



**HAL**  
open science

## Direct oral anticoagulants are associated with limited damage of endothelial cells of the blood-brain barrier mediated by the thrombin/PAR-1 pathway

Clémentine Puech, Xavier Delavenne, Zhiguo He, Valérie Forest, Patrick Mismetti, Nathalie Perek

### ► To cite this version:

Clémentine Puech, Xavier Delavenne, Zhiguo He, Valérie Forest, Patrick Mismetti, et al.. Direct oral anticoagulants are associated with limited damage of endothelial cells of the blood-brain barrier mediated by the thrombin/PAR-1 pathway. *Brain Research*, 2019, 1719, pp.57-63. 10.1016/j.brainres.2019.05.024 . hal-02309490

**HAL Id: hal-02309490**

**<https://hal.science/hal-02309490v1>**

Submitted on 9 Oct 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Direct oral anticoagulants are associated with limited damage of endothelial cells of the blood-brain barrier mediated by the thrombin/PAR-1 pathway**

**Clémentine Puech<sup>a,b,\*</sup>, Xavier Delavenne<sup>a,b,c</sup>, Zhiguo He<sup>b,d</sup>, Valérie Forest<sup>e</sup>, Patrick Mismetti<sup>a,b,f</sup>, Nathalie Perek<sup>a,b</sup>,**

<sup>a</sup> INSERM, U1059 Sainbiose, Dysfonction Vasculaire et Hémostase, Saint-Etienne, France

<sup>b</sup> Université de Lyon, Saint-Etienne, F-42023, France

<sup>c</sup> Laboratoire de Pharmacologie Toxicologie, CHU Saint-Etienne, F-42055 Saint-Etienne, France

<sup>d</sup> EA 2521, Biologie, Ingénierie et Imagerie de la Greffe de Cornée (BIIGC), Saint-Etienne, France

<sup>e</sup> Mines Saint-Etienne, Univ Lyon, Univ Jean Monnet, INSERM, U 1059 Sainbiose, Centre CIS, F-42023 Saint-Etienne, France

<sup>f</sup> Unité de Recherche Clinique Innovation et Pharmacologie, CHU de Saint-Etienne, F-42055 Saint Etienne, France

\* Corresponding author: Clémentine Puech e-mail: [c.puech@univ-st-etienne.fr](mailto:c.puech@univ-st-etienne.fr)

Postal address: 10 rue de la Marandière BP 80019, Faculté de Médecine, 42270 Saint-Priest-en-Jarez, France

Tel: (+33)4.77.42.14.43

## **Abstract**

Anticoagulant therapy presents iatrogenic effects such as intracerebral hemorrhage (ICH). The latest anticoagulants on the market, direct oral anticoagulants (DOACs) such as apixaban, dabigatran and rivaroxaban, are reported to cause less ICH than other anticoagulants. Next to the ICH area, the thrombin is accumulated and the blood-brain barrier (BBB) is opened. The effects of thrombin on the BBB are largely mediated by the protease activated receptor (PAR) family, especially the PAR-1 isoform. Our hypothesis is that DOACs may limit the effects of thrombin on endothelial cells (of the BBB) alteration by a mechanism probably involving PAR-1 activation. To test this hypothesis *in vitro*, we used HBEC-5i human brain endothelial cells as a human BBB model. The effects of thrombin under warfarin, heparin, rivaroxaban, apixaban, and dabigatran treatment on endothelial cells were then investigated by measuring of permeability and junction proteins: ZO-1 and VE-cadherin expressions and PAR-1 cleavage.

Depending on the anticoagulant used, we observed three profiles of response of the endothelial cells after thrombin exposure: i) dabigatran treatment allowed maintaining the tightness of the endothelial monolayer; ii) other DOACs limited thrombin-induced alteration of the endothelial monolayer; and iii) pretreatment with warfarin and heparin did not protect from thrombin-induced BBB breakdown. Pretreatment with DOACs clearly limited the impact of thrombin on PAR-1 cleavage in our model, contrary to other anticoagulants, associated with ZO-1 and VE-cadherin expressions. In conclusion, DOACs seem to limit the alteration of the monolayer of endothelial cells of the BBB mediated by the thrombin/PAR-1 pathway.

***Keywords: Endothelial cells monolayer; Blood-brain barrier; Direct oral anticoagulants; Protease activated receptor-1; Thrombin; VE-cadherin; ZO-1***

## 1. **Introduction**

In the last decade, direct oral anticoagulants (DOACs) have emerged as alternatives to other anticoagulants, such as vitamin K antagonists (VKAs) and low molecular weight heparin (LMWH), in the prevention of thromboembolic events. DOACs are reported to cause less intracerebral hemorrhage (ICH) effect than VKAs (Ezekowitz et al., 2007; Miller et al., 2012; Ruff et al., 2014). There are two types of DOACs: direct thrombin inhibitor (dabigatran) and factor Xa inhibitors (rivaroxaban and apixaban).

ICH is a known iatrogenic effect occurring under anticoagulation therapy. In the literature, phase III clinical studies have shown a decrease in intracranial bleeding under DOACs compared to VKAs (warfarin) in patients suffering from atrial fibrillation (Connolly et al., 2009; Granger et al., 2011; Ogawa et al., 2011). It has also been reported in an *in vivo* murine model that rivaroxaban and dabigatran prevented hemorrhage in comparison to warfarin (Ploen et al., 2014; Sun et al., 2013). In ICH, thrombin, a known multifunctional serine protease and a fundamental component in the coagulation cascade to produce fibrin clots, has been largely involved. The activity of thrombin is the target of anticoagulant therapy.

During ICH, the blood-brain barrier (BBB) disruption and more precisely the alteration of the endothelial monolayer is an important contributor to brain hematoma expansion. The BBB is a physical and metabolic barrier which separates the central nervous system (CNS) from the blood circulation (Arai et al., 2006; Ishida et al., 2006; Yin et al., 2010). The BBB is composed of endothelial cells, pericytes, astrocytes, and neurons, the whole forming the neurovascular unit. Endothelial cells are the main components of the BBB because they restrict and control the passage of molecules inside and outside the brain. During ICH, the level of thrombin has been shown to increase and provoke disruption of endothelial cells of the BBB (Brailoiu et al., 2017; Hawkins et al., 2015).

Integrity barrier properties are ensured by the close opposition between brain microvascular cells. These properties are mainly provided by tight junction proteins, with the most important in the BBB being zonula occludens1 (ZO-1). As with other vascular barriers, thrombin significantly compromises BBB integrity, mediates ZO-1 decrease associated with damage to an adherent junction protein specific of vascular cells, i.e., vascular endothelial cadherin (VE-cadherin) (Festoff et al., 2016; Rabiet et al., 1996).

The effects of thrombin are largely mediated by the protease activated receptor (PAR) family composed of several isoforms, PAR-1 to 4. It has been reported that thrombin exposure

causes barrier disruption via activation of PAR-1 (Cheng et al., 2014; Guan et al., 2004; Xi et al., 2003).

If clinical studies have shown that DOACs induced fewer ICHs than VKAs (Miller et al., 2012; Ogawa et al., 2011; Ruff et al., 2014), the cellular mechanisms have not yet been clearly established for human BBB. Hawkins et al. (2015) demonstrated that a 24-h pretreatment with dabigatran limited the effects of thrombin on an *in vitro* rodent BBB model (Hawkins et al., 2015). It has also been described that thrombin exposure activates PAR-1 receptor and provokes BBB disruption. As dabigatran is a direct thrombin inhibitor, we could suppose that dabigatran limits PAR-1 activation and prevents BBB disruption (Chen et al., 2015).

In this study, our hypothesis was that DOACs may limit the effects of thrombin on brain endothelial cells by a mechanism probably involving PAR-1 activation. To investigate the effects of anticoagulant agents on thrombin-induced damage to human BBB, HBEC-5i human brain endothelial cells were used as a BBB model. Several DOACs, i.e., dabigatran (direct thrombin inhibitor), rivaroxaban and apixaban (direct Xa factor inhibitor), were compared to warfarin and heparin, two indirect inhibitors of thrombin.

Firstly, pretreatment with different anticoagulants were performed prior to the addition of thrombin. Trans endothelial electrical resistance (TEER) and apparent permeability ( $P_{app}$ ) were investigated. Secondly, we evaluated the activation of the receptor of thrombin, PAR-1, and the expression of VE-cadherin and ZO-1 junction proteins.

## **2 Results**

### **2.1 Impact on the tightness**

Cells were treated with or without thrombin (1-5-10 U/mL) for 1 h. TEER,  $P_{app}$  with Na-Fl and dextran-FITC were evaluated (Figure 1). The highest thrombin concentration (10U/mL) decreased TEER by 26% (Figure 1A) and increased  $P_{app}$  5-fold to Na-Fl or dextran-FITC compared to control (Figure 1B-C). Our results clearly demonstrated that thrombin produced an alteration of the endothelial cell monolayer, the TEER and  $P_{app}$  values were modified in a concentration-dependent manner. Pretreatment effects with anticoagulant alone on endothelial cells of the BBB showed no effects on TEER and permeability values (Figure 1CD).

We then evaluated the pretreatment of anticoagulant during 24h with the highest thrombin concentration of 10U/mL on the endothelial monolayer (Figure 2). Depending on the anticoagulant used, we observed three responses of the endothelial cells after thrombin exposure.

Dabigatran pretreatment allowed to maintain the BBB monolayer exposed to thrombin: TEER=  $40.3 \pm 1.7 \text{ } \Omega \cdot \text{cm}^2$  (Figure 2A) and  $P_{\text{app}} = 7.4 \pm 0.8 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  for Na-Fl and  $4.7 \pm 0.2 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  for dextran-FITC (Figure 2BC).

Treatment with other DOACs limited the alteration of endothelial cells induced by the thrombin. With apixaban and rivaroxaban, we obtained TEER values of  $36.2 \pm 0.7 \text{ } \Omega \cdot \text{cm}^2$  and  $37.2 \pm 0.7 \text{ } \Omega \cdot \text{cm}^2$  respectively (Figure 2A),  $P_{\text{app}}$  value for Na-Fl of  $16.9 \pm 1.17 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  and  $20.5 \pm 0.31 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  for Na-Fl respectively (Figure 2B). We obtained  $P_{\text{app}}$  value of  $12.4 \pm 0.8 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  and  $11.8 \pm 0.2 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  for dextran-FITC respectively (Figure 2C).

On the contrary, thrombin under treatment with warfarin and heparin provoked an alteration of endothelial cells, as represented by a low TEER ( $32.3 \pm 0.9$  and  $32.5 \pm 1.2 \text{ } \Omega \cdot \text{cm}^2$  respectively) (Figure 2A) and an increase in  $P_{\text{app}}$  ( $27.9 \pm 0.70 \times 10^{-6}$  and  $28.8 \pm 2.82 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  for Na-Fl (Figure 2B) and  $15.9 \pm 0.6 \times 10^{-6}$  and  $16.9 \pm 0.4 \times 10^{-6} \text{ cm} \cdot \text{s}^{-1}$  for dextran-FITC respectively (Figure 2C).

Our hypothesis was that the breakdown of the BBB is mediated by the modulation of the activation of the receptor PAR-1 by thrombin.

## 2.2 Thrombin/PAR-1 pathway

First, we used an activator (SFLLRNPNDKYEPF) and an inhibitor (vorapaxar) of PAR-1 to observe the effects on TEER and  $P_{\text{app}}$ .

Vorapaxar alone had no impact on TEER and permeability (Figure 3). Preincubation of vorapaxar blocked the effects of thrombin on the endothelial cells. Effectively, in this condition, we found the same result as the control for TEER (Figure 3A),  $P_{\text{app}}$  for Na-Fl (Figure 3B) and Dextran-FITC (Figure 3C). As expected, the use of the activator of PAR-1 induced an alteration of the endothelial monolayer, in the same manner as for the thrombin 10IU/mL exposure (Figure 3ABC).

Then we evaluated the release of the soluble fraction of PAR-1, since to be activated PAR-1 must be cleaved (Figure 4). The soluble fraction of PAR-1 increased after thrombin exposure in a dose-dependent manner (Figure 4A). The lowest concentration of thrombin allowed a cleavage of PAR-1 to  $196 \pm 21 \text{ pg}$  soluble fraction of PAR-1/ $\mu\text{g}$  protein; and this value was increased approximately 2.5 times with the highest concentration of thrombin to obtain a high cleavage value of  $476 \pm 6.4 \text{ pg}$  soluble fraction of PAR-1/ $\mu\text{g}$  protein.

Anticoagulants alone did not cleave PAR-1, as observed for the control condition (Figure 4B). Depending on the anticoagulant used, we observed three responses of PAR-1 cleavage after thrombin exposure (Figure 4C). Under dabigatran, we obtained a low cleavage:  $51.6 \pm 7.8$  pg soluble fraction of PAR-1/ $\mu$ g protein. Under rivaroxaban and apixaban, we observed a limited cleavage (half of thrombin alone values). Under heparin, the cleavage fraction was as important as that observed with thrombin exposure alone. Pretreatment with DOACs clearly limited the impact of thrombin on PAR-1 cleavage in our model.

### 2.3 Effect of thrombin under anticoagulant on brain endothelial cell junctions

We verified that decrease in BBB tightness was associated with an alteration of cell junctions, the most represented being ZO-1 and VE-cadherin. Thrombin exposure (10 U/mL) provoked a 60% decrease in ZO-1 and a 40% decrease in VE-cadherin expressions with whole cell ELISA assay.

The DOACs rivaroxaban and dabigatran prevented the decrease of both ZO-1 and VE-cadherin expressions with whole cell ELISA assay (Figure 5AB). For the other anticoagulants, warfarin, heparin and apixaban, we observed a 60% decrease in ZO-1 (Figure 5A) and a 40% decrease in VE-cadherin expression (Figure 5B), similar to that observed in the thrombin condition. Immunofluorescence ZO-1 assays confirmed whole cell assays, the BBB showed more important disruption linearity at cell-cell junction zones (white arrows, Figure 5C) under warfarin, compared to DOACs. Immunofluorescence assays allowed to observe disrupted ZO-1 and VE-cadherin linearity at cell-cell junctions (Figure 5CD). Immunofluorescent staining showed a decreased expression in VE-cadherin with thrombin. It seemed that there was more important disruption linearity at cell-cell junction zones (white arrows, Figure 5D) under warfarin, compared to DOACs.

## 3 Discussion

Iatrogenic effects of anticoagulants are associated with BBB breakdown. Thrombin is a key factor in this rupture of the BBB (Krenzlin et al., 2016). In the literature, it was described that DOACs cause less intracranial bleeding compared to other classes of anticoagulants (Connolly et al., 2009; Miller et al., 2012; Ruff et al., 2014). The involvement of the BBB in the limited number of hemorrhages under DOACs was observed *in vivo* in rodent models with a lower blue Evans diffusion (Ploen et al., 2014). Few studies on the biological mechanisms are available to date. It seems that the activation of PAR-1 and other PARs such as PAR-3 and PAR-

4 are implicated in the alteration or the maintaining of the barrier integrity (Festoff et al., 2016; Luo et al., 2007; Rohatgi et al., 2004). Our objective was to study the effects of thrombin/PAR-1 on the endothelial cells of the BBB model under different anticoagulants and to evaluate the activation of PAR-1.

The concentration of thrombin was based on the literature; we wanted a short but sufficient time to induce effects and to observe a modulation (Alabanza and Bynoe, 2012; Hawkins et al., 2015). In human endothelial cells, maximal permeability is obtained after a minimum of 60 min of treatment with thrombin (Alabanza and Bynoe, 2012). First, we checked the effects of thrombin on BBB permeability with different parameters such as TEER and  $P_{app}$ . Our results were in agreement with the literature, we observed a decrease in TEER and an increase in permeability to small molecules in a thrombin concentration-dependent manner (Alabanza and Bynoe, 2012; Hawkins et al., 2015).

The concentration of dabigatran was based on Hawkins' works, it is a clinically relevant concentration (Hawkins et al., 2015). For warfarin, apixaban and rivaroxaban, we chose the same concentration as dabigatran. Heparin can be used in many models as an adjuvant to reinforce BBB properties; we chose a concentration without any impact on BBB tightness. Pretreatment with anticoagulants before exposure of endothelial cells to thrombin was evaluated, and there was no effect on the tightness in the endothelial monolayer.

We used different types of anticoagulant: DOACs, warfarin, an antivitamin K which corresponded to an older generation of oral anticoagulants, and heparin sodium an injectable anticoagulant. To our knowledge, no publication has compared all these anticoagulants at the cellular level to date.

In our studies, we observed different responses depending on the anticoagulant used. dabigatran, a direct inhibitor of thrombin, blocked the effects of thrombin on the BBB as described in the literature (Hawkins et al., 2015).

The other DOACs apixaban and rivaroxaban are inhibitors of the factor Xa in the coagulation cascade, limiting the effects of thrombin on the BBB with maintaining of the integrity of the monolayer. The other anticoagulants warfarin and heparin at this concentration, did not limit the effects of thrombin on the BBB integrity. In the literature, an *in vivo* model with rodent showed a decrease of blue Evans permeability in rivaroxaban treated animals compared to warfarin treatment after a mechanic induction of a stroke (Ploen et al., 2014). This observation seems in agreement with ours.

Our hypothesis was that the cleavage is modulated by different anticoagulants, so we evaluated the cleavage of PAR-1. As expected, in our model, thrombin cleaved the receptor PAR-



1 (Chen et al., 2015; Luo et al., 2007). As for the alteration of the endothelial monolayer, we observed different responses depending on the anticoagulant used. In the literature it has been demonstrated that the expression of the protein PAR-1 can be modulated in the presence of warfarin, this mechanism has not been observed with DOACs (Moriyama et al., 2017).

As described in the literature, the thrombin-bound dabigatran blocks the cleavage of PAR-1 preventing its activation that leads to BBB breakdown (Chen et al., 2015). We obtained the same results with dabigatran in our model.

The other DOACs limited the cleavage of PAR-1 by thrombin. The other anticoagulants (warfarin, heparin) did not limit the cleavage of PAR-1. Our results are original and have not been reported in the literature on the BBB. These results are in agreement with the modulation of the tightness of the endothelial cells in a BBB model.

We have studied the ZO-1 tight junction protein which plays a key role in the tightness of the BBB. It has been described that this junction is impacted in response to treatment with thrombin on endothelial cells (Alabanza and Bynoe, 2012; Brailoiu et al., 2017; Machida et al., 2017). VE-cadherin is an adherent junction protein characteristic of vascular endothelial cells. It has been shown that this junction protein is the first to be altered in response to thrombin treatment on endothelial cells; its alteration can induce ZO-1 alteration in a second time (Kustermann et al., 2014; Rabiet et al., 1996). It seems that under the action of thrombin, mediated by PAR-1 activation, the VE-cadherin and ZO-1 junction proteins are phosphorylated and removed from the membrane for ZO-1, which would decrease their availability to bind to other junction proteins (Sabath et al., 2008; Timmerman et al., 2010). The activation of the receptor PAR-1 can also result in the endocytosis of junctions (Keep et al., 2018). In our model, thrombin induced a modification in expression of ZO-1 and VE-cadherin, and this modulation was limited by pretreatment with dabigatran and rivaroxaban before thrombin exposure; whereas the other anticoagulants did not limit this effect. The results obtained with the cell ELISA assay can be explained by the non possibility of the antibody to bind to the protein due to phosphorylation or internalization of junctions. This result is in agreement with the observation in immunostaining assays.

#### **4 Conclusion**

DOACs seem to act on PAR-1 cleavage and is directly related to the permeability of the cell barrier. DOACs limit the rupture of the BBB mediated by the thrombin/PAR-1 pathway.

All these different responses between DOACs and other classes of anticoagulants reinforce our hypothesis of a limitation, under DOACs, especially rivaroxaban and dabigatran, of the BBB breakdown mediated by the thrombin/PAR-1 pathway.

In this study, we have only observed the effect of anticoagulants and thrombin on the endothelial cell. To better understand the result of clinical studies that have shown that DOACs induced fewer ICHs than VKAs, a more complex model with other cells of the BBB is necessary.

## **5 Material and methods**

### **5.1 Chemicals and reagents**

Bovine serum albumin (BSA), sodium fluorescein (Na-FI), BCA assay kit QuantiPro, DMEM-F12 HAM (Dulbecco's Modified Eagle Medium: nutrient Mixture F-12), Hank's balanced salt solution (HBSS), Dulbecco's phosphate buffered saline without sodium and magnesium (DPBS), fetal bovine serum (FBS), trypsin, antibiotic and antimycotic solution, fluorescein isothiocyanate dextran (dextran-FITC 4kDa), HEPES buffer, 3,3',5,5'-tetramethylbenzidine (TMB), hydrogen peroxide solution (H<sub>2</sub>O<sub>2</sub>), Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe (SFLLRNPNDKYEPF) peptide were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Rabbit anti-ZO-1 (ab59720) and rabbit anti-VE cadherin (ab33168) antibodies were purchased from Abcam (Cambridge, United Kingdom). Secondary antibodies for cell ELISA were purchased from Santa Cruz Biotechnology (Dallas, USA). The F2R ELISA kit for PAR-1 measurement was purchased from Aviva Systems Biology (San Diego, USA). Rivaroxaban, dabigatran, apixaban and vorapaxar were purchased from Alsachim (Illkirch, France). Heparin sodium was purchased from Panpharma (Luitré, France). Coumadin (warfarin) was purchased from Bristol-Myers Squibb (New York, USA). Bovine thrombin was purchased from Siemens Healthcare (Erlangen, Germany). Normal goat supplement (NGS) was purchased from Millipore (Burlington, USA). Secondary antibody (Alexa Fluor 555 goat anti-rabbit (A-21429)) and 4', 6-diamidino-2-phenylindole (DAPI) for microscopy were purchased from Thermo Fischer Scientific (Massachusetts, USA). Vectashield Mounting Medium (H-1000) was purchased from Vector Laboratories (Burlingame, USA).

Cell culture inserts for 24-well plates (high density pore, 0.4 µm pore diameter size, translucent or translucent polyterephthalate ethylene (PET) membrane) were purchased from Becton Dickinson (Franklin Lakes, USA). The EVOM voltohmmeter system was purchased from World Precision Instruments (Hertfordsire, UK). 25 cm<sup>2</sup> culture flasks and companion plates were purchased from Dominique Dutscher (Strasbourg, France).

## 5.2 HBEC-5i BBB culture

The model was composed of HBEC-5i endothelial cells (from ATCC-Manassas, VA, USA) cultivated with conditioned medium from human astrocytes(HA from ScienCell Research Laboratories, Carlsbad, CA, USA) as previously described (Puech et al., 2018b).Cells were cultured in DMEM-F12 HAM, supplemented with 10% FBS and 1% antibiotic antimycotic solution. Cells were maintained in a humidified incubator at 5% CO<sub>2</sub> and 37°C.

## 5.3 Treatments

Cells were incubated with or without dabigatran, rivaroxaban, apixaban, warfarin (at 0.5 µM physiological dose based on Hawkins (Hawkins et al., 2015)), and heparin (at 10 IU/mL) for 24 h. Cells were then treated with or without thrombin at 1, 5, or 10 IU/mL for 1 h to mimic a haemorrhagic event(Alabanza and Bynoe, 2012; Hawkins et al., 2015).We used vorapaxar and SFLLRNPNDKYEPF as inhibitor and activator of the thrombin/PAR-1 pathways respectively.

## 5.4 Barrier properties

The apparent permeability ( $P_{app}$ ) was assessed using two hydrophilic fluorescent molecules: Na-Fl (MW = 376 Da) or dextran-FITC (MW= 4 kDa). HBSS transporter buffer supplemented with 10 mM HEPES containing 10µg/mL of Na-Fl or dextran-FITC was loaded onto the apical side of the insert and incubated at 37°C for 1 h. Fluorescence was measured with a fluorescence spectrophotometer (FluoroskanAscent™, Thermo Fischer scientific, France) with 485 nm excitation and 530 nm emission wavelengths. The permeability for all molecules was computed using the following formula:

$$P_{app} = \frac{V_r}{C_0} \times \frac{1}{S} \times \frac{C_1}{t} \text{ (Formula 1)}$$

Where  $P_{app}$  is the apparent permeability,  $V_r$  is the volume of medium in the receiver chamber,  $C_0$  is the concentration of fluorescent compound in the donor chamber at  $T_0$ ,  $S$  is the surface area of the monolayer,  $C_1$  is the concentration of fluorescent molecule in the receiver chamber after 1 h of incubation and  $t$  is the incubation time. The results are express in  $\text{cm}\cdot\text{s}^{-1}$  (Puech et al., 2018a).

Trans endothelial electrical resistance (TEER) was recorded using an EVOM® resistance meter with STX-2 electrode to characterize the formation of a tight endothelial cell monolayer. The measurement of a blank filter, *i.e.* without cells, was subtracted from those of a filter with cells. Then the resulting value was multipliate by the membrane area to obtain the TEER measurement in  $\Omega\cdot\text{cm}^2$ .

## 5.5 Thrombin, PAR-1 pathway

PAR-1 is activated by proteolytic cleavage of its extracellular N-terminus domain, resulting in the formation of a novel N-terminus (with sequence: SFLLRNPN) that serves as a tethered ligand folding back into the ligand-binding pocket of the receptor PAR-1. PAR-1 activation is evaluated with the cleavage of PAR-1 based on standard sandwich ELISAs. Measurements were realized according to the manufacturer's recommendation. The result was normalized by micro BCA total protein assay according to the manufacturer's instructions.

## 5.6 Protein expression

### 5.6.1 Whole cell ELISA

Cells were seeded onto a 96-well plate at a density of 10,000 cells/well. At confluence, cells were treated with anticoagulants for 24 h and/or thrombin at 10 IU/mL for 1 h. Then cells were fixed with 4% paraformaldehyde and permeabilized with methanol with 3% H<sub>2</sub>O<sub>2</sub>. ZO-1 and VE-cadherin expressions were evaluated in our model with primary polyclonal antibodies diluted 1/200 and incubated at room temperature. Secondary IgG antibody diluted at 1/500 was incubated at room temperature. Then, streptavidin was added for 20 min at room temperature. TMB substrate was added in each well. At the end the reaction was stopped by addition of hydrochloric acid 1N and was measured with a spectrophotometer (Multiskan<sup>TM</sup> RC, Thermo Fischer Scientific, France) at 450 nm. Our results were normalized with the total protein content as assessed by Coomassie blue total protein assay, according to the manufacturer's instructions.

### 5.6.2 Immunofluorescence studies

BBB on inserts were fixed in glacial methanol for 6 min and rehydrated. Following a blocking step, the inserts were cut and placed in 24-well plates. Primary antibodies were diluted at 1/200 and incubated for 1 h. Secondary antibodies were diluted 1/500 and incubated with an insert membrane for 45 min. Nuclei were finally counterstained with DAPI in PBS at room temperature for 30 min. The staining of all biomarkers had been imaged using an epifluorescence inverted microscope IX81 (Olympus, Tokyo, Japan) equipped with CellSens imaging software (Olympus, Munster, Germany). FIJI software was used to treat the image.

## 5.7 Statistical Analysis

Graphpad software was used for data analysis and graphic outputs. Data were expressed as the mean  $\pm$  standard deviation. Statistical analyses were performed using Graphpad software (Graphpad Software Incorporation, San Diego, USA). A Mann-Whiney test was used. Treated samples were compared to untreated control. The differences were considered statistically significant when p-value<0.05.

## **6 Funding:**

This work was supported by Jean Monnet University (Saint Etienne, France).

## **7 Acknowledgements**

We thank Fina Liu for her correction of the manuscript text

## **8 References**

- Alabanza, L.M., Bynoe, M.S., 2012. Thrombin induces an inflammatory phenotype in a human brain endothelial cell line. *Journal of Neuroimmunology* 245, 48–55. <https://doi.org/10.1016/j.jneuroim.2012.02.004>
- Arai, T., Miklossy, J., Klegeris, A., Guo, J.-P., McGeer, P.L., 2006. Thrombin and prothrombin are expressed by neurons and glial cells and accumulate in neurofibrillary tangles in Alzheimer disease brain. *J. Neuropathol. Exp. Neurol.* 65, 19–25.
- Brailoiu, E., Shipy, M.M., Yan, G., Abood, M.E., Brailoiu, G.C., 2017. Mechanisms of modulation of brain microvascular endothelial cells function by thrombin. *Brain Res* 1657, 167–175. <https://doi.org/10.1016/j.brainres.2016.12.011>
- Chen, B., Soto, A.G., Coronel, L.J., Goss, A., van Ryn, J., Trejo, J., 2015. Characterization of thrombin-bound dabigatran effects on protease-activated receptor-1 expression and signaling in vitro. *Mol. Pharmacol.* 88, 95–105. <https://doi.org/10.1124/mol.114.096446>
- Cheng, Y., Xi, G., Jin, H., Keep, R.F., Feng, J., Hua, Y., 2014. Thrombin-induced cerebral hemorrhage: role of protease-activated receptor-1. *Transl Stroke Res* 5, 472–475. <https://doi.org/10.1007/s12975-013-0288-8>
- Connolly, S.J., Ezekowitz, M.D., Yusuf, S., Eikelboom, J., Oldgren, J., Parekh, A., Pogue, J., Reilly, P.A., Themeles, E., Varrone, J., Wang, S., Alings, M., Xavier, D., Zhu, J., Diaz, R., Lewis, B.S., Darius, H., Diener, H.-C., Joyner, C.D., Wallentin, L., RE-LY Steering Committee and Investigators, 2009. Dabigatran versus warfarin in patients with atrial fibrillation. *N. Engl. J. Med.* 361, 1139–1151. <https://doi.org/10.1056/NEJMoa0905561>
- Ezekowitz, M.D., Reilly, P.A., Nehmiz, G., Simmers, T.A., Nagarakanti, R., Parcham-Azad, K., Pedersen, K.E., Lionetti, D.A., Stangier, J., Wallentin, L., 2007. Dabigatran with or without concomitant aspirin compared with warfarin alone in patients with nonvalvular atrial fibrillation (PETRO Study). *Am. J. Cardiol.* 100, 1419–1426. <https://doi.org/10.1016/j.amjcard.2007.06.034>
- Festoff, B.W., Sajja, R.K., van Dreden, P., Cucullo, L., 2016. HMGB1 and thrombin mediate the blood-brain barrier dysfunction acting as biomarkers of neuroinflammation and progression to neurodegeneration in Alzheimer’s disease. *J Neuroinflammation* 13, 194. <https://doi.org/10.1186/s12974-016-0670-z>
- Granger, C.B., Alexander, J.H., McMurray, J.J.V., Lopes, R.D., Hylek, E.M., Hanna, M., Al-Khalidi, H.R., Ansell, J., Atar, D., Avezum, A., Bahit, M.C., Diaz, R., Easton, J.D., Ezekowitz, J.A., Flaker, G., Garcia, D., Geraldes, M., Gersh, B.J., Golitsyn, S., Goto, S., Hermosillo, A.G.,

- Hohnloser, S.H., Horowitz, J., Mohan, P., Jansky, P., Lewis, B.S., Lopez-Sendon, J.L., Pais, P., Parkhomenko, A., Verheugt, F.W.A., Zhu, J., Wallentin, L., ARISTOTLE Committees and Investigators, 2011. Apixaban versus warfarin in patients with atrial fibrillation. *N. Engl. J. Med.* 365, 981–992. <https://doi.org/10.1056/NEJMoa1107039>
- Guan, J.-X., Sun, S.-G., Cao, X.-B., Chen, Z.-B., Tong, E.-T., 2004. Effect of thrombin on blood brain barrier permeability and its mechanism. *Chin. Med. J.* 117, 1677–1681.
- Hawkins, B.T., Gu, Y.-H., Izawa, Y., del Zoppo, G.J., 2015. Dabigatran abrogates brain endothelial cell permeability in response to thrombin. *J Cereb Blood Flow Metab* 35, 985–992. <https://doi.org/10.1038/jcbfm.2015.9>
- Ishida, Y., Nagai, A., Kobayashi, S., Kim, S.U., 2006. Upregulation of protease-activated receptor-1 in astrocytes in Parkinson disease: astrocyte-mediated neuroprotection through increased levels of glutathione peroxidase. *J. Neuropathol. Exp. Neurol.* 65, 66–77.
- Keep, R.F., Andjelkovic, A.V., Xiang, J., Stamatovic, S.M., Antonetti, D.A., Hua, Y., Xi, G., 2018. Brain endothelial cell junctions after cerebral hemorrhage: Changes, mechanisms and therapeutic targets. *J. Cereb. Blood Flow Metab.* 38, 1255–1275. <https://doi.org/10.1177/0271678X18774666>
- Krenzlin, H., Lorenz, V., Danckwardt, S., Kempfski, O., Alessandri, B., 2016. The Importance of Thrombin in Cerebral Injury and Disease. *Int J Mol Sci* 17. <https://doi.org/10.3390/ijms17010084>
- Kustermann, S., Manigold, T., Ploix, C., Skubatz, M., Heckel, T., Hinton, H., Weiser, T., Singer, T., Suter, L., Roth, A., 2014. A real-time impedance-based screening assay for drug-induced vascular leakage. *Toxicol. Sci.* 138, 333–343. <https://doi.org/10.1093/toxsci/kft336>
- Luo, W., Wang, Y., Reiser, G., 2007. Protease-activated receptors in the brain: receptor expression, activation, and functions in neurodegeneration and neuroprotection. *Brain Res Rev* 56, 331–345. <https://doi.org/10.1016/j.brainresrev.2007.08.002>
- Machida, T., Takata, F., Matsumoto, J., Miyamura, T., Hirata, R., Kimura, I., Kataoka, Y., Dohgu, S., Yamauchi, A., 2017. Contribution of thrombin-reactive brain pericytes to blood-brain barrier dysfunction in an in vivo mouse model of obesity-associated diabetes and an in vitro rat model. *PLOS ONE* 12, e0177447. <https://doi.org/10.1371/journal.pone.0177447>
- Miller, C.S., Grandi, S.M., Shimony, A., Fillion, K.B., Eisenberg, M.J., 2012. Meta-analysis of efficacy and safety of new oral anticoagulants (dabigatran, rivaroxaban, apixaban) versus warfarin in patients with atrial fibrillation. *Am. J. Cardiol.* 110, 453–460. <https://doi.org/10.1016/j.amjcard.2012.03.049>
- Morihara, R., Yamashita, T., Kono, S., Shang, J., Nakano, Y., Sato, K., Hishikawa, N., Ohta, Y., Heitmeier, S., Perzborn, E., Abe, K., 2017. Reduction of intracerebral hemorrhage by rivaroxaban after tPA thrombolysis is associated with downregulation of PAR-1 and PAR-2. *J. Neurosci. Res.* 95, 1818–1828. <https://doi.org/10.1002/jnr.24013>
- Ogawa, S., Shinohara, Y., Kanmuri, K., 2011. Safety and efficacy of the oral direct factor xa inhibitor apixaban in Japanese patients with non-valvular atrial fibrillation. -The ARISTOTLE-J study-. *Circ. J.* 75, 1852–1859.
- Ploen, R., Sun, L., Zhou, W., Heitmeier, S., Zorn, M., Jenetzky, E., Veltkamp, R., 2014. Rivaroxaban does not increase hemorrhage after thrombolysis in experimental ischemic stroke. *J. Cereb. Blood Flow Metab.* 34, 495–501. <https://doi.org/10.1038/jcbfm.2013.226>
- Puech, C., Delavenne, X., Perek, N., 2018a. The expected characteristics of an in vitro human Blood Brain Barrier model derived from cell lines, for studying how ABC transporters influence drug permeability. *Journal of Drug Delivery Science and Technology* 45, 159–167. <https://doi.org/10.1016/j.jddst.2018.03.002>
- Puech, C., Hodin, S., Forest, V., He, Z., Mismetti, P., Delavenne, X., Perek, N., 2018b. Assessment of HBEC-5i endothelial cell line cultivated in astrocyte conditioned medium as a human blood-brain barrier model for ABC drug transport studies. *International Journal of Pharmaceutics* 551, 281–289. <https://doi.org/10.1016/j.ijpharm.2018.09.040>
- Rabiet, M.-J., Plantier, J.-L., Rival, Y., Genoux, Y., Lampugnani, M.-G., Dejana, E., 1996. Thrombin-Induced Increase in Endothelial Permeability Is Associated With Changes in Cell-to-Cell Junction Organization. *Arteriosclerosis, Thrombosis, and Vascular Biology* 16, 488–496. <https://doi.org/10.1161/01.ATV.16.3.488>

- Rohatgi, T., Sedehizade, F., Reymann, K.G., Reiser, G., 2004. Protease-activated receptors in neuronal development, neurodegeneration, and neuroprotection: thrombin as signaling molecule in the brain. *Neuroscientist* 10, 501–512. <https://doi.org/10.1177/1073858404269955>
- Ruff, C.T., Giugliano, R.P., Braunwald, E., Hoffman, E.B., Deenadayalu, N., Ezekowitz, M.D., Camm, A.J., Weitz, J.I., Lewis, B.S., Parkhomenko, A., Yamashita, T., Antman, E.M., 2014. Comparison of the efficacy and safety of new oral anticoagulants with warfarin in patients with atrial fibrillation: a meta-analysis of randomised trials. *Lancet* 383, 955–962. [https://doi.org/10.1016/S0140-6736\(13\)62343-0](https://doi.org/10.1016/S0140-6736(13)62343-0)
- Sabath, E., Negoro, H., Beaudry, S., Paniagua, M., Angelow, S., Shah, J., Grammatikakis, N., Yu, A.S.L., Denker, B.M., 2008. G $\alpha$ 12 regulates protein interactions within the MDCK cell tight junction and inhibits tight-junction assembly. *J Cell Sci* 121, 814–824. <https://doi.org/10.1242/jcs.014878>
- Sun, L., Zhou, W., Ploen, R., Zorn, M., Veltkamp, R., 2013. Anticoagulation with dabigatran does not increase secondary intracerebral haemorrhage after thrombolysis in experimental cerebral ischaemia. *Thromb. Haemost.* 110, 153–161. <https://doi.org/10.1160/TH12-12-0942>
- Timmerman, I., Hordijk, P., Van Buul, J., 2010. Phosphorylation at endothelial cell-cell junctions: Implications for VE-cadherin function. *Cell Health and Cytoskeleton* 23. <https://doi.org/10.2147/CHC.S9590>
- Xi, G., Reiser, G., Keep, R.F., 2003. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? *J. Neurochem.* 84, 3–9.
- Yin, X., Wright, J., Wall, T., Grammas, P., 2010. Brain endothelial cells synthesize neurotoxic thrombin in Alzheimer's disease. *Am. J. Pathol.* 176, 1600–1606. <https://doi.org/10.2353/ajpath.2010.090406>

## Figures legends

Figure 1: Effect of thrombin and anticoagulants on the endothelial cells of the blood brain barrier (BBB) model. **A:** Mean Trans endothelial electrical resistance (TEER) values for BBB model with three concentrations of thrombin after an incubation of 1h. Mean  $\pm$  SD n= 3 N=5. **B:** Apparent permeability ( $P_{app}$ ) values for sodium fluorescein (Na-Fl) (full circle) and dextran-FITC (empty circle) in the same configuration. **C:** Mean TEER values for BBB model with several anticoagulants after an incubation of 24 h n=3. **D:**  $P_{app}$  values for Na-Fl with the same anticoagulant. \* p-value<0.05; \*\* p-value<0.01; \*\*\*p-value<0.005 compared to untreated control as determined by a Mann-Whitney test.

Figure 2: Effect of anticoagulant pretreatment on the endothelial cells of blood brain barrier (BBB) damage caused by thrombin. **A:** Mean trans endothelial electrical resistance (TEER) values for the effect of pretreatment with anticoagulant for 24 h before an exposure of thrombin at 10 IU/mL for 1 h Mean  $\pm$  SD n=3 N=2. Apparent permeability ( $P_{app}$ ) values for sodium fluorescein (Na-Fl) (**B**) and dextran-FITC (**C**) on the same configuration. Mean  $\pm$  SD n=3. \* p-value<0.05; \*\* p-value < 0.01; \*\*\*p-value<0.001 compared to untreated control as determined by a Mann-Whitney test.

Figure 3: Effect of activator and inhibitor of the protease activated receptor-1 (PAR-1) pathways on the blood brain barrier (BBB). **A:** Mean Trans endothelial electrical resistance (TEER) values for vorapaxar (inhibitor of PAR-1) for 24 h alone or with thrombin for 1h and SFLLRNDKYEPF (activator of PAR-1) for 1 h. Mean  $\pm$  SD n=3, N=2. Apparent permeability ( $P_{app}$ ) values for sodium fluorescein (Na-Fl) (**B**) and dextran-FITC (**C**) on the same configuration. Mean  $\pm$  SD n=3. \* p-value<0.05; \*\* p-value < 0.01; \*\*\*p-value<0.001 compared to untreated control as determined by a Mann-Whitney test.

Figure 4: Effect of thrombin with or without anticoagulant on the cleavage of the protease activated receptor-1 (PAR-1). **A:** Evaluation of the expression of soluble fraction of PAR-1 in the supernatant of HBEC-5i after exposure to different concentrations of thrombin for 1h. **B:** Evaluation of the expression of the soluble fraction of PAR-1 in the supernatant of HBEC-5i after exposure to anticoagulants for 24 h. **C:** Evaluation of the expression of the soluble fraction of PAR-1 in the supernatant of HBEC-5i after a pretreatment with anticoagulant before



thrombin exposure. Mean  $\pm$  SD n= 4. \* p-value < 0.05; \*\*p-value<0.01 compared to untreated control as determined by a Mann-Whitney test.

Figure 5: Evaluation of the effect of thrombin under anticoagulant exposure on ZO-1 and VE-cadherin junction proteins. **A:** Cell ELISA for ZO-1 expression with thrombin alone or after a pretreatment with anticoagulant. Mean  $\pm$  SD n=4 N=2. **B:** Cell ELISA for VE-cadherin expression in the same configuration. \* p-value < 0.05; \*\* p-value < 0.01; \*\*\*p-value<0.005 compared to untreated control as determined by a Mann-Whitney test.

Immunostaining of ZO-1( **C**) or VE-cadherin (**D**) in control condition (1) and after 10IU/mL thrombin incubation (2), or pretreatment before thrombin: dabigatran (3), rivaroxaban (4), apixaban (5), warfarin (6). Scale bar=50 $\mu$ m.

Figure 1

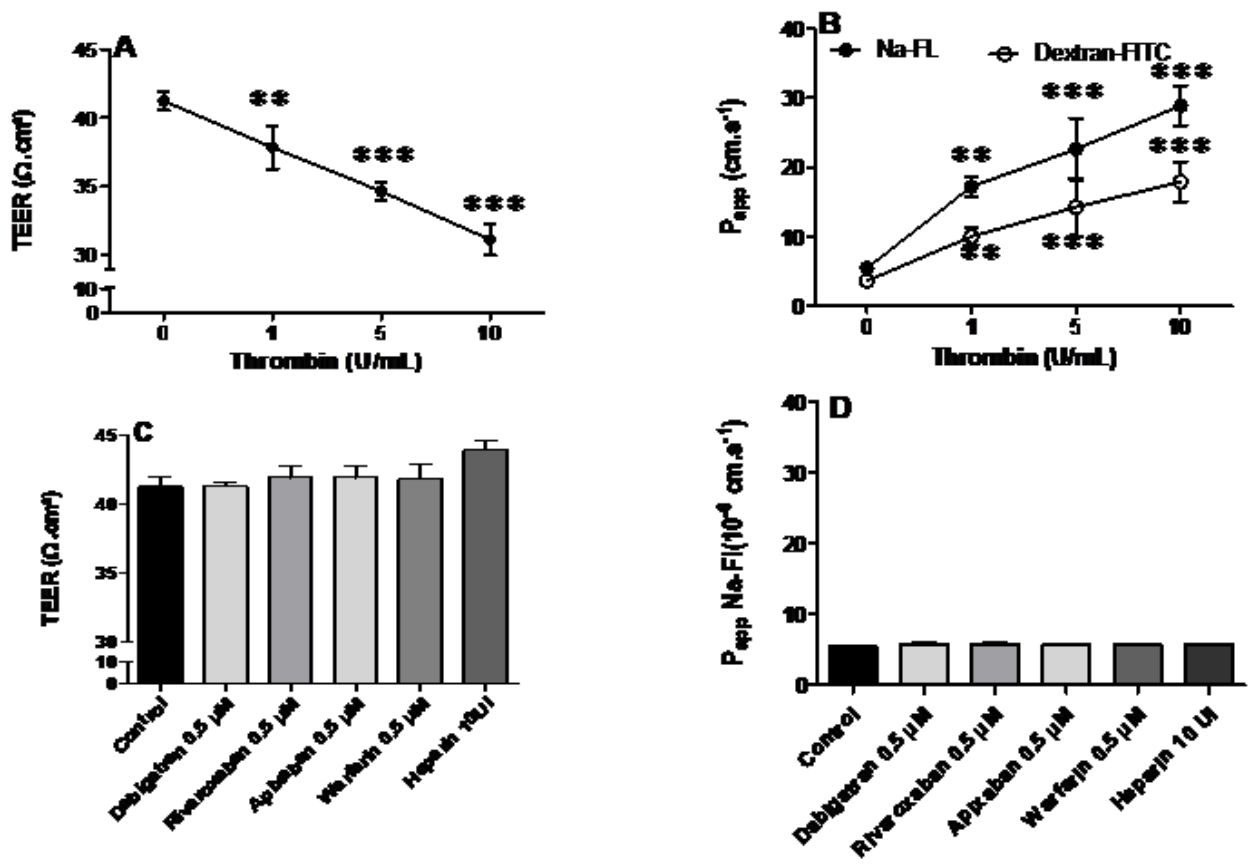


Figure 2

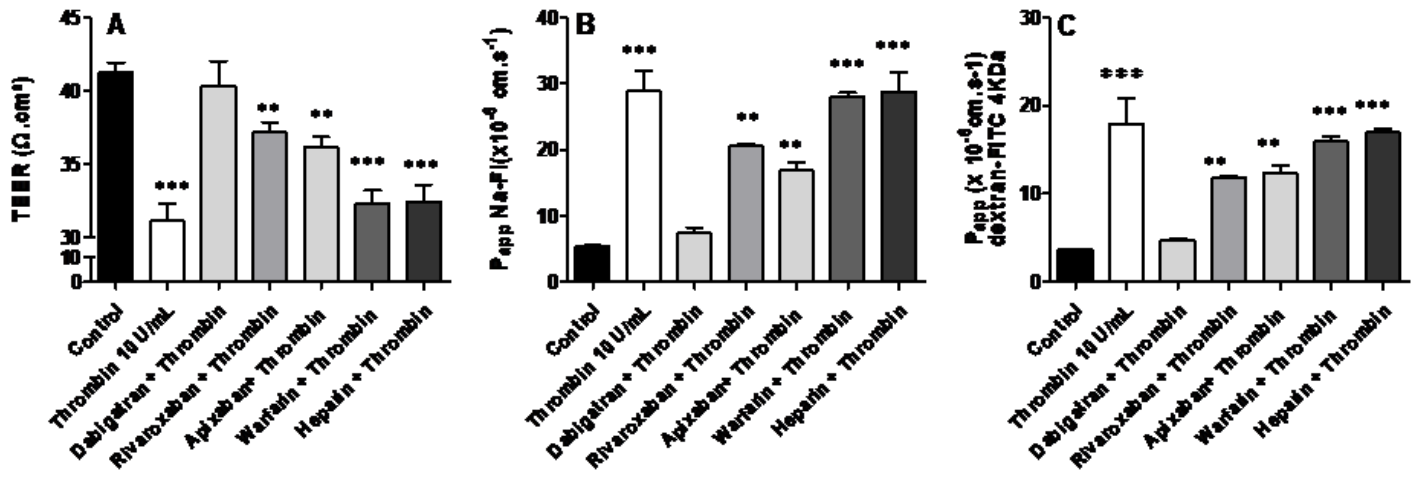


Figure 3

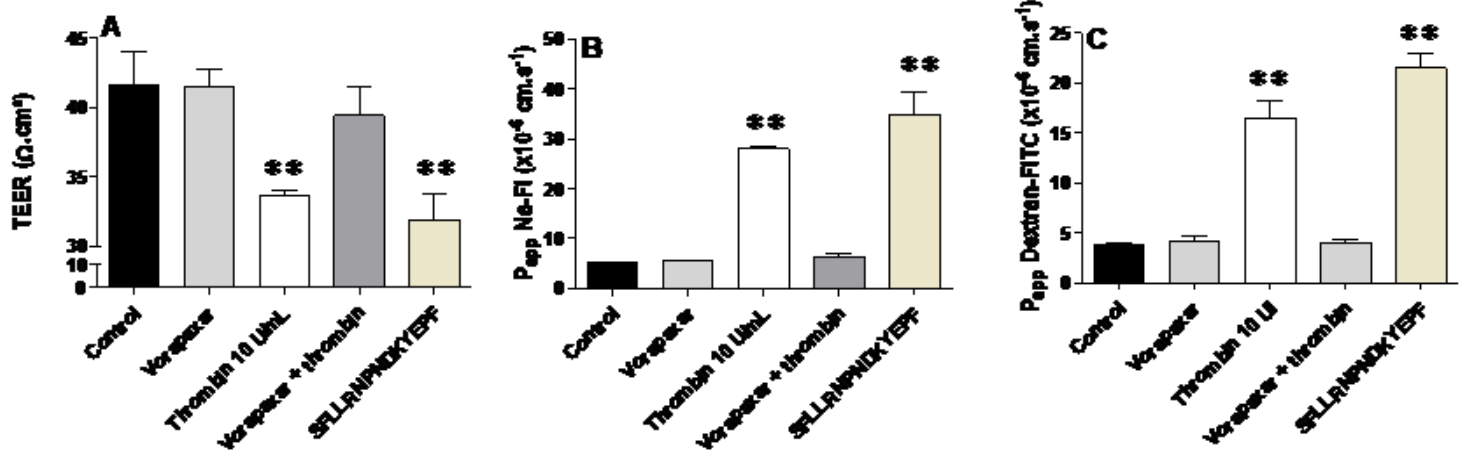


Figure 4

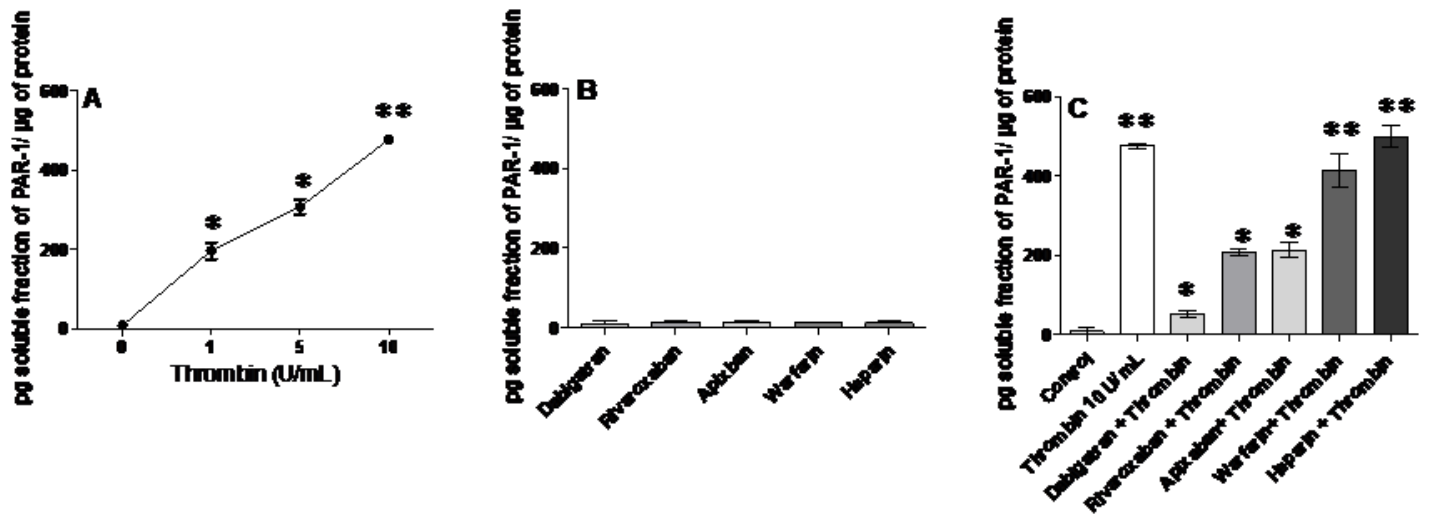
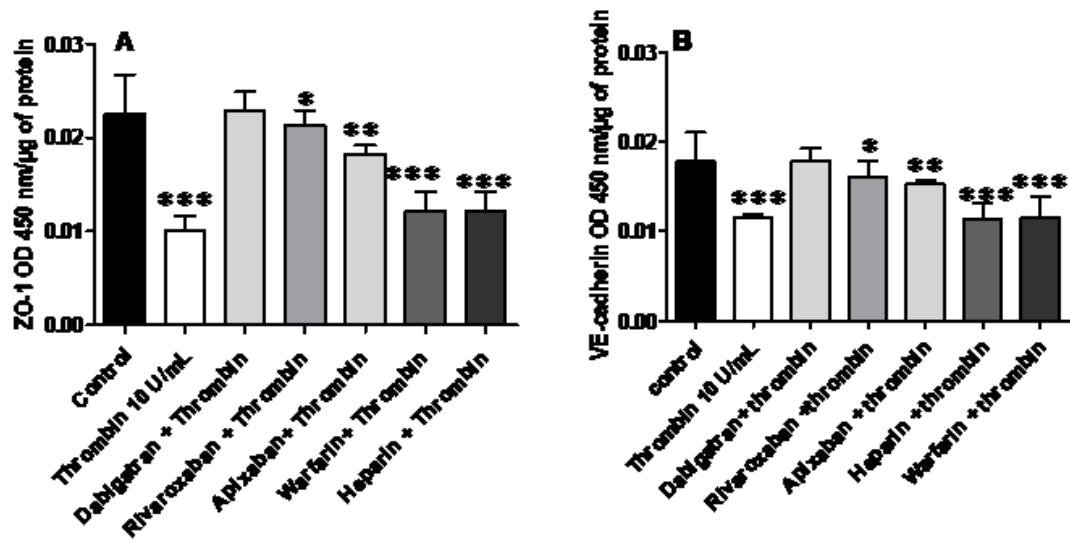
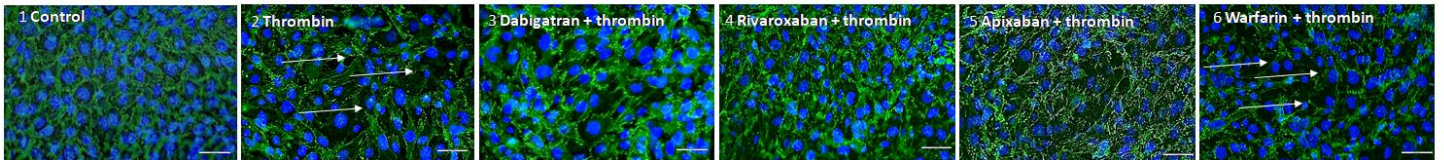


Figure 5



C. ZO-1



D. VE-Cadherin

