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## **Thrombin generation on vascular cells in the presence of factor VIII and/or emicizumab.**

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## Essentials

- Thrombin generation by factor VIII and emicizumab on vascular cells was investigated
- Endothelial and smooth muscle cells support FVIII- and emicizumab-mediated thrombin generation
- Emicizumab activity is limited by an excess of factor IX when phospholipids are limiting
- Anticoagulant components play an important role in controlling FVIII and emicizumab activity

## Abstract

**Background:** The effect of factor VIII (FVIII) or emicizumab on thrombin generation is usually assessed in assays using synthetic phospholipids. Here, we assessed thrombin generation at the surface of human arterial cells (aortic endothelial cells (hAECs) and aortic vascular smooth muscle cells (hVSMCs)).

**Aim:** Exploring the capacity of hAECs (resting or stimulated) and hVSMCs to support thrombin generation by FVIII or emicizumab.

**Methods:** Primary hVSMCs and hAECs were analyzed for tissue factor (TF)-activity and antigen, phosphatidylserine (PS)-exposure, tissue factor pathway inhibitor (TFPI)-content and thrombomodulin expression. Cells were incubated with FVIII-deficient plasma spiked with FVIII, emicizumab, activated prothrombin complex concentrate (aPCC) or combinations thereof.

**Results:** TF-activity and PS-exposure were present on both hVSMCs and hAECs. In contrast, thrombomodulin and TFPI were expressed on hAECs, while virtually lacking on hVSMCs, confirming the procoagulant nature of hVSMCs. TNF $\alpha$ -mediated stimulation of hAECs increased not only TF-antigen, TF-activity and PS-exposure, but also TFPI and thrombomodulin expression. As expected, FVIII and emicizumab promoted thrombin generation on non-stimulated hAECs and hVSMCs, with more thrombin being generated on hVSMCs. Unexpectedly, FVIII and emicizumab increased thrombin generation to a lesser extent on stimulated hAECs compared to non-stimulated hAECs. Finally, adding emicizumab to FVIII did not further increase thrombin generation, whereas the addition of emicizumab to aPCC resulted in exaggerated thrombin generation.

**Conclusion:** TNF-stimulation of hAECs increases both pro- and anti-coagulant activity. Unexpectedly, the increased anti-coagulant activity is sufficient to limit both FVIII- and emicizumab-induced thrombin generation. This protective effect disappears when emicizumab is combined with aPCC.

## Introduction

Hemophilia A is an X-linked bleeding disorder caused by defects in the gene encoding factor VIII (FVIII), affecting 1-2 per 10,000 male births.<sup>1</sup> Replacement therapy using plasma-derived or recombinant FVIII-concentrates has proven to be effective in the management of bleedings in hemophilia A.<sup>2</sup> Nevertheless, replacement therapy is limited by the need for frequent intravenous administrations and the development of neutralizing antibodies (inhibitors) in 15-30% of the severe hemophilia A patients.<sup>3,4</sup> Bleeding episodes and need for surgery in inhibitor patients were traditionally managed using bypassing agents that act independently of FVIII in the coagulation cascade in order to generate thrombin. These agents include recombinant activated factor VII (rFVIIa) and activated prothrombin complex concentrate (aPCC). However, both aPCC and rFVIIa do not eliminate bleeding or successfully control acute bleeds in all patients with inhibitors.<sup>5,6</sup>

To overcome these limitations, novel treatment strategies that act independently of FVIII options have been or are becoming available. Some of these are based on restoring the hemostatic balance by targeting components of the anticoagulant pathway, such as tissue factor pathway inhibitor (TFPI), activated protein C or antithrombin.<sup>7-9</sup> Alternatively, bispecific antibodies have been developed that aim to mimic FVIII cofactor function. Examples hereof are emicizumab and Mim8.<sup>10,11</sup> Emicizumab (approved for clinical use by both FDA and EMA) has shown clinical efficacy in multiple clinical trials, with significantly reduced annual bleeding rates and improved quality of life.<sup>12-16</sup>

*In vitro* activities of both FVIII and emicizumab are usually determined in assays using synthetic phospholipids, such as chromogenic activity assays and thrombin generation assays.<sup>17-22</sup> However, emicizumab and FVIII are exposed to vascular cells within the circulation, like endothelial cells and, upon vascular breaching, smooth muscle cells. These cells display a different phospholipid signature compared to synthetic phospholipids, while also expressing both pro- and anti-coagulant proteins, including TFPI, thrombomodulin and tissue factor. In this study, we therefore aimed to explore the contribution of primary vascular arterial cells to emicizumab- and FVIII-supported thrombin generation. Our data show that both endothelial cells and vascular smooth muscle cells support FVIII- and to a lesser extent emicizumab-dependent thrombin generation. Interestingly stimulation of endothelial cells by tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) resulted in an increase of both pro- and anti-coagulant activity, resulting in a net reduced potential to stimulate FVIII- and emicizumab-dependent thrombin generation. In all cases, combining emicizumab with aPCC resulted in exaggerated thrombin generation.

## Materials and Methods

### *Cell culture*

Primary human aortic endothelial and vascular smooth muscle cells (hAECs and hVSMCs), human umbilical vein endothelial cells (hUVECs) and human microvascular endothelial cells (hMVECs) were purchased from Lonza and grown in Endothelial Cell Growth Medium-2 BulletKit™ and SmGM™-2 Smooth Muscle Cell Growth Medium-2 BulletKit™ (Lonza, Basel, Switzerland), respectively during 3 passages. Batches of three different individuals were used in this study. Cells were used between passages 3 to 7 to reduce phenotypic drift. Cells (6,500-7,500/well) were seeded in 96-well tissue culture flat-bottom plates, (MICROTEST-96), grown to sub-confluence a day before use. We have previously established that cells seeded above 2500/well generates homogeneous thrombin generation parameters.<sup>23</sup> Cells were washed with 20 mM HEPES, 140 mM NaCl, pH 7.35 containing 5 mg/mL albumin (HBS-buffer) before use. In some experiments, hAECs were activated by incubating with 5 nM TNF-alpha in growth medium for 4 hours before use.

### *Flow cytometry*

Cells were harvested with trypsin-EDTA and then incubated 1h with an anti-Endothelial Protein C receptor (EPCR) antibody (cat # 563624; BD Biosciences), an anti-thrombomodulin antibody (cat # MAB3947; R&D Systems) or an anti-tissue factor pathway inhibitor (TFPI) antibody (cat Ab260041; Abcam) in PBS/BSA 1%. After washing, cells were resuspended in PBS/BSA1% and measurement was performed with Celesta Sorp flow cytometer (BD Biosciences). Cells (10,000) were routinely counted, and cell population was defined based on forward-side scatter characteristics. Data were analyzed with the Kaluza analysis 2.1 software. Non-labelled cells were used as negative controls

### *Antigen and activity assays*

Tissue factor (TF) and tissue factor pathway inhibitor (TFPI) activities were measured in 200 µL HBS incubated for 1 h at room temperature with hAECs or hVSMCs using the Actichrome-TF and Actichrome-TFPI activity assay respectively (American Diagnostica, Stamford, CT). To measure cell surface TF or TFPI activity, washed adherent cells were overlaid with HBS-buffer. The reaction was stopped with glacial acetic acid and the absorbance read at 405 nm. The chromogenic assay measuring the phosphatidylserine (PS)-equivalent in hAECs and hVSMCs was performed as described previously for VSMCs. Briefly, washed adherent cells were incubated with 50 µL of 20 mM HEPES, pH 7.35 containing 60 mg/mL bovine serum albumin (BSA), 50 µL of FXa-FVa-CaCl<sub>2</sub> and 50 µL of bovine prothrombin (6 µM) plus Z-Gly-Gly-Arg-AMC substrate (1.25 mM) in 20 mM HEPES pH 7.35 containing 60 mg/mL BSA. The

plate was placed in the Fluoroskan Ascent fluorometer and allowed to warm to 37°C for 5 min before kinetic readings were taken over 10 min. Phospholipid concentration was estimated from the initial rate of thrombin formation by reference to a standard curve constructed with a mixture of phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (20:20:60 mol%), and expressed as PS equivalents.

The Quantikine Human Thrombomodulin Immunoassay (cat # BDCA-3; R&D Systems) was used to measure human Thrombomodulin in cell culture lysates as instructed.

### *Thrombin generation*

Lyophilized FVIII deficient plasma (SYSMEX/HYPHEN Biomed) was reconstituted with distilled water and kept for 30 min at room temperature.

Thrombin generation was essentially performed as described by Hemker et al.<sup>24</sup> In brief, 20 µl of 20 mM Hepes (pH 7.4)/140 mM NaCl/5 mg/ml bovine serum albumin (HBS-BSA) and 80 µl FVIII-deficient plasma, alone or supplemented with FVIII (0.25-1.5 U/ml), emicizumab (10-80 µg/ml) or aPCC (1 U/ml) was added to wells (polypropylene 96-well microtiter plates; Nunc, Thermo Fischer Scientific) containing non-stimulated hAECs, TNF $\alpha$ -stimulated hAECs or hVSMCs previously washed with HBS-BSA. Cell-counts were between 10,000 and 11,000 hVSMCs/well and between 8,000 and 9,000 hAECs/well. After incubation for 5 min at 37°C, thrombin generation was initiated by the addition of 20 µl pre-warmed Fluo-substrate (20 mM Hepes (pH7.4)/0.1 M Z-Gly-Gly-Arg-AMC/2.5 mM CaCl<sub>2</sub>/60 mg/ml BSA). Thrombin generation curves were recorded continuously with a microtiter-plate fluorometer (Fluoroskan Ascent, Thermo Labsystems, Helsinki, Finland) under control of Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands), filtered for excitation at 390 nm and emission at 460 nm. A calibrator assay was done using FVIII deficient plasma included on wells with a 20-µl solution that contained a known concentration of thrombin- $\alpha$ 2-macroglobulin complex (thrombin calibrator, Stago, France). In total, four to six experiments were performed in duplicate at three different dates (with 1-2 experiments/day) for each cell-type. Control experiments using normal pooled plasma were performed to establish reproducibility of the cell-based thrombin generation assay. Intra-assay coefficients of variability (CV) for hVSMCs based on 30 values were 6.1%, 2.5%, 6.4% and 5.5% for lag-time, endogenous thrombin potential (ETP), peak height and time to peak, respectively. Inter-assay CV for hVSMCs based on 35 values were 13.0%, 8.6%, 10.8% and 7.9% for lag-time, endogenous thrombin potential (ETP), peak height and time to peak, respectively. Intra-assay coefficients of variability (CV) for hAECs based on 30 values were 5.8%, 7.6%, 8.1% and 6.0% for lag-time, endogenous thrombin potential (ETP), peak height and time to peak, respectively. Inter-assay CV for hAECs

based on 35 values were 10.3%, 9.9%, 13.7% and 11.8% for lag-time, endogenous thrombin potential (ETP), peak height and time to peak, respectively.

### **FX activation**

Phospholipid vesicles (60% phosphatidylcholine, 20% phosphatidylserine, 20% phosphatidylethanolamine) were incubated in 25 mM Hepes pH 7.2, 120 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 0.1% BSA with FIXa in the absence or presence of FIX for 10 min at 37°C in 60% of the final volume. We then added FVIII in the presence of thrombin or emicizumab. After 1 min, FXa generation was initiated by the addition of the substrate FX and hirudin (to inactivate thrombin). At this point, volume was at 100% for all incubations, with the final concentrations being: phospholipids 50 nM or 1 μM, 9 nM FIXa, 90 nM FIX, 0.1 nM FVIII, 5 nM thrombin, 50 μg/ml emicizumab, 120 nM FX and 2 U/ml hirudin. At various times after initiation, samples were taken and FXa generation was quenched by the addition of EDTA (10 mM), and FXa concentrations were determined using the synthetic substrate pNAPEP-1065. An active-site titrated FXa preparation was used to relate substrate hydrolysis to FXa concentrations.

### **Data analysis**

All data were presented as mean ± standard deviation (SD) unless stated otherwise.

The statistical analyses were performed using Prism-9 for MacOS (Graphpad Software; San Diego, USA) as specified in the legends.

## Results

### *Characterization of vascular cells*

Two types of human cells of aortic origin were used in this study: endothelial cells (hAECs) and vascular smooth muscle cells (hVSMCs). Flow cytometric analysis showed that hAECs were positive for thrombomodulin, TFPI and EPCR, whereas hVSMCs only proved positive for EPCR (Fig. 1). Quantitative analysis further revealed that hVSMCs displayed about 10-fold more TF antigen than hAECs ( $1.1 \pm 0.12$   $\mu\text{g}/\text{mg}$  cell protein versus  $0.13 \pm 0.04$   $\mu\text{g}/\text{mg}$  cell protein), whereas TF activity was approximately 2-fold higher in hVSMCs compared to hAECs ( $19 \pm 3$  pM vs  $8 \pm 1$  pM; Table 1). Both cell types expressed similar levels of phosphatidylserine (PS)-equivalent ( $34 \pm 7$  nM vs  $53 \pm 20$  nM; Table 1). With regard to anti-coagulant proteins, hVSMCs exposed low levels of thrombomodulin and TFPI just above the limit of detection ( $1 \pm 1$  ng/mg cell protein and  $0.04 \pm 0.01$   $\mu\text{g}/\text{mg}$  cell protein), much less than in hAECs ( $9 \pm 1$  ng/mg cell protein and  $0.31 \pm 0.04$   $\mu\text{g}/\text{mg}$  cell protein, respectively; Table 1).

A stimulation of hAECs by  $\text{TNF}\alpha$  (5 nM; 4 hours) resulted in an 11-fold increase in TF antigen (from  $0.13 \pm 0.04$   $\mu\text{g}/\text{mg}$  cell protein to  $1.4 \pm 0.1$   $\mu\text{g}/\text{mg}$  cell protein), with a relative minor increase in TF activity being noted ( $10 \pm 2$  pM). Furthermore, a 4-fold increase in PS-equivalent ( $197 \pm 99$  nM) was measured. Perhaps unexpectedly, an increase in thrombomodulin and TFPI expression was also observed ( $16 \pm 1$  ng/mg cell protein and  $0.47 \pm 0.04$   $\mu\text{g}/\text{mg}$  cell protein, respectively). These data suggest that the pro- and anticoagulant balance in hAECs remains seemingly unchanged upon stimulation by  $\text{TNF}\alpha$ . Furthermore, hVSMCs display a more procoagulant profile compared to hAECs, due to a lack of anticoagulant components thrombomodulin and TFPI.

### *Thrombin generation on vascular cells in FVIII-deficient plasma*

To investigate the effect of FVIII concentrations on thrombin generation at the surface of vascular cells, FVIII-deficient plasma was spiked with different concentrations of recombinant FVIII. No external phospholipids, TF or FXIa were added to the reactions, which were therefore dependent on the amounts of TF and phospholipids exposed by the cells. Thrombin generation was first determined in FVIII-deficient plasma alone to establish background values. In the absence of any addition, minimal amounts of thrombin were generated when using hAECs or  $\text{TNF}\alpha$ -stimulated hAECs, even when this was slightly increased in the stimulated cells (Fig. 2A-B). ETP values were  $35 \pm 9$  nM·min and  $120 \pm 45$  nM·min for hAECs and  $\text{TNF}\alpha$ -stimulated hAECs, respectively (see Table 2). At the surface of hVSMCs, thrombin generation was significantly higher (ETP  $768 \pm 70$  nM·min; Table 2; Fig. 2C), in keeping with the more procoagulant nature of these cells.



### *Thrombin generation on vascular cells in the presence of FVIII*

The addition of FVIII resulted in a dose-dependent increase in thrombin generation (Fig. 2A-C). Thrombin generation-related parameters (lag-time, thrombin peak and ETP) changed in a dose-dependent manner for all three cell-types (p-trend <0.05; Fig. 2D-L; summarized in Table 2). Between the lowest and highest concentration of FVIII tested (0.25 and 1.5 U/ml), the ETP increased from 1022±19 to 1475±112 nM·min on hAECs, from 574±71 to 1166±247 nM·min on TNF $\alpha$ -stimulated hAECs, and from 1079±65 to 1287±55 nM·min on hVSMCs. Thus, the extent of thrombin generation was dependent on the amount of FVIII for each of the cell-types investigated.

### *Thrombin generation on vascular cells in the presence of emicizumab*

In a next series of experiments, FVIII-deficient plasma was spiked with emicizumab (10-80  $\mu$ g/ml), knowing that a concentration of 50  $\mu$ g/ml represents the average therapeutic emicizumab level. The presence of 10  $\mu$ g/ml emicizumab did not increase thrombin generation above the levels of FVIII-deficient plasma when tested in TNF-stimulated hAECs (Fig. 3B). Concentrations of 25, 50 and 80  $\mu$ g/ml increased thrombin generation for all three cell types. Interestingly, the largest increase in thrombin generation was observed for non-stimulated hAECs, increasing ETP from 35±9 nM·min to 686±195 nM·min for emicizumab at 50  $\mu$ g/ml (Fig. 3A; Table 3). Increased thrombin generation was also observed for TNF $\alpha$ -stimulated hAECs in the presence of 50  $\mu$ g/ml emicizumab, albeit to a lesser extent (ETP: from 120±45 nM·min to 262±59 nM·min). When added to FVIII-deficient plasma exposed on hVSMCs, emicizumab induced a less pronounced increase in thrombin generation as compared to the endothelial cells, although the absolute ETP was higher than for both other conditions. For example, the ETP increased from 768±70 nM·min to 900±38 nM·min at 50  $\mu$ g/ml emicizumab (Table 3). Together, this shows that, emicizumab is less efficient in comparison to FVIII in promoting thrombin generation at the surface of vascular cells. Nevertheless, it shares with FVIII that its capacity to enhance thrombin generation is dependent on cell type.

### *Effect of FIX on FIXa-mediated FXa generation*

The PS-equivalent on hVSMCs and hAECs is lower compared to the PS-equivalent used in traditional thrombin generation assays (30-200 nM versus 1.6  $\mu$ M). We tested therefore if the limiting phospholipid-surface affects FXa-generation by FIXa/FVIIIa or FIXa/emicizumab in the absence or presence of FIX using purified proteins. With regard to the FIXa/FVIIIa complex, FXa generation was unaffected by the presence of FIX, irrespective whether a low or high PS-equivalent was used (Fig. 4). In contrast, when FIXa was combined with emicizumab, it appeared that FXa generation was more efficient in the absence compared to the presence of

FIX ( $1.0 \pm 0.2$  nM/min vs  $2.2 \pm 0.5$  nM/min;  $p < 0.0001$ ; Figure 5). This inhibitory effect of FIX was elevated when using 1  $\mu$ M PS-equivalent ( $33 \pm 8$  nM/min vs  $32 \pm 4$  nM/min;  $p = 0.73$ ). Apparently, under conditions where phospholipids are limiting, the presence of FIX reduces the efficiency by which the FIXa/emicizumab complex is able to generate FXa.

#### *Thrombin generation on different endothelial cells*

Since endothelial cells may vary in phenotype, we tested thrombin generation also using two other endothelial cells: hUVECs and hMVECs. Levels of thrombomodulin, TFPI, EPCR, TF activity, TF antigen and PS-equivalent were similar as for the unstimulated hAECs (Table 1; Supplementary Figure S1). In addition, thrombin generation was in the same order of magnitude for these cells, irrespective whether FVIII (0.25 or 1.0 U/ml) or emicizumab (50  $\mu$ g/ml) was used (Supplementary Table S1). This suggests that the data obtained using hAECs might be extrapolated to various other types of endothelial cells.

#### *Thrombin generation on vascular cells in the presence of both FVIII and emicizumab*

We next investigated the effect of combined FVIII and emicizumab on thrombin generation using the same three cell-types. FVIII-deficient plasma spiked with both FVIII (1.0 U/ml) and emicizumab (50  $\mu$ g/ml) generated thrombograms that were similar to those of FVIII alone, irrespective of the cell types on which thrombin generation was analyzed (Fig. 5; Table 3; Supplementary Table 1). Apparently, the contribution of emicizumab to thrombin generation in the presence of physiological FVIII concentrations is limited, if present at all under these conditions.

#### *Thrombin generation of vascular cells in the presence of emicizumab and aPCC*

Based on previous findings that combining emicizumab and aPCC have a synergistic effect on thrombin generation when using synthetic phospholipids<sup>25</sup>, it was of interest to evaluate their combination on vascular cells as well. The addition of aPCC alone (1 U/ml) increased thrombin generation on non-stimulated and TNF $\alpha$ -stimulated hAECs (ETP: from  $35 \pm 9$  nM·min and  $120 \pm 45$  nM·min to  $575 \pm 36$  nM·min and  $794 \pm 108$  nM·min, respectively; Fig. 5; Table 3; Supplementary Table 1). On hVSMCs, the addition of aPCC resulted in a minor increase in thrombin generation (ETP: from  $768 \pm 70$  nM·min to  $845 \pm 70$  nM·min; Fig. 5; Table 3). However, combining aPCC (1 U/ml) with emicizumab (50  $\mu$ g/ml) resulted in a massive increase in thrombin generation on each of these three cell types. ETP values were  $2087 \pm 332$  nM·min,  $2704 \pm 135$  nM·min and  $2623 \pm 39$  nM·min for hAECs, TNF $\alpha$ -stimulated hAECs and hVSMCs, respectively. These data confirm that the combination of aPCC and emicizumab provokes

exaggerated thrombin generation, and that this process may also occur at the surface of vascular cells.

## Discussion

We have analyzed the capacity of human aortic vascular cells (smooth muscle cells (hVSMCs) and endothelial cells (hAECs)) to support thrombin generation in the presence of FVIII and/or emicizumab. We observed that independent of the cell type analyzed, low concentrations of FVIII (0.25 U/ml) are more efficient than emicizumab to enhance thrombin generation. Adding emicizumab to FVIII at physiological concentrations (1 U/ml) does not seem to further increase thrombin generation. However, combining emicizumab with aPCC resulted in markedly increased thrombin generation, similar as what has previously been observed when using synthetic phospholipid vesicles.

For this study, primary cells were used originating from the aorta, isolated from at least three different individuals. Characterization of the hAECs and hVSMCs revealed a clear difference in balance between pro- and anti-coagulant proteins, with TFPI and thrombomodulin being virtually absent in hVSMCs. The lack of anticoagulant potential was visible when comparing the background thrombin generation using FVIII-deficient plasma: the ETP was 20-fold higher in hVSMCs compared to hAECs. These data are illustrative for the interest of exploring cell-based thrombin generation assays compared to the standard assays that use synthetic phospholipids. The extent of phospholipid-exposure and the absence or presence of pro- and anticoagulant cellular components may modulate FVIII- and emicizumab-dependent thrombin generation. This was particularly apparent when testing similar TF concentrations (10 pM) in combination with synthetic phospholipids. FVIII-deficient plasma, FVIII (0.25 or 1 U/ml) and emicizumab all generated similar amounts of thrombin (Supplementary Table S3). Indeed, there is sufficient TF/FVIIa complex to bypass FIXa-dependent FX activation under these conditions and cellular anticoagulant components are absent to dampen this process. Together, these data provide insight into how local conditions (the balance between pro- and anti-coagulant components) within the vascular tree are of relevance to control thrombin generation

TNF $\alpha$ -mediated stimulation of hAECs resulted in a 15-fold increase in TF antigen and a 1.8-fold increase in PS-exposure. Nevertheless, thrombin generation remained very low in FVIII-deficient plasma, with peak levels being  $3\pm 1$  nM and  $4\pm 1$  nM for non-stimulated and TNF $\alpha$ -stimulated hAECs, respectively. In the absence of FVIII or emicizumab, a minor increase in ETP for the stimulated cells was observed, which is most likely due to the increased phospholipid-exposure. Increased phospholipid-exposure also allowed for a more sensitive response to different doses of FVIII. In non-stimulated hAECs ( $53\pm 20$  nM PS-equivalent), no significant difference in thrombin generation parameters were observed between 0.25 and 1.0

U/ml. In contrast, a significant difference in peak height ( $p=0.006$ ), ETP ( $p=0.046$  and time to peak ( $p=0.0013$ ) was observed for  $\text{TNF}\alpha$ -stimulated hAECs.

Unexpectedly, when FVIII-deficient plasma was spiked with FVIII or emicizumab, thrombin generation on  $\text{TNF}\alpha$ -stimulated hAECs was consistently lower compared to non-stimulated hAECs. A potential explanation is that despite the 15-fold increase in TF antigen, TF activity was only minorly increased, from 8 to 10 pM. Indeed, TF can exist in an active or a cryptic non-functional form, and it has been reported that the majority of the TF exposed at the cellular surface is in its cryptic conformation.<sup>26</sup> It seems conceivable that the majority of the newly synthesized TF is in its cryptic conformation and does not contribute to coagulation. A second, not mutually exclusive explanation is that the increased pro-coagulant potential is compensated by the increased anti-coagulant potential by virtue of increased TFPI and thrombomodulin levels. In particular, the increased TFPI availability may interfere with FVIIa and FXa activity on the  $\text{TNF}\alpha$ -stimulated hAECs. It is tempting to speculate that the increased anti-coagulant potential may exist to avoid exaggerated coagulation to occur under conditions of inflammation.

An intriguing question that came up during the study was whether or not the use aortic endothelial cells would be representative for other endothelial cell types. In particular, expression of thrombomodulin may differ between tissues, as for instance its expression is particular limited in brain tissue.<sup>27</sup> However, when testing additional endothelial cell types (hUVECs and hMVECs), we observed that not only thrombomodulin levels, but also TFPI, TF and phosphatidyl-serine exposure was similar to those of hAECs. The thrombomodulin levels being similar is in keeping with a previous report showing that thrombomodulin expression is not controlled by vessel type, but rather that thrombomodulin expression is highest on continuous endothelium and reduced on fenestrated or sinusoidal endothelium.<sup>28</sup> In addition, similar thrombin generation data were observed by using these cells. This suggests that our observations seem to be representative for a large portion of the endothelial cell population throughout the vascular tree.

Under all conditions tested, emicizumab promoted significantly lower thrombin generation than FVIII (25%-150% rFVIII), whether it be on hVSMCs or hAECs (Supplementary Table S2). This seems in contrast to results obtained using classic thrombin generation assays using synthetic phospholipids, in which the addition of emicizumab to hemophilia A plasma nearly or completely normalizes the ETP.<sup>20,21</sup> Classic thrombin generation assays differ from cell-based thrombin generation assays by the absence of cellular components (cell-derived TFPI and thrombomodulin) and the different concentrations of TF and/or factor XIa that are used to

trigger thrombin generation. It is further important to mention that the PS-equivalent on the vascular cells used in this study is markedly lower compared to regular thrombin generation assays: 30-200 nM *versus* 1.6  $\mu$ M, suggesting that the phospholipid-surface may become a limiting factor in the formation of the enzyme/substrate complex. Indeed, levels of FIX will exceed those of FIXa (probably in a 10:1 ratio, with FIX levels being 80-100 nM), and the affinities of both for PS-containing phospholipids are 15-20 nM.<sup>29,30</sup> It is likely that FIX rather than FIXa will occupy most of the available binding sites in the absence of FVIIIa. In contrast, FIXa binds with higher affinity (about 2 nM) in the presence of FVIIIa (which itself binds phospholipids with an affinity of <1 nM), and will display preferential binding over FIX. In addition, the presence of both FVIIIa and FIXa reduces the affinity of FX for phospholipids about 10-fold.<sup>30</sup> Emicizumab differs from FVIIIa in that it will not enhance binding of FIXa over FIX to the limited phospholipid-surface, which will therefore result in less efficient FXa generation compared to what is seen in traditional thrombin generation assays. This explanation is supported by our experiments using purified proteins (Figure 5). At low PS-equivalent (50 nM), FIX reduced the efficiency of FIXa/emicizumab-mediated FXa generation but not that of FIXa/FVIIIa-mediated FXa generation. At high PS-equivalent (1  $\mu$ M), the presence of FIX did not affect FXa generation by either complex. Similar observations were made when we analyzed thrombin generation under conditions of low and high PS-equivalent (Supplementary table S3).

Several studies have evaluated the effect of aPCC on thrombin generation in the presence of emicizumab.<sup>19-22,31</sup> Consistent findings in these studies is the exaggerated thrombin formation when combining emicizumab and aPCC. A similar synergistic effect of aPCC and emicizumab has also been observed in our study. The mechanism by which aPCC enhances emicizumab-mediated thrombin formation could be multiple: First, increased concentrations of the substrate of the prothrombinase-complex, i.e. prothrombin, promote thrombin generation.<sup>32</sup> Second, by increasing concentrations of FIX and FX, more ternary complex with emicizumab can be formed.<sup>33</sup> Third, aPCC contains substantial concentrations of FIXa, which will also result in higher concentrations of the active ternary complex. One question that is of course relevant to address is whether such synergistic thrombin generation also occurs when combining aPCC and emicizumab *in vivo*. Recently, Kizilocak and colleagues administered aPCC (up to 75 U/kg) to hemophilia A patients who were treated with emicizumab. Twenty minutes after infusion, blood samples were taken, and plasma samples were analyzed for thrombin generation. In none of the samples tested, exaggerated thrombin generation was observed, in contrast to samples from the same patients that were spiked with aPCC *in vitro*.<sup>34</sup> This can potentially be explained by the rapid elimination of FIXa when infused into the circulation.<sup>35</sup> Not

only is FIXa rapidly inhibited by antithrombin, but both the FIXa/antithrombin complex and free FIXa are ligands for the clearance-receptor LRP1.<sup>36</sup>

If the FIXa component of aPCC is rapidly cleared, how then to explain the development of thrombotic microangiopathy in patients that received aPCC in combination with emicizumab?<sup>12</sup> An important difference between the Haven-1 study (in which those complications were first described) and the study by Kizilocak *et al.*, is that the patients who developed thrombotic microangiopathy not only received higher doses of aPCC (100 U/kg) but also multiple doses over a 24h-period.<sup>12</sup> This may have resulted in an accumulation of FIXa. It should also be noted that not all patients developed such complications, suggesting that perhaps other factors have played a role. In our study, we observed that emicizumab-mediated thrombin generation in the presence of aPCC was particularly amplified when using TNF $\alpha$ -stimulated hAECs (10-fold) compared to non-stimulated hAECs and hVSMCs (both 3-fold). It is possible therefore that an inflammatory status of endothelial cells could have contributed to the amplifying effect of aPCC when added to emicizumab.

A limitation of our study is that we have not investigated the role of another anticoagulant component in this study, *i.e.* heparan sulphate proteoglycans, which may promote antithrombin-mediated inhibition of FIXa, FXa and thrombin. The presence of these molecules may differ between cell types, and thereby their capacity to inhibit the activity of these proteases.

In conclusion, by testing thrombin generation on primary vascular cells, we have found that hAECs that line the aortic wall display a low procoagulant potential, mainly due to the presence of sufficient anticoagulant components, like thrombomodulin and TFPI. This is in line with previous findings that the FIXa/FVIIIa complex plays a little, if any, role in the basal activation of coagulation *in vivo*, but is relevant for thrombin generation upon injury.<sup>37</sup> Furthermore, we show that emicizumab shows lower procoagulant activity compared to FVIII on non-stimulated- and TNF $\alpha$ -stimulated hAECs as well as on hVSMCs.

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**Author contributions:** SA, CS, JL & VR performed experiments; PL, P.J.L., C.V.D., O.D.C. & VR designed and supervised the study; P.J.L. & O.D.C. wrote the manuscript. All authors contributed to the editing of the manuscript.



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## Figure legends

### **Figure 1: Cell-surface expression of TFPI, thrombomodulin and EPCR on hAECs and hVSMCs.**

Cell-surface expression of Tissue Factor Pathway Inhibitor (TFPI), Thrombomodulin (TM) and Endothelial Protein C-Receptor (EPCR) on non-stimulated human aortic endothelial cells (hAECs) and human aortic vascular smooth muscle cells (hVSMCs) was determined by flow cytometry. Shown are representative flow cytograms of 3-5 analyses. Red cytograms represent cells incubated with isotype-control antibodies, and blue cytograms represent cells incubated with TFPI-, TM- or EPCR-specific antibodies.

### **Figure 2: Thrombin generation in the presence of FVIII.**

Vascular cells (hAECs, TNF $\alpha$ -stimulated hAECs and hVSMCs) were incubated with FVIII-deficient plasma (black lines) supplemented or not with various concentrations of recombinant full-length FVIII (Pink: 0.25 U/ml; Green: 0.5 U/ml; Blue: 1.0 U/ml and Red: 1.5 U/ml) for 5 min before initiation of thrombin generation. Thrombin generation was initiated by the addition of CaCl<sub>2</sub>, without any other activating agents. Panels A-C depict representative thrombograms for each of the conditions tested. Panels D-F show the calculated lag-times, panels G-I the calculated thrombin peak and panels J-L the endogenous thrombin potential (ETP). P-trend was calculate using a one-way ANOVA test with trend analysis.

### **Figure 3: Thrombin generation in the presence of emicizumab.**

Vascular cells (hAECs, TNF $\alpha$ -stimulated hAECs and hVSMCs) were incubated with FVIII-deficient plasma (black lines) supplemented or not with various concentrations of emicizumab (Pink: 10  $\mu$ g/ml; Green: 25  $\mu$ g/ml; Blue: 50  $\mu$ g/ml and Red: 80  $\mu$ g/ml) for 5 min before initiation of thrombin generation. Thrombin generation was initiated by the addition of CaCl<sub>2</sub>, without any other activating agents. Panels A-C depict representative thrombograms for each of the conditions tested. Panels D-F show the calculated lag-times, panels G-I the calculated thrombin peak and panels J-L the endogenous thrombin potential (ETP). P-trend was calculate using a one-way ANOVA test with trend analysis. Data for FVIII-deficient plasma are similar to those presented in Figure 2.

### **Figure 4: FXa generation by FIXa/FVIIIa and FIXa/emicizumab in the absence or presence of FIX**

FX (120 nM) was activated by FIXa (9 nM) in the presence of FVIIIa (0.1 nM; panels A & B) or emicizumab (50  $\mu$ g/ml; panels C & D). Mixtures contained phospholipids (PS-equivalent 50 nM

for panels A & C or PS-equivalent 1  $\mu$ M for panels B & D). FXa generation was performed in the absence or presence of FIX (90 nM). FXa generation was quantified employing the synthetic substrate S-2222.

**Figure 5: Thrombin generation in the presence of emicizumab in combination with FVIII or aPCC**

Vascular cells (hAECs, TNF $\alpha$ -stimulated hAECs and hVSMCs) were incubated with FVIII-deficient plasma (black lines) supplemented or not with emicizumab (Green: 50  $\mu$ g/ml), FVIII (Red: 1.0 U/ml), aPCC (Red: 1 U/ml) or with a combination of emicizumab with FVIII (Blue: 50  $\mu$ g/ml & 1.0 U/ml) or emicizumab with aPCC (Blue: 50  $\mu$ g/ml & 1.0 U/ml). Data for FVIII-deficient plasma, emicizumab and FVIII were taken from figures 2 & 3.

**Table 1: Expression of pro-and anticoagulant proteins in different cell types**

	hAECs				
	hVSMCs	Non-stimulated	TNF $\alpha$ -stimulated	hUVECs	hMVECs
TF antigen ( $\mu\text{g}/\text{mg}$ cell protein)	$1.1 \pm 0.1$ (n=7)	$0.11 \pm 0.08$ (n=10)	$1.4 \pm 0.10$ (n=6)	$0.11 \pm 0.09$ (n=6)	$0.13 \pm 0.09$
TF activity (pM)	$19 \pm 3$ (n=5)	$8 \pm 1$ (n=6)	$10 \pm 2$ (n=6)	$10 \pm 1$ (n=6)	$7 \pm 1$ (n=6)
PS-exposure (nM)	$34 \pm 7$ (n=18)	$53 \pm 20$ (n=14)	$197 \pm 99$ (n=12)	$65 \pm 6$ (n=6)	$53 \pm 5$ (n=6)
Thrombomodulin (ng/mg cell protein)	$1 \pm 1$ (n=7)	$9 \pm 1$ (n=11)	$16 \pm 1$ (n=6)	$9 \pm 1$ (n=6)	$8 \pm 1$ (n=6)
TFPI ( $\mu\text{g}/\text{mg}$ cell protein)	$0.04 \pm 0.01$ (n=4)	$0.31 \pm 0.04$ (n=4)	$0.47 \pm 0.04$ (n=4)	$0.35 \pm 0.03$ (n=6)	$0.33 \pm 0.03$ (n=6)

hAECs: human aortic endothelial cells; hVSMCs: human aortic vascular smooth muscle cells; hUVECs: human umbilical endothelial cells; hMVECs: human dermal microvascular endothelial cells; TF: tissue factor; PS-exposure: phosphatidylserine-exposure; TFPI: tissue factor pathway inhibitor. N represents number of independent wells have been analyzed for each of the components.

**Table 2: Summary of thrombin generation parameters in FVIII-deficient plasma spiked with FVIII**

	hVSMCs	hAECs	
		Non-stimulated	TNF $\alpha$ -stimulated
<b>FVIII</b>			
<i>Lag time, min</i>			
FVIII deficient plasma	12 $\pm$ 1 (n=6)	30 $\pm$ 4 (n=4)	26 $\pm$ 5 (n=4)
+ FVIII (0.25 U/ml)	12 $\pm$ 1 (n=6)	15 $\pm$ 4 (n=6)	14 $\pm$ 1 (n=6)
+ FVIII (0.5 U/ml)	11 $\pm$ 1 (n=6)	14 $\pm$ 2 (n=6)	15 $\pm$ 1 (n=6)
+ FVIII (1.0 U/ml)	10 $\pm$ 1 (n=6)	12 $\pm$ 2 (n=6)	13 $\pm$ 1 (n=6)
+ FVIII (1.5 U/ml)	11 $\pm$ 1(n=6)	10 $\pm$ 2 (n=6)	11 $\pm$ 2 (n=6)
<i>Peak height, nM</i>			
FVIII deficient plasma	27 $\pm$ 2 (n=6)	4 $\pm$ 1 (n=4)	3 $\pm$ 1 (n=4)
+ FVIII (0.25 U/ml)	45 $\pm$ 1 (n=6)	42 $\pm$ 10 (n=6)	20 $\pm$ 3 (n=6)
+ FVIII (0.5 U/ml)	50 $\pm$ 1 (n=6)	49 $\pm$ 8 (n=6)	23 $\pm$ 4 (n=6)
+ FVIII (1.0 U/ml)	57 $\pm$ 2 (n=6)	58 $\pm$ 16 (n=6)	30 $\pm$ 4 (n=6)
+ FVIII (1.5 U/ml)	61 $\pm$ 1 (n=6)	93 $\pm$ 8 (n=6)	62 $\pm$ 23 (n=6)
<i>Endogenous Thrombin Potential, nM·min</i>			
FVIII deficient plasma	771 $\pm$ 46 (n=6)	35 $\pm$ 9 (n=4)	120 $\pm$ 45 (n=4)
+ FVIII (0.25 U/ml)	1099 $\pm$ 36 (n=6)	1022 $\pm$ 191 (n=6)	574 $\pm$ 97 (n=6)
+ FVIII (0.5 U/ml)	1170 $\pm$ 50 (n=6)	1015 $\pm$ 99 (n=6)	610 $\pm$ 85 (n=6)
+ FVIII (1.0 U/ml)	1179 $\pm$ 45 (n=6)	1085 $\pm$ 187 (n=6)	721 $\pm$ 125 (n=6)
+ FVIII (1.5 U/ml)	1279 $\pm$ 40 (n=6)	1475 $\pm$ 112 (n=6)	1166 $\pm$ 247 (n=6)
<i>Time to Peak, min</i>			
FVIII deficient plasma	26 $\pm$ 1 (n=6)	57 $\pm$ 4 (n=4)	52 $\pm$ 3 (n=4)
+ FVIII (0.25 U/ml)	24 $\pm$ 1 (n=6)	26 $\pm$ 5 (n=6)	28 $\pm$ 1 (n=6)
+ FVIII (0.5 U/ml)	23 $\pm$ 1 (n=6)	23 $\pm$ 3 (n=6)	27 $\pm$ 2 (n=6)
+ FVIII (1.0 U/ml)	19 $\pm$ 1 (n=6)	21 $\pm$ 4 (n=6)	24 $\pm$ 2 (n=6)
+ FVIII (1.5 U/ml)	19 $\pm$ 2 (n=6)	16 $\pm$ 2 (n=6)	19 $\pm$ 4 (n=6)

Mean and standard deviation (SD) of values for Lag time, Peak Height, Endogenous Thrombin Potential, and Time to Peak are shown.

**Table 3: Summary of thrombin generation parameters in FVIII-deficient plasma spiked with Emicizumab ± FVIII or aPCC.**

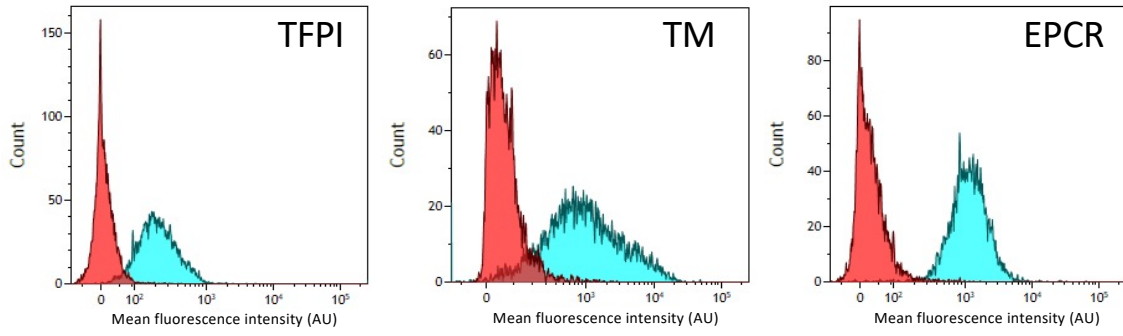
	hVSMCs	hAECs	
		Non-stimulated	TNF $\alpha$ -stimulated
<b>Emicizumab</b>			
<i>Lag time, min</i>			
emicizumab (10 $\mu$ g/ml)	NT	NT	30 $\pm$ 4 (n=6)
emicizumab (25 $\mu$ g/ml)	11 $\pm$ 1 (n=6)	26 $\pm$ 6 (n=6)	27 $\pm$ 7 (n=6)
emicizumab (50 $\mu$ g/ml)	12 $\pm$ 1 (n=6)	27 $\pm$ 9 (n=6)	26 $\pm$ 5 (n=6)
emicizumab (80 $\mu$ g/ml)	13 $\pm$ 1 (n=6)	24 $\pm$ 6 (n=6)	23 $\pm$ 4 (n=5)
FVIII (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	12 $\pm$ 1 (n=6)	14 $\pm$ 3 (n=6)	13 $\pm$ 1 (n=6)
aPCC (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	8 $\pm$ 1 (n=6)	7 $\pm$ 1 (n=6)	6 $\pm$ 1 (n=6)
<i>Peak height, nM</i>			
emicizumab (10 $\mu$ g/ml)	NT	NT	2 $\pm$ 1 (n=6)
emicizumab (25 $\mu$ g/ml)	30 $\pm$ 1 (n=6)	12 $\pm$ 5 (n=6)	4 $\pm$ 1 (n=6)
emicizumab (50 $\mu$ g/ml)	32 $\pm$ 1 (n=6)	15 $\pm$ 6 (n=6)	5 $\pm$ 1 (n=6)
emicizumab (80 $\mu$ g/ml)	32 $\pm$ 1 (n=6)	19 $\pm$ 7 (n=6)	5 $\pm$ 2 (n=5)
FVIII (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	54 $\pm$ 13 (n=6)	57 $\pm$ 8 (n=6)	27 $\pm$ 3 (n=6)
aPCC (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	151 $\pm$ 6 (n=6)	121 $\pm$ 12 (n=6)	128 $\pm$ 4 (n=6)
<i>Endogenous Thrombin Potential, nM·min</i>			
emicizumab (10 $\mu$ g/ml)	NT	NT	99 $\pm$ 25 (n=6)
emicizumab (25 $\mu$ g/ml)	820 $\pm$ 45 (n=6)	573 $\pm$ 131 (n=6)	177 $\pm$ 57 (n=6)
emicizumab (50 $\mu$ g/ml)	900 $\pm$ 38 (n=6)	686 $\pm$ 195 (n=6)	262 $\pm$ 59 (n=6)
emicizumab (80 $\mu$ g/ml)	955 $\pm$ 50 (n=6)	793 $\pm$ 211 (n=6)	247 $\pm$ 68 (n=5)
FVIII (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	1163 $\pm$ 35 (n=6)	1355 $\pm$ 217 (n=6)	713 $\pm$ 74 (n=6)
aPCC (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	2632 $\pm$ 34 (n=6)	2087 $\pm$ 332 (n=6)	2704 $\pm$ 135 (n=6)
<i>Time to Peak, min</i>			
emicizumab (10 $\mu$ g/ml)	NT	NT	52 $\pm$ 4 (n=6)
emicizumab (25 $\mu$ g/ml)	25 $\pm$ 1 (n=6)	49 $\pm$ 11 (n=6)	49 $\pm$ 9 (n=6)
emicizumab (50 $\mu$ g/ml)	27 $\pm$ 1 (n=6)	48 $\pm$ 13 (n=6)	43 $\pm$ 7 (n=6)
emicizumab (80 $\mu$ g/ml)	28 $\pm$ 1 (n=6)	43 $\pm$ 11 (n=6)	44 $\pm$ 6 (n=5)
FVIII (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	22 $\pm$ 1 (n=6)	22 $\pm$ 4 (n=6)	24 $\pm$ 1 (n=6)
aPCC (1.0 U/ml) & emicizumab (50 $\mu$ g/ml)	15 $\pm$ 1 (n=6)	12 $\pm$ 1 (n=6)	12 $\pm$ 1 (n=6)

Mean and standard deviation (SD) of values for Lag time, peak height, Endogenous Thrombin Potential, and time to peak are shown. NT: not tested



Figure 1  
Atsou et al

hAECs



hVSMCs

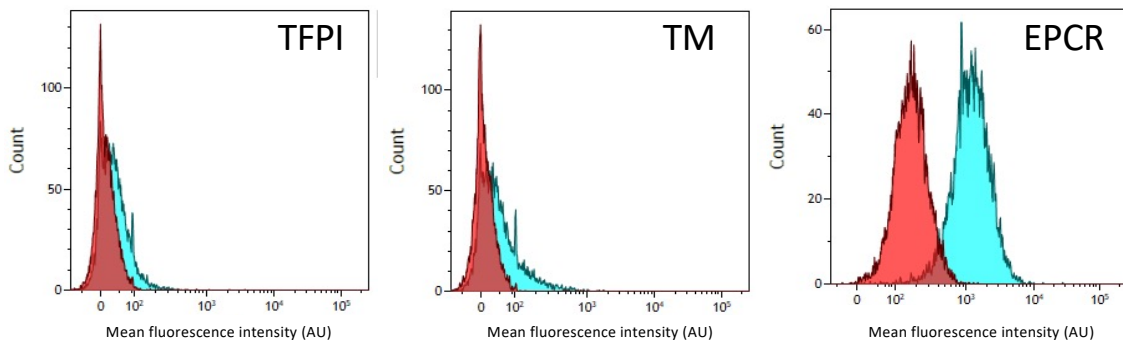


Figure 2  
Atsou et al

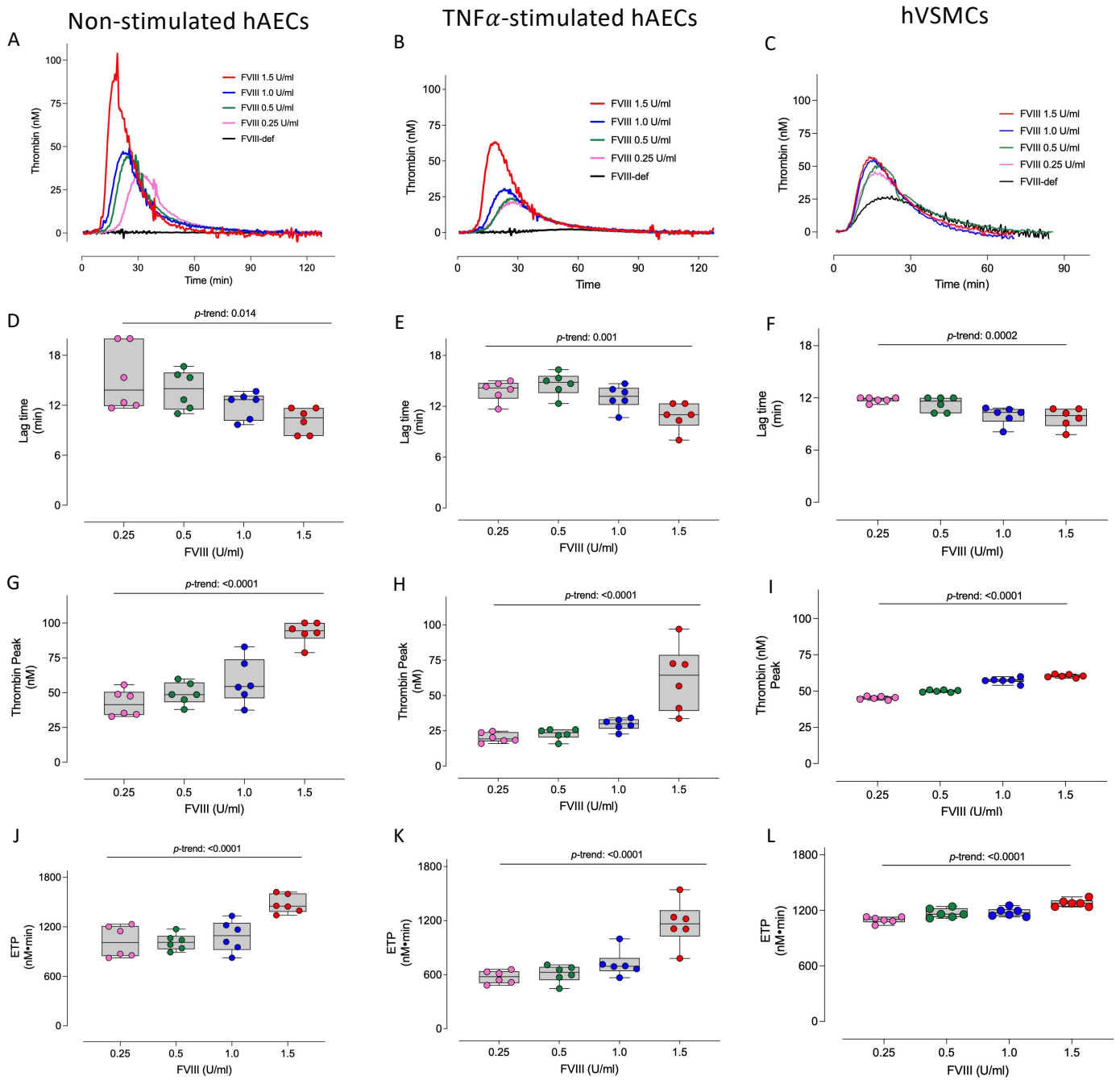


Figure 3  
Atsou et al

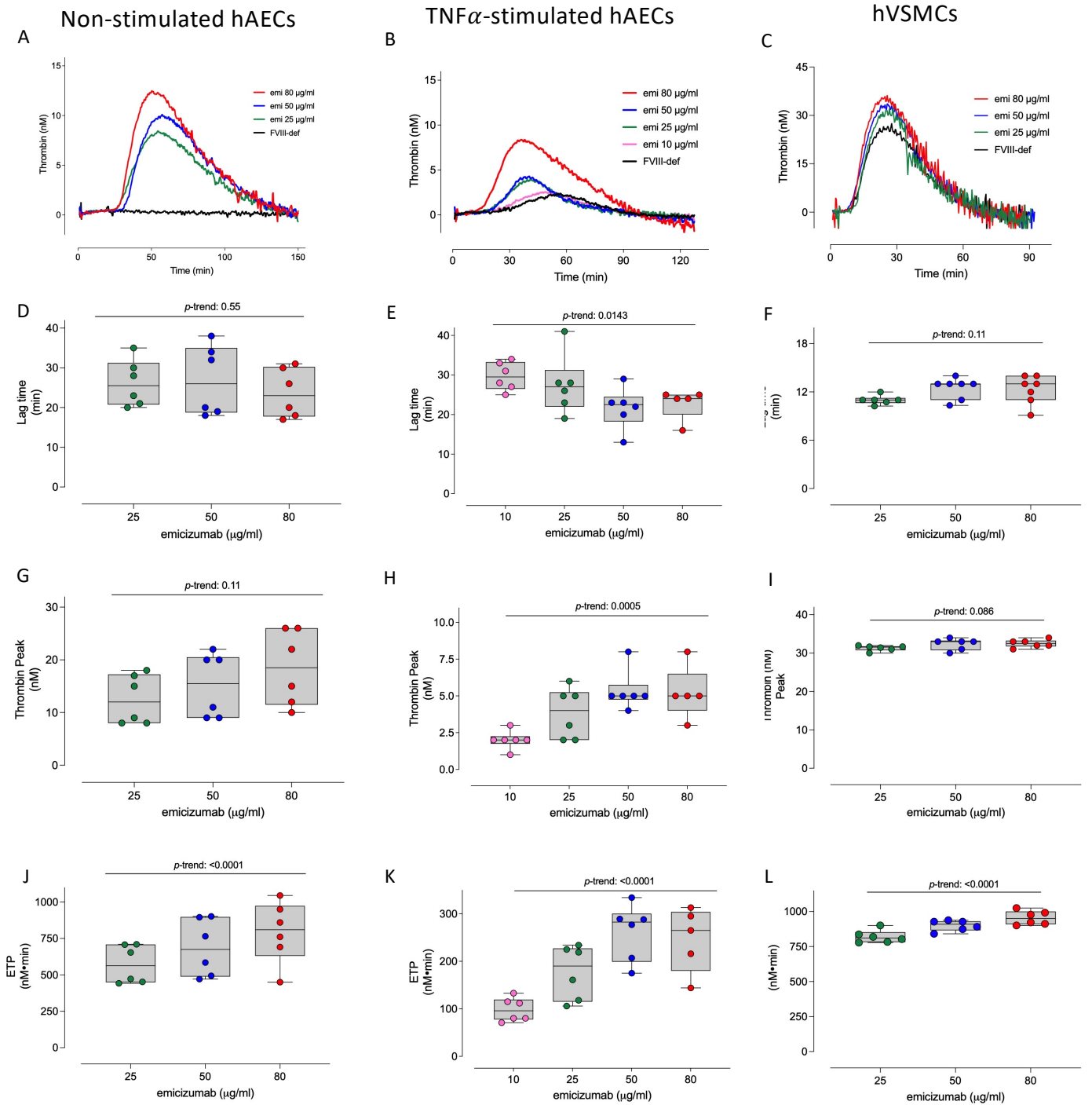


Figure 4  
Atsou et al

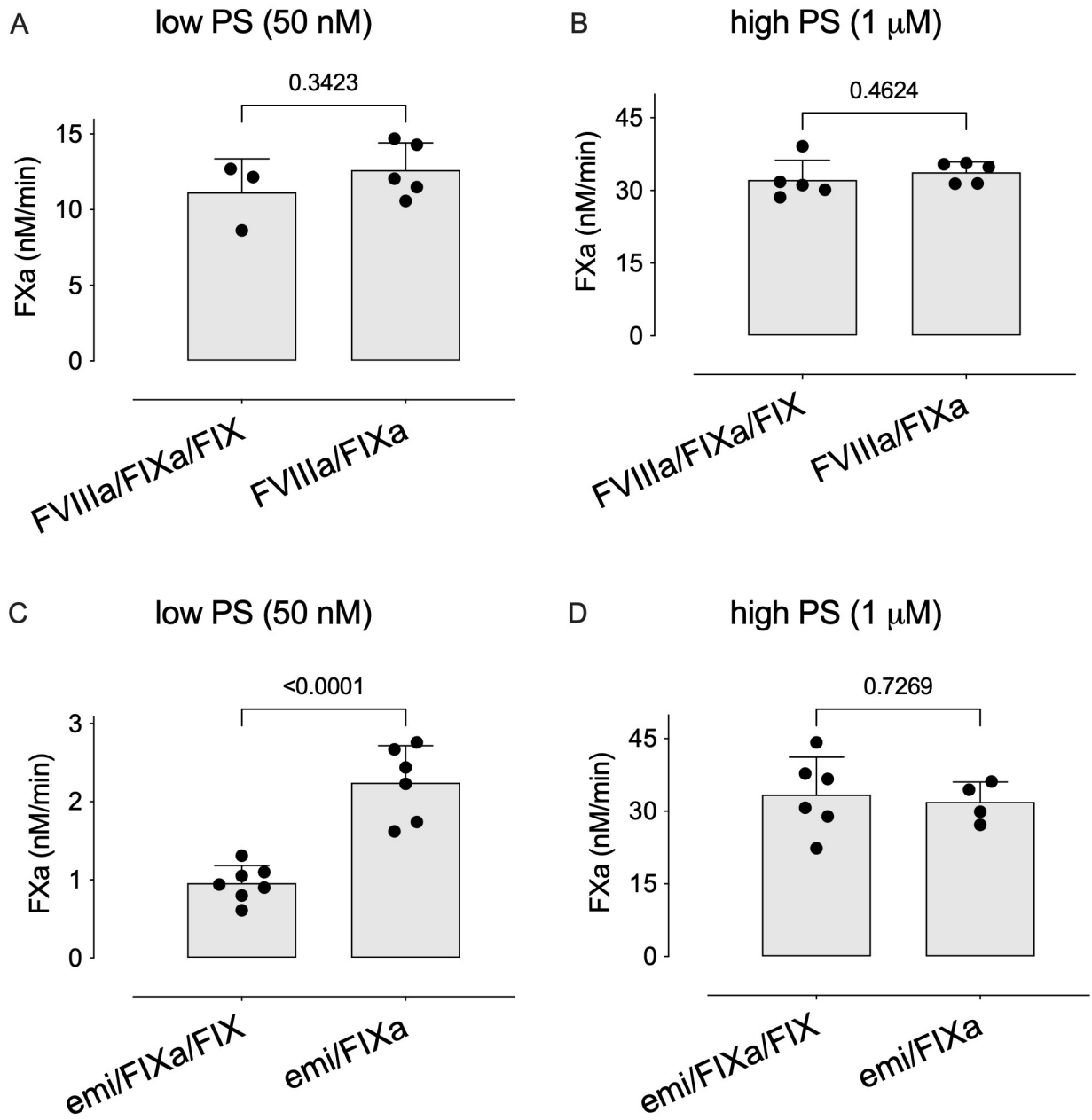


Figure 5  
Atsou et al

