 and 3, whereas APC released into the circulation downregulates thrombin formation through proteolytic inactivation of FVa (activated factor V) and FVIIIa (activated factor VIII).[3,4] The proteolytic activity of APC towards FVa and FVIIIa is enhanced by complex formation with the cofactor PS (protein S).[5]

The activity of the PC pathway shows a high degree of interindividual variability depending on the strength

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For Sources of Funding and Disclosures, see page 118.
*B. Pötzsch and H. Rühl are joint senior authors.

Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb

Nonstandard Abbreviations and Acronyms

APC	activated protein C
AUC	area under the curve
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EC	endothelial cell
ECFC	endothelial colony–forming cell
EPCR	endothelial protein C receptor
F1+2	prothrombin fragment 1+2
FI	fluorescence intensity
FVa	activated factor V
FVIIIa	activated factor VIII
FVL	factor V Leiden
GA-1000	gentamicin/amphotericin
hEGF	human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
hFGF-B	human basic fibroblast growth factor
HUVEC	human umbilical vein endothelial cell
IL-1β	interleukin 1 beta
IQR	interquartile range
OECA	oligonucleotide-based enzyme capture assay
PC	protein C
PECAM-1	platelet endothelial cell adhesion molecule 1
PS	protein S
R3-IGF-1	R3 insulin-like growth factor-1
rFVIIa	recombinant activated factor VII
RT	room temperature
TAT	thrombin-antithrombin complex
TF	tissue factor
TM	thrombomodulin
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor 2

Highlights

- We have developed a novel, ex vivo model of the anticoagulant and anti-inflammatory protein C pathway using patient-specific endothelial cells and autologous plasma.
- This autologous model allows for selective analysis of the impact of plasma and cellular dysfunctions of the protein C pathway in a patient-specific manner.
- Disease-specific analysis of the protein C system demonstrates increased activated protein C formation rates in carriers of the factor V Leiden mutation.

of the procoagulant and anticoagulant drivers operating within the plasma and on the APC formation potential of the endothelium.[6] This is demonstrated by the variability of the clinical phenotypes of the FVL (factor V Leiden) mutation.[7–9] Although all FVL carriers share an identical mutation, the individual thrombotic risk ranges from a lifelong asymptomatic course without any thrombotic event, to recurrent venous thromboembolism starting from a young age. Recently we have shown that APC response rates, measured following in vivo coagulation activation induced by low-dose rFVIIa (recombinant activated factor VII) infusion, differ between FVL carriers and noncarriers and between asymptomatic and symptomatic FVL carriers.[10,11] In this in vivo model, higher APC formation rates in FVL carriers than in noncarriers could be attributed to higher thrombin formation rates, as indirectly indicated by a greater increase of F1+2 (prothrombin fragment 1+2)[10] or TAT (thrombin-antithrombin complex)[11] on cohort level but with great interindividual variation. However, direct monitoring of free thrombin was not possible in the in vivo model due to its low concentration in plasma. Moreover, differences in APC generation between asymptomatic and symptomatic FVL carriers could not be explained by differences in thrombin formation, as F1+2 and TAT formation kinetics did not differ between cohorts.[11] Therefore, it can be hypothesized that differences in APC generation by the endothelium may cause these discrepancies in APC response rates. Further investigation of this hypothesis requires an ex vivo model allowing for analysis of both the extent of thrombin generation and the ability of the endothelium to generate APC in a patient-specific manner.

Human umbilical vein ECs (HUVECs) have been used previously to define several important components of the molecular and cellular features of the PC pathway including the PAR signaling cascade mechanism and the discovery of EPCR and its functions.[12,13] A major prerequisite to establish a personalized PC pathway model, however, is the availability of both plasma and ECs from one single donor. Although HUVECs can be used to investigate the general function of TM and EPCR, ECs from affected individuals would be required to study the impact of mutations in these endothelial surface proteins on the PC pathway. Here, we show that endothelial colony–forming cells (ECFCs), formerly referred to as blood outgrowth ECs, can be routinely isolated and used to establish an autologous ex vivo model of the PC pathway. This approach relies on 3 steps: (1) incubation of confluent monolayers of ECFCs with plasma of the same donor, (2) initiation of endogenous thrombin generation by addition of TF (tissue factor), and (3) measurement of time-dependent generation of thrombin and APC using highly sensitive oligonucleotide-based enzyme capture assays (OECAs; Figure 1). This model accurately reflects

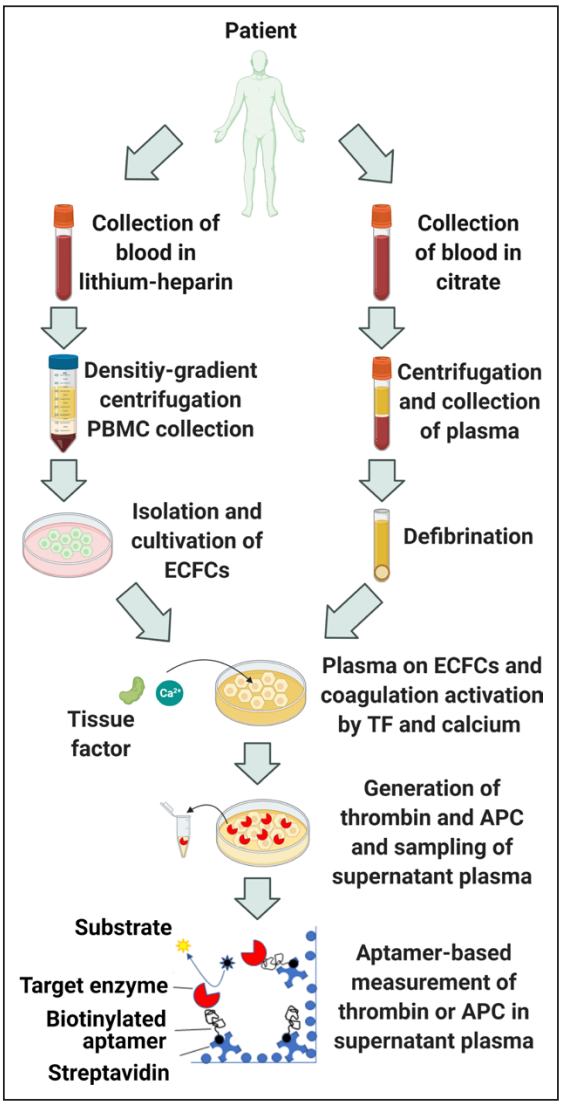

Figure 1. Principle of the autologous protein C pathway model.
Endothelial colony–forming cells (ECFCs) were isolated from peripheral blood mononuclear cells (PBMCs). After cell propagation, confluent ECFC monolayers were overlaid with defibrinated plasma, and the coagulation cascade was activated through addition of TF (tissue factor; in the presence of calcium). Formation of both thrombin and APC (activated protein C) were monitored over time using oligonucleotide-based enzyme capture assays.

the impaired APC response caused by cytokine-induced downregulation of TM and EPCR expression, indicating the general ability of the model to simulate procoagulant EC dysfunction. Furthermore, we used this model to study kinetics of thrombin and APC formation in FVL carriers. The results showed increased APC response rates to thrombin in FVL carriers compared to noncarriers. These data confirm and extend our previous in vivo findings. Most importantly, however, these data give direct evidence that this autologous system can be used to investigate the PC pathway on a personalized level without exposing them to rFVIIa as in the previously used in vivo model.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. This prospective study was conducted from February 2020 through September 2022 at the Institute of Experimental Hematology and Transfusion Medicine, University Hospital Bonn, Germany. The study proposal was approved by the Institutional Review Board and Ethics Committee of the Medical Faculty of the University of Bonn. Written informed consent was obtained from all participants in compliance with the declaration of Helsinki. The procedures followed were in accordance with institutional guidelines.

Materials

Human α-thrombin was obtained from Haematologic Technologies (Essex Junction, VT). Protein C Concentrate (Human) was obtained from Baxter (Heidelberg, Germany). Argatroban was obtained from Mitsubishi Pharma (Düsseldorf, Germany). Human recombinant TM was obtained from Sigma-Aldrich (Taufkirchen, Germany). Aprotinin was purchased from PanReac AppliChem ITW Reagents (Darmstadt, Germany). Bivalirudin was obtained from The Medicines Company (Oxfordshire, United Kingdom). rAPC (recombinant APC) was from Eli Lilly (Indianapolis, IN). Batroxobin reagent was obtained from Siemens Healthcare Diagnostics Products (Marburg, Germany). Normal platelet-poor plasma was prepared in-house by pooling citrated plasma of at least four healthy blood donors. The TF reagent for thrombin generation (platelet-poor plasma–reagent low) was purchased from Stago (Asnières-sur-Seine, France). Ficoll-Paque Plus was obtained from GE Healthcare (Solingen, Germany). Dulbecco's phosphate-buffered saline (DPBS), 0.5 % trypsin-EDTA solution (10×), and FBS were purchased from Thermo Fisher Scientific (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from WAK Chemie Medical (Steinbach, Germany). Rat tail collagen type I was purchased from Corning (Wiesbaden, Germany). The EBM-2 Endothelial Cell Growth Basal Medium-2 BulletKit (basal medium+supplements) was obtained from Lonza (Basel, Switzerland). HUVECs and Endothelial Cell Growth Medium Kit (basal medium+supplemental mix) were purchased by Promocell (Heidelberg, Germany). Liquid Plate Sealer was purchased from Candor Bioscience (Wangen, Germany). Biotinylated aptamers were synthesized and purified by Microsynth (Balgach, Switzerland). The fluorogenic peptide substrate Boc-Asp(OBzl)-Pro-Arg-AMC (I-1560) was obtained from Bachem (Weil am Rhein, Germany) while the fluorogenic substrate Pyroglu-Pro-Arg-AMC (Pefafluor PCa) was purchased from Pentapharm (Basel, Switzerland). Fluorescently labeled, recombinant anti-human (REAfinity) antibodies against CD31, CD309, CD45, CD201, and CD141 were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). IL-1β, TNF-α (tumor necrosis factor alpha), and lipopolysaccharide were obtained from Sigma-Aldrich

(Taufkirchen, Germany). Recombinant activated factor VII (INN-eptacog alfa [activated]) was purchased from NovoNordisk (Bagsvaerd, Denmark). Please see the Major Resources Table in the Supplemental Material.

Blood Collection

Blood was collected from FVL carriers (n=6; 5 heterozygous and 1 homozygous) and noncarriers (n=5), in whom other thrombophilic risk factors (prothrombin mutation 20210G>A, deficiencies of antithrombin, PC, or PS, or the presence of antiphospholipid antibodies) were previously ruled out. Inclusion criteria were a normal body mass index (18–27 kg/m^2) and adequate peripheral venous access. Exclusion criteria consisted of antiplatelet or anticoagulant medication within 2 weeks, and/or venous thromboembolism within 6 months before blood sampling, arterial cardiovascular or malignant diseases, renal or hepatic disorders, and for female participants, pregnancy and breastfeeding. General characteristics of the study population are shown in Table S1.

Blood samples were obtained by venipuncture of an antecubital vein using 21-gauge winged infusion sets (Sarstedt, Nümbrecht, Germany). After discarding the first 2 mL, blood was drawn into citrate tubes (10.5 mmol/L final concentration, Sarstedt) and platelet-poor plasma was obtained by centrifugation (2600×*g*, 10 minutes) within 30 minutes and stored at below −70 °C until further processing.

ECFC Isolation, Culture, and Proinflammatory Stimulation

For isolation of ECFCs, blood was drawn into lithium-heparin tubes (16 IE/mL blood, Sarstedt) and processed within 20 minutes of collection as described elsewhere.[14,15] In brief, heparinized blood was diluted with DPBS and layered over Ficoll-Paque Plus solution. Subsequently, tubes were centrifuged for 20 minutes at 1000×*g* at room temperature (RT) without brake. The buffy coat containing mononuclear cells was then collected, diluted in DPBS, and centrifuged for 7 minutes at 540×*g* at RT. After discarding the supernatant, the cellular pellet was resuspended in EBM-2 Endothelial Cell Growth Basal Medium-2 (Lonza) supplemented with hydrocortisone, hFGF-B (human basic fibroblast growth factor B), VEGF (vascular endothelial growth factor), R3-IGF-1 (R3 insulin-like growth factor-1), ascorbic acid, hEGF (human epidermal growth factor), gentamycin/amphotericin (GA-1000), heparin and 18 % FBS and centrifuged again for 7 minutes at 540×*g* at RT. Afterward, the supernatant was removed, the pellet resuspended in the above-described medium, and cells seeded in collagen-coated 48-well plates. After 24 hours of incubation at 37 °C in a 95% air/5% CO_2 atmosphere saturated with H_2O (cell incubator), nonadherent cells and debris were removed, and the medium was exchanged twice weekly. The appearance of cell colonies with a cobblestone-like appearance was monitored on a weekly basis. A nearly confluent colony on a 48-well plate was then transferred to a collagen-coated 24-well-plate and subsequently further expanded in collagen-coated T75 flasks. When 6 T75 flasks derived from one donor were nearly confluent, the cells were resuspended in FBS containing 5% DMSO and cryopreserved in liquid nitrogen.

Quality control of cultured cells included cobblestone morphology as examined by light microscopy (Axiovert 25 or Axio Observer, both Carl Zeiss Microscopy, Oberkochen, Germany; Figure S1), positive staining for CD31 (PECAM-1 [platelet EC adhesion molecule 1]), CD309 (VEGFR-2 [VEGF receptor 2]), CD201 (EPCR), and CD141 (TM) and negative staining for CD45 analyzed by flow cytometry (Figure S1) as described.[15] Briefly, after cell dissociation using 0.5% trypsin-EDTA, cells were resuspended in staining buffer (DPBS, pH 7, 2 % FBS, 0.5 mol/L EDTA) and 10^5 cells per mL were stained with fluorescently labeled antibodies for 30 minutes at RT in the dark. Cells were washed by centrifugation at 300×*g* for 5 minutes and resuspended in staining buffer. All cytometric analyses were performed using a Navios EX flow cytometer (Beckman Coulter Life Sciences, Brea, CA).

For cell experiments, frozen EC-lines were thawed and washed in the respective culture medium by centrifugation for 7 minutes at 540×*g* at RT. ECFCs were cultivated in EBM-2 Endothelial Cell Growth Basal Medium-2 (Lonza) as described above. HUVECs were cultivated in Endothelial Cell Growth Medium (Promocell) supplemented with hydrocortisone, hFGF-B, EC growth supplement, hEGF, heparin, and 2% FBS. Both cell types were cultured at 37 °C in the cell incubator on collagen-coated plates. For cytokine stimulation of ECFCs, the culture medium was replaced by EBM-2 Endothelial Cell Growth Basal Medium-2 supplemented with IL-1β, TNF-α, or lipopolysaccharide to reach final concentrations of 1 ng/mL, 10 ng/mL, or 1 µg/mL, respectively, and cells incubated for 24 hours at 37 °C. All cellular assays were performed with cells seeded on 24-well plates and grown until reaching confluency. Cells of passage number 7 were used in all experiments.

Measurement of APC and Thrombin

Free thrombin and APC were measured using highly sensitive OECAs that were initially described by Müller et al.[16,17] and applied in other studies.[10,11,18–22] In brief, Maxisorp Fluorononunc microtiter modules (Nunc A/S, Roskilde, Denmark) were coated with 10 µg/mL of bovine serum albumin (BSA)–biotin (100 µL/well). After incubation at 4 °C overnight, wells were washed, and a solution of 10 µg/mL streptavidin was added and incubated for 1 hour at RT. After washing and blocking with 2% BSA for 2 hours at RT, wells were treated with Liquid Plate Sealer and stored at 4 °C until used. For running the OECAs, 3'-biotinylated aptamers (HD1-22 for the thrombin-OECA or HS02-52G for the APC-OECA) were added to primed wells and, after washing, samples were added. After incubation and washing, enzyme-specific fluorogenic peptide substrates were added to the wells for detection of captured thrombin or APC, respectively. Changes in fluorescence over time were measured using a fluorescence plate reader (Synergy 2, BioTek Instruments, Bad Friedrichshall, Germany). Calibration curves (in the same matrix as the samples) were processed in parallel which covered a ½-log10 concentration range from 0 to 10 ng/mL of thrombin (0-272 pmol/L) or 0 to 50 ng/mL of rAPC (0-910 pmol/L). Data obtained from the calibrators were interpolated by 4-parameter curve fit and used to calculate the thrombin or APC concentration in the samples. F1+2 and TAT were determined using the Enzygnost F1+2 (monoclonal) assay (Siemens Healthcare Diagnostics Products, Marburg,

Germany), and the TAT microassay (Siemens Healthcare Diagnostics Products, Marburg, Germany), respectively.

APC Generation in the Purified System

For generation of APC in the cell-free system, a buffer solution (10 mM HEPES, pH 7.4, 137 mM NaCl, 4 mM KCl, 11 mM glucose, 4 mg/mL BSA [buffer A]) containing $CaCl_2$ and PC at variable concentrations (0–4 mmol/L and 0–100 nmol/L, respectively) was prepared. Subsequently, thrombin and TM were added to the reaction mixture to achieve final concentrations of 1 and 2 nmol/L, respectively. After incubation for 30 minutes at RT, the reaction was stopped by addition of argatroban (2.5 µmol/L final concentration), and the fluorogenic substrate Pefafluor PCa was added. Changes in fluorescence over time were measured using the Synergy 2 plate reader.

For generation of APC on cells, cells were washed once with DPBS and once with buffer A before addition of buffer A with added 2 mmol/L $CaCl_2$, 100 nmol/L PC, and 1 nmol/L thrombin. At predefined time points, the reaction was stopped by adding argatroban (2.5 µmol/L final concentration). The fluorogenic substrate Pefafluor PCa was then added to all wells and changes in fluorescence over time were measured (Synergy 2).

APC Generation in Plasma

Plasma was defibrinated using Batroxobin reagent (9+1 parts) by incubating the mixture for 1 hour at 37 °C under occasional agitation and subsequent centrifugation for 2 minutes at 2320×*g*. After washing the cells twice with DPBS, the defibrinated plasma was added to EC cultures on a collagen-coated 24-well plate. Subsequently, thrombin and APC generation was initiated by triggering the extrinsic pathway (addition of 16.6 mmol/L $CaCl_2$ and 1 pmol/L TF/4 µmol/L phospholipids [final concentrations]) at RT. For measurement of thrombin generation over time, reactions were stopped, and thrombin stabilized by diluting aliquots of the supernatant plasma 1:100 in PBS containing 200 µmol/L argatroban, 3 mmol/L $MgCl_2$ and 0.1 % BSA. For measurement of generated APC, reactions were stopped and generated APC stabilized by diluting aliquots of the supernatant plasma 1:10 in Tris-buffered saline containing aprotinin (1000 KIU/mL), bivalirudin (0.5 mg/mL), 1 mmol/L $MgCl_2$, 7.5 mmol/L $CaCl_2$, and 0.1 % BSA. All samples were stored at below −70 °C until further processing.

APC Generation in Vivo

Study participants were required to fast overnight before morning administration of 15 µg/kg rFVIIa as a single IV bolus injection. Blood samples were drawn immediately before and 10, 30, 60, and 120 minutes after rFVIIa administration. Each blood sample was obtained from a new venipuncture as described above. Citrate tubes additionally contained argatroban (100 µmol/L final concentration) for thrombin measurement and aprotinin and bivalirudin (final concentration of 10 µmol/L and 250 µg/mL, respectively) for APC measurement.

Statistics

Data are presented as mean±SD or as median and interquartile range (IQR). The area under the curve (AUC) was estimated using the least squares method. Concentrations of APC and thrombin generated in the presence of ECFCs, HUVECs, and in the absence of cells were compared using the Friedman test, followed by pairwise comparison using Nemenyi procedure. The two-sided Mann-Whitney test was used to assess differences between ECFC surface marker expression, thrombin, F1+2, TAT and APC concentrations, APC and thrombin AUC, and the ratio thereof, in FVL carriers and noncarriers. $P \leq 0.05$ were considered significant. All calculations were performed using the XLSTAT statistical and data analysis solution software (Addinsoft, Boston, MA). H.R. had full access to all the data in the study and takes responsibility for its integrity and the data analysis.

RESULTS

Isolation of Primary ECFC Cell Lines

All established ECFC cell lines showed the typical cobblestone-shaped cell monolayer and stained positive for CD31 (PECAM-1, median fluorescence intensity [FI] 120.8, IQR, 111.9–154.8), CD309 (VEGFR-2, 46.2, 37.9–58.8), CD201 (EPCR, 75.4, 66.3–80.6), and CD141 (TM, 32.8, 24.1–53.2), whereas CD45 expression was not detectable (Figure 2 and Figures S2 and S3). The FI of all studied surface proteins in ECFCs obtained from FVL carriers compared to FVL noncarriers did not yield significant differences. In addition to the 11 successful sample preparations from FVL carriers and noncarriers, there were 2 homozygous and one heterozygous FVL carriers, and one noncarrier, that failed to yield ECFC colonies.

ECFC-Dependent APC Formation

Incubation of ECFCs with purified PC in the presence of thrombin resulted in a time-dependent increase in APC, whereas without cells there was no measurable increase in APC activity over time (Figure 3A). After 60 minutes of incubation, this approach yielded APC concentrations in FVL carriers (4.12; 3.64–5.08 nmol/L) and noncarriers (2.70; 2.55–4.56 nmol/L), that did not differ significantly (Mann-Whitney test; Figure 3B). To assess EC-dependent APC formation rates in the plasma matrix, cells were incubated with defibrinated plasma. Addition of TF (in the presence of calcium) induced thrombin formation, reaching a median (IQR) peak level of 27.3 (24.8–29.7) nmol/L at 10 minutes in ECFCs (Figure 3C). The maximal concentration of thrombin did not differ significantly between ECFCs and HUVECs, but it was lower in the presence of ECFCs than in the absence of cells (P=0.014, Friedman test followed by Nemenyi procedure). Peak levels of APC were achieved 30 minutes after the thrombin peak in ECFCs and 20 minutes after the thrombin peak in the HUVEC model (Figure 3D). Median peak levels of APC in ECFCs and in HUVECs did not differ

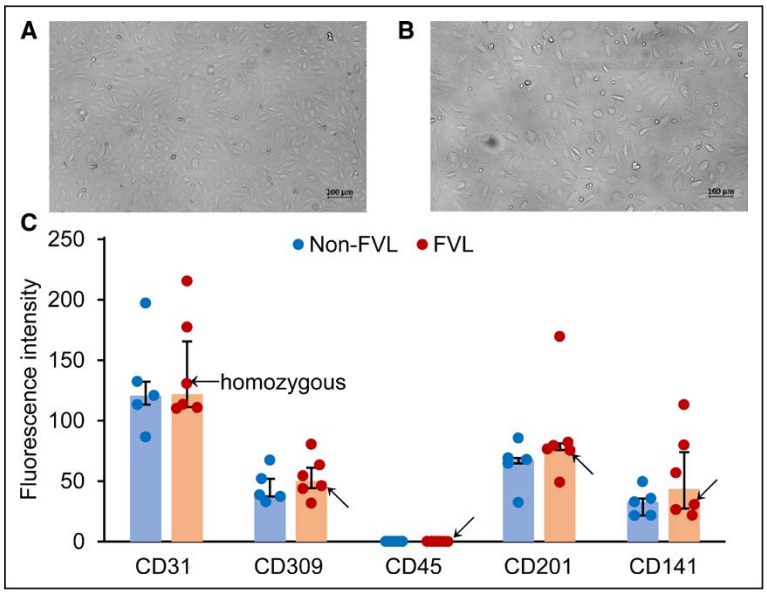

Figure 2. Phenotypic characterization of endothelial colony–forming cells (ECFCs). ECFC cell lines showed cobblestone-like morphology, which is typical for endothelial cells (ECs), independent of FVL (factor V Leiden) carrier status. Representative micrographs of ECFSs from (**A**) an FVL noncarrier and (**B**) an FVL carrier are shown (Axio Observer, camera Axiocam 702 mono, Carl Zeiss Microscopy). **C**, Expression levels of CD31, CD309, CD45, CD201, and CD141 of 5 FVL noncarriers (non-FVL, blue) and 6 FVL carriers (FVL, red, thereof 5 heterozygous and 1 homozygous, indicated by an arrow) were measured by flow cytometry. Columns show the median, whereas error bars show the interquartile range. Fluorescence intensities were compared using the Mann-Whitney test. Statistically significant differences ($P \leq 0.05$) between FVL carriers and noncarriers were not observed. Cobblestone-like appearance, positive staining for CD31, CD309, CD201, CD141, and negative staining for CD45 confirm the EC origin of the ECFCs.

significantly (Friedman test). In the absence of cells, APC generation was negligible.

Proinflammatory Stimulation of ECFCs

Stimulation of ECFCs with the proinflammatory cytokines IL-1ß, TNF-α, or lipopolysaccharide changed the ECFC morphology from a round cobblestone-like appearance to an elongated form in a dose-dependent and cytokine-dependent manner. The change in morphology was more pronounced in cells stimulated with TNF-α and lipopolysaccharide compared to cells stimulated with IL-1β cells (Figure 4A). Surface expression of CD141 (TM) was significantly reduced by TNF-α and IL-1β stimulation (FI of 0.14±0.05 and 2.12±0.15 in control cells, Kruskal-Wallis test followed by pairwise correction using the Dunn procedure and Bonferroni correction, P=0.020). At the same time, expression of CD31 (PECAM-1) and CD201 (EPCR) were reduced significantly (P=0.002) by TNF-α (Figure 4B). Measurement of CD309 (Figure S4A) and CD45 (Figure S4B) yielded a low FI after cytokine stimulation near the typical range of the isotype controls, with a maximal mean FI of 0.17 and 0.32, respectively. Based on these results, IL-1ß was selected in all subsequent experiments to induce EC dysfunction, because with this cytokine maximal TM reduction was achieved with the lowest effect on cell morphology.

Peak thrombin levels in experiments with stimulated cells (median, 283.5; IQR, 281.2–283.5 nmol/L) were within the ranges observed in unstimulated cells (267.4; 251.1–283.5 nmol/L; Figure 4C). However, observed ranges of APC peak levels on stimulated cells (median 1.12; IQR, 1.09–1.14 nmol/L) and unstimulated cells (3.64; 3.60–3.66 nmol/L) did not overlap (Figure 4D).

PC Pathway Modeling in FVL Carriers

When the autologous PC pathway model was applied to FVL carriers and noncarriers, higher peak APC levels were observed in FVL carriers as compared to noncarriers, whereas peak levels of thrombin were comparable between FVL carriers and noncarriers (Figure 5A). Corresponding with this increase in free thrombin, an increase in F1+2 and TAT was observed (Figure S5A and S5B). This finding correlates with the in vivo data obtained after low-dose rFVIIa infusion, in which higher plasma levels of APC were observed over 120 minutes in FVL carriers as compared to noncarriers (Figure 5B). Also consistent with ex vivo data, in vivo kinetics of TAT (Figure 5B) and F1+2 (Figure S5C) were comparable in FVL carriers and noncarriers. Due to the short half-life of thrombin, thrombin concentrations measured in vivo were several magnitudes lower than in the autologous PC pathway model, especially in FVL noncarriers, thereby precluding analysis of their kinetics (Figure S5D). To compare the total amount of thrombin and APC formed over time, the AUC was calculated. The AUC of thrombin formation in the ex vivo model was comparable in FVL carriers and noncarriers (median 2.1; IQR, 1.6–2.3 versus 2.2; 2.1–3.0 µmol·min/L, Mann-Whitney test P=0.429), whereas the AUC of APC formation was greater (158; 135–196 versus 61; 39–124 nmol·min/L, Mann-Whitney test P=0.030). In addition, the ratio between the AUC of APC and thrombin was greater in FVL carriers than in noncarriers (0.09; 0.08–0.09 versus 0.03; 0.02–0.05, Mann-Whitney test P=0.017; Figure 5C). Ex vivo APC and thrombin formation rates were both higher in the only homozygous FVL carrier than in the heterozygous individuals, resulting in a ratio of APC AUC/thrombin

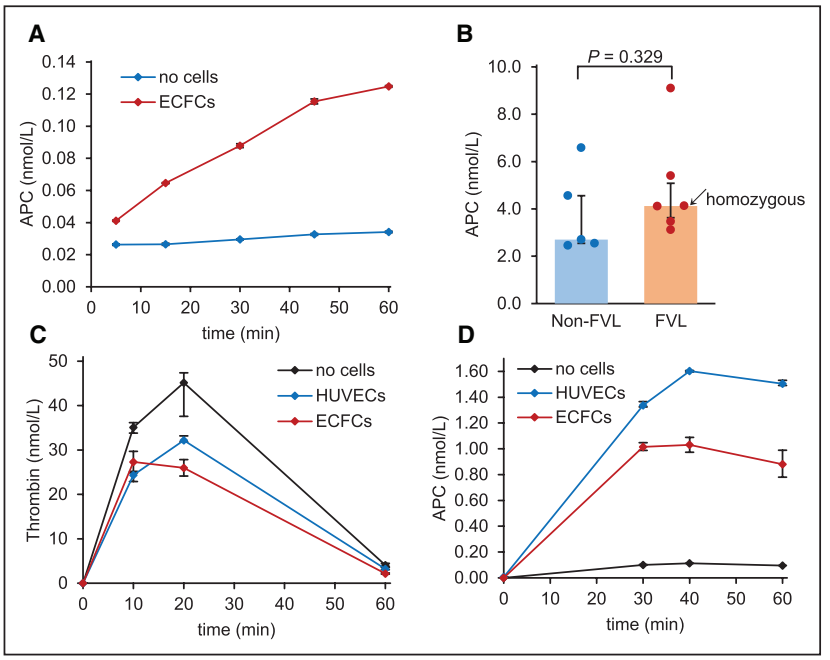

Figure 3. APC (activated protein C) generation capacities of endothelial colony–forming cells (ECFCs).
A, To assess the APC formation capacity of ECFCs, confluent monolayers (red symbols) were overlaid with purified thrombin (1 nmol/L) and purified protein C (100 nmol/L) in HEPES buffer. Time-dependent APC formation was measured using the APC oligonucleotide-based enzyme capture assay. Cell-free wells (blue symbols) were used as controls. Data points show the median of four measurements, error bars show the interquartile range (IQR). **B**, The APC concentration after 60 minutes of incubation in FVL (factor V Leiden) carriers (red, n=6, thereof five heterozygous and one homozygous) and non-FVL carriers (blue, n=5) was compared using the Mann-Whitney test. Columns and error bars show median and IQR. To study APC formation in plasma, confluent monolayers were overlaid with defibrinated normal human plasma and thrombin formation induced by addition of tissue factor. Time-dependent formation of (**C**) thrombin and (**D**) APC were monitored over time in ECFCs (red symbols) and human umbilical vein endothelial cells (HUVECs, blue symbols). Cell-free wells (black symbols) were used as controls. Data points show the median of four measurements, error bars show the IQR. Experiments were conducted in the 24-well format.

AUC within the range observed in heterozygous FVL carriers.

DISCUSSION

EC-based in vitro models are a key element for the investigation of the complex interactions between the various plasma and cellular components that activate and regulate the PC pathway. The aim of this study was to establish a model for personalized and patient-specific PC pathway analysis by combining patient-derived ECs with autologous plasma.

Mature ECs shed from the vasculature and end up in circulation. However, with a mean value of 80 cells/mL blood, the cell yield achievable by routine blood sampling is too low to perform functional studies.[23] ECFCs are a second EC fraction that is found in blood in minute amounts, but due to their proliferative nature, their number can be expanded via cell culture, thereby achieving an adequate cell yield for performing functional analysis.[24] This is confirmed by the data presented in the present study. Starting with 27 mL lithium-heparinized blood obtained during routine peripheral blood sampling, stable ECFC cell lines were established. These cell lines showed an EC-typical phenotypic signature including the expression of the EC markers and cellular components of the PC pathway TM and EPCR, suggesting that ECFCs can promote PC activation in a thrombin-dependent manner. This was confirmed by showing a dose- and time-dependent activation of purified PC by thrombin-stimulated ECFCs. Using cells derived from human cord blood, Smadja et al[25] have previously described an ECFC model allowing the generation of APC on a fibrin matrix in the presence of exogenously added purified PC, and our findings are in line with this previous study. In our study population, expression of EC surface markers, especially of TM and EPCR, did not differ significantly between FVL carriers and noncarriers. Consistent with these findings, fixed amounts of thrombin and PC yielded comparable APC generation rates in both cohorts.

Purified thrombin and PC are used in the majority of studies that investigate EC-dependent APC activation

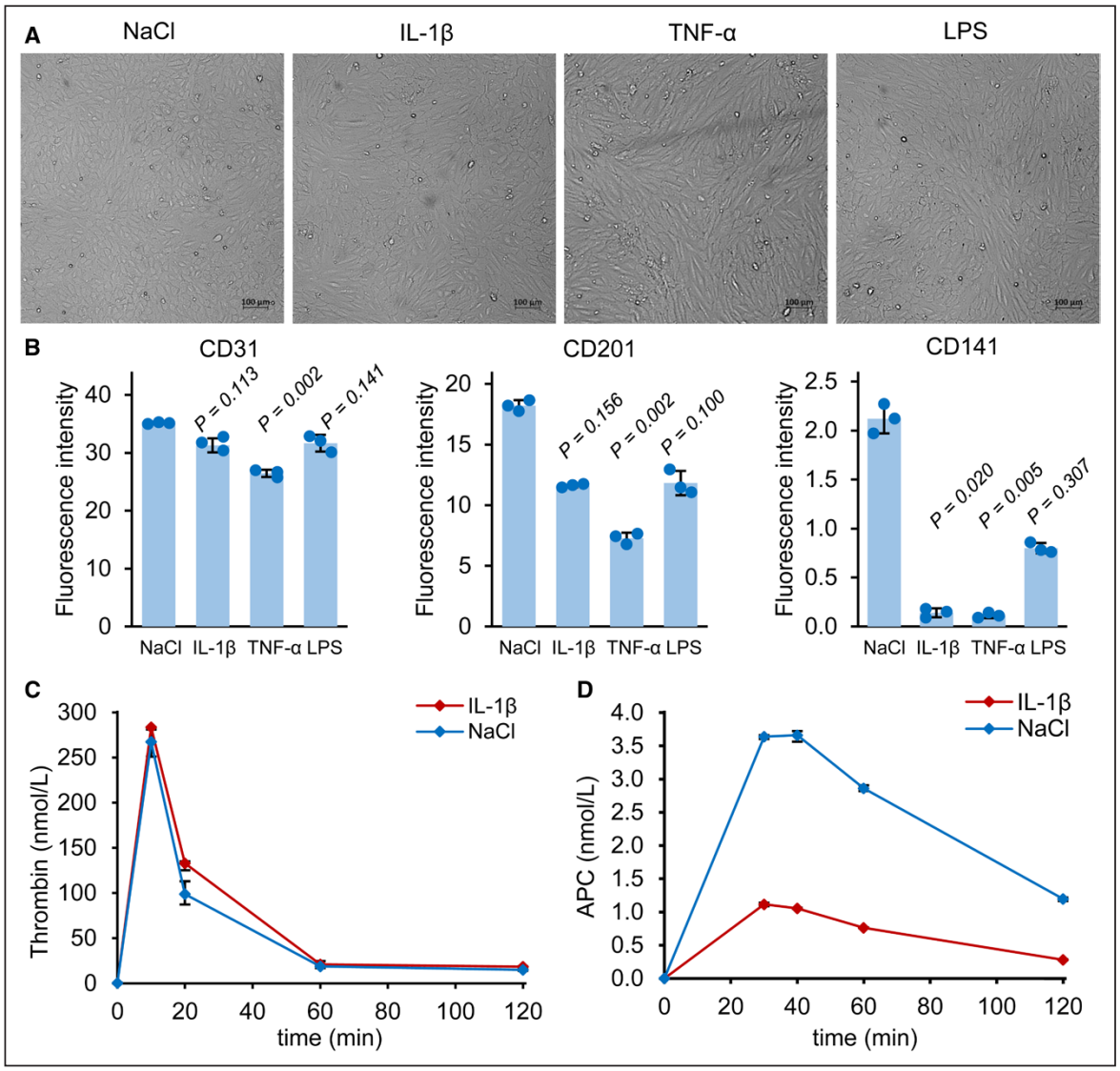

Figure 4. Endothelial cell (EC)–dependent downregulation of APC (activated protein C) formation.
Confluent endothelial colony–forming cells (ECFCs) were cultured in the presence of (**A**) 1 ng/mL IL-1β (interleukin 1 beta), 10 ng/mL TNF-α (tumor necrosis factor alpha), 1 µg/mL lipopolysaccharide (LPS), or NaCl for 24 hours to induce downregulation of TM (thrombomodulin) and EPCR (endothelial protein C receptor) and thereby simulate procoagulable EC dysfunction. Morphology of representative cell cultures under bright field microscopy (Axio Observer, camera Axiocam 702 mono, Carl Zeiss Microscopy) is shown. Flow cytometry analysis of (**B**) CD31, CD201, and CD141 expression on stimulated and unstimulated ECFCs is shown. Columns show the isotype-corrected mean of three measurements, error bars show the standard deviation *P* values indicate differences between stimulated and unstimulated ECFCs and were calculated by the Kruskal-Wallis test followed by pairwise comparison using the Dunn procedure with Bonferroni correction. **C**, Thrombin and (**D**) APC formation were measured in autologous plasma on ECFCs that were either stimulated with IL-1β (1 ng/mL; red symbols) or unstimulated (blue symbols). Data points show the median of 4 measurements, error bars show the interquartile range.

in vitro. Although this approach has some advantages, it differs from the in vivo situation in which the amount of thrombin that reaches the EC surface is balanced by the procoagulant and anticoagulant potential of plasma. To more closely reflect the in vivo situation, purified proteins were replaced by plasma, and thrombin formation was induced by TF, the physiological activator of the coagulation cascade. To avoid fibrin clot formation as a result of thrombin formation, plasma was defibrinated using the snake venom batroxobin. Batroxobin selectively depletes fibrinogen from plasma while levels of other coagulation factors remain unchanged.[26] Major challenges of the plasma approach include the complexity of the plasma matrix and the relatively low concentrations of thrombin and APC that are yielded, requiring highly sensitive and specific

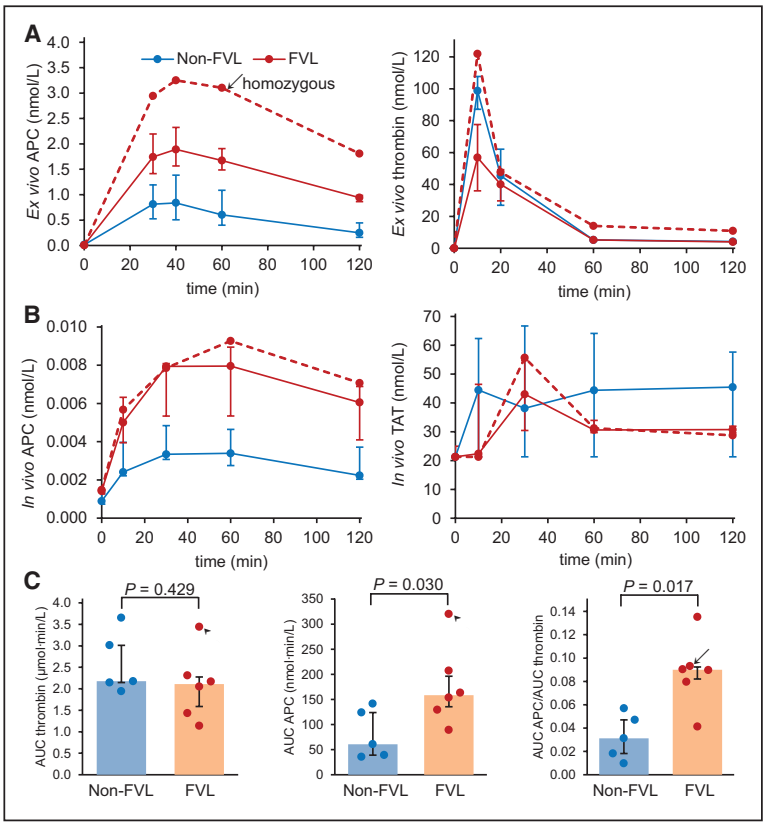

Figure 5. Disease-specific modeling of the PC (protein C) pathway.
A, The autologous PC pathway model was used to analyze APC (activated PC) and thrombin formation kinetics in FVL (factor V Leiden) carriers (n=6, red symbols, thereof 5 heterozygous and one homozygous, indicated by an arrow) and noncarriers (n=5, blue symbols) in plasma. Data points show the median, error bars show the interquartile range (IQR). **B**, In vivo formation of APC and TAT (thrombin-antithrombin complex) was measured in the same individuals after infusion of low-dose recombinant activated factor VII. **C**, Area under the curve (AUC) of ex vivo thrombin formation in plasma, the AUC of ex vivo APC formation in plasma, and the ratio between both. Columns and error bars show median and IQR. *P* values were calculated using the Mann-Whitney test.

detection methods. In previous studies, we have shown that an aptamer-based detection platform fulfills these requirements in terms of assay sensitivity and specificity. Using this technology, peak thrombin levels in ECFCs were observed 10 minutes after addition of TF, whereas APC peaked 30 minutes later. The kinetics of thrombin and subsequent APC formation are comparable to the results obtained by continuous thrombin generation measurement in plasma in the presence of purified TM, with the exception that peak levels were significantly lower.[27] Both thrombin and APC formation declined after 60 minutes, indicating that thrombin and APC are cleared in the plasma matrix by endogenous inhibitors and that thrombin-TM complexes are internalized by ECs.[28]

Although these data confirm the ability of the ECFC-plasma approach to study the reactivity of the PC pathway, it is unclear whether the model can accurately reflect differences in EC-dependent APC formation. To investigate this, cellular expression levels of TM and EPCR were downregulated by stimulation of ECFCs with proinflammatory cytokines. The close correlation between the expression levels of these cellular components of the PC pathway and the decrease in APC formation indicate that the model can be used to study prothrombotic EC dysfunction.

After low-dose administration of rFVIIa, we found that FVL carriers had significantly higher APC response rates compared to noncarriers. To confirm that these disease-specific differences are reproducible in the autologous model of the PC pathway, we performed an ex vivo analysis of APC formation in FVL carriers and noncarriers and once again observed that APC formation rates were significantly higher in FVL carriers, indicating that the autologous PC model is indeed able to reflect the in vivo situation.

It is known that the FVL mutation prolongs the half-life of FVa, resulting in increased prothrombinase complex activity. It was, therefore, a matter of discussion whether increased rates of thrombin formation are the major driver of increased APC responses in FVL carriers.[10] In the in vivo setting, this hypothesis could not be directly addressed because the very short half-life of thrombin limits the in vivo detection of free thrombin.[18] In sufficiently large cohorts, rFVIIa induced a greater increase of indirect thrombin markers in FVL carriers than in noncarriers (F1+2, n=24; TAT, n=45)[10,11] However, differences in APC formation rates between asymptomatic and symptomatic FVL carriers could not be explained by differences in indirect thrombin markers,[11] suggesting that additional cellular or plasmatic factors modulate APC generation. In extension of these studies, the ex vivo model enabled us to directly monitor activation and inactivation kinetics of free thrombin. Although the interindividual variation in ex vivo thrombin formation

appeared less pronounced than that of TAT and F1+2 in the in vivo approach, no statistically significant difference in thrombin formation rates was observed between FVL carriers and noncarriers. This is most probably due to the smaller sample size in comparison to our previous in vivo studies.[10,11] Nevertheless these results confirm and extend the results of previous studies showing the ability of ECFC-based approaches to assess changes in thrombin generation in vascular diseases.[29,30]

In conclusion, the autologous model of the PC pathway using ECFCs and plasma from one single donor represents a significant progress to personalized investigation of the PC pathway. As a proof of concept, we confirmed downregulation of APC formation in response to cytokine-induced EC dysfunction. Furthermore, the increased APC response rates in FVL carriers demonstrate the ability of the autologous approach to analyze disease-specific phenotypic differences of the PC pathway and confirm earlier results obtained in vivo. In future studies, the ex vivo approach can be used to investigate yet unexplained differences in APC generation between symptomatic and asymptomatic FVL carriers without the need for exposition to in vivo coagulation activation. On a broader scope, it can be used to study the impact of defects in EC surface proteins, such as mutations in EPCR or TM, on the APC generation capacity of the endothelium.

ARTICLE INFORMATION

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Disclosures

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Supplemental Material

Table S1
Figures S1–S5
Major Resources Table

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SUPPLEMENTAL MATERIALS

Ex Vivo Modeling of the Protein C Pathway Using Endothelial Cells and Plasma: A Personalized Approach

Nadine Schwarz[1], PD Dr. Jens Müller[1], Dr. Hamideh Yadegari[1], Hannah L. McRae[1], Dr. Sara Reda[1], Dr. Nasim Shahidi Hamedani[1], Prof. Dr. Johannes Oldenburg[1], Prof. Dr. Bernd Pötzsch[1], PD Dr. Heiko Rühl[1]

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Table S1. Characteristics of endothelial colony forming cell (ECFC) donors

	Sex	Age, years	FVL	History of VTE
Control #1	male	59	non-carrier	No
Control #2	female	62	non-carrier	No
Control #3	female	51	non-carrier	No
Control #4	female	27	non-carrier	No
Control #5	female	23	non-carrier	No
FVL #1	female	57	heterozygous	No
FVL #2	female	59	heterozygous	Yes
FVL #3	female	23	heterozygous	No
FVL #4	female	26	heterozygous	No
FVL #5	female	53	heterozygous	No
FVL #6	male	50	**homozygous**	Yes

FVL, factor V Leiden; VTE, venous thromboembolism.

Supplementary Figures

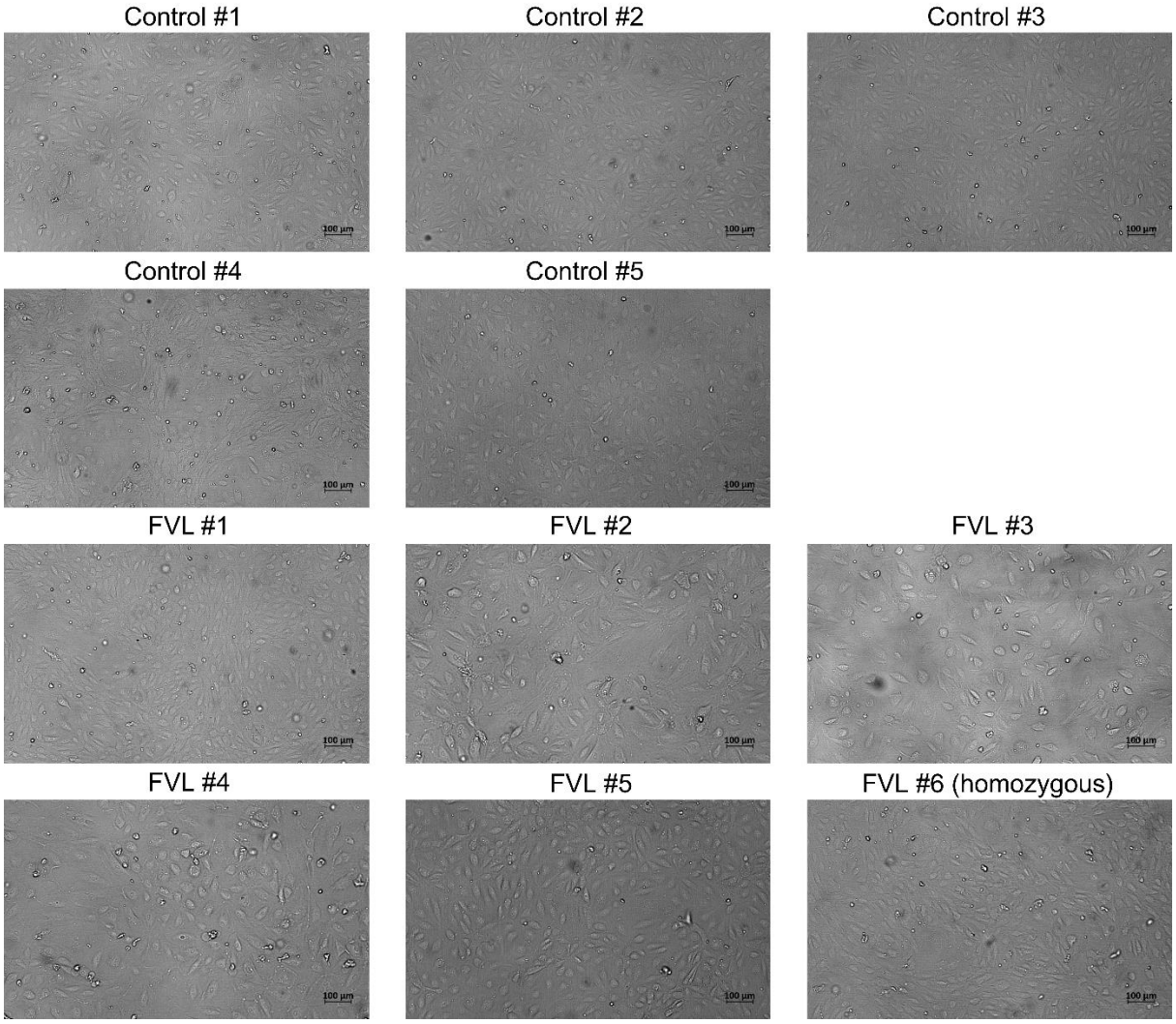

Figure S1. Micrographs of endothelial colony forming cells. Controls were factor V Leiden (FVL) non-carriers. FVL carriers #1 - #5 were heterozygous. Micrographs were obtained with an Axio Observer microscope using an Axiocam 702 mono camera, both Carl Zeiss Microscopy, Oberkochen, Germany.

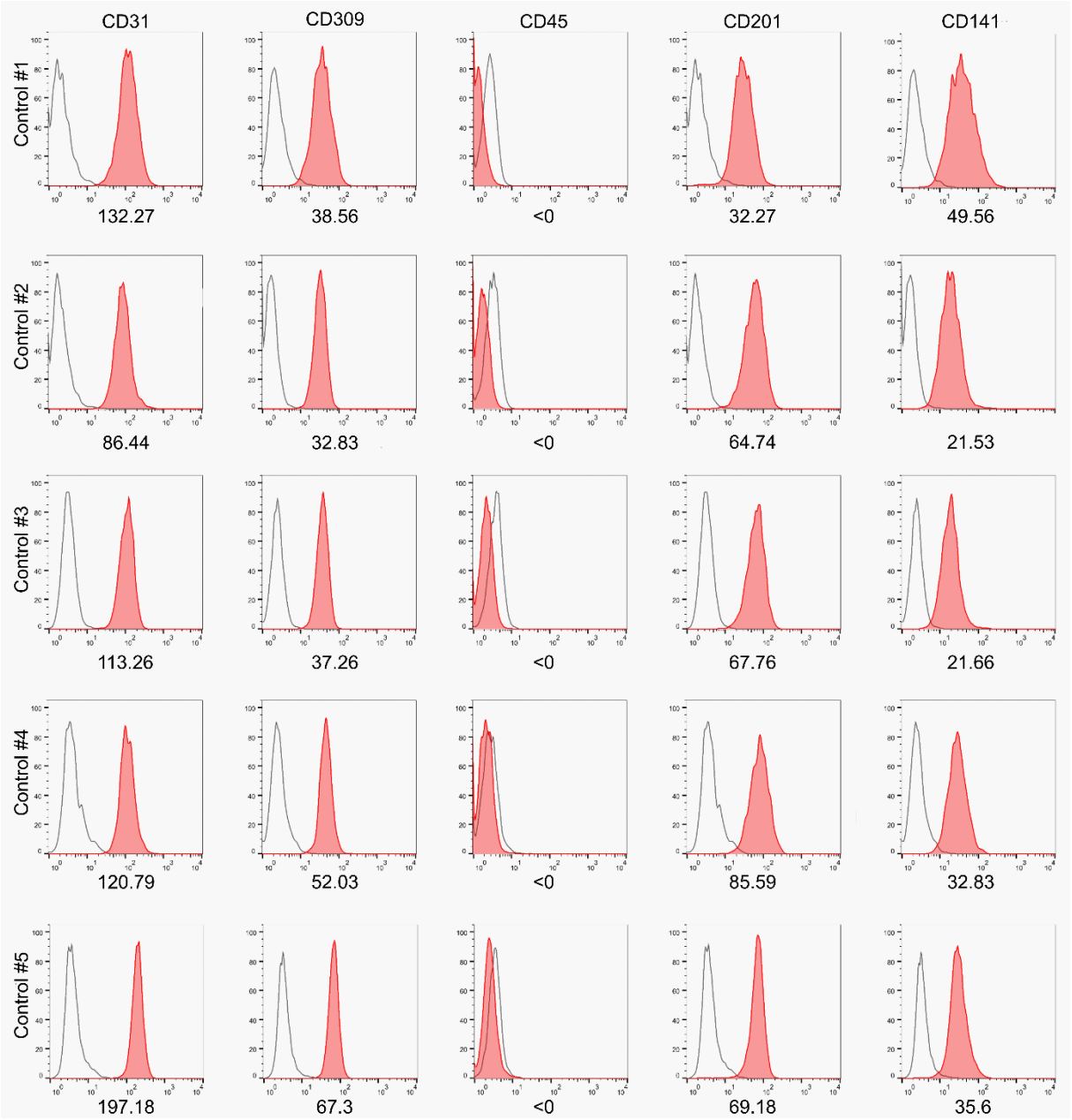

Figure S2. Flow cytometry measurement of surface markers on endothelial colony forming cells in non-FVL carriers. All graphs show the logarithmic fluorescence intensity (FI) versus the number of events measured in flow cytometry. The signal from the respective fluorescently labelled antibody of interest is shown in red while the white overlaid peak shows the isotype control labelled with the same fluorophore, respectively. The mean FI shown below the charts indicates the mean shift on the x-axis after isotype correction. Measurements were performed on a Navios EX flow cytometer (Beckman Coulter, Brae, CA, USA).

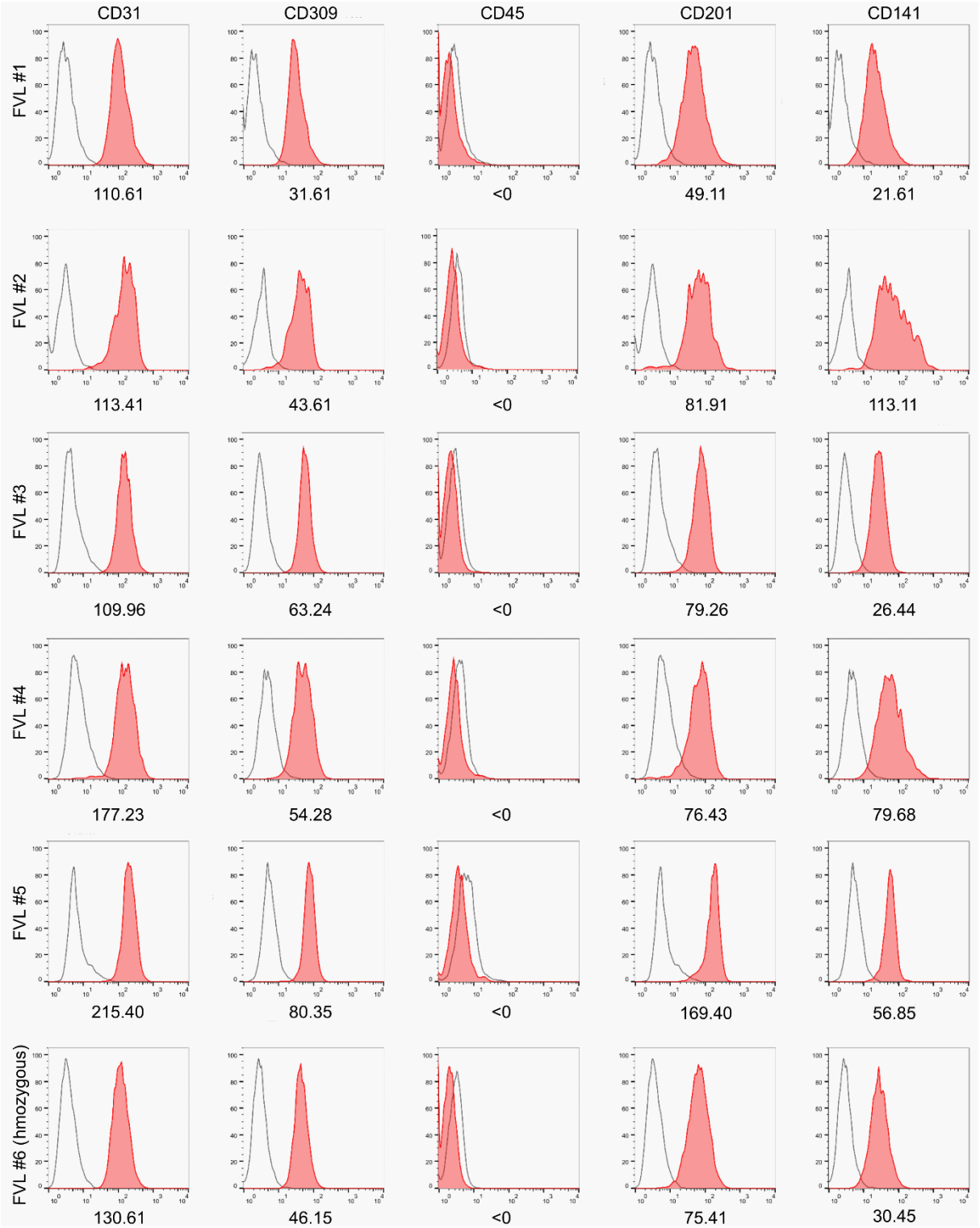

Figure S3. Flow cytometry measurement of surface markers on endothelial colony forming cells in FVL carriers. All graphs show the logarithmic fluorescence intensity (FI) versus the number of events measured in flow cytometry. The signal from the respective fluorescently labelled antibody of interest is shown in red while the white overlaid peak shows the isotype control labelled with the same fluorophore, respectively. The mean FI shown below the charts indicates the mean shift on the x-axis after isotype

correction. Measurements were performed on a Navios EX flow cytometer (Beckman Coulter, Brae, CA, USA)

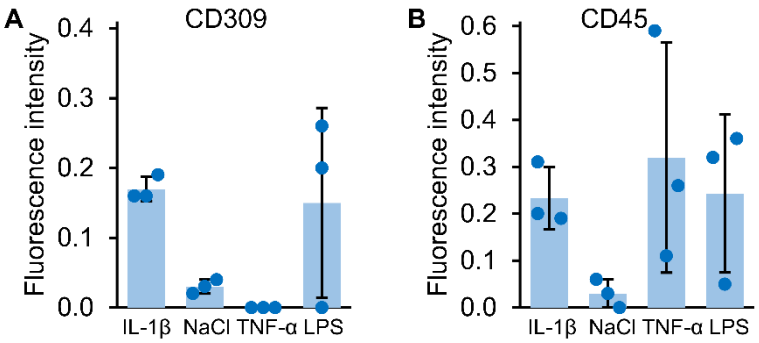

Figure S4. Endothelial colony forming cell (ECFC) surface markers under inflammatory stimulation. (A) CD309 and (**B**) CD45 were measured by flow cytometry on non-FVL ECFCs stimulated with 1 ng/mL interleukin 1 beta (IL-1β), 10 ng/mL tumor necrosis factor alpha (TNF-α), or 1 µg/mL lipopolysaccharide (LPS). NaCl was used as negative control. Stimulation experiments were performed in triplicates. Columns show the isotype-corrected mean of three measurements, error bars show the standard deviation.

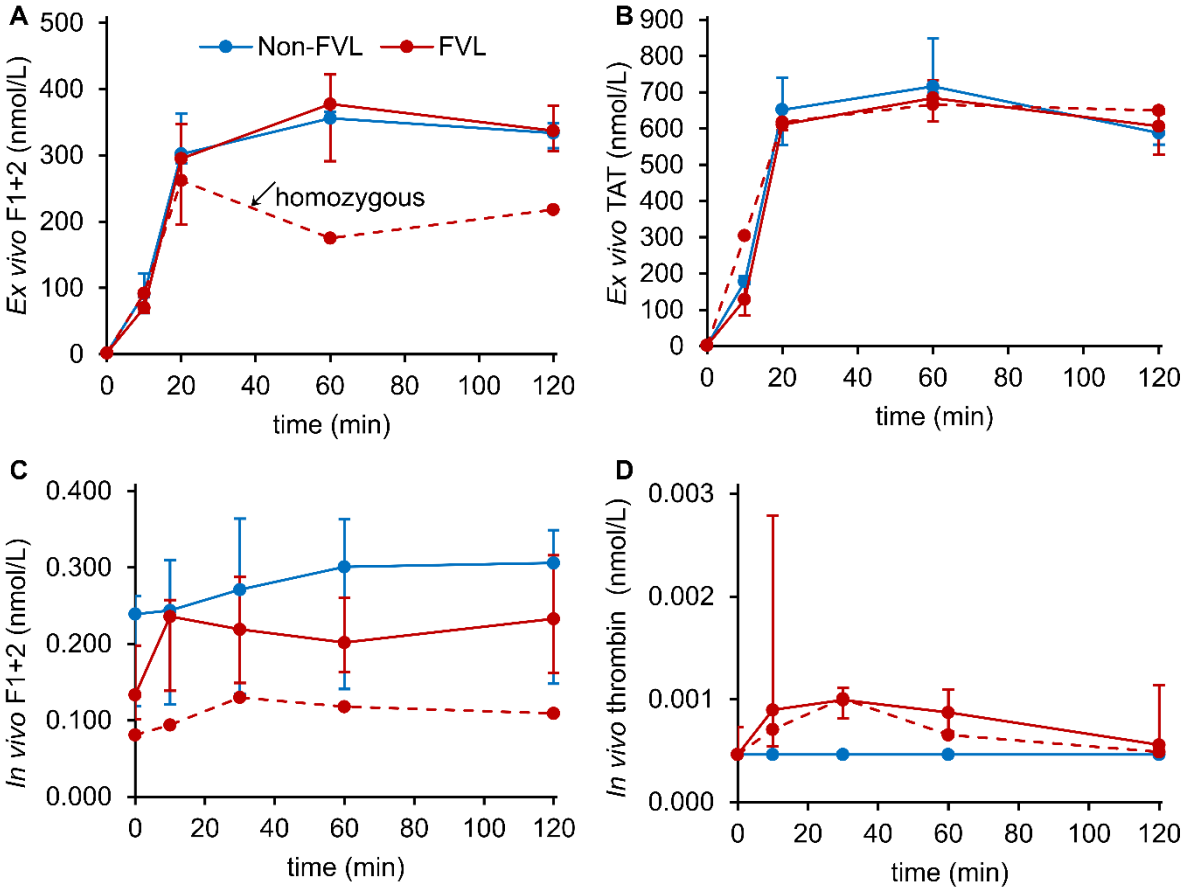

Figure S5. Additional thrombin formation markers. (**A**) Prothrombin fragment 1+2 (F1+2) and (**B**) thrombin-antithrombin complex (TAT) formation kinetics in factor V Leiden (FVL) carriers (n = 6, red symbols, thereof 5 heterozygous and one homozygous, indicated by an arrow) and non-carriers (n = 5, blue symbols) were analyzed in plasma in the *ex vivo* PC pathway model. Data points show the median, error bars show the interquartile range (IQR). *In vivo* formation of (**C**) F1+2 and (**D**) free thrombin was measured in the same individuals after infusion of low-dose recombinant activated factor VII.

5.4 Appendix D

Preprint and supplement to chapter 2.4

The Endothelium Modulates the Prothrombotic Phenotype of Factor V Leiden: Evidence from an Ex Vivo Model

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ABSTRACT

Background: Clinical expressivity of the thrombophilic factor V Leiden (FVL) mutation is highly variable. Increased activated protein C (APC) formation in response to thrombin formation has been observed in asymptomatic FVL carriers in vivo. Here we further explored this association using a recently developed endothelial colony forming cell (ECFC)-based ex-vivo model.

Methods: ECFCs and citrated plasma were obtained from FVL carriers with/without previous venous thromboembolism (VTE+/-, n=7 each) and seven healthy controls. Coagulation was activated by tissue factor in defibrinated recalcified plasma added to confluent cell cultures. Thrombin and APC concentration were measured over time and the respective areas under the curve (AUC) calculated. Additionally, inhibition kinetics of exogenously added APC and APC sensitivity of the prothrombinase complex were measured in plasma. Expression of thrombomodulin and endothelial protein C receptor (EPCR) on ECFCs was assessed using cell-based enzyme-linked immunosorbent assays.

Results: In autologous plasma on ECFCs, the APC response to thrombin formation (AUC APC/AUC thrombin), was higher in FVL VTE- than FVL VTE+ patients (0.138 versus 0.028, *P*=0.026). APC inactivation kinetics, APC sensitivity, and thrombomodulin/EPCR expression on ECFCs did not differ between these cohorts and compared to healthy controls. Cross-over experiments with plasma from FVL VTE- and FVL VTE+ patients on non-FVL ECFCs yielded indistinguishable results. In contrast, in normal plasma on FVL VTE- ECFCs the APC response remained significantly higher than on FVL VTE+ ECFCs (0.052 versus 0.022, *P*=0.011).

Conclusions: Consistent with results from previous in vivo experiments, APC response rates to thrombin formation were higher in asymptomatic FVL carriers compared to those with previous VTE. Our observations suggest that this increased APC response is driven by the endothelium. Further studies are warranted to elucidate yet unknown endothelial mechanisms that might modulate the clinical expressivity of FVL.

Keywords: endothelium, protein C, thrombin, venous thromboembolism, thrombophilia

Non-standard Abbreviations and Acronyms

APC, activated protein C; APC-sr, APC sensitivity ratio; AUC, area under the curve; AT, antithrombin; BSA, bovine serum albumin; cell-ELISA, cell-based enzyme-linked immunosorbent assay; DPBS, Dulbecco's phosphate-buffered saline; ECFC; endothelial colony forming cell; EDTA, ethylenediaminetetraacetic acid; EPCR, endothelial protein C receptor; FBS, fetal bovine serum; FII, prothrombin; FIX, factor IX; FV, factor V; FVa, activated factor V; FVII, factor VII; FVIII, factor VIII; FVIIIa, activated factor VIII; FVL, factor V Leiden; FX, factor X; FXa, activated factor X; FXI, factor XI; horseradish peroxidase (HRP); IQR, interquartile range; OECA, oligonucleotide-based enzyme capture assay; OD, optical density; PBS, phosphate-buffered saline; PC, protein C; PS, protein S; TBS, Tris buffered saline; TM, thrombomodulin; VTE, venous thromboembolism.

INTRODUCTION

The factor V Leiden (FVL) mutation is the most common genetic risk factor for venous thromboembolism (VTE) with a prevalence from 3% to 15%, depending on the geographical location.[1,2] Known under the concept of activated protein C (APC) resistance, the mutant FVL gene product lacks the APC cleavage site within its heavy chain resulting in impaired proteolytic inactivation of activated factor V (FVa)[3,4] and, eventually in an approximately 7-fold (respectively 80-fold) increased thrombotic risk of heterozygous (respectively homozygous) carriers.[5] However, the majority of FVL carriers remain asymptomatic throughout their lives. This highly variable clinical expressivity of FVL challenges our understanding of the interplay between genetic predisposition and molecular interactions contributing to the development of VTE.

The protein C (PC) pathway, which becomes impaired by FVL, is unique amongst anticoagulant mechanisms, since APC formation is directly linked to the amount of thrombin formed and thereby

counterbalances coagulation activation with an anticoagulant response. More detailed, PC binds to the endothelial protein C receptor (EPCR) and is activated by a complex formed between thrombin and the endothelial receptor thrombomodulin (TM).[6,7] APC, upon its release into the circulation, acts as an anticoagulant by proteolytic inactivation of FVa and activated factor VIII (FVIIIa).[8,9] As a cofactor, circulating protein S (PS) enhances the proteolytic activity of APC.[10,11] FVL carriers have been shown to exhibit higher thrombin and APC formation rates in vivo than non-FVL carriers after coagulation activation by low-dose recombinant activated factor VII.[12,13] The most interesting finding in these studies was the observation that the anticoagulant APC response to thrombin formation was significantly increased in asymptomatic FVL carriers in comparison to those with a history of VTE.[13,14]

Potential explanations of this observation include alterations of plasmatic and/or endothelial components of the PC system that might modulate thrombin and APC formation rates. Among the plasmatic variables are, besides levels of involved pro- and anticoagulant proteins, other influencing factors of APC resistance[15] and APC inactivation kinetics, the latter since elevated APC inhibitor levels have been associated with an increased thrombotic risk.[16–18] Endothelial variables include, in particular, EPCR and TM, since their downregulation or variants in their encoding genes (*PROCR* respectively *THBD*) have been suggested as thrombotic risk factors.[19–23]

We have recently introduced an ex vivo model of the PC system, in which endothelial colony forming cells (ECFCs) and autologous plasma are utilized for personalized assessment of the functionality of the PC pathway.[19] In the present study this experimental approach amongst others was used to further investigate plasmatic and endothelial factors that potentially modulate the APC response to thrombin in FVL carriers with or without previous VTE, in order to better understand the variable clinical expressivity of FVL.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. A list of the materials and commercially available assays used is provided in the **Supplemental Methods**. This prospective study was conducted from February 2020 through June 2023 at the Institute of Experimental Hematology and Transfusion Medicine, University Hospital Bonn, Germany. The study proposal was approved by the Institutional Review Board and Ethics Committee of the Medical Faculty of the University of Bonn. Written informed consent was obtained from all participants in compliance with the declaration of Helsinki. The procedures followed were in accordance with institutional guidelines.

Study Participants

FVL carriers with and without history of thrombosis and FVL non-carriers were recruited from the thrombophilia outpatient clinic of our institution and from our blood donation service, respectively. Other thrombophilic risk factors including *F2* 20210G>A, deficiencies of antithrombin (AT), PC, or PS, or the presence of antiphospholipid antibodies were ruled out prior to inclusion. Molecular genetic testing for variants in *F5* (factor V, FV), *F2* (prothrombin, FII), *PROC* (PC), *PROS* (PS), *PROCR* (EPCR), *THBD* (TM) and other genes involved in the PC system was performed as described in the **Supplemental Methods**. Exclusion criteria were antiplatelet or anticoagulant medication within two weeks, and/or VTE within six months prior to blood sampling, arterial cardiovascular or malignant diseases, renal or hepatic disorders, and for female participants, pregnancy, and breast feeding. The collection and processing of blood samples is described in the **Supplemental Methods**.

Isolation and Cultivation of Endothelial Colony Forming Cells

ECFCs were isolated and cultured as described elsewhere.[20,21] In brief, heparinized blood was diluted with Dulbecco's phosphate-buffered saline (DPBS) and layered over Ficoll-Paque Plus solution.

Subsequently, tubes were centrifuged for 20 minutes at 1,000 x g at room temperature without brake. The buffy coat containing mononuclear cells was then collected, diluted in DPBS and centrifuged for 7 minutes at 540 x g at room temperature. The cellular pellet was resuspended in EBM™-2 Endothelial Cell Growth Basal Medium-2 supplemented with the EGM™-2 SingleQuots™ Supplements (hydrocortisone, human basic fibroblast growth factor, vascular endothelial growth factor, R3 insulin like growth factor-1, ascorbic acid, human epidermal growth factor, gentamycin/amphotericin-1000, heparin) and 18% fetal bovine serum (FBS) and centrifuged again for 7 minutes at 540 x g at room temperature. Afterwards, cells were resuspended in the same medium and cells seeded in collagen-coated 48-well plates. After 24 hours of incubation at 37°C in a 95% air/5% CO_2 atmosphere saturated with H_2O, non-adherent cells and debris were removed and the medium exchanged twice weekly. The appearance of cell colonies with a cobblestone-like appearance was monitored daily after two weeks of culture. ECFC cultures were expanded by dissociation using 0.5% trypsin-ethylenediaminetetraacetic acid (EDTA) and transference to 24-well-plates, subsequently to 6-well plates, and eventually to T75 flasks, all collagen-coated. When nearing confluence, cells of six T75 flasks derived from one donor were resuspended in FBS containing 5% dimethyl sulfoxide and cryopreserved in liquid nitrogen. For experiments, frozen ECFCs were thawed, washed, and cultivated in the medium described above. All cellular assays were performed with ECFCs of passage number 7 seeded on collagen-coated 24-well, 48-well, or 96-well plates and grown until reaching confluency.

Assessment of Endothelial Cell-Dependent Thrombin and Activated Protein C Formation

The evaluation of thrombin and APC formation in plasma or in a purified system on ECFCs was performed as described previously[19], with modifications. For the variant using plasma, cells were washed twice with DPBS in 24-well plates and 400 µL citrated platelet-poor plasma, previously defibrinated using Batroxobin, was added to the cell culture. Thrombin and subsequent APC formation were initiated by addition of $CaCl_2$, tissue factor, and phospholipids at final concentrations of 16.6 mmol/L, 1 pmol/L, and

4 µmol/L, respectively. For monitoring of thrombin and APC generation over time, reactions were stopped and active enzymes in the reaction mixture stabilized by diluting aliquots of the supernatant plasma 1:100 into phosphate-buffered saline (PBS) containing 200 µmol/L argatroban, 3 mmol/L $MgCl_2$, and 0.1 % bovine serum albumin (BSA, for thrombin measurement), or 1:10 into tris-buffered saline (TBS), containing 1,000 KIU/mL aprotinin, 0.5 mg/mL bivalirudin, 1 mmol/L $MgCl_2$, 7.5 mmol/L $CaCl_2$, and 0.1 % BSA (APC sample buffer). All samples were stored at below -70°C until measured using oligonucleotide-enzyme-capture-assays (OECAs) which were initially described by Müller *et al*.[22,23]

Monitoring of Activated Protein C Inactivation Kinetics in Plasma

For measurement of APC inhibition in plasma over time, human APC at a final concentration of 89 pmol/L (5 ng/mL) was added to citrated plasma and the mixture incubated at 37°C under agitation (300 rpm). Aliquots were taken over time and the reaction stopped by adding 5,000 KIU/mL aprotinin and 2,5 mg/ml bivalirudin (final concentration). Samples were stored at below -70°C until measured using the APC-OECA.

Assessment of Activated Protein C Sensitivity Using a Prothrombinase-based Assay

To evaluate APC resistance independent from influencing factors other than factor V (FV), a prothrombinase-based assay was performed as described before[24] with modifications. In brief, citrated plasma was diluted 1:2,000 into a phospholipid-containing buffer solution (20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 137 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, 5 mg/mL BSA, 5 µg/ml phospholipids) and all FV in the reaction mixture activated by incubation with 200 mU/mL Russel's viper venom factor V activator at 37°C for 10 minutes. The mixture was divided into two parts and APC (0.64 nmol/L final concentration) was added to one part of the mixture while an equal volume of the buffer solution was added to the other part to serve as a control. Both mixtures were incubated at 37°C until reactions were stopped after 20 minutes by addition of an APC-inhibiting aptamer (HS02-52G, 10 nmol/L

final concentration).[25] Subsequently, activated factor X (FXa, 10 pmol/L), FII (10 nmol/L), and a thrombin-specific fluorogenic substrate (Boc-Asp(OBzl)-Pro-Arg-AMC, 100 µmol/L) were added and the formation of thrombin monitored over 10 minutes using a fluorescence plate reader (Synergy 2, BioTek Instruments, Bad Friedrichshall, Germany). The APC sensitivity ratio (APC-sr) was determined by dividing measured optical density (OD) to the OD of controls.

Cell-based Enzyme Linked Immunosorbent Assay

Surface expression of EPCR and TM was measured using a cell-based enzyme-linked immunosorbent assay (cell-ELISA). ECFCs were washed and fixated with 1 % glutaraldehyde for 15 minutes on ice. Afterwards, the cells were washed and blocked with 1 % BSA for 1 hour at room temperature. A murine IgG isotype control, anti-TM antibody, or anti-EPCR antibody, labelled with horseradish peroxidase (HRP) using a HRP Conjugation Kit (Abcam, Cambridge, United Kingdom), was incubated on cells at a concentration of 0.25 µg/mL for 20 minutes at room temperature. After washing the cells twice, 3,3',5,5'-tetramethylbenzidine substrate was added and the reaction stopped after 5 minutes. The OD at 450 nm was measured using a plate reader and background absorbance of unstained controls subtracted. Subsequently, cells were stained using Janus Green stain according to the manufacturer's instructions and the OD of HCl-eluted stain was measured at 595 nm. To normalize for cell density, the ratio of the background-corrected OD at 450 nm and the OD at 595 nm was calculated. Calibration curves of HRP-labelled control antibody were processed in parallel covering a range from 0 to 0.5 ng/mL.

Data Analysis

Data are generally presented as median and interquartile range (IQR). The area under the curve (AUC) was estimated using the least squares method. Normality of data was assessed using the Shapiro-Wilk test. The Kruskall-Wallis test followed by pairwise comparison using the Dunn procedure or the

Mann-Whitney test were used to compare datasets. The Bonferroni method was used to correct for multiple comparisons. Two-sided, unpaired tests were used and *P* values ≤ 0.05 were considered significant. Calculations were performed using GraphPad Prism version 9.5 (GraphPad Software, Inc., San Diego, USA). N.S. had full access to all the data in the study and takes responsibility for its integrity and the data analysis.

RESULTS

In the cohorts of asymptomatic FVL carriers (FVL VTE-), FVL carriers with a history of unprovoked VTE, (FVL VTE+), and FVL non-carriers (non-FVL), success rates of ECFC isolation and cell cultivation parameters were comparable, yielding a final study population of n=7 in each cohort, with a slightly higher number of females and of comparable age (**Table 1**).

The Activated Protein C Response to Thrombin Formation is Increased in Asymptomatic Factor V Leiden Carriers

Thrombin and APC formation rates were assessed in the ECFC-based model of the PC pathway using autologous plasma (**Figure 1A**). In all cohorts, thrombin formation peaked at 10 minutes and levels almost fully declined after 60 minutes (**Figure 1B**) while APC peaked at 40 minutes and increased levels persisted throughout the observation time of 120 minutes (**Figure 1C**). As a measure of APC formation in response to thrombin formation, the respective AUCs were calculated for each individual subject and the ratio between the corresponding AUCs (AUC APC/AUC thrombin) was compared between cohorts. While the ratio did not differ between the non-FVL and FVL VTE+ subgroups (Kruskall-Wallis test *P*>0.999), it was found to be 3-fold higher in asymptomatic FVL VTE- compared to non-FVL carriers (median, IQR of 0.138, 0.110-0.151 versus 0.047, 0.025-0.054; *P*=0.007) and 5-fold higher compared to the FVL VTE+ group (0.028, 0.022-0.092; *P*=0.026) (**Figure 1D**). Of note, homozygous FVL carriers showed higher AUCs of both thrombin and APC formation than heterozygous FVL carriers within the FVL

VTE- group (AUC thrombin: >2209 versus <1775 nmol·min·L^{-1}, AUC APC: >305 versus <265 nmol·min·L^{-1}) and the FVL VTE+ group (AUC thrombin: 3441 versus <3269 nmol·min·L^{-1}, AUC APC: 286 versus <208 nmol·min·L^{-1}), but the ratio between AUCs lay near the median of the respective groups (**Table S1, Figure 1F**).

In addition to FVL, other gene variants were detected in the study population, including *F5* 6755A>G (indicative for the FV HR2 haplotype), *THBD* 1418C>T, *PROCR* 4600A>G, and *PROCR* 4678G>C. Their allele frequencies in the FVL VTE-, FVL VTE+, and non-FVL carriers are listed in **Table S1**, along with the respective ranges of the APC response (AUC APC/AUC thrombin). These ranges in homozygous *PROCR* 4678G>C carriers, and in any carriers of other gene variants lay within the ranges observed in respective non-carriers in both FVL cohorts (**Table S1**).

Examinations of Plasmatic Determinants Potentially Influencing the Activated Protein C Response

To study whether plasmatic variables might be causative for an increased APC response in asymptomatic FVL carriers, we compared coagulation factor and inhibitor levels, the plasmatic APC-inhibitory capacity, and APC resistance between the FVL VTE-, FVL VTE+, and non-FVL cohort. Plasma levels of coagulation factors (FII, FV, factor VII, factor VIII, factor X, and factor XI) and inhibitors (AT, PC, and PS) did not differ statistically significantly between cohorts (Kruskall-Wallis test P>0.05) (**Figure 2A**). The APC half-life was found to be virtually identical (Kruskall-Wallis test P>0.05) when APC was added to plasma and decline of the enzyme was followed over time, with median (IQR) of 19.2 (18.8-20.7) minutes in non-FVL, 19.8 (17.4-21.5) minutes in FVL VTE-, and 20.9 (19.4-21.8) minutes in FVL VTE+ cohorts (**Figure 2B**). As a measure of APC resistance, we performed a prothrombinase-based assay in diluted plasma under targeted FV-activation and determined the APC-sr via measurement of thrombin formation over time with and without incubation with APC. As expected, the median (IQR) APC-sr in the non-FVL cohort (22.9, 17.8-22.9%) was significantly lower when compared to the FVL VTE- subgroup

(49.4, 47.7-64.4%; Kruskall Wallis test *P*=0.012) and to the FVL VTE+ subgroup (54.0, 52.4-57.3%; *P*=0.002) whereas the APC-sr did not differ between FVL VTE- and FVL VTE+ (*P*>0.999) (**Figure 2C**). The APC-sr also showed no difference between FVL VTE- and FVL VTE+, when homozygous FVL carriers, who showed a higher APC-sr than heterozygous carriers, were excluded (*P*=0.636).

Modifying the autologous PC pathway model with focus on plasmatic variability, FVL VTE- or FVL VTE+ plasma from each study participant was added to the same non-FVL ECFC culture and the APC response was evaluated via the AUC APC/AUC thrombin ratio. In contrast to the results from the autologous system, the APC response did not differ statistically significantly between the FVL VTE- and FVL VTE+ groups (Mann-Whitney test *P*=0.053), which suggests that differences in the autologous approach are not driven by plasmatic variation (**Figure 3A**).

Endothelial-dependent Activated Protein C response in Healthy Control Plasma is Higher in Asymptomatic Factor V Leiden Carriers

Modifying the autologous PC pathway model with focus on endothelial variability, the same pooled normal plasma was added to ECFC cultures from FVL VTE- or FVL VTE+ individuals and the APC response was evaluated via the AUC APC/AUC thrombin ratio. As measured in the autologous system and in contrast to the varying plasma approach, the APC response in asymptomatic FVL VTE- carriers was found to be significantly higher compared to the FVL VTE+ subgroup in this endothelial-dependent approach (Mann-Whitney test *P*=0.011) (**Figure 3A**). This observation suggests that the increased APC response in asymptomatic FVL carriers is driven by the endothelium rather than by plasma components.

For evaluation of this phenomenon, we compared characteristics of ECFCs obtained from non-FVL, FVL VTE-, and FVL VTE+ carriers and further studied individual endothelial factors with potential influence on the APC response. ECFC morphology in light microscopy (**Figure 3B, Figure S1**), total protein amount of a confluent colony (**Figure S2A**), and characteristic surface marker expression in flow

cytometry (**Figure S2B**) did not differ between cohorts. The surface expression of EPCR and TM on ECFCs was quantitatively measured by cell-ELISA. EPCR expression was found to be higher in non-FVL carriers compared to the FVL VTE+ subgroup (Kruskall-Wallis test *P*=0.037) but did not differ between non-FVL and FVL VTE- (*P*=0.635), and between FVL VTE- and FVL VTE+ (*P*=0.635) (**Figure 3C**). In addition, we found that TM expression on the cell surface did not differ between cohorts (Kruskall-Wallis test *P*>0.05) (**Figure 3C**).

DISCUSSION

In the common understanding on how FVL affects thrombotic risk, thrombogenicity is driven by the prolonged half-life of FVa, resulting in increased prothrombinase complex activity. Accordingly, the thrombotic risk is higher in homozygous FVL carriers, who have higher levels of APC-resistant FVa, than in heterozygous carriers.[5] Provided that the endothelial PC pathway is intact, higher thrombin formation rates should result in higher APC formation rates. Indeed, using an ECFC-based ex vivo model of the PC pathway, we have recently shown that the APC response to thrombin formation was increased in FVL carriers, the majority of whom had no history of VTE.[19] In the present study, using the same model but distinguishing between symptomatic and asymptomatic FVL carriers, we observed an increased APC response only in the latter cohort. Of note, while homozygous FVL carriers showed the highest AUCs of thrombin in their respective cohorts (VTE+ and VTE-) and the highest AUCs of APC overall, the ratio of AUC APC/AUC thrombin in homozygous FVL carriers lay near the median of their respective cohorts. This can be explained by the ex vivo model being a dynamic system, in which APC formation downregulates thrombin formation as does the PC pathway itself. The ex vivo results are in line with previous in vivo findings of increased APC response rates after injection of rFVIIa in asymptomatic FVL carriers compared to those with a history of VTE.[13] While this further strengthens the validity of the ECFC-based model of the PC pathway, the question remains which factors are causative for the observed differences in the APC response.

To narrow down potentially influencing factors, additional experiments were conducted using a single ECFC line from a non-FVL carrier and plasma from FVL carriers with and without a history of VTE. As a result, the differences in the APC response between FVL VTE+ and FVL VTE- disappeared, speaking against a role of plasmatic factors in the observed differences in the APC response. Further investigation of potential influencing factors in plasma revealed no differences in coagulation factors and inhibitors, APC inhibition kinetics, or APC resistance between the cohorts of FVL carriers. The APC response in carriers of the FV HR2 haplotype (one in FVL VTE-, two in FVL+), which is known to modulate APC resistance in FVL carriers,[26,27] lay within the ranges of the APC resistance in both cohorts. To sum up, the observed differences in the APC response could not be explained by the examined plasmatic variables.

When ECFC lines from the FVL VTE+ and FVL VTE- cohorts were combined with normal pooled plasma in the ECFC model, the differences in the APC response observed in the autologous approach, could be reproduced, albeit at a lesser extent. Taken together, the obtained data using the ECFC-based model of the PC pathway suggest that endothelial factors modulate the APC response.

Well established endothelial determinants of APC formation are the receptors TM and EPCR. Since PC activation is directly linked to the amount of TM and EPCR presented on the endothelial cell surface,[28,29] we studied their expression on ECFCs using cell-ELISAs. Although we observed no difference in TM or EPCR expression between asymptomatic and symptomatic FVL carriers in a resting state, the localization of receptors across the endothelial cell membrane could change upon coagulation activation. While they are usually presented on the cell surface, TM and EPCR can be endocytosed in response to stimulation making them unavailable for further ligand binding and, at the same time, bound ligands are cleared from the circulation. Thrombin induces the internalization of TM which is followed by degradation of thrombin.[30–32] Similarly, ligand binding to EPCR promotes endocytosis of the receptor. In vitro studies showed that PC, APC, FVII, and FVIIa bind to EPCR with a similar affinity[33] and are internalized via endocytosis in a similar rate.[34] Therefore, EPCR endocytosis downregulates both the

anticoagulant response of the PC pathway and the procoagulant response of the extrinsic pathway. While in theory, internalization of endothelial receptors should have a proportionate effect on pro- and anticoagulant mechanisms, one might speculate if a dysbalanced internalization could explain the observed differences in the APC response.

Gene variants in *THBD* or *PROCR* have also been postulated to affect APC formation and thrombotic risk. Navarro et al[35] reported an association of the *THBD* c.1418 C>T polymorphism with reduced VTE risk and increased circulating APC levels in carriers of the 1418T allele. In addition, they performed functional studies in cultured human umbilical vein endothelial cells and observed increased PC activation on cells carrying the 1418T allele. Furthermore, different *PROCR* haplotypes and their functional phenotypes have been characterized. The H1 (or A1) haplotype, tagged by the 4678 G>C sequence, was associated with reduced EPCR shedding and decreased VTE risk.[36,37] Medina et al[38] showed that FVL carriers with the *PROCR* H1 haplotype have a reduced thrombotic risk, although no association between APC levels and the polymorphism could be established. In opposite, the H3 (or A3) haplotype, tagged by the 4600 A>G sequence, was associated with lower PC activation, increased EPCR shedding and higher VTE risk.[39,40] While the association between an increased thrombotic risk and the *PROCR* 4600 A>G variant was confirmed by larger trials and meta-analyses,[41–43] these studies did not find an association between the common *THBD* c.1418C>T polymorphism[44–47] and the *PROCR* 4678 G>C variant[39,41] and reduced risk of VTE. In our study, the APC response in symptomatic and asymptomatic FVL carriers, who were carriers of the aforementioned *THBD* or *PROCR* variants, lay within the range of the APC response in the respective cohorts. Therefore, they cannot explain the observed differences in the APC response. However, a potential effect *THBD* or *PROCR* variants on the APC response cannot be excluded but would require examination in study populations who would need to be accordingly selected.

In conclusion, our findings confirm previous in vivo evidence that the extent of APC formation in response to thrombin modulates the thrombotic risk in FVL and might therefore at least partially explain

the variable clinical expressivity of this thrombophilic mutation. Furthermore, they underline the validity of the utilized ECFC-based ex vivo model in assessing the functionality of the PC pathway regarding clinically relevant endpoints on a personalized level. Although the underlying mechanism remains yet to be identified, the obtained data strongly suggest that endothelial variables are a major driver of variation in the APC response in symptomatic and asymptomatic FVL carriers. In addition to further elucidation of causative factors, further studies are warranted to examine a potential role of the endothelium in other types of thrombophilia.

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Figure 1A and 3A have been created with BioRender.com.

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Supplemental Material

Supplemental Methods

Table S1

Figure S1-S2

Major Resources Table

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TABLES

Table 1. Isolation of endothelial colony forming cells and study population

	Non-FVL	**FVL VTE-**	**FVL VTE+**
Successful ECFC isolations, n (%)	7/16 (44%)	7/12 (58%)	7/13 (54%)
ECFC colonies, n	1.9 (1-3)	1.4 (1-3)	1.3 (1-3)
Days in culture, n	38 (35-42)	38 (32-48)	39 (31-45)
Sex, males/females, n	3/4	2/5	3/4
Age, years, n	44 (27-60)	39 (22-60)	45 (28-57)
FVL homozygous, n	-	2	1
FV HR2 haplotype, n	-	1	2

Number of endothelial colony forming cell (ECFC) colonies, days in culture, and age are presented as mean and range. FV, factor V; FVL, factor V Leiden; VTE, venous thromboembolism.

FIGURES AND FIGURE LEGENDS

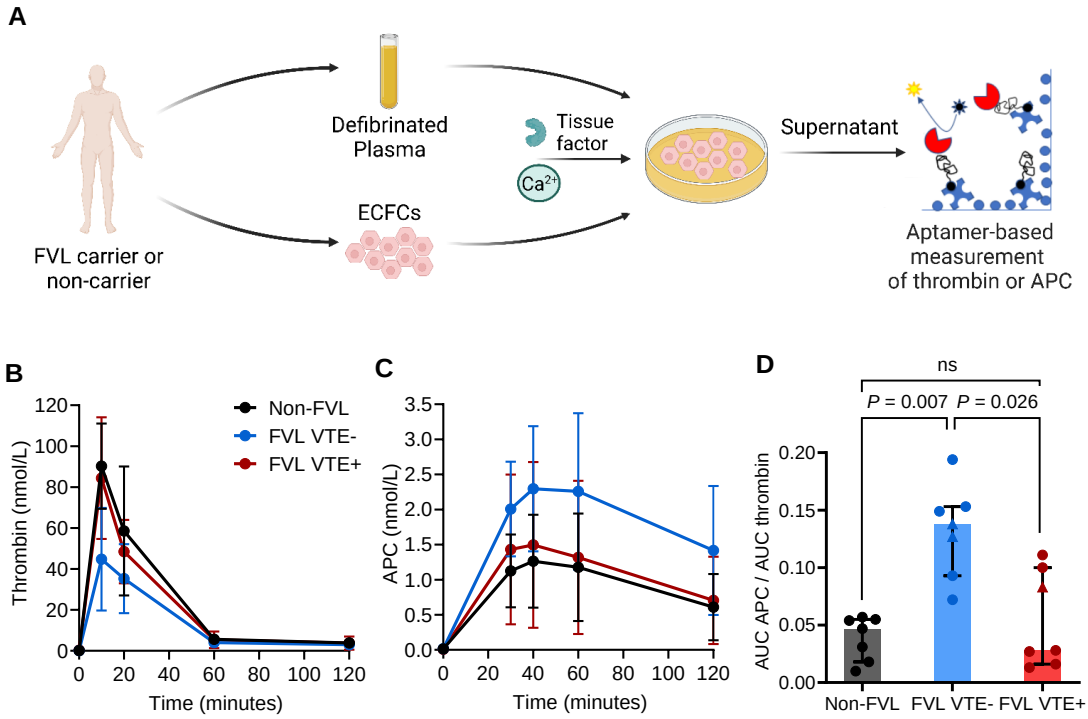

Figure 1. Protein C pathway modelling using endothelial cells and autologous plasma in factor V Leiden carriers and healthy controls. A, To assess the endothelial activated protein C (APC) formation capacity, confluent monolayers of endothelial colony forming cells (ECFCs) were overlaid with autologous defibrinated, citrated plasma and thrombin formation induced by addition of tissue factor and $CaCl_2$. In the supernatant, time-dependent formation of (**B**) thrombin and (**C**) APC were monitored in non-factor V Leiden (FVL) carriers (depicted in black), FVL carriers with a history of venous thromboembolism (VTE, FVL VTE+, red) or asymptomatic FVL carriers (FVL VTE-, blue, n=7 each). **D**, The ratio between the area under the curve (AUC) of thrombin formation and the AUC of APC formation (AUC APC/AUC thrombin) as measure of the APC response were compared between cohorts using the Kruskall-Wallis test followed by pairwise comparison using the Dunn procedure. Data are shown as median and interquartile range. Measurement results in homozygous FVL carriers are distinguished by triangles. ns, not significant.

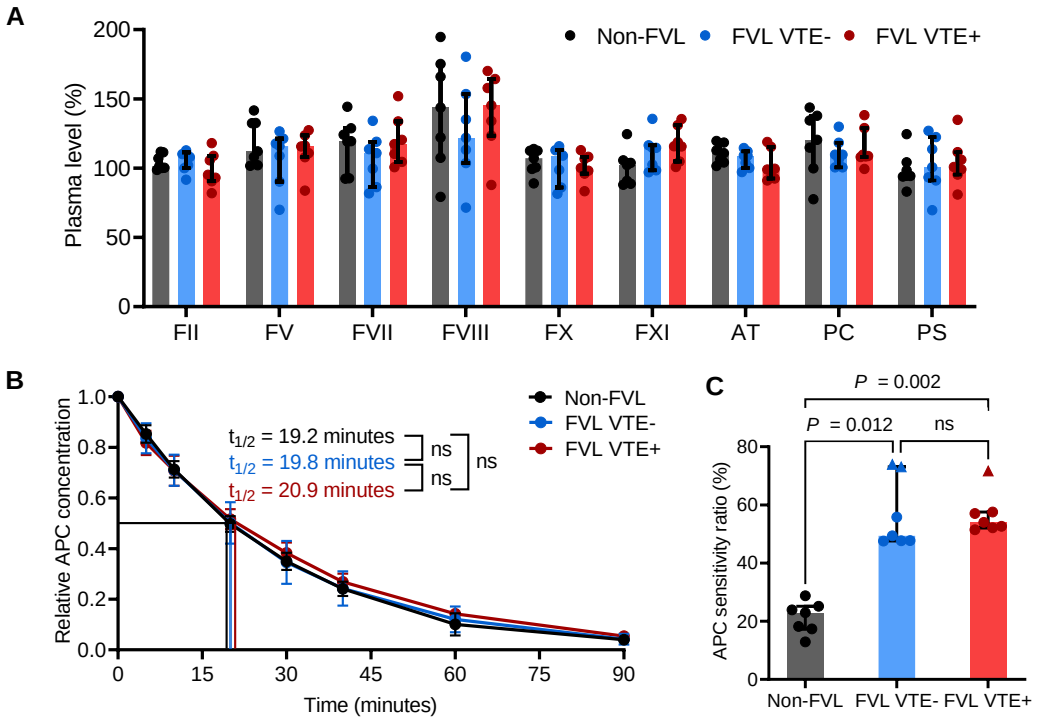

Figure 2. Evaluation of plasmatic factors potentially affecting the activated protein C response. Data obtained in non-factor V Leiden (FVL) carriers (depicted in black), FVL carriers with a history of venous thromboembolism (VTE, FVL VTE+, red) or asymptomatic FVL carriers (FVL VTE-, blue, n=7 each) are shown as median and interquartile range. **A**, Plasma levels of coagulation factors and inhibitors were compared (*P*>0.05 each). **B**, Activated protein C (APC) inhibition kinetics were evaluated by addition of APC (5 ng/mL final concentration) to citrated plasma and monitoring of residual plasma concentrations over time by an oligonucleotide-based enzyme capture assay. The calculated half-life time ($t_{1/2}$) was compared between cohorts. **C**, The APC resistance of activated factor V (FVa) was assessed by measuring the residual prothrombinase activity after inactivation by APC and the APC sensitivity ratio was determined. Data are shown as median and interquartile range. Measurement results in homozygous FVL carriers are depicted by triangles. Comparisons between cohorts were performed using the Kruskall-Wallis test followed by pairwise comparison using the Dunn procedure. AT, antithrombin; FII-XI, factor II-XI; ns, not significant; PS, protein S.

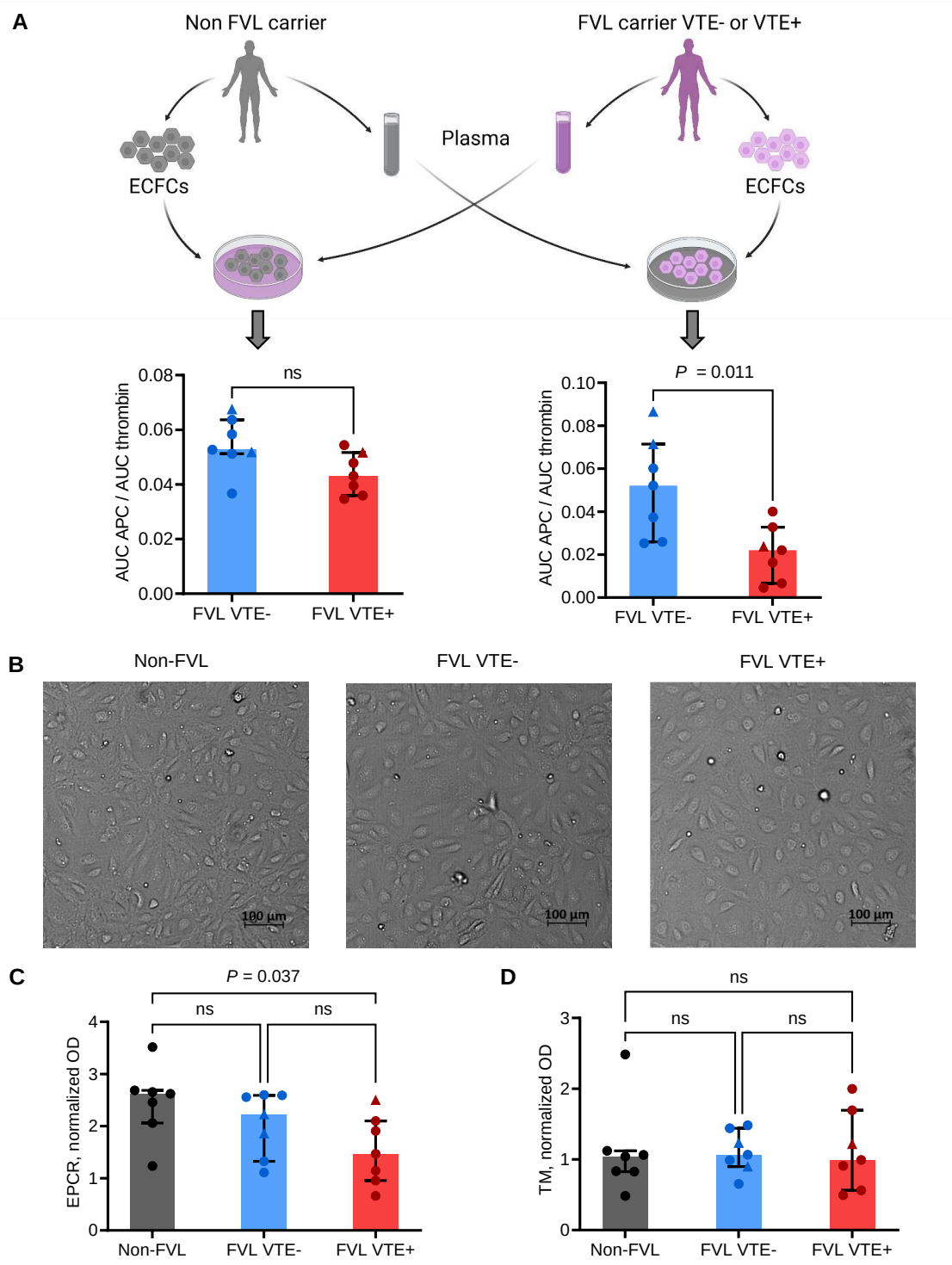

Figure 3. Evaluation of endothelial factors potentially affecting the activated protein C response.

A, The activated protein C (APC) response was studied in the endothelial cell-based protein C pathway model using either endothelial colony-forming cells (ECFCs) from a non-FVL carriers and plasma from

FVL VTE- and FVL VTE+ individuals; or ECFCs from FVL VTE- and FVL VTE+ individuals and pooled normal plasma. The ratio between the area under the curve (AUC) of APC formation (AUC APC) and the AUC of thrombin formation (AUC thrombin) as measure of the APC response was compared between cohorts using the Mann Whitney test. **B**, Representative micrographs of endothelial colony forming cells (ECFCs) from a non-factor V Leiden (FVL) carrier, a FVL carrier with a history of venous thromboembolism (VTE, FVL VTE+), and an asymptomatic FVL carrier (FVL VTE-) are shown (Axio Observer, camera Axiocam 702 mono (Carl Zeiss Microscopy). The following data, obtained in non-FVL (black), FVL VTE- (blue), and FVL VTE+ (red) individuals (n=7 each), are shown as median and interquartile range, and were compared using the Kruskall-Wallis test followed by pairwise comparison using the Dunn procedure. Measurement results in homozygous FVL carriers are distinguished by triangles. Expression of (**C**) endothelial protein C receptor (EPCR) and (**D**) thrombomodulin (TM) on ECFCs was studied by cell-based enzyme-linked immunosorbent assays, in which ECFCs were fixated and incubated with the respective antibody. The background corrected optical density (OD) was normalized for cell density assessed by Janus Green staining. ns, not significant.

NOVELTY AND SIGNIFICANCE

What is known?

- The factor V Leiden (FVL) mutation impairs inactivation of procoagulant factor V by anticoagulant activated protein C (APC).
- FVL is the most prevalent hereditary risk factor for venous thromboembolism (VTE) with a highly variable clinical expressivity.
- In vivo, asymptomatic FVL carriers have been shown to form more APC upon coagulation activation than those with previous VTE.

What new information does this article contribute?

- An increased APC response in asymptomatic FVL carriers was confirmed in an ex vivo model using patient-specific endothelial cells and autologous plasma.
- Differences in the APC response were driven by the patients' endothelial cells rather than variables in plasma.
- Endothelial mechanisms might modulate the thrombotic risk in FVL carriers.

The FVL mutation alters the site at which this procoagulant plasma protein is cleaved by the anticoagulant APC, which is formed on the endothelial surface upon coagulation activation. While it is the most common hereditary thrombophilia, its clinical phenotype – venous thrombosis and pulmonary embolism – shows a highly variable expressivity. Recently, we have shown in vivo, that asymptomatic FVL carriers form more APC after extrinsic coagulation activation than those with a history of VTE. Using an ex vivo model based on patient-specific endothelial cells and autologous plasma we were able to confirm these differences in the APC response, that depended on the clinical phenotype, in FVL carriers. Selective analysis of endothelial and plasmatic variables that might affect APC formation we found that the increased APC formation in asymptomatic FVL carriers was driven by the patients' endothelium. These data suggest that endothelial mechanisms might modulate the thrombotic risk in FVL carriers.

SUPPLEMENTAL MATERIALS

Increased Activated Protein C Response to Thrombin Formation in Asymptomatic Factor V Leiden Carriers is Driven by the Endothelium: Evidence from and Endothelial Cell-based Ex Vivo Model

SUPPLEMENTAL METHODS

Materials

Human α-thrombin, protein C (PC) and activated PC (APC) were obtained from CellSystems (Troisdorf, Germany). Argatroban was obtained from Mitsubishi Pharma (Düsseldorf, Germany). Aprotinin and Streptavidin were purchased from PanReac AppliChem ITW Reagents (Darmstadt, Germany). Bivalirudin was obtained from The Medicines Company (Oxfordshire, UK). Biotinylated aptamers were synthesized and PAGE-purified by Microsynth (Balgach, Switzerland). Liquid Plate Sealer® and HRP Protector™ were purchased from Candor Bioscience (Wangen, Germany). The fluorogenic peptide substrate Boc-Asp(OBzl)-Pro-Arg-AMC (I-1560) was obtained from Bachem (Weil am Rhein, Germany) while the fluorogenic substrate Pyroglu-Pro-Arg-AMC (Pefafluor PCa) was purchased from Pentapharm (Basel, Switzerland). Batroxobin reagent was obtained from Siemens Healthineers (Marburg, Germany). Normal platelet-poor plasma was prepared in-house by pooling citrated plasma of at least four healthy blood donors. The tissue factor reagent for thrombin generation (PPP-reagent low) was purchased from Stago (Asnières-sur-Seine, France). Phospholipid emulsion containing phosphatidylserine, phosphatidylcholine and sphingomyelin was obtained from Rossix (Mölndal, Sweden). Ficoll Paque Plus was obtained from GE Healthcare (Solingen, Germany). Biotinylated bovine serum albumin (BSA), Dulbecco's phosphate-buffered saline (DPBS), ethylenediaminetetraacetic acid (EDTA), 0.5 % trypsin-EDTA solution (10x), glutaraldehyde 10 % solution, mouse IgG isotype control, fetal bovine serum (FBS), and Pierce™ BCA Protein Assay Kit were purchased from Thermo Fisher Scientific (Darmstadt, Germany). Dimethyl sulfoxide was purchased from WAK Chemie Medical (Steinbach, Germany). Rat tail

collagen type I was purchased from Corning (Wiesbaden, Germany). EBM™-2 Basal Medium and EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ were obtained from Lonza (Basel, Switzerland). Russel's viper venom factor V activator was purchased from Loxo (Dossenheim, Germany). Mouse monoclonal antibodies against endothelial protein C receptor and thrombomodulin, and Janus Green cell normalization stain were purchased from abcam (Cambridge, United Kingdom). BSA and 3,3',5,5'-tetramethylbenzidine were obtained from Sigma Aldrich (St. Louis, United States). Fluorescently labelled, recombinant anti-human (REAfinity™) antibodies against CD31, CD309, CD201, CD141, CD34, and CD45 were obtained from Miltenyi Biotech (Bergisch Gladbach, Germany).

Collection and Processing of Blood Samples

Blood samples were obtained by venipuncture of an antecubital vein using 21-gauge winged infusion sets (Sarstedt, Nümbrecht, Germany). After discarding the first 2 mL, blood was drawn into EDTA tubes, lithium-heparin tubes (16 IU/mL), and citrate tubes (10.5 mmol/L, Sarstedt). Platelet-poor plasma was obtained from citrate tubes by centrifugation (2,600 x g, 10 minutes) within 30 minutes and stored at below -70°C until further processing.

Genetic analysis

Genomic DNA was isolated from EDTA blood using the Blood Core Kit (Qiagen, Hilden, Germany). The sequencing analyses were carried out on a Mini-Seq genome sequencer (Illumina, Santa Clara, CA, USA), which was used for next generation sequencing using a Nextera Rapid Capture Custom Enrichment (Illumina) including the following genes: *F2, F5, F7, F8, F9, F10, F11, F13A1, F13B, FGA* (fibrinogen alpha chain), *FGB* (fibrinogen beta chain), *FGG* (fibrinogen gamma chain), *VWF, GGCX, VKORC1, LMANT, MCFD2, SERPINC1* (antithrombin), *PROS1* (protein S), *PROC* (PC), *THBD* (thrombomodulin), and *PROCR* (endothelial protein C receptor, EPCR). Data were evaluated by SeqPilot (JSI medical systems, Ettenheim, Germany) software. For the description of sequence variations, the

guidelines of the Human Genome Variation Society were applied, and variants were filtered according to minor allele frequency (MAF<1% in gnomAD).

Quality Control of Cultured Endothelial Colony Forming Cells

Cobblestone morphology of endothelial colony forming cells (ECFCs) was examined by light microscopy (Axiovert 25 or Axio Observer, both Carl Zeiss Microscopy, Oberkochen, Germany). Measurement of the total protein was performed amount using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany) and staining of CD31 (platelet endothelial cell adhesion molecule, PECAM-1), CD309 (vascular endothelial growth factor receptor 2, VEGFR-2), CD201 (EPCR), and CD141 (thrombomodulin), CD34, and CD45 analyzed by flow cytometry as described elsewhere.[23] Briefly, cells were dissociated, resuspended in staining buffer (DPBS, pH 7, 2 % FBS, 0.5 mol/L EDTA), and 10^5 cells were stained with fluorescently labelled antibodies for 30 minutes at room temperature in the dark. Cytometric measurements were performed using a Navios EX flow cytometer (Beckman Coulter Life Sciences, Brea, CA, USA) and analysis was performed using the FlowJo™ Software version 10.8 (BD Life Sciences, Ashland, USA).

Aptamer-based Measurement of Thrombin and Activated Protein C

Maxisorp Fluoronunc microtiter modules (Nunc A/S, Roskilde, Denmark) were coated with 10 µg/mL BSA-biotin, loaded with 10 µg/mL streptavidin, and blocked using 2 mg/ml BSA: Primed plates were treated with Liquid Plate Sealer® and stored in aluminium bags at 4°C until being used. For running the oligonucleotide-based enzyme capture assays (OECAs), pre-coated plates were incubated with 3'-biotinylated aptamers (HD1-22 for the thrombin-OECA or HS02-52G for the APC-OECA) and washed. Thrombin samples were further diluted 1:10 and diluted thrombin and APC samples were added to the aptamer-coated plates, respectively. After incubation and washing, detection of captured thrombin or APC was performed using the respective enzyme-specific fluorogenic peptide substrates (Boc-Asp(OBzl)-Pro-Arg-AMC for the thrombin-OECA or Pyroglu-Pro-Arg-AMC for the APC-OECA). Changes in fluorescence

over time were measured using a fluorescence plate reader (Synergy 2, BioTek Instruments, Bad Friedrichshall, Germany). Calibration curves (in the same matrix as the samples) were processed in parallel covering a ½-log10 concentration range (0 to 10 ng/mL of thrombin, 0-272 pmol/L; or 0 to 50 ng/mL of APC, 0-910 pmol/L). Data obtained from the calibrators were interpolated by 4-parameter curve fit and used to calculate the thrombin or APC concentration in the samples.

Assessment of Endothelial Cell-Dependent Activated Protein C Formation in a Purified System

For generation of APC on ECFCs in a purified system, a buffer solution (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 137 mmol/L NaCl, 4 mmol/L KCl, 11 mmol/L glucose, 2 mmol/L $CaCl_2$, 4 mg/mL BSA) was prepared. Cells were washed once with DPBS and once with buffer before addition of 50 nmol/L PC, and 0.1 U/mL thrombin in buffer (200 µL/well in 48-well plates). After one hour incubation at room temperature, the reaction was stopped, and generated APC was stabilized by diluting aliquots of the supernatant 1:10 into APC sample buffer. Samples were stored at below -70°C until APC was measured by OECA.

Measurement of Coagulation Factors and Inhibitors in Plasma

Plasma levels of factor II, factor V, factor VII, factor VIII, factor IX, factor X, factor XI, antithrombin, PC, and PS were measured on the Atellica® COAG 360 System using corresponding reagents (Siemens Healthineers, Erlangen, Germany).

SUPPLEMENTAL TABLES

Table S1. Gene variants and range of activated protein C response in the autologous ex vivo model

		Non FVL *		FVL VTE- †		FVL VTE+ ‡	
		n	AUC APC/ AUC thrombin	n	AUC APC/ AUC thrombin	n	AUC APC/ AUC thrombin
***F5* 1691G>A (Factor V Leiden**	GG	7	0.010-0.057	0	-	0	-
	GA	0	-	5	0.072-0.194	6	0.013-0.111
	AA	0	-	2	0.127-0.138	1	0.083
***F5* 6755A>G (HR2 haplotype)**	AA	7	0.010-0.057	6	0.072-0.194	5	0.016-0.111
	AG	0	-	1	0.153	2	0.028-0.100
***THBD* 1418C>T**	CC	4	0.047-0.057	3	0.072-0.194	5	0.016-0.111
	CT	3	0.010-0.031	1	0.093	1	0.013
	TT	0	-	3	0.127-0.153	1	0.027
***PROCR* 4600A>G**	AA	4	0.018-0.057	6	0.072-0.194	6	0.013-0.111
	AG	3	0.010-0.031	1	0.127	1	0.016
***PROCR* 4678G>C**	GG	2	0.047-0.057	3	0.093-0.153	3	0.013-0.111
	CG	4	0.010-0.055	3	0.072-0.194	2	0.027-0.100
	CC	1	0.031	1	0.149	2	0.028-0.083
All		7	0.010-0.057	7	0.072-0.194	7	0.013-0.111

* In one subject (AUC APC/AUC thrombin = 0.031) the additional variants *THBD* 1477G>T and *F10* 424G>A were detected. † In one subject (AUC APC/AUC thrombin = 0.153) the additional variant *FGA* 1823G>C was detected. ‡ In one subject (AUC APC/AUC thrombin = 0.100) the additional variants *F13* 1730C>T and *FGB* 794C>T were detected. APC, activated protein C; AUC, area under the curve; FVL, factor V Leiden mutation; VTE, venous thromboembolism.

SUPPLEMENTAL FIGURES

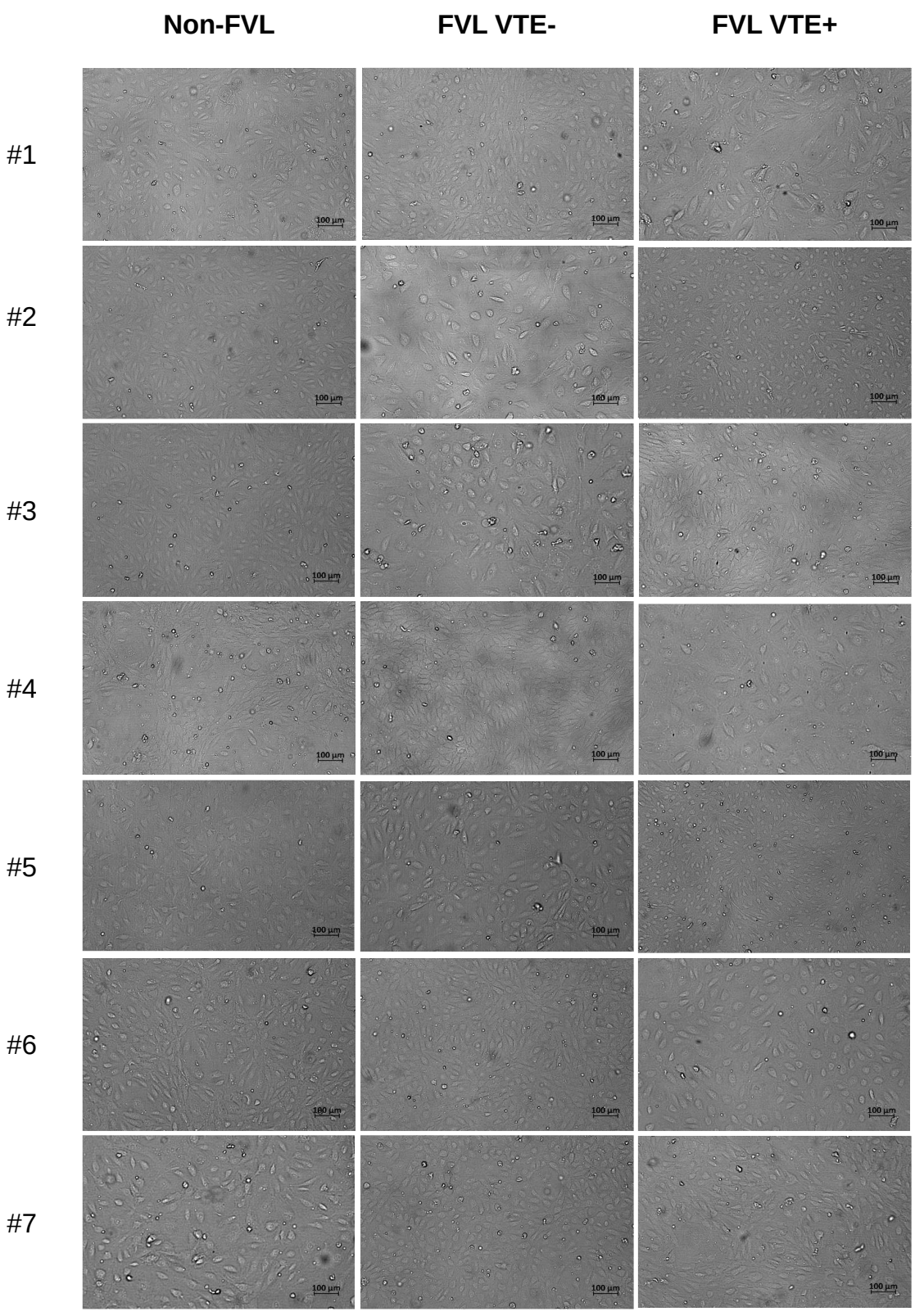

Figure S1. Representative micrographs of endothelial colony forming cells. Micrographs were obtained with an Axio Observer microscope using an Axiocam 702 mono camera (both Carl Zeiss Microscopy, Oberkochen, Germany). FVL, factor V Leiden; VTE, venous thromboembolism.

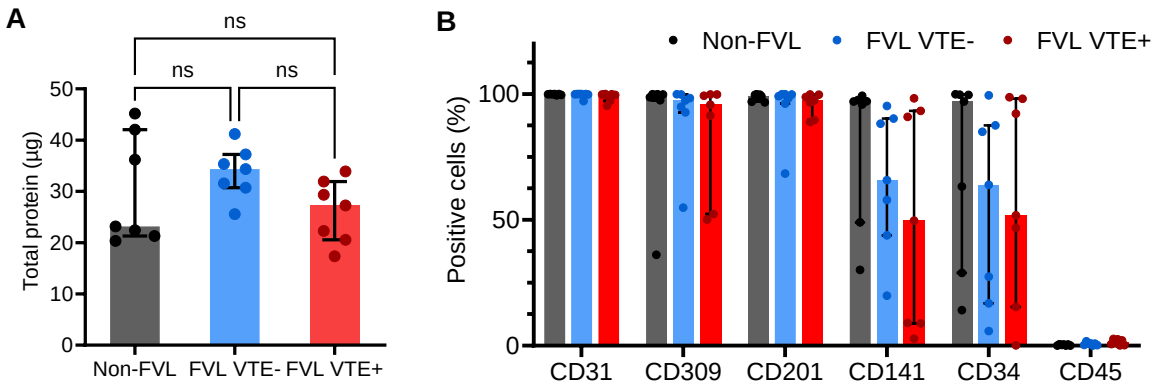

Figure S2. Quality control of endothelial colony forming cells. A, Cell lysates were obtained from confluent cell cultures on a 24-well plate using lysis buffer containing Triton X-100 (50 mM Tris HCl, 150 mM NaCl, 1 % Triton X-100, pH 8). Total protein amount was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany). **B**, Cells were dissociated, resuspended in staining buffer, and 10^5 cells were stained with fluorescently labelled antibodies against CD31, CD309, CD201, CD141, CD34, and CD45 for 30 minutes. Cytometric measurements were performed using a Navios EX flow cytometer (Beckman Coulter Life Sciences, Brea, CA, USA) and analysis was performed using the FlowJoTM Software version 10.8 (BD Life Sciences, Ashland, USA). Data are shown as median and interquartile range. Differences between cohorts were assessed using the Kruskall-Wallis test followed by pairwise comparison using the Dunn procedure. The Bonferroni method was used to correct for multiple comparisons. No statistically significant differences between cohorts were observed. FVL, factor V Leiden mutation; ns, not significant; VTE venous thromboembolism.