FACTOR VII ACTIVATING PROTEASE (FSAP) IN THROMBOSIS AND HAEMOSTASIS *IN VIVO*

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by

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Giessen 2014

“This thesis is dedicated to my beloved parents”

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ABBREVIATIONS

AA	Arachidonic acid
ADP	Adenosine-5`-diphosphatase
aPTT	Partial thromboplastin time
AT-III	Anti-thrombin III
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CFT	Clot formation time
COX	Cyclooxygenase
CP	Ceruloplasmin
CRP	Collagen related peptides
CT	Clotting time
CvX	Convulxin
DVT	Deep vein thrombosis
ECAM-1	Endothelial cell adhesion molecule
EDTA	Ethylenediaminetetraacetic acid
EE	Embolic events
EGF	Epithelial growth factor
ExTEM	Extrinsic thromboelastometry
FDP	Fibrin degradation product
$FeCl_3$	Ferric chloride
FSAP	Factor VII activating protease
FVII	Factor VII (zymogens)
FVIIa	Activated factor VII
FX	Factor X (zymogens)
FXa	Activated factor X
GPIb	Glycoprotein Ib
GPV	Glycoprotein V
GPVI	Glycoprotein VI
H&E	Hematoxylin & eosin
HABP2	Hyaluronic acid binding protein 2
HCT	Hematocrit
HE buffer	High end buffer
HGF-A	Hepatic growth factor -A
ICAM-1	Intercellular adhesion molecule 1
IVC	Inferior vena cava
KCl	Potassium chloride
$KHCO_3$	Potassium bicarbonate
MCF	Maximum clot firmn
$MgCl_2$	Magnesium chloride
MI	Marburg I
MPV	Mean platelet volume
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NH_4Cl	Ammonium chloride
NO	Nitric oxide
OCT	Optimum cutting temperature
PAI-1	Plasminogen activator inhibitor 1
PAR	Proteinase-activated receptor
PBS	Phosphate buffer saline
PDGF-BB	Platelet derived growth factor BB
PE	Pulmonary embolism
PGI_2	Prostaglandin I_2
PHBP	Plasma hayaluran binding protein
PLA_2	Phopholipase A_2
PLG	Plasminogen
PPP	Platelet poor plasma

PRP	Platelet rich plasma	TFPI	Tissue factor pathway inhibitor
PT	Prothrombin time	TIT	Thrombus initiation time
PVDF	Polyvinylidene difluoride	tPA	Tissue plasminogen activator
RBC	Red blood cells	TTO	Time to occlusion
RhoD	Rho-related GTP-binding protein	TXA_2	Thromboxane A_2
RT	Room temperature	UFH	Unfractionated heparin
RT-PCR	Real time-polymerase chain reaction	uPA	Uroplasminogen activator
SD	Standard deviation	VCAM-1	Vascular cell adhesion protein 1
SDS	Sodium dodecyl sulfate	VTE	Venous thromboembolism
SNP	Single nucleotide polymorphism	vWF	Von willibrand factor
TAFI	Thrombin activatable fibrinolysis inhibitor	WBC	White blood cells
TF	Tissue factor	HEPES	Hydroxyethyl piperazine ethanesulfonic acid

SYMBOLS

α	Alpha	°	Degree
β	Beta	™	Trademark
γ	Gamma	±	Plus or minus
δ	Delta		

1. Introduction

Formation of haemostatic plugs after the vascular injury is a critical survival response for the prevention of excessive blood loss (Westrick et al., 2007). The coagulation process that leads to haemostasis involves a complex cascade of reactions. This includes conversion of prothrombin to thrombin which in turn converts soluble fibrinogen to an insoluble fibrin network, which further forms a complex with platelets leading to a stable thrombus formation. Inherited or acquired disparity in the coagulation process leads to an increased risk of thrombosis. There are two distinctive forms of thrombosis, venous thrombosis and arterial thrombosis, each of them can be represented by several subcategories based on the occurrence sites (e.g. renal, jugular vein, cerebral venous, carotid artery, etc.). Thrombotic complications and the associated vascular diseases are the leading cause of mortality and morbidity worldwide. Heart attack and stroke are the two major consequences. These are typically acute events and are primarily caused by thrombus formation that prevents blood flow to regions of the heart or brain.

1.1. Haemostasis

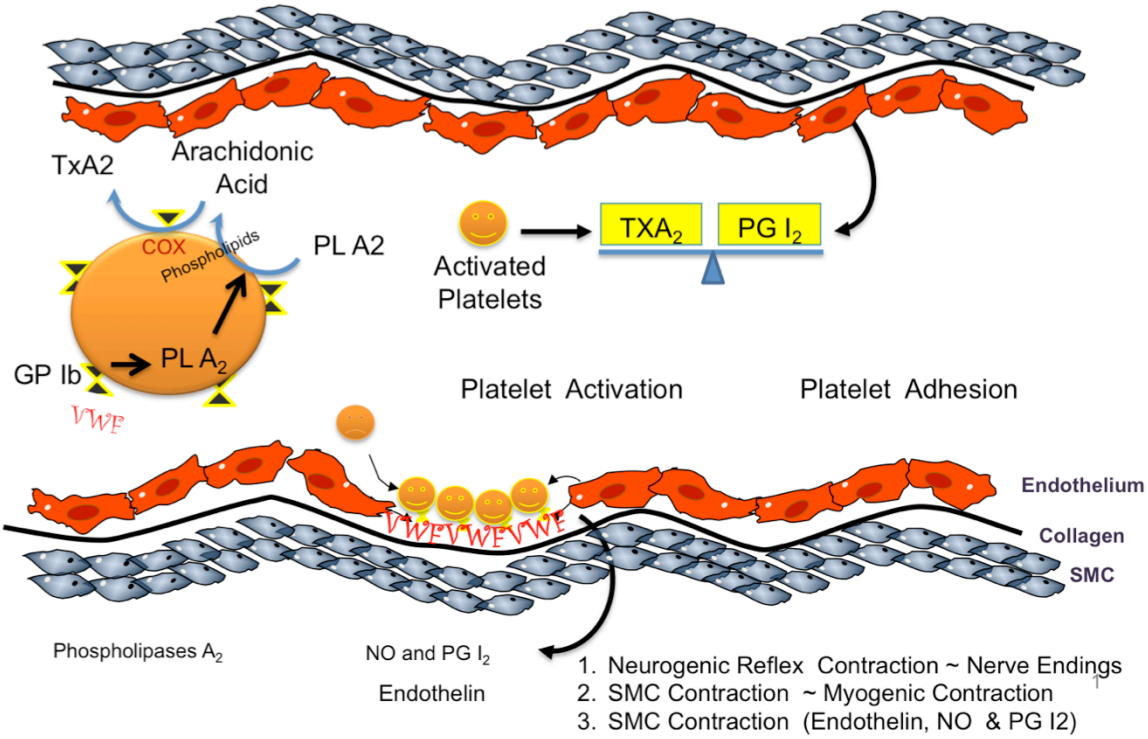
Blood is the transport system that brings nutrients and oxygen to support living cells and simultaneously facilitates the scavenging of carbon dioxide and other waste products from the body. If the pressurized circulatory system gets injured, a cascade of reactions is initiated to seal the system. This prevents the loss of blood as well as blocks the entrance of foreign organisms. Thus, blood borne cells and plasmatic proteins play an important defensive role in our body. This process is called haemostasis and it acts quickly and effectively to prevent excessive bleeding. This process depends on an intricate series of events involving platelets, leucocytes, coagulation proteins and a host of other components. Injury to a blood vessel triggers the coagulation cascade that is divided into the intrinsic, the extrinsic and the common pathway. The endothelium controls the pro and anticoagulant factors to limit haemostasis at the site of vascular injury (Golan, 2007).

1.2. Platelets in primary haemostasis

Vascular injury exposes the collagen and vWF (von willebrand factor) in the vessel wall to the circulatory system. Adherence of platelets to the extracellular matrix, especially collagen, is the initial step in primary haemostasis. Activated platelet monolayer is formed on the surface (rolling, adhering and spreading) of the exposed

collagen matrix when it comes in contact with vWF, secreted by the endothelial cells (Brass, 2003).

Figure 1.1: Primary platelet plug: activation of platelet and platelet adhesion: *vWF interaction with*



GPIb leads to platelet activation. This inturn stimulates the downstream pathway to release TXA_2. TXA_2 and PGI_2 must be balanced in order to maintain haemostasis. vWF: von Willebrand factor; TXA_2: Thromboxane A_2; PGI_2: Prostaglandin I2; NO: Nitric oxide; SMC: Smooth muscle cells; GPIb: Glycoprotein Ib; PLA_2: Phospholipase A_2; COX: Cyclooxygenase.

The initial platelet adhesion is mediated by the interaction between Glycoprotein (GP)Ib/V/IX receptor complexes on the surface of platelets with vWF as well as Glycoprotein (GP)VI and GPIb with collagen, at the sites of vascular injury. Under normal conditions, soluble as well as normal endothelial vWF does not interact significantly with GPIb/V/IX complexes. Interestingly, at the sites of injury, vWF released from the damaged endothelium is immobilized on the collagen surface, which acts as a strong adhesive substrate for platelet adhesion thereby leading to its activation (Jennings, 2009). This in turn initiates phosphorylation reaction within the platelets which leads to the further release of platelet granules. This also leads to activation of the platelet membrane phospholipase A_2 to form arachidonic acid *via* platelet phospholipids. This arachidonic acid is further converted to thromboxane A_2 (TXA_2), with the help of cyclooxygenase (COX) which propagates further platelet activation and vasoconstriction by stimulating, the granule release reaction **(Fig. 1.1)**. There are two types of granules

released by platelets namely delta (δ) granules (serotonin, adenosine diphosphate (ADP) and Ca^{2+}) and alpha (α) granules (coagulation factors, fibrinogen and platelet derived growth factors). These platelet derived factors further activate other platelets eventually a platelet plug is formed (Kottke-Marchant, 2010). TXA_2 and ADP play a potential role in

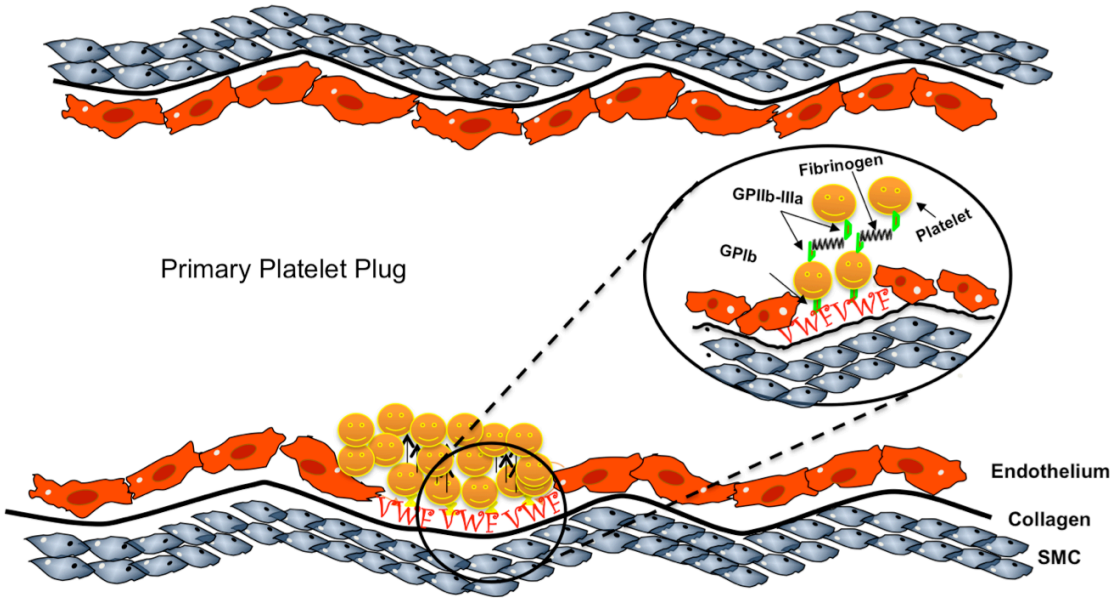


Figure 1.2: Primary platelet plug and receptor complexes: *Receptor complexes differ based on the type of interactions involved. During the platelet adhesion process, vWF interacts through GPIb receptor with platelets. Whereas during the platelet aggregation process, platelets aggregate via GPIIb-IIIa receptors mediated by fibrinogen. GPIIb-IIIa: Glycoprotein IIb-IIIa; SMC: Smooth muscle cells; GPIb: Glycoprotein Ib.*

leading to platelet aggregation. Platelets aggregate to each other *via* a binding molecule, fibrinogen, which has multiple binding sites for functional GPIIb-IIIa (Suehiro et al., 1996)**(Fig. 1.2)**. However, this primary haemostatic platelet plug is unstable, the stability being inversely proportional to the diameter, internal flow rate and flow pressure of the vessel (Reininger et al., 2006).

1.3. Secondary haemostasis

Secondary haemostasis is also described as the coagulation cascade. The main function of this cascade is to stabilize the primary platelet plug *via* cross-linked fibrin via a process that involves a sequence of enzymatic reactions **(Fig. 1.3).** Firstly, the circulating inactive proenzymes, which are synthesized by the liver, are proteolytically cleaved in order to become active. These activated factors lead to a chain of catalytic activation of other downstream factors. As a result of this catalytic activation, large

amounts of fibrin are formed in a short period of time at the injured sites. Secondly, the negatively charged phospholipids, such as phosphatidylserine (PS), help to form complexes of zymogens, cofactors and activators on the platelet surface. Normally, PS is present in the inner leaflet of the plasma membrane but due to cell death or other active processes they translocate to the outer leaflet of the membrane. Calcium is also required for the enzyme, substrate and co-factor to assemble in a specific conformation for the activation of subsequent proenzymatic coagulation factors by proteolytic cleavage. The coagulation cascade is divided into two major pathways namely intrinsic pathway and extrinsic pathway. The extrinsic pathway is considered to be physiologically more important pathway for the initiation of coagulation *in vivo* (Dahlback, 2000; Mackman et al., 2007) as it leads to a faster activation of factor VII *via* tissue factor (Mann et al., 1998) thereby forming a complex which further activates Factor X.

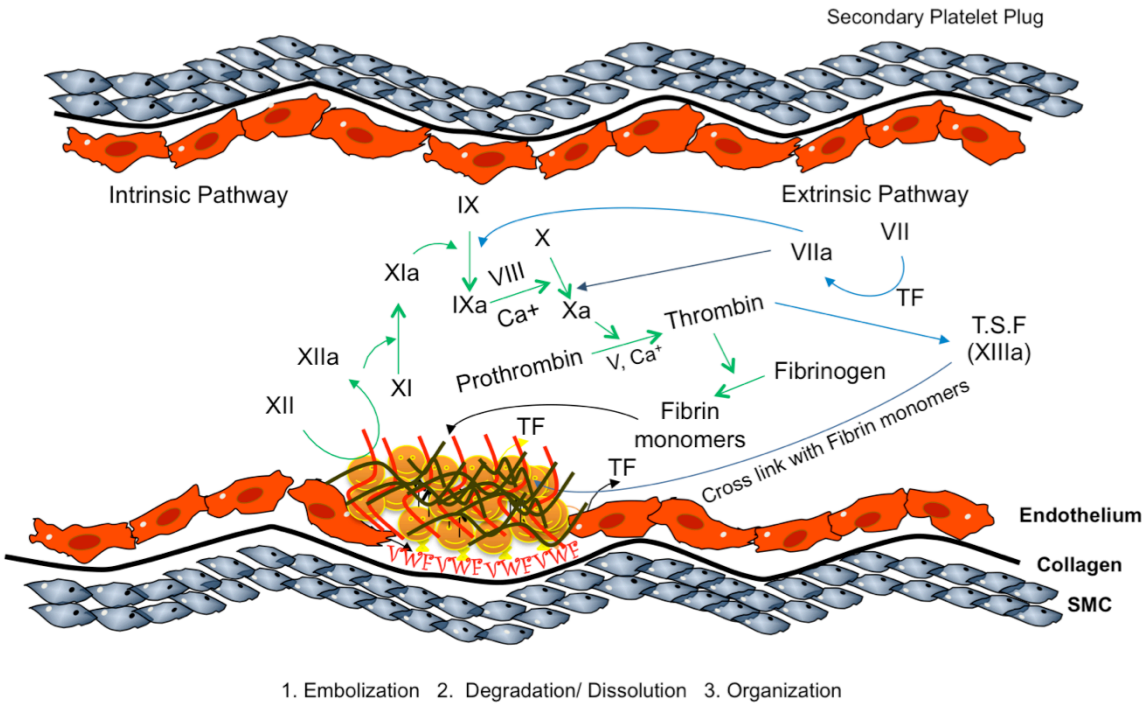


Figure 1.3: Secondary platelet plug: *Activation of coagulation cascade is the primary step in the secondary platelet plug formation. Activation of intrinsic (contact factors) and extrinsic (tissue factors) pathways play a major role in stable fibrin cross-links formation. TF: Tissue factor; TSF: Tissue fibrin stabilizing factor; SMC: Smooth muscle cells.*

Factor X can be however activated by both intrinsic and extrinsic pathways **(Fig. 1.3).** The intrinsic pathway involves activation of factor XII which undergoes auto-activation to form factor XIIa on a negatively charged surface. However, charged surfaces, and the developing thrombus can also activate the intrinsic pathway *in vivo*

(Renne et al., 2005; Schmaier and McCrae, 2007). This activated factor XII activates factor XI which leads to activation of factor IX which in the presence of factor VIII and calcium activates factor X thereby forming Xa. This activated factor Xa further forms a complex with factor V in the presence calcium leading to the proteolytic cleavage of factor II (Prothrombin) to factor IIa (Thrombin) (Lane et al., 2005). In the pathology of thrombosis, thrombin plays several roles, which includes catalytic promotion of the clot cascades (factors V and VIII activation), fibrin formation, and activation of factor XIII. Most importantly, it acts as a strong activator of platelets, causing the release of granules, platelet derived microparticles release and also platelet aggregation. This effect of thrombin is mediated through a series of G protein-coupled protease-activated receptors (PARs)(Martorell et al., 2008). Activation of these receptors on endothelial cells leads to the release of nitric oxide (NO), PGI_2, fibrinolytic substances (uPA and tPA) and PAI-1 at the site of vessel injury(Verhamme and Hoylaerts, 2006).

1.4. Regulation of haemostasis

The endothelium initiates four major mechanisms to maintain constant blood flow in the systemic circulation. Firstly, endothelial secretions of NO, prostacyclin (PGI2) and ADP dephospatase inhibits activated platelets. Secondly, activation of AT-III *via* endothelial-heparan sulphate leads to the formation of a complex, which inactivates thrombin as well as Xa and IXa. In the third mechanism the endothelial cell surface thrombomodulin blocks the coagulation cascade *via* thrombomodulin-protein C complex. Protein C and S are vitamin K dependent proteins that slow down the coagulation pathway by cleavage and inactivation of FVa and FVIIIa (Dahlback, 1995; Kottke-Marchant and Comp, 2002; Lane et al., 2005).

In addition to the activation protein C, the TM/Thrombin complex also activates thrombin activatable fibrinolysis inhibitor (TAFI), which regulates fibrin degradation (Myles et al., 2003)**.** Lastly, secretion of plasminogen activators (tPA and uPA) activate the fibrinolytic pathway. These plasminogen activators convert the plasminogen into plasmin to degrade the fibrin cross-links to fibrin degradation products (FDP). When the local concentrations of thrombin and inflammatory cytokines (IL-1 and TNF α) are increased, endothelial cells release plasminogen activator inhibitor (PAI), which inhibits tPA, leading to less activation of plasminogen **(Fig. 1.4).**

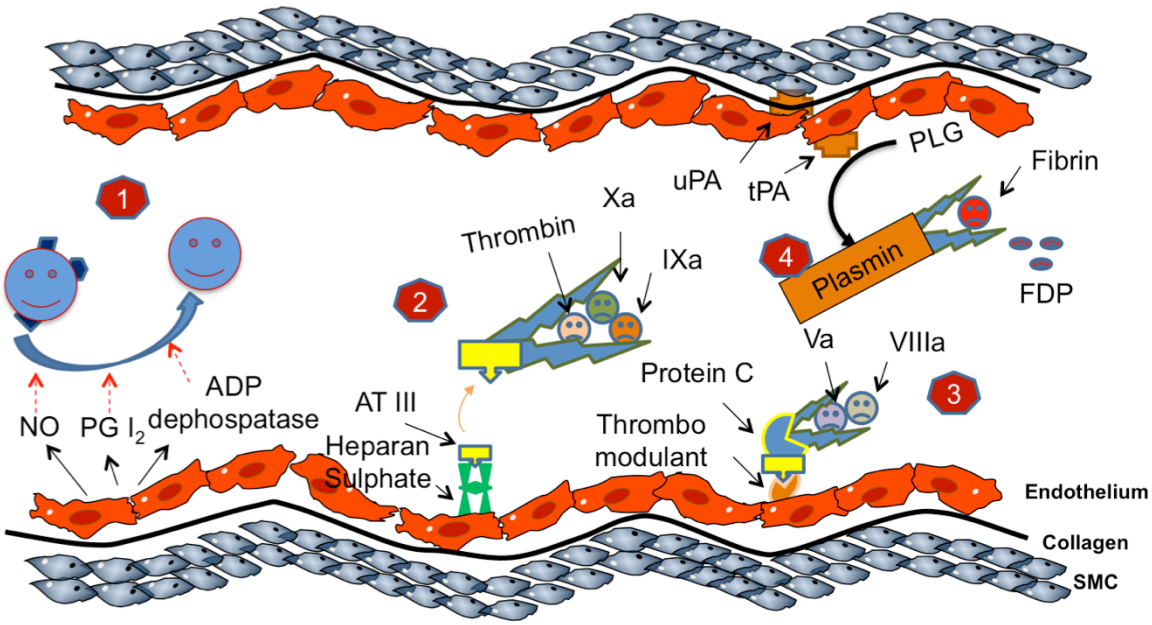


Figure 1.4: Normal defense mechanism to prevent clotting in the circulatory system*: Four major functions are involved in platelet inactivation. (i) release of NO, PGI2 an ADP dephospatase (ii) activation of AT-III via Heparan sulphate,(iii) protein C activation via thrombomodulant and (iv) activation of fibrinolysis pathway. ADP: Adenosine 5 diphosphatase; uPA: Urokinase plasminogen activator; tPA: Tissue plasminogen activator; FDP: Fibrin degradation product; PGI_2: Prostaglandin I_2; NO: Nitric oxide; AT III:Anti-thrombin III; SMC: Smooth muscle cells; PLG: Plasminogen.*

1.5. Tissue factor pathway inhibitor

TFPI is an endogenous protein (40-55 kDa), which is a specific anticoagulant expressed by endothelial cells, vascular smooth muscle cells, platelets, monocytes/macrophages and cardiac myocytes (Bajaj et al., 1990; Caplice et al., 1998; Maroney et al., 2007; Novotny et al., 1988; Novotny et al., 1991; van der Logt et al., 1994; Werling et al., 1993) As its name implies, it limits the action of tissue factor (TF). Three isoforms of TFPI exists in mice (α, β and γ) among which TFPIβ is the most predominant (Maroney et al., 2008). The coagulation cascade is initiated when activated VIIa complex along with TF is exposed or activated at the site of injury. This complex catalytically further activates factor X. TFPI inhibits VIIa/TF *via* two-step process. Firstly, it interacts with activated factor Xa forming TFPI/Xa complex which in turn interacts with the VIIa/TF complex through second *kunitz* domain of TFPI leading to formation of quaternary complex. This quaternary complex inactivates the factor VIIa:TF mediated activation of factor X. TFPI may also down regulate coagulation cascade by internalizing cell surface TF/VIIa complexes.

1.6. Fibrinolysis

The fibrinolytic system is responsible for the degradation of fibrin clots. This leads to restoration of blood flow in the obstructed vessels. Plasminogen is the central factor of fibrinolysis system, which is activated by urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA) to plasmin **(Fig. 1.6)**. This activated plasmin is responsible for the degradation of fibrin into fibrin degradable products (FDP). Two major inhibitors PAI-1 (Loskutoff et al., 1983) and α_2-antiplasmin (Collen, 1976; Moroi and Aoki, 1976; Mullertz and Clemmensen, 1976), play a vital role in the fibrinolytic system. uPA is also believed to be involved in the cell migration, cell invasion and metastasis (Carmeliet and Collen, 1996).

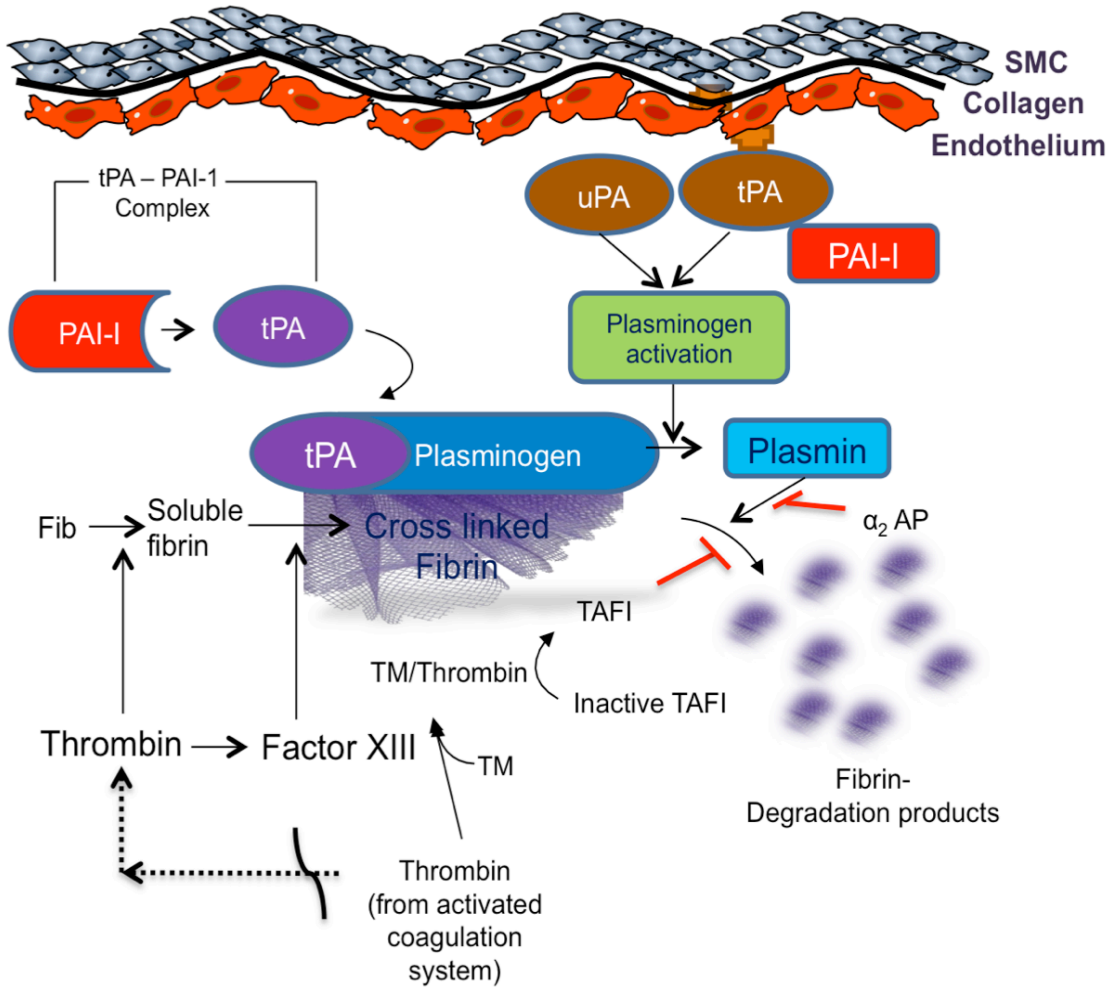


Figure 1.5: Fibrinolytic system: *Fibrinolysis system also regulates coagulation by generation of plasmin, which dissolves fibrin clots. Fibrinolysis system is down regulated by plasminogen activator inhibitor-1(PAI-1). A delicate balance between coagulation and anticoagulation is necessary to prevent pathophysiological conditions such as excessive bleeding or thrombosis. tPA: tissue plasminogen activator; PAI: Plasminogen activator inhibitor; AP: Anti-plasmin; uPA: Urokinase plasminogen activator;TM: Thrombomodulin; Fib: Fibrinogen.*

The thrombin-activated fibrinolysis inhibitor (TAFI) is also a known regulator of fibrinolysis. TAFI is activated by thrombin/thrombomodulin complex (Bajzar et al., 1996; Wang et al., 2000). Active TAFI removes lysine residues from fibrin, which are essential for the binding of tPA, plasminogen, and plasmin. (Dempfle, 2007). The fibrin thus loses its co-factor activity leading to less plasmin, and the residual plasmin finds less fibrin binding sites, resulting in an increased resistance of the clot towards plasmin proteolysis (Dempfle, 2007).

1.7. Arterial and venous thrombosis

Arterial and venous thrombosis are distinct diseases with respect to their epidemiology and treatment strategies. Arterial thrombus occurs at the sites of arterial injury and high shear stress. Whereas, venous thrombi occurs at the sites of undamaged vein wall and low shear stress. Arterial thrombus results in white thrombi (platelets rich), whereas venous thrombus results in red thrombi (red cells rich). In this context, classical arterial thrombosis-associated risk factors such as hypertension have not been shown to influence venous thrombosis. Likewise, venous thrombotic risk factors such as hyper activation of FVIII or imbalance in fibrinolysis does not aggravate the risk of arterial thrombosis (Lijfering et al., 2011).

Arterial thrombosis is a formation of thrombus within an artery, which leads to myocardial infarction, ischemic stroke, and manifestation of peripheral arterial diseases (Albers et al., 2008; Van de Werf et al., 2008). It occurs after the rupturing or slow destruction of unstable atherosclerotic plaques in the coronary circulation(Jackson, 2011). Risk factors for arterial thrombosis include high blood pressure, age, genetic factors and smoking. Although coagulation cascade activation is indisputably implicated in arterial thrombosis, platelet activation plays a primary role in arterial thrombosis. Hence, the use of anti-platelet drugs is the most effective therapy for the arterial thrombosis and its complications (Lange and Hillis, 2004).

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are collectively known as venous thromboembolism (VTE). Contrary to arterial thrombosis, venous thrombosis and its complication are not usually linked with vascular pathologies. Instead, venous thrombosis is associated with the dysregulation of coagulation proteins or venous stasis. In 1870, Rudolf Virchow postulated the Virchow's triad, which has been the base for understanding the pathophysiology of venous thrombosis. The triad proposed the

changes in blood flow, vessel wall and blood composition **(Fig. 1.5)**. A major complication of venous thrombosis is PE, which occurs when the thrombus breaks away

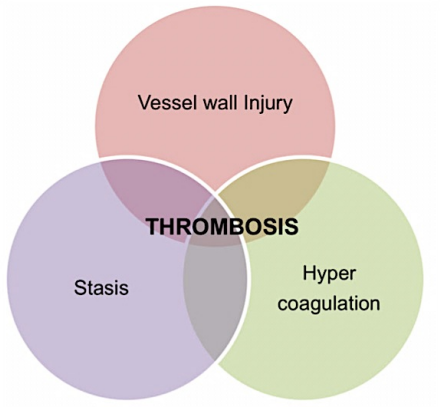


Figure 1.6: Virchow's Triad: *Rudolf Virchow's triad proposing the changes in blood flow, vessel wall injury and hypercoagulation, the prime factors causing venous thrombosis.*

and translocates to the minor vasculature areas, especially pulmonary artery (Owens and Mackman, 2010). Numerous studies have been reported that show the increased risk of VTE with age. Human bleeding disorders and its related genetic deficiencies are fairly known, but complete knowledge of genetic factors related to the increased susceptibility towards thrombosis remains unclear.

1.8. Factor VII activating protease (FSAP)

Factor VII activating protease (FSAP) belongs to a serine protease family and circulates in plasma at a concentration of 12 μg/ml (Choi-Miura et al., 1996; Kannemeier et al., 2001). The structure of FSAP consists of various structural modules, including three-epidermal growth factor (EGF) like domains, a kringle and a serine protease domain, which mimics the high structural homology to uPA, plasminogen and hepatocyte growth factor (Choi-Miura et al., 1996; Hashimoto et al., 1997). FSAP derived its name because of its functional ability to activate factor VII independent of TF (Romisch et al., 1999). In addition, due to the binding ability of FSAP to hyaluronic acid, it is also called hyaluronan-binding protein 2 (HABP2). As it regulates pro-urokinase (pro-uPA) and factor VII activation (VIIa) activation, a role in the regulation of both coagulation and fibrinolytic system has been proposed (Romisch et al., 1999). Various serine protease inhibitors like, serpins, α_1-proteinase inhibitor, α_2-plasmin inhibitor, C1-inhibitor as well as plasminogen activator inhibitor-1 (PAI-1) and protease nexin-1(PN-1), inhibit activated FSAP rapidly (Choi-Miura et al., 2001). As yet the specific in vivo functions of FSAP are unknown **(Fig. 1.7).**

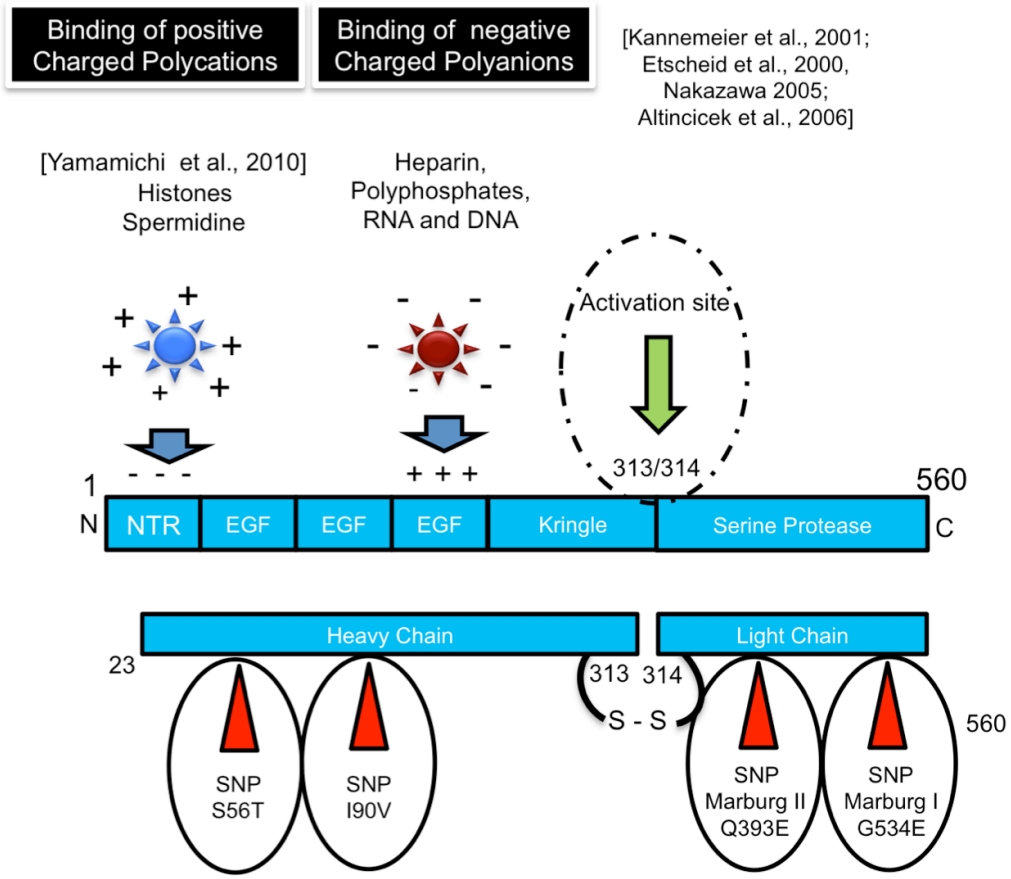


Figure 1.7: Structure of factor VII activating protease (FSAP): *FSAP has 560 amino acids representing a 23 amino acid signal peptide trailed by three epidermal growth factor domains (EGF 1-3), a kringle domain, and a catalytic serine protease domain. The activation through cleavage of the Arg313–Ile314 located in front of the serine protease domain results in a 50 kDa heavy chain and 25 kDa light chain. Both the chains are bound by a disulfide bridge. The enzyme is inactivated through two additional cleavages, one located in the EGF-3 domain (between Lys169 and Arg170) and the other located in the serine protease domain (Arg480). The heavy chain is cleaved in two fragments of 25 kDa whereas the light chain is cleaved into fragments of 18 and 8 kDa.* ***(Kanse et al., 2012).***

1.9. Single nucleotide polymorphisms and FSAP gene

There are two single nucleotide polymorphisms (SNP) existing in the FSAP encoding gene i.e., G534E (Marburg I) and E393Q (Marburg II) that lead to an exchange of a single amino acid in the protease domain leading to the reduction of enzymatic activity (Romisch, 2002). About 5% of the Western Europeans are carriers of these SNPs (Romisch, 2002). MI-SNP carriers are susceptible to getting late complications of atherosclerosis (Gardener et al., 2011), carotid stenosis (Willeit et al., 2003), vascular calcification (Zakai et al., 2011), liver fibrosis (Wasmuth et al., 2009) and stroke (Trompet et al., 2011). In one study, it was shown that Marburg-I SNP (MI-SNP) results in 50-80% decline in the proteolytic activity of FSAP (Sedding et al., 2006). Earlier, Romisch and colleagues demonstrated that FSAP-MI had reduced activity towards pro-

uPA activation *in vitro* but there was no change in the activation of FVII (Romisch, 2002). However, FVII activation by FSAP remains a contradictory issue (Stavenuiter et al., 2012).

In order to further investigate the functional differences between FSAP and Marburg-I carriers, another study was carried out where a wire induced arterial stenosis was performed in mice. Application of Marburg I variant exhibited significantly higher neointima formation compared to wild-type FSAP (Sedding et al., 2006). Several studies demonstrated the effects of FSAP outside the coagulation system. Recently a protective role of FSAP in liver fibrosis (Borkham-Kamphorst et al., 2013) and the regulation of endothelial permeability has been reported (Mambetsariev et al., 2010).

1.10. FSAP in thrombosis and haemostasis

FSAP has high structural homology to plasma haemostasis factors like plasminogen, urokinase and hepatocytes growth factor activator (Choi-Miura et al., 1996; Hashimoto et al., 1997). This information primarily has driven us to search for its functions in coagulation cascade and fibrinolytic pathway **(Fig. 1.8)**.

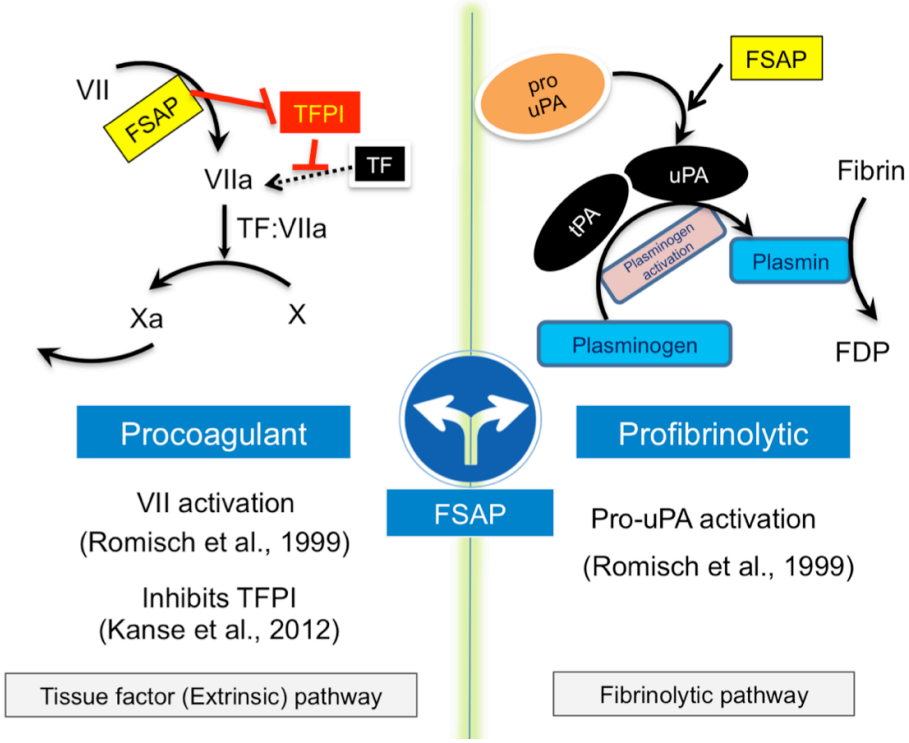


Figure 1.8: Dual function of FSAP in vitro: *FSAP acts as a procoagulant due to its role in factor VII activation and inhibition of TFPI.It also exihibits its profibrinolytic properties via pro-uPA activation. TFPI: Tissue factor pathway inhibitors; Pro-uPA: Pro-urokinase plasminogen activator; FDP: Fibrin degradation products.*

Initial studies identified FSAP to be responsible for factor VII and pro-uPA activation (Romisch et al., 1999). However, prothrombin time (PT; extrinsic pathway) and plasma recalcification time were reduced with the addition of exogenous FSAP and there was no influence on partial thromboplastin time (pTT; intrinsic pathway). This confirms that FSAP functions majorly through the extrinsic pathway and not by the intrinsic pathway (Romisch, 2002). FSAP Marburg-I variant represents a general cardiovascular risk factor and might also be involved in venous thromboembolism (Hoppe et al., 2005; Ireland et al., 2004). However, others could not reproduce these findings (Franchi et al., 2006; Gulesserian et al., 2006; van Minkelen et al., 2005). It was later proposed by Kanse et al., that FSAP inhibits TFPI *in vitro* (Kanse et al., 2012). Thus, these studies were unclear with respect to the pathophysiological significance of FSAP in thrombosis and haemostasis. Sidelmann and colleagues observed elevated levels of ciruclating FSAP in deep vein thrombosis patients (Sidelmann et al., 2008). Recently, deep vein thrombosis is also associated with an elevation in the ciruclating FSAP (Sidelmann et al., 2008). MI-SNP was also found to be strongly associated to the late complications of carotid stenosis indicating that FSAP is involved in the development of atherosclerosis (Willeit et al., 2003) and stroke (Trompet et al., 2011).

2. AIM OF THE STUDY

Due to the association of Marburg-1(MI) SNP with venous thrombosis and other vascular diseases, we intended to define its function in thrombosis and haemostasis *in vivo*. The major aim of this study was to characterize FSAP deficient (knockout) mice using well-established mouse models for thrombosis and haemostasis.

The main questions addressed in this thesis are as follows:

1. Does endogenous FSAP play a role in haemostasis?
2. What is the role of endogenous FSAP in thrombosis?
3. Does FSAP activate factor VII *in vivo*?
4. Does FSAP inactivate TFPI *in vivo*?

3. Materials and Methods

3.1. Animal study: FSAP$^{-/-}$ mice

FSAP$^{-/-}$(Knockout) mice were generated as described previously (Borkham-Kamphorst et al., 2013). These mice were then backcrossed with C57BL/6J mice for 10 generations (F10) and heterozygous crosses were set up to generate FSAP$^{-/-}$ mice and littermate controls. Age and gender matched animals were used in all the experiments. Animals underwent an acclimatization period of one week before the experimental procedures. The animals were maintained in the same room under specific pathogen free (SPF) conditions. All interventions were performed in full compliance with the institutional and German protection laws and approved by the local animal welfare committee. (V54-19c 20-15 (1) GI 20/2 Nr. 90/2010 andV54-19c 20-15 (1) GI 18/11 Nr.60/2008).

3.2.Tail-bleeding assay

Mice (5-6 weeks) were matched for sex and weight and anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine cocktail. After the complete anesthesia, mice were placed on the heating pad (37°C). The tails were then amputed (approximately 4-5 mm length) using a sharp scalpel and immediately immersed (approx. 2 cm) into a 15 ml tube contained pre-warmed buffer (isotonic normal saline) at 37°C **(Fig. 3.1)**. This buffer was constantly changed every minute, till 12 minutes.

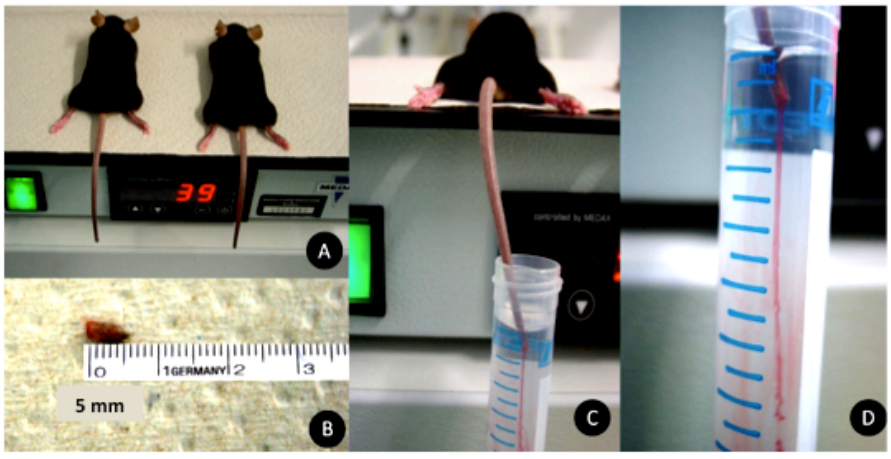


Figure 3.1: Murine model of haemostasis: Tail bleeding times of WT and FSAP$^{-/-}$ mice (n=10) were monitored by positioning the resected (5 mm) tail in a tube containing saline at 37°C which was changed every minute. The experimental set up was for 12 min after which the hemoglobin content was measured spectrophotometrically. **(A)** Animals placed on the warm plate at 37°C **(B)** Amputation of the tail tip **(C)** Amputed tail tip immersed immediately in to the normal saline tubes **(D)** Visualization of bleeding pattern.

To measure hemoglobin content, samples were centrifuged at 600*g* for 5 minutes at 20°C and resuspended in 3 mL of red blood cell (RBC) lysis buffer (8.3M NH_4Cl, 1M $KHCO_3$, 3.7 mM EDTA, pH 7.3). After a standing period of 5 minutes at room temperature, the tubes were again centrifuged at 600*g*, for 5 minutes at 20°C. The supernatant was collected and the hemoglobin content was determined spectrophotometrically at 560 nm. The primary (initial arrest) and re-bleeding (initial arrest and re-bleeding) times were established from the experimental data.

3.3. Ferric chloride ($FeCl_3$) induced venous thrombosis

The $FeCl_3$ induced inferior vena cava (IVC) thrombosis model was used as previously described (Wang et al., 2006). A midline laparotomy was performed on anesthetized mice (10-12 weeks). The intestines were exteriorized and covered with

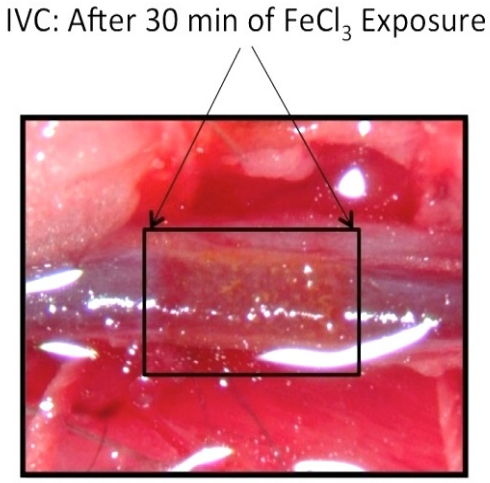


Figure 3.2: $FeCl_3$ induced venous thrombosis: Venous thrombosis was induced by topical application of a filter paper soaked with 3.5% $FeCl_3$ on the IVC for 3 min. Thrombus protein content was measured after 30 min.

gauze soaked in physiologic saline to prevent desiccation. A section of IVC, between the renal and left common iliac veins was cleared and separated from the aorta by dissection and a piece of Whatmann filter paper (1.0 × 1.0 mm) pre-saturated in a solution of 3.5% $FeCl_3$ in 1% phosphate buffer saline (PBS) was placed on the surface of the IVC for 3 minutes. After 30 minutes of the initial application of filter paper, the entire section of IVC (5-6 mm) was removed for downstream processing **(Fig. 3.2).** Excessive moisture was removed before weighing the thrombus. Thrombi were placed in 200 µL of Tris buffer (pH 7.5) supplemented with 20 µL Proteinase K (Fermentas) for overnight

digestion at 50°C for 8-10 h, and total thrombus protein content was measured at an optical density of 280 nm. In this model the effects of administration of the anti-coagulant, heparin was also determined. 0.9% saline vehicle or heparin (0.1 IU/g, i.v.) was applied 15 minutes before the $FeCl_3$ application. Blood samples were collected in the citrated (3.8 % sodium citrate) tubes and processed for plasma separation.

3.4. $FeCl_3$ induced carotid artery thrombosis

Mice (10-15 weeks) were anesthetized and the experiment was performed as described by Pozgajova et al. with slight modifications (Moller and Tranholm, 2010; Pozgajova et al., 2006). Midline cervical incision was performed to expose the right common carotid artery of anesthetized mice and the normal carotid blood flow was measured for 5 min using Ultrasound Doppler transonic flow probe (Transonic systems, USA). Vascular injury was made with 10% $FeCl_3$ (0.62M on 0.5 × 0.5mm filter paper) for 3 min, washed with warm saline and the blood flow was measured for 45 min. This blood flow (mL/min) was recorded using Transonic T420 model 0.5 VB probe with a chart recorder (Servogor 220, BBC Goerz, Austria). In both models, the time to occlusion (TTO) was defined as the time between $FeCl_3$ application on the indicated vessel and the lags in flow for 2 min. Embolization was defined as a rapid increase in blood flow (at least 0.2 mL/min in 5 min) after stable occlusion occurred. Blood was drawn, either 10 min after stable occlusion or after 45 min without stable occlusion from the IVC into 3.8% sodium citrate buffer and processed to platelet poor plasma (PPP) by centrifugation (5000*g* for 10 min). The normal and thrombotic carotid arteries were frozen at -80°C for further downstream processes **(Fig. 3.3)**.

3.5. Application of exogenous FSAP

In order to achieve the normal circulating FSAP levels, a blood volume of 2mL was assumed in a 25g mouse and 1.2mg/kg body weight of human plasma FSAP was applied. The control group was administered with FSAP carrier buffer (high-end (HE) buffer; 5 mM Citrate pH 4.5, 0.2 M Arginine and 0.2 M Lysine). FSAP/HE buffer administration done was *via* the lateral tail vein. The injection volume was 100 µL and this was done 15 min before the induction of carotid thrombosis. Blood was then drawn, either 10 min after stable occlusion or 45 min without stable occlusion from the IVC into 3.8% sodium citrate and processed to platelet poor plasma (PPP) by centrifugation at

5000*g* for 10min. The normal and thrombotic carotid arteries were then frozen at -80°C for further downstream processes **(Fig. 3.3)**.

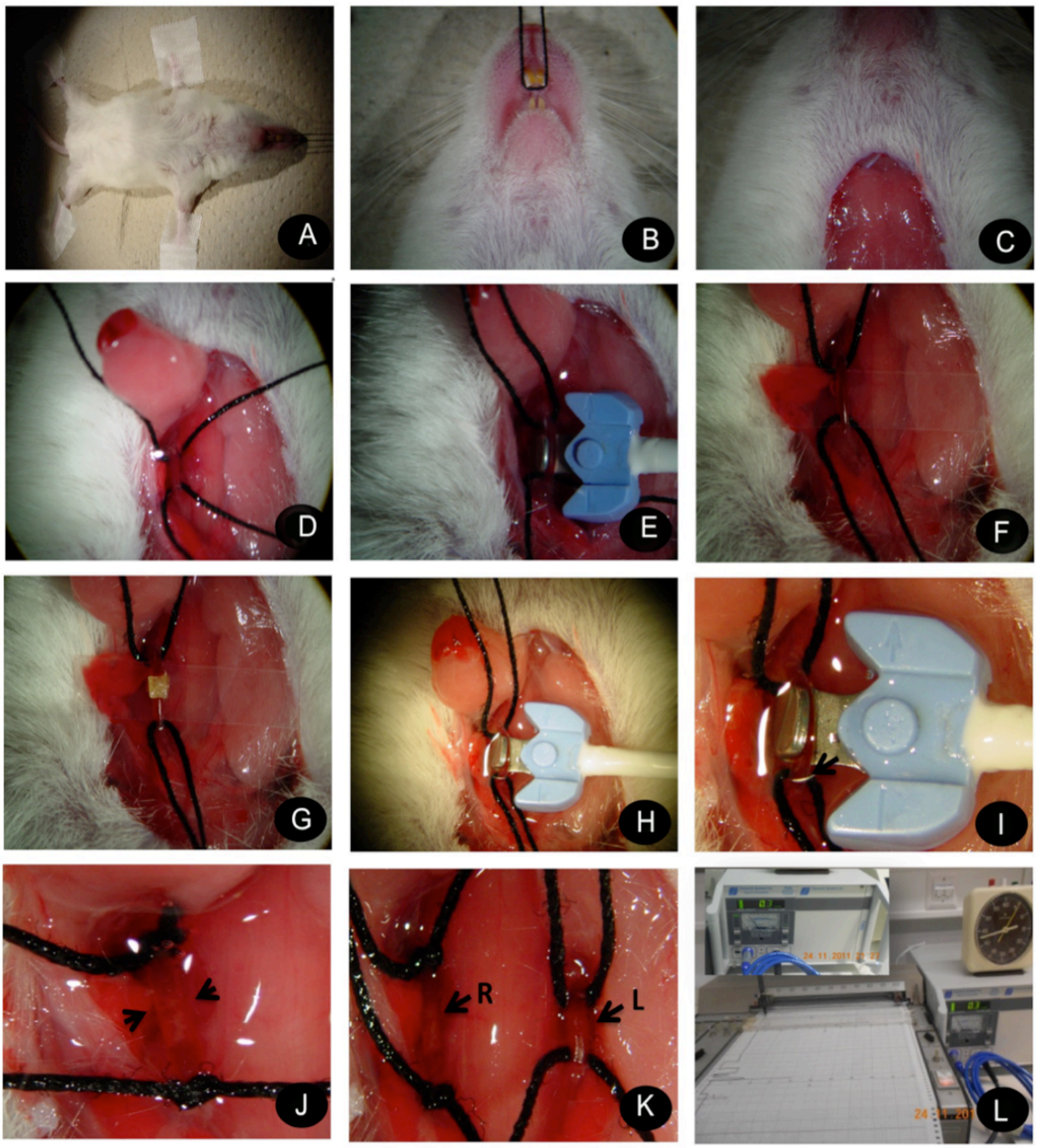


Figure 3.3: Carotid artery thrombosis: **(A)** After anesthesia the mice was placed in a supine position. **(B)**Mandible to suprasternal notch area was cleaned by disinfectant. **(C)**A midline incision extended from mandible to suprasternal notch was made to expose the anterior cervical triangle which was then blunt dissected to free the right carotid artery from the surrounding tissue. **(D)** Free right carotid artery was separated by surgical suture. **(E)**Normal blood flow was measured using flow probe (Transonic T420 model 0.5 VB). **(F)**Transparent flexible spacer was placed below the right carotid artery to avoid the dilution of $FeCl_3$. **(G)**.Whatmann filter paper (1.5 x 1.5 mm) saturated with 10% $FeCl_3$ was placed over the adventia of the right carotid artery for 3 min.**(H&I)**Blood flow measured using flow probe after $FeCl_3$ exposure. **(J)** Indicated the complete occlusion of carotid artery and arrows indicates visible aggregated platelets). **(K)** Normal and thrombotic carotid arteries (Right carotid artery- R; Left carotid artery - L). **(L)** Chart recorder connected with the transonic flow meter.

3.6. $FeCl_3$ induced mesenteric arteriole thrombosis

Mice (5-6weeks) were anesthetized and the experiment was performed as described by Pozgajova et al. with slight modifications (Pozgajova et al., 2006). In brief, 2µg 488-Dylight–coupled GPIX-IgG derivative (X488; Emfret Analytics) in 150 µL PBS was injected retro-orbitally and the mesentery was gently exteriorized using a midline abdominal incision approach. Arterioles (35 to 60µm diameter) were visualized with a Zeiss Axiovert 200 inverted microscope (10X) equipped with a 100-W HBO fluorescent

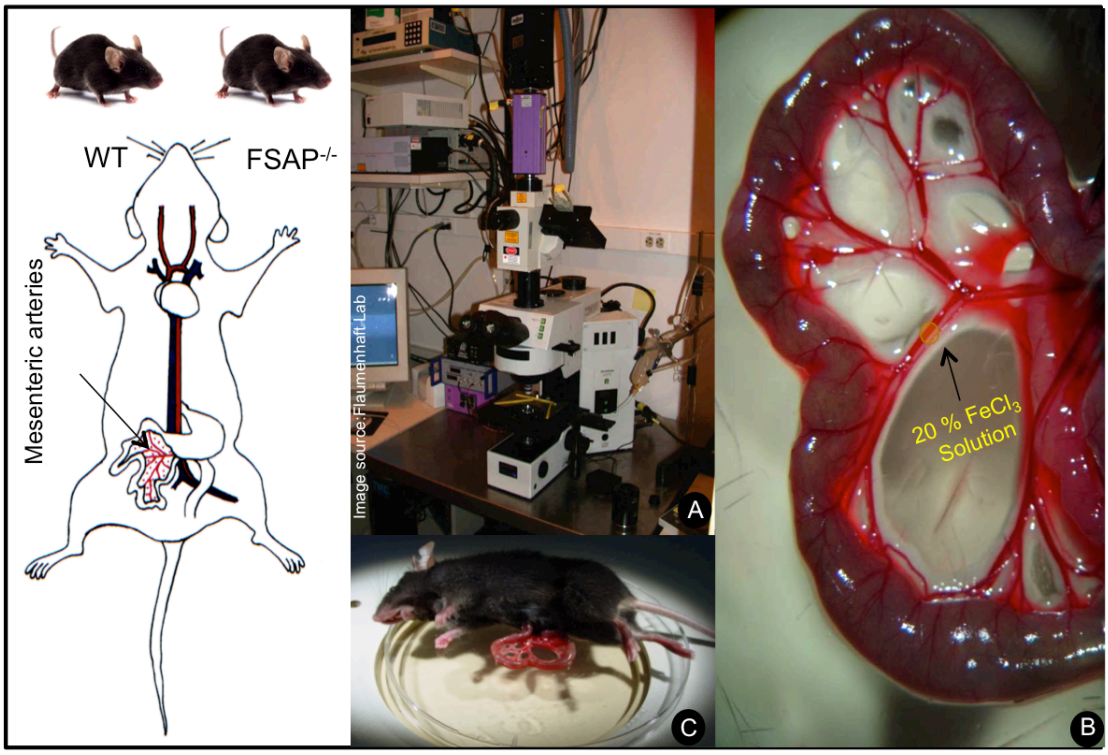


Figure 3.4: $FeCl_3$ Induced thrombosis in the mesentery: Vascular injury created in mesenteric arterioles by topic application of 20% $FeCl_3$ **(A &B)**. Before initiating the vascular injury, mesenteric arterioles were exteriorized and spread on the transparent Petridish as displayed **(C)**. Live images and videos were captured with a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Jena, Germany) with a 63 X/0.75 Ph1 objective, a 100-W HBO fluorescent lamp source, and a CCD camera (CV-M300; Visitron Systems, (puchheim, Germany) connected to an AG-7355 S-VHS video recorder (Panasonic, Matsushita Electric, Osaka, Japan). Video taped images were analyzed using a computer assisted image analysis program, Meta View Version 5.0 (Visitron Systems).

lamp source and a CCD camera (CV-M300) connected to an S-VHS video recorder (AG-7355; Panasonic, Matsushita Electric, Tokyo, Japan). A drop of $FeCl_3$ solution (20%) was used to create vascular injury in the arterioles. Arterioles were monitored for 40 min or until complete occlusion (blood flow stoppage for more than 1 min). Firm platelet adhesion was determined by the number of fluorescently labeled platelets that deposited on the vessel wall, 5 min after injury. A thrombus was defined as a platelet aggregate

larger than 10μm in diameter. Thrombus stability was characterized as the number of embolus events (thrombi larger than 10 μm in diameter) that detached during the observation period from the viewing field **(Fig. 3.4).** These experiments were performed in collaboration with Prof. Bernd Nieswandt (Universität Wurzburg, Germany).

3.7. Collagen/ epinephrine model of venous thromboembolism

Mice (10-12 weeks) were anesthetized and maintained on pre-warmed heating pad (37°C). Collagen (0.8 mg/kg, Horn collagen, Nycomed, Germany) and epinephrine (60 μg/kg, Jenaphrama, Germany) cocktail was injected through jugular vein (Pozgajova et al., 2006). Breathing time was monitored and the time taken for the complete respiratory arrest was recorded. Blood was collected immediately in citrate buffer and processed for obtaining plasma. Mice were then perfused with cold PBS (1%). Lungs were retrieved and immediately processed for optimum cutting temperature (OCT) followed by freeze storage at -80°C. H&E staining was performed and the thrombus load was assessed.

3.8. FSAP activity assay

Total FSAP activity was measured by an immunocapture activity with slight modifications (Kanse et al., 2012b). 96 well plate was coated with 10mg/mL anti-FSAP rabbit polyclonal antibody (2μg/mL) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) over night at 4°C, followed by blocking with standard buffer (20 mM Na-citrate, 150 mM NaCl, 100mM Arginin, pH 6.0) containing 3% BSA. Standard plasma and mouse plasma were diluted (1:200) in a standard buffer containing 0.1% Tween 80, 1% BSA and 100U/mL unfractionated heparin (Ratiopharm, Germany) and applied on the plate, incubated for 1 h at room temperature (RT) and washed three times in TBS with 0.1% Tween 20. Recombinant single-chain pro-uPA (1 μg/mL; Saruplase, Grünenthal, Stolberg, Germany in TBS with 0.1% Tween 80, 2 mM $CaCl_2$, pH 7.2) was added and incubated for 5 min followed by chromogenic substrate S-2444 (2.5mM) L-pyroglutamyl-glycyl-L-arginine-p-nitroanilinedihydro-chloride;HaemochromDiagnostica, Essen, Germany) and further incubation at 37°C. Absorbance was recorded at 405 nm, every min with micro plate reader EL808 (Biotek Instruments, Winooski, OR, USA) for 60 min (37°C). For the assay, calibration curves were established through a dilution series of Standard Human Plasma (SHP, Siemens Diagnostics, Marburg, Germany). SHP served as reference for the measured FSAP activity, which was defined as 1 plasma equivalent unit (1000 mU/mL).

3.9. Fibrin plate assay

A solution containing fibrinogen (9.5 μM) from human plasma (Sigma) was laid over dishes and clotted by the addition of human thrombin 15 IU (Enzyme Research Laboratory). Fresh plates were prepared and incubated at room temperature for 30 min before the experiment. 10 μL of uPA (3ng) and FSAP (10 nM, 30 nM, 60 nM and 90 nM) alone or together with uPA and plasma euglobulins of normal and experimental plasma (10 μL) were pipetted onto the surface of the fibrin plate (n=3), and incubated further at room temperature overnight. Plasma euglobulin preparation: Plasma (150 μL) was acidified (1:10 dilution) with 1% acetic acid (pH 5.9) on ice. After 15-20 min on ice, the precipitate was centrifuged (800*g* for 5 min at 0°C) and dissolved in 150μL of tris buffer (pH 7.5). The lytic areas were determined as a measurement of fibrinolytic activity. To determine the pro-uPA activation of pure human FSAP, 0.5% of agarose solution (5 mL) was used additionally to create solid matrix well and to measure the exact pro-uPA activity.

3.10. Flow cytometry and hematological parameters

Heparinized whole blood was diluted 1:20 with modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.0) containing 5 mM glucose, 0.35% bovine serum albumin (BSA), and 1 mM $CaCl_2$. For glycoprotein expression and platelet count, blood samples were incubated with appropriate fluorophore-conjugated monoclonal antibodies for 15 min at room temperature and were directly analyzed on a FACS calibur (Becton Dickinson, Heidelberg, Germany).Whole blood, drawn from the inferior vena cava of anesthetized mice into a one-tenth volume of 0.1 M citrate, was used for complete blood cell analysis.

3.11. *In situ* casein zymography

The assay was performed essentially as described in the standard protocol (BODIPY FL E6638, Green Fluorescence). Cryostat sections (5 μm in thickness) of unfixed thrombotic carotid artery tissue were air-dried for 2 min. Mastermix was prepared and 30 μL was dropped on each section and covered with small cover slip. The slides were then incubated immediately at 37°C for 4 h and pictures were taken using a fluorescent microscope (Leica). Preparation of master mix for 15 sections: 1% agarose (500μL) was prepared under sterile conditions and prewarmed at 37°C. To this 10μg/mL

plasminogen (2.6 µL), 100 µg/mL DAPI (0.5 µL), casein E6638 (50 µL) was added. 30 µL of the same mixture was then poured on each of the sections. For negative control, prior to the master mix, sections were pre-incubated for 30 min with 0.5 mM amiloride (uPA inhibitor). This assay demonstrated the total substrate turnover at specified time point.

3.12. Casein zymography

Casein zymography is used to measure the plasminogen activators (uPA and tPA). Mice plasma samples (0.5µL) were loaded under a non-reduced and non-boiled condition onto a 10% SDS polyacrylamide gel. The gel was washed at 25°C for 30 min with 2.5% Triton X-100 to remove SDS and overlayed on the polymerized agarose matrix plate followed by incubation at 37°C. Agarose matrix plate preparation: Mix 1% agarose solution (3 mL) with 8% milk solution (1.5 mL), 1 M PBS (4.5 mL), 10% sodium azide (100µL) and plasminogen (40 mg/mL)at 37°C on a pre-warmed station, spread the whole solution (9 mL) quickly on the GelBond film (85 x 100 mm, thickness 0.2 mm, Germany) and allow it to polymerize for 30 min. Activity of plasminogen activators, were measured by the zone of clearance, according to their molecular weight.

3.13. Measurement of circulating factors and blood parameters

Whole blood, collected from the inferior vena cava of anesthetized mice in 0.1 M citrate (one tenth volume), was used to prepare plasma. Circulating coagulation factor activities in citrated mouse plasma were measured as prothrombin time-PT (FII, FV, FVII, FVIIa, FX) or activated partial thromboplastin time-aPTT (FVIII, FIX, FXI, FXII) in single factor-depleted human plasma relative to that of human standard plasma (Siemens Healthcare, Marburg, Germany). Briefly, mouse plasma samples were pre-diluted to three different concentrations in imidazole buffer (50 mM imidazole, 150 mM NaCl, 1% BSA, pH 7.4) to obtain clotting times within the range of the human reference plasma. The prothrombin time was measured at a final 1:6600 dilution of the tissue factor/phospholipid reagent (Thromborel S, Siemens Healthcare) to achieve reasonably long clotting times (human control plasma: about 140-180 s). The aPTT reagent was used without pre-dilution. The clotting was measured in a micro plate reader with increase in turbidity at 405 nm.

PT assay*:* 40 µL imidazole buffer was mixed with 60 µl of diluted plasma sample, 20 µL depleted plasma, 30 µL Thromborel S (1:1000 dilution in imidazole buffer), and

incubated for 8 min at 37°C. 50 µL 20 mM $CaCl_2$ was added to start the clotting reaction. FVIIa was quantified in the PT assay using a soluble recombinant tissue factor/phospholipid mix as described recently(Etscheid et al., 2012b).

aPTT assay*:* 30 µL imidazole buffer was mixed with 10 µL of the diluted mouse plasma or human reference plasma, 30 µL single factor-depleted plasma and 30 µL of a PTT reagent (Actin FS; Siemens Healthcare). The incubation condition, clotting start and measurement were as described previously for the PT.

TFPI Assay**:** TFPI activity in mouse plasma was quantified as described recently(Kanse et al., 2012a) using the Actichrome TFPI activity assay (American Diagnostica; Pfungstadt, Germany). This assay measures in TFPI-depleted human plasma, the ability of mouse plasma TFPI to inhibit the activation of FX by the TF/FVIIa complex. Human TFPI reference standard in TFPI-depleted human plasma served as calibration curve. To test if mouse FSAP can inactivate mouse TFPI we used a polyclonal antibody to capture FSAP from mouse plasma. Thereafter, mouse recombinant TFPI (R&D Systems, Wiesbaden, Germany) was incubated in the wells for 60 min at RT and TFPI activity was measured as described above. Dr. Michael Etscheid made all these measurements from the Paul Ehrlich Institute, Langen, Germany.

3.14. Histology and immunohistochemistry

Harvested tissues were immediately embedded in OCT compound (Tissue-Tek, Miles Inc), snap-frozen in pre-cooled 2-methyl butane, and stored at -80°C until further analysis. 5 µm cross sections were made through the whole frozen tissue and stained with Hematoxylin and Eosin (Adler lab: http://www.jhu.edu/adlerlab/protocols/histo_HE) and Carstairs stain (online reference protocol from Electron Microscopy Sciences (EMS):http://www.emsdiasum.com/microscopy/technical/datasheet/26381.aspx). Carotid artery thrombosis vessels were stained with TFPI (H-120, 200 µg/mL, 1:100; Santa Cruz Biotech),vWF (Rabbit polyclonal, 400µg/mL; 1:500, DAKO, Denmark), fibrinogen (Rabbit polyclonal, 400µg/mL; 1:500, DAKO, Denmark), Fibronectin (Rabbit polyclonal, 1mg/mL; 1:400, Abcam). Light microscopy and fluorescence microscope images were captured using Leica Microsystems, Wetzlar, Germany equipped with a High speed VISitron systems camera using MetaMorph imaging software version series 7.0.

3.15. SDS-PAGE and western blotting

Plasma or tissue extracts were separated by 10 or 12% SDS-PADE under non-reducing conditions. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and probed with primary antibodies. Bound antibodies were detected using horseradish peroxidase conjugated secondary antibodies (Swine anti rabbit polyclonal; P0217, DAKO, Denmark) and the enhanced chemiluminescence detection system (Amersham-Pharmacia, GE Healthcare, Germany). Blots were re-probed for either the plasma protein ceruloplasmin or actin as appropriate. Primary antibodies used: TFPI (Rabbit polyclonal; sc-28861, Santa Cruz Biotech, Germany), plasminogen (Rabbit polyclonal; A0081, DAKO, Denmark), ceruloplasmin (Rabbit polyclonal; A0031, DAKO, Denmark) and FSAP (Rabbit Anti-mouse; ME New).

3.16. Measurement of active TFPI by ligand blotting with Biotinylated FXa

Factor X (Xa, 2 U/µL, Invitrogen) was dialysed against 100 mM sodium phosphate buffer (pH 7.4). A 20 mg/mL Sulfo-NHS-LC-Biotin (ThermoFisher scientific, Germany) stock solution in DMSO (stock solution) was added to the protein solution to obtain a 10-fold molar excess of biotinylation reagent over protein. The biotinylation reaction was allowed to proceed for 60 min and the unbound biotin was removed through dialysis. This biotinylated factor Xa was used as a primary probe for the ligand blot to detect the active TFPI on PVDF membranes.

3.17. Statistical Analysis

Results were shown as mean ± SD. Statistical analysis between WT and $FSAP^{-/-}$ mice was assessed by nonparametric Mann-Whitney U test. Differences in time course of processes was analysed using Kaplan Meier plots with log-rank (Mantel-Cox) test comparisons. More than 2 groups are analyzed by one-way ANOVA followed by Bonferroni posthoc test. *P<0.05, **P<0.01, ***P<0.001 and n.s. indicates not significant. Western blots were quantified using Image J, NIH software, (version 1.62).

4. RESULTS

4.1. Breeding and genotyping of FSAP$^{-/-}$ (knockout) mice

We have previously reported the generation of FSAP$^{-/-}$ mice (Borkham-Kamphorst et al., 2013). FSAP$^{+/-}$ mice from each generation were crossed with clear C57BL/6J mice for at least 10 generations (F10). FSAP$^{-/-}$ mice showed no difference in the normal breeding parameters like gestation period, litter size, weaning period etc. Cage activity, food intake, water intake, nature of the feces, etc., were identical. There were no differences in fertility, development and survival rate between FSAP$^{-/-}$ mice and WT littermates. FSAP genotypes were confirmed by PCR using genomic DNA extracted from ear punch samples (NucleoSpin® Tissue kit, Macherey-Nagel, Germany) with oligonucleotides 5′- GAA TTC ATG GAG GCT TTG -3′ for both targeted neomycin gene and endogenous gene in combination with 5′-CCG TGT CCT GCT GCT AAT-3′ for endogenous gene or 5′-GCA GCG CAT CGC CTT CTA TC-3′ targeted neomycin gene.

4.2. Phenotypic difference between WT and FSAP$^{-/-}$ mice

FSAP$^{-/-}$ mice displayed no spontaneous bleeding events and blood pressure and heart rates were normal. Gross and microscopic histological evaluation revealed no notable differences in the FSAP$^{-/-}$ mice when compared to WT mice. Deletion of FSAP had no significant impact on peripheral platelet counts and basic blood cell

Table 4.1: Basic blood parameters in WT and FSAP$^{-/-}$ mice

BLOOD CELLS	WT	FSAP$^{-/-}$ mice
Platelets (x 10^3/µL)	988.75 ± 194.75	971.25 ± 104.04$^{n.s}$
MPV (fL)	5.36 ± 0.15	5.24 ± 0.11$^{n.s}$
RBC (x 106/ µL)	9.56 ± 2.12	9.81 ± 1.61$^{n.s}$
WBC (x 10^3/µL)	14.56 ± 2.59	13.30 ± 3.60$^{n.s}$
HCT (%)	52.22 ± 11.91	52.64 ± 9.00$^{n.s}$

Basic blood parameters were assessed using diluted whole blood with sysmex hematology analyzer in WT and FSAP$^{-/-}$ mice. Results were expressed as mean ± SD (n=6). *p<0.05. n.s. indicates not significant. HCT, hematocrit; RBC, Red blood cells; WBC, White blood cells; MPV, Mean platelet volume. Experiment performed with the help of Ina Thielman and Martina Morowski at University of Wurzburg, Germany. fl: Femtolitre; µL: microliter.

counts of RBCs and WBCs **(Table 4.1).** Also, there were no significant differences in the expression of the prominent platelet surface receptors including integrins, GPVI, GPV, etc., **(Table.4.2).**

Table 4.2: Levels of platelet glycoproteins in wild type and $FSAP^{-/-}$ mice

EXPRESSION (MFI)	WT	$FSAP^{-/-}$
GPIb	331.00 ± 16.73	$337.50 \pm 7.58^{n.s}$
GPV	297.33 ± 12.48	$286.83 \pm 3.60^{n.s}$
GPIX	414.67 ± 6.92	$419.00 \pm 2.53^{n.s}$
GPVI	16.33 ± 0.52	$16.17 \pm 0.75^{n.s}$
CLEC-2	146.50 ± 5.75	$140.33 \pm 5.75^{n.s}$
α_2	47.83 ± 1.72	$48.33 \pm 1.03^{n.s}$
α_5	21.67 ± 1.75	$21.20 \pm 0.45^{n.s}$
β_1	121.50 ± 3.45	$118.67 \pm 3.61^{n.s}$
β_3	216.83 ± 7.39	$208.33 \pm 7.55^{n.s}$
$\alpha IIb\beta_3$	524.33 ± 10.33	$519.83 \pm 12.21^{n.s}$
CD9	955.00 ± 25.04	$982.33 \pm 18.28^{n.s}$

Expression of glycoproteins on the platelet surface was determined by flow cytometry. Diluted whole blood was incubated with FITC-labeled antibodies at saturating concentrations for 15 min at RT and platelets were analyzed directly. Results were expressed as mean fluorescence intensity (MFI) ± SD (n=6). *P<0.05 and n.s. indicates not significant. α_2, integrin α_2; β_1, integrin β_1; $\alpha IIb\beta_3$, integrin $\alpha IIb\beta_3$. Experiment performed with the help of Ina Thielman and Martina Morowski at University of Wurzburg, Germany.

4.3. FSAP is essential for normal primary haemostasis

In a tail-bleeding model **(Fig. 4.1)** a significant haemostatic defect was observed in $FSAP^{-/-}$ mice. $FSAP^{-/-}$ mice exhibited prolonged initial bleeding compared to littermate WT mice in a tail-bleeding test (≈ 400s and 200s respectively), although the differences were not significant **(Fig. 4.1A)**. Within the 15 min time scale of the experiment, about 50% $FSAP^{-/-}$ mice (≈ 600s) displayed re-bleeding compared to 10% of the WT littermates (≈300s) **(Fig. 4.1B)**. The total blood loss was measured

spectrophotometrically and it showed significantly higher blood loss **(Fig. 4.1C)** in FSAP$^{-/-}$ mice due to the frequent re-bleeding events. These results led us to further investigate the progression of thrombosis in FSAP$^{-/-}$mice.

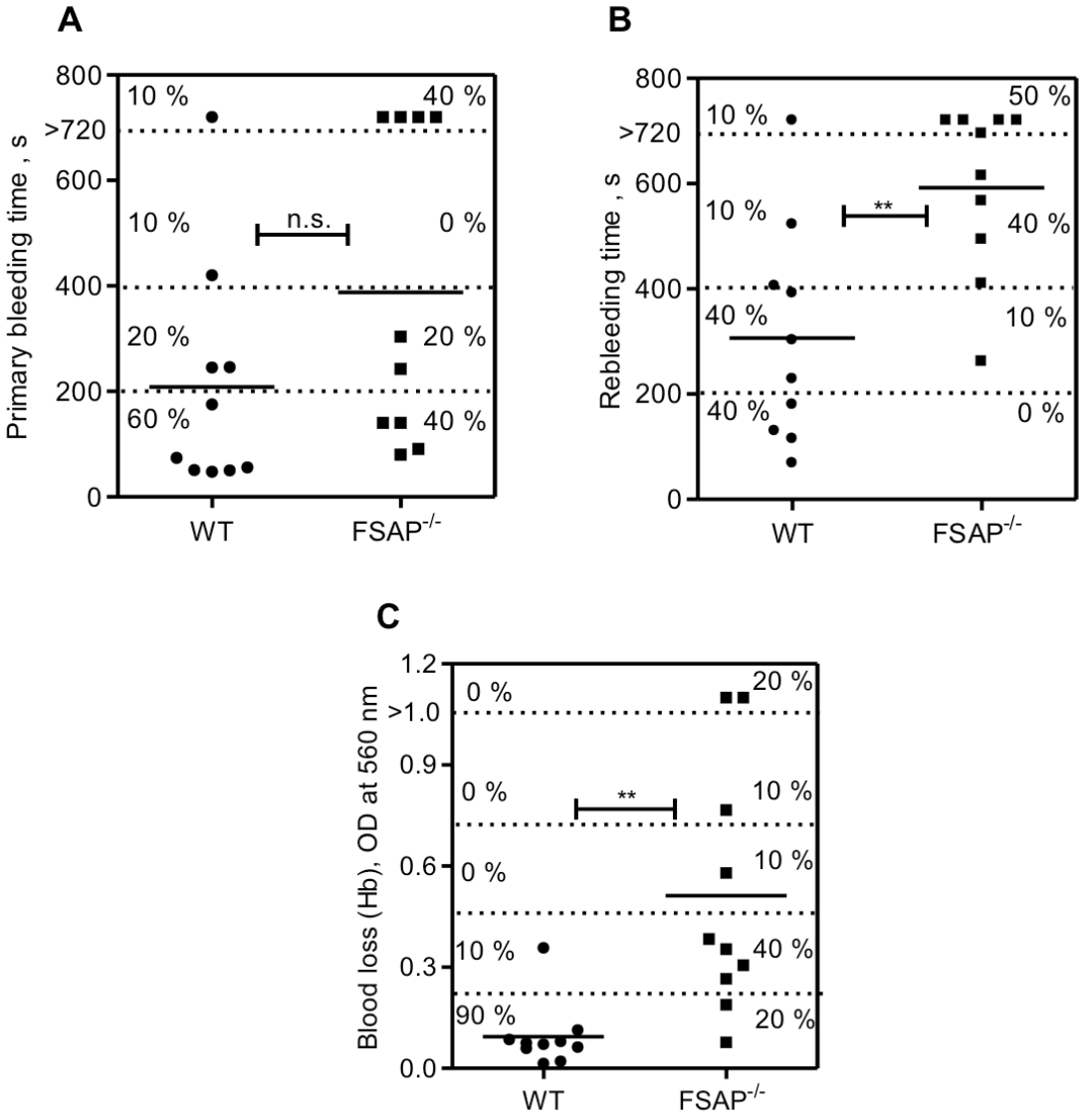


Figure 4.1: Haemostasis and thrombosis in FSAP$^{-/-}$ mice. Tail bleeding time of WT and FSAP$^{-/-}$ mice (n=10) were monitored by positioning the resected tail in a tube containing saline at 37°C and these tubes were changed in every min. The experiment was stopped after 12 min and hemoglobin content was measured spectrophotometrically. **(A)** primary bleeding and **(B)** re-bleeding was recorded for each mouse during the course of the experiment and plotted as in percentage blocks. **(C)** Total blood loss over the 12 min period was recorded for each mouse and expressed in percentage blocks. Data were analyzed by Welch's t-test. *P<0.05, **P<0.01 and n.s. indicates not significant.

4.4. Reduced thrombus stability in FSAP$^{-/-}$ mice

4.4.1. $FeCl_3$ induced venous thrombosis: Reduced thrombus volume in FSAP$^{-/-}$ mice

Venous thrombosis was induced in the inferior vena cava (IVC) using 3.5% $FeCl_3$ The thrombus protein content as well as thrombus weight was higher in WT mice compared to FSAP$^{-/-}$ mice (**Fig. 4.2A and B),** although these differences were not statistically significant. Thrombus formations in both strains was reduced by

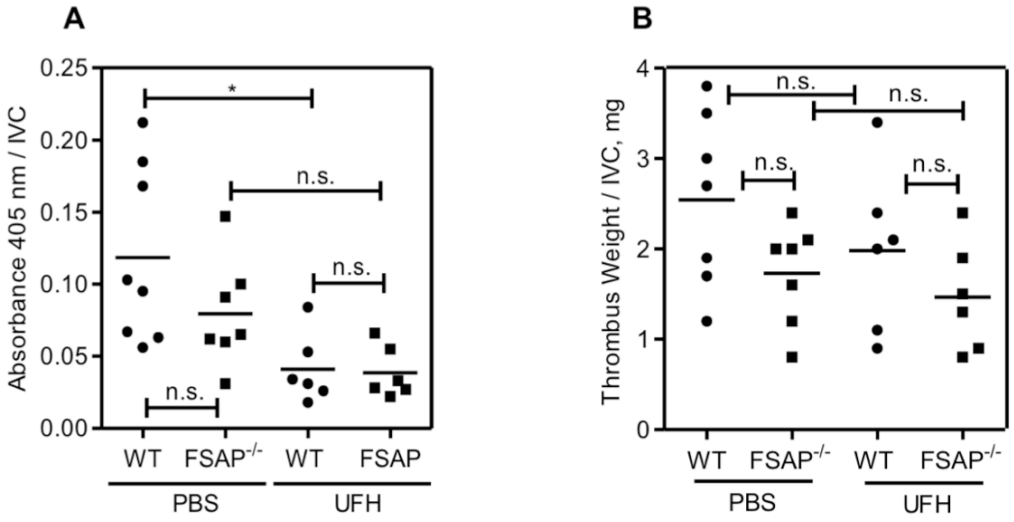


Figure 4.2: $FeCl_3$ **induced venous thrombosis in WT and FSAP$^{-/-}$ mice:** Venous thrombosis was induced using a topically applied filter paper soaked with 3.5% $FeCl_3$ to the IVC for 3 min. Thrombus protein content (A) and thrombus weight (B) were measured after 30 min. Data were expressed in scatter plot with mean bar. *P<0.05 and n.s. indicates not significant.

heparin application (100 U/kg, intravenously), consistent with the fact that this model relies strongly on the plasmatic coagulation factors **(Fig. 4.2A and B).**

4.4.2. Collagen /epinephrine induced pulmonary thromboembolism

To investigate the consequences of FSAP deficiency *in vivo*, we examined WT littermates and FSAP$^{-/-}$ mice in a model of lethal pulmonary thromboembolism induced by infusion of collagen and epinephrine through jugular vein. Mice of both genotypes died within 4 to 8 min except that the onset of death was significantly later in the FSAP$^{-/-}$ mice **(Fig. 4.3A)**. The deposits of platelet/fibrin complexes were examined through histological section of lung tissue through H&E staining and it showed reduced platelet/fibrin complex in FSAP$^{-/-}$ mice compared to WT mice **(Fig. 4.3B)**. Although there were no differences in number of minor occluded vessels in both the genotypes, the major vessels were

obstructed in WT mice. Thus, lack of endogenous FSAP, playing a protective role in models of venous thrombosis and pulmonary thromboembolism.

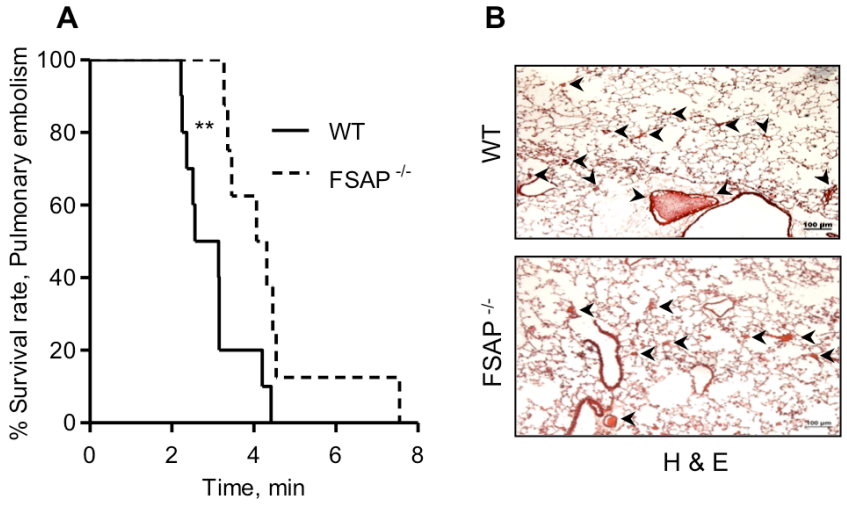


Figure 4.3: Pulmonary thromboembolism: (A) Kaplan Meier plot of mortality associated with the injection of collagen (0.8 mg/ Kg body weight.) and epinephrine (60 µg/Kg body weight) via jugular vein in WT (n=10) and FSAP$^{-/-}$ (n=10) mice. **(B)** Cross section (5 µm) of a collagen epinephrine induced pulmonary embolism model lung tissue stained with hematoxylin and eosin from WT (n=3) and FSAP$^{-/-}$ (n=3) mice. Arrow indicates the micro vascular thrombosis. Data were analyzed with Kaplan Meier plot and expressed as % survival rate. *P < 0.05, **P < 0.01 and n.s. indicates not significant.

4.4.3. $FeCl_3$ induced carotid artery thrombosis

Thrombus formation was also induced in the carotid artery by 10% $FeCl_3$ application and occlusion was determined by measuring blood flow using a Doppler flow probe **(Fig. 4.4B).** Blood flow decreased progressively and led to occlusion of the artery in 95% of the animals of the WT genotype. Mean initial flow rates ranged from 0.6 to 1.2 mL/min in WT and FSAP$^{-/-}$ mice. In FSAP$^{-/-}$ mice, thrombus formation was considerably delayed ($P< 0.05$) and thrombus was stable only in 56% of the mice **(Fig. 4.4A).** Cross sections of thrombi from WT and FSAP$^{-/-}$ mice were stained with H&E as well as Carstairs staining to visualize matrix (fibrin, collagen, platelets and smooth muscle cells) and thrombotic tissue. WT mice had even platelet aggregation whereas FSAP$^{-/-}$ mice had disturbed aggregation with a loose structure **(Fig. 4.4C and D)**. Immunohistochemistry analysis of carotid artery thrombosis was also performed to investigate the role of procoagulant mediators. In the entire thrombus vWF, fibrinogen and PDGF-BB showed no changes.

However, changes were observed in FSAP$^{-/-}$ mice with respect TFPI (described under section 4.6).

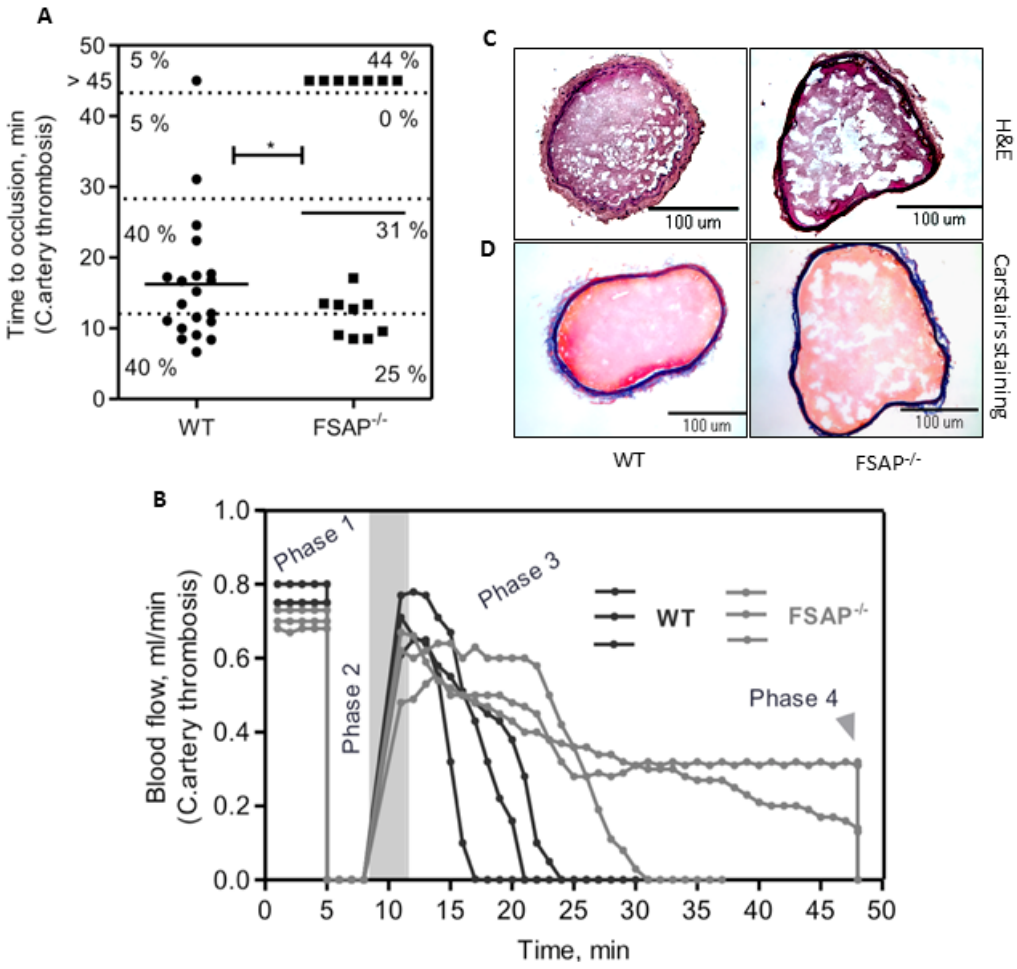


Figure 4.4. $FeCl_3$ induced arterial thrombosis in $FSAP^{-/-}$ mice: Vascular injury was induced in carotid artery by topic application of 10% $FeCl_3$. **(A)** The scatter plot shows time to occlusion (TTO) for carotid arteries in WT (n=20) and $FSAP^{-/-}$ (n=16) mice. TTO was calculated based on time taken for flow to stop completely after vascular injury. Data were represented as percentage blocks. **(B)** Blood flow in WT and $FSAP^{-/-}$ mice during the course of the experiment (n=3). After the carotid artery exposure, normal carotid blood flow was measured (Phase 1) then mice were subjected to carotid artery injury with 10 % $FeCl_3$ solution saturated paper for 3 min (Phase 2). After exposure, vessels were cleaned with normal saline and the flow probe was placed around the carotid artery, above the injury site. These processes take approximately 2 min, which is shown in the graph as gray scale. Time taken to occlusion (TTO) was measured after the vascular injury (Phase 3). If no occlusion was observed till 45 min, then this was designated as not occluded and the experiment was stopped manually (Phase 4). **Carotid artery thrombosis:** Representative cross section (5 µm) of carotid artery thrombi (n=5) from WT and $FSAP^{-/-}$ mice after hematoxylin and eosin (H&E) staining **(C)** as well as Carstairs staining representative images **(D)** which represents fibrin (bright red), collagen (bright blue) and platelets (navy blue). WT: Wild-type mice; $FSAP^{-/-}$: FSAP knockout mice. Data were analyzed by nonparametric Mann-Whitney U test. *p < 0.05 and n.s. indicates not significant.

4.4.4. Vascular injury in mesenteric arterioles: Intravital study

$FeCl_3$ induced oxidative vascular injury in mesenteric arterioles model was used to test the robustness of these findings. Occlusive thrombus formation was induced in mesenteric arterioles by $FeCl_3$ application and assessed by intravital fluorescence

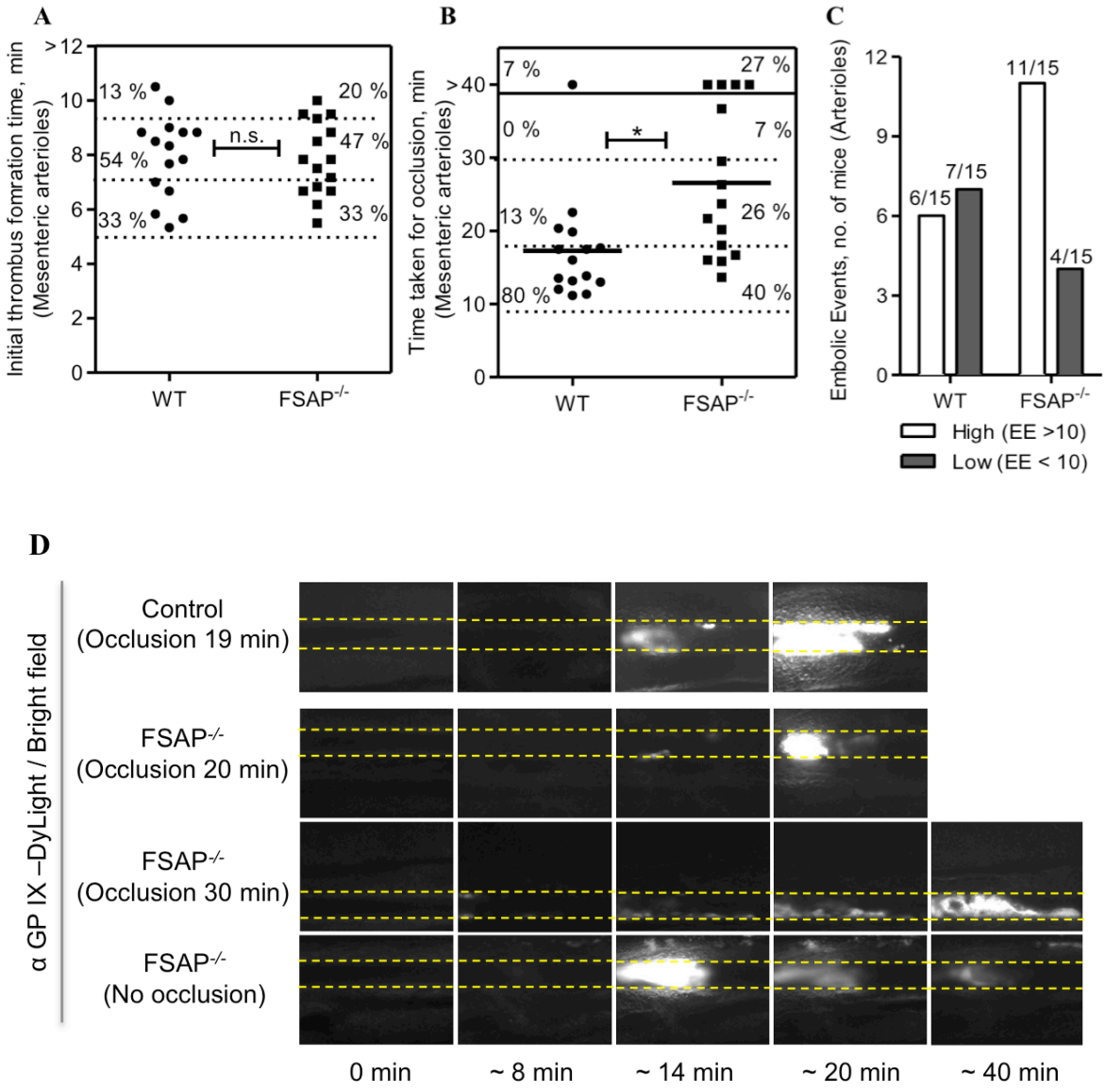


Figure 4.5: $FeCl_3$ induced thrombosis in the mesentery: Vascular injury was induced in mesenteric arterioles by topic application of $FeCl_3$. **(A)** Initial thrombus formation was measured (>10 µm) between WT and $FSAP^{-/-}$ mice. Data were expressed as scatter plot in percentage blocks. Each dot represents individual animals. The scatter plot **(B)** shows time taken for the occlusion (TTO) of mesenteric arterioles in WT (n=15) and $FSAP^{-/-}$ (n=15) mice. Data were expressed in percentage blocks. **(C)** Embolic events (EE) were classified according to the frequency of the events. More than 10 embolic events were considered to be a high incidence (EE > 10) and less than 10 embolic events were considered to be low incidence (EE < 10) in WT (n=15) and $FSAP^{-/-}$ (n=15) mice. **(D)** Representative phase contrast images were shown for the better illustration. Indicated time points represent min after $FeCl_3$ induced injury. Images were captured with a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Jena, Germany) with a 63 X/0.75 Ph1 objective, a 100-W HBO fluorescent lamp source, and a CCD camera (CV-M300; Visitron Systems, (Germany) connected to an AG-7355 S-VHS video recorder (Panasonic, Matsushita Electric, Osaka, Japan). Video tapped images were analyzed using a computer assisted image analysis program, Meta View Version 5.0. (Experiment performed with the help of Ina Thielman at University of Wurzburg, Germany). Data were analyzed by nonparametric Mann-Whitney U test. *$p < 0.05$ and n.s. indicates not significant. WT: Wild-type mice; $FSAP^{-/-}$: FSAP knockout mice.

microscopy of labeled platelets (Dylight–coupled GPIX-IgG derivative). Adhesion of labeled platelets at the site of vascular injury was normal demonstrating that platelet response intact in the absence of endogenous FSAP **(Fig. 4.5).** Time to initial thrombus (TIT) formation was indistinguishable in both strains of mice **(Fig. 4.5A)**, where both WT littermates and FSAP$^{-/-}$ mice showed approximately 8 to 12 min. Time to occlusion (TTO) or stable thrombus formation in FSAP$^{-/-}$ mice was delayed and in 27% of the cases the thrombi was not stable and there was no occlusion of the arterioles when compared to 7% of WT mice **(Fig. 4.5B)**. Thrombi were unstable, loose and detached from the vessel wall and the number of embolic events (EE) was higher in FSAP$^{-/-}$ mice compared to WT littermates **(Fig. 4.5C)**. Representative intravital microscopy photomicrographs of thrombi from WT and FSAP$^{-/-}$ mice are as shown **(Fig. 4.5D)**. Some of the FSAP$^{-/-}$ mice occluded like WT littermates, but the intensity of the thrombi was less in FSAP$^{-/-}$ mice **(Fig. 4.5D)**. Thus, the facts from different mouse models demonstrate that endogenous FSAP is required for the stabilization of clots *in vivo*.

4.5. FSAP activity assay

A direct chromogenic substrate immuno-capture activity assay for FSAP was used to measure the circulatory FSAP concentration through its ability to activate pro-uPA. In all the above-described three mouse model experiments, plasma samples were collected

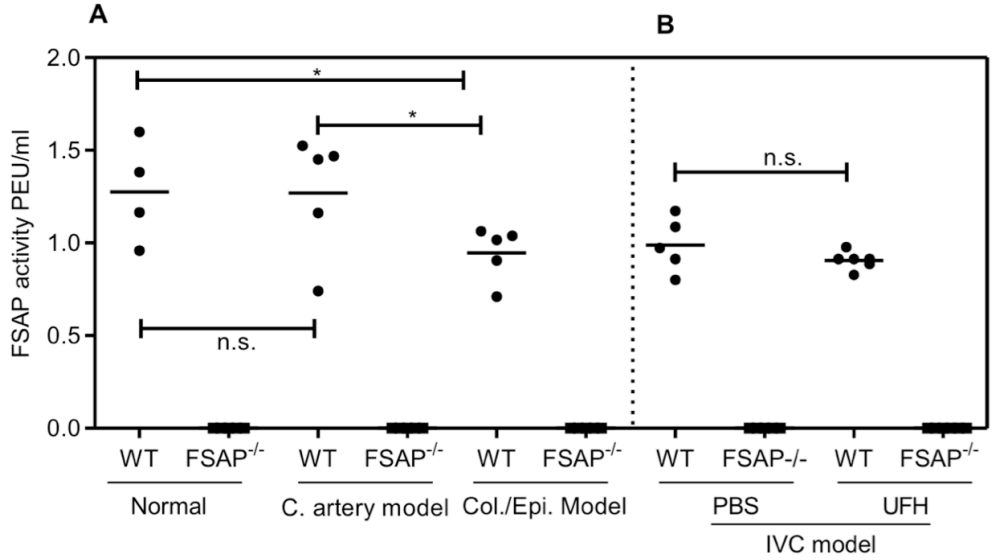


Figure 4.6: FSAP activity assay: FSAP activity was measured in plasma samples from all the experiments performed in this study. Data were expressed in plasma equivalent units (PEU) where standard human plasma represents 1 PEU/mL. Data were represented as scatter plots **(A & B)** with mean bars (n= 4-6) and analyzed with one-way ANOVA followed by *Bonferroni post hoc* test *p < 0.05 and n.s. indicates no significance. WT: Wild-type mice; FSAP$^{-/-}$: FSAP knockout mice. C.artery model: Carotid artery model; IVC: Inferior vena cava; Col./Epi. model: Collagen/Epinephrine or pulmonary embolism model.

and FSAP activity was measured by immunocapture assay (Borkham-Kamphorst et al., 2013; Kanse et al., 2012b). This assay was also used to confirm the genotype of the mice. The carotid artery thrombosis experiment did not show any changes in systemic FSAP activity levels in thrombotic WT littermates compared to non-thrombotic littermates. Whereas, collagen and epinephrine induced thromboembolism model showed reduced systemic FSAP activity levels in thrombotic WT mice compared to non-thrombotic mice **(Fig. 4.6A)**. IVC thrombosis experiment did show reduced systemic FSAP activity level after unfractionated heparin treatment as compared to PBS treatment in WT mice but it was statistically insignificant **(Fig. 4.6B)**.

4.6. The role of TFPI in the *in vivo* effects of endogenous FSAP

Our previous findings showed that TFPI was proteolytically cleaved and inactivated by FSAP *in vitro* (Kanse et al., 2012a). We thus further wanted to investigate if this was clinically reliable *in vivo* using mouse model. There are three types of TFPI isoforms in mice (α, β and γ), TFPIβ being the prime form in mouse adult tissue (Massberg et al., 2010). TFPIβ lacks the *Kunitz*-3 domain; as a result, the heparin-releasable pool of TFPI in mice is very small. Foremost, we showed that immune-captured mouse FSAP from mouse plasma could inactivate recombinant mouse TFPIα **(Fig 4.7A**). We next investigated whether FSAP degrades TFPI during arterial thrombosis *in vivo*. Carotid thrombi were exclusively stained for mouse TFPI and intense staining was observed in $FSAP^{-/-}$ mice compared to WT littermates **(Fig. 4.7B).** In carotid artery tissue extracts **(Fig. 4.8A)** and Western blotting analysis of mouse plasma **(Fig. 4.7C)**, we obtained only one TFPI band at 55 kDa in WT littermates as well as $FSAP^{-/-}$ mice. In mice plasma from $FeCl_3$ carotid artery thrombosis as well as venous thrombosis, TFPI was unaffected in $FSAP^{-/-}$ mice compared to WT littermates **(Fig. 4.7C, D).** Carotid artery tissue extracts of WT and $FSAP^{-/-}$ mice showed no differences in TFPI content in vessels with thrombosis or without thrombosis **(Fig. 4.8A).** Application of unfractionated heparin tends to decrease the levels of plasma TFPI **(Fig. 4.7D).**

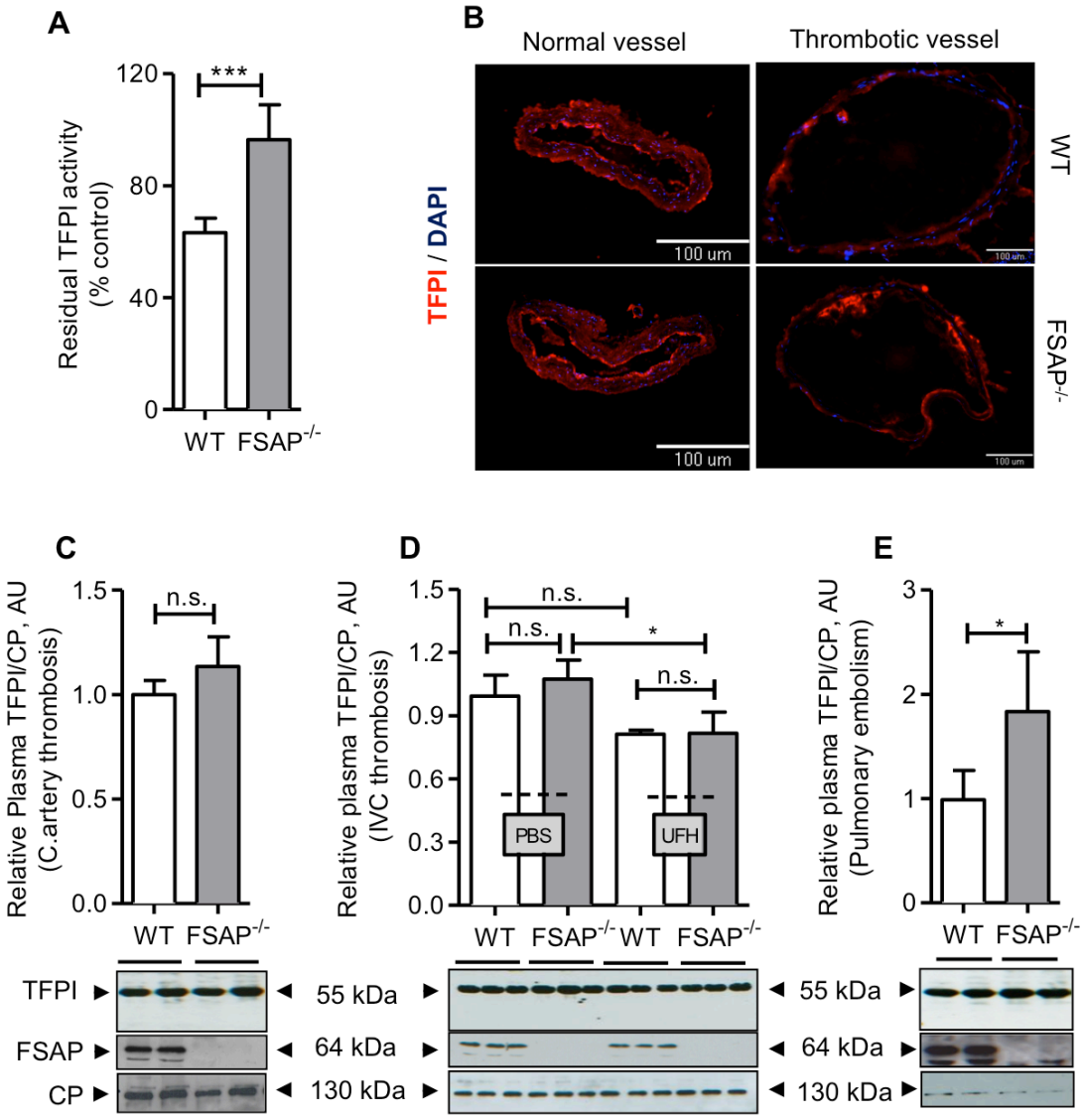


Figure 4.7. Endogenous TFPI in FSAP$^{-/-}$ mice: Mouse plasma FSAP was immune-captured using anti mouse FSAP antibody. **(A)** Recombinant mouse TFPI β was added and residual was measured (% activity of TFPI). **(B)** Frozen sections of carotid artery with thrombosis (5μm) were stained for TFPI in WT and FSAP$^{-/-}$ mice (n=3). TFPI in plasma of WT and FSAP$^{-/-}$ mice with **(C)** carotid artery thrombosis **(D)** venous thrombosis and **(E)** pulmonary thromboembolism were analyzed by Western blotting (n=4). Plasma from WT and FSAP$^{-/-}$ mice was compared with respect to ceruloplasmin (CP) (loading control). Bands were quantified and the intensity of the TFPI band is represented in arbitrary units (AU) in relation to the CP bands. (D) mice of both strains were given PBS or unfractionated heparin (100 U/Kg body weight) 10 min before induction of thrombosis. Data were expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001 and n.s. indicates not significant. C.artery thrombosis: Carotid artery thrombosis; IVC: Inferior vena cava; Col./Epi. model: Collagen/Epinephrine or pulmonary embolism model .

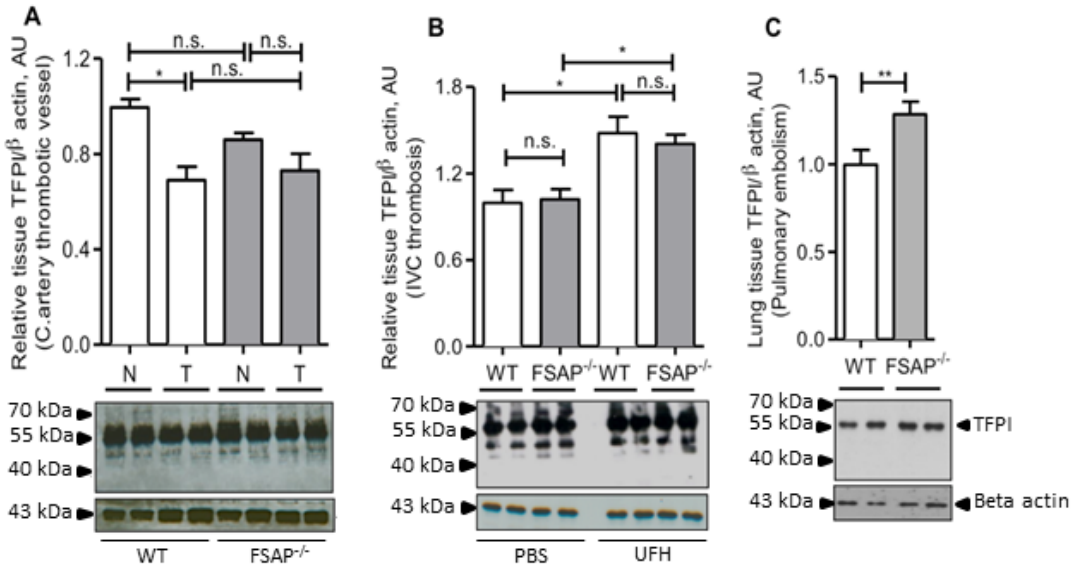


Figure 4.8: (A) TFPI in tissue extracts of carotid artery thrombi: Carotid artery thrombosis was induced by topical application of filter paper soaked in 10 % $FeCl_3$ solution for 3 min (T; grey bars). Contralateral vessels were used as controls (N; white bars). Thrombi/ vessels were collected and TFPI was analyzed by western blot (n=4). **(B) Reduced venous thrombus formation:** Venous thrombosis was induced by applying filter paper soaked in 5 % $FeCl_3$ topically onto the IVC for 3 min. Thrombus vessels were collected and the vessel TFPI was measured by western blot (n=4). **(C) Pulmonary embolism:** Lungs from pulmonary embolism model were analysed for TFPI by western blotting (n=4). Data were expressed as mean ± SD. *p<0.05, **p<0.01 and n.s. indicates not significant. C.artery thrombosis: Carotid artery thrombosis; IVC: Inferior vena cava.

These findings were further confirmed by the IVC tissue extracts i.e. TFPI and levels were determined by Western blotting. Unfractionated heparin treatment followed by IVC induced vessels displayed significantly higher TFPI **(Fig. 4.8B)** compared to PBS treated (control) group. In the collagen/epinephrine induced pulmonary thromboembolism model, the plasma TFPI in $FSAP^{-/-}$ mice was 1.8-fold higher than WT littermates signifying that a more systemic change in the thrombosis status is required to observe the effect of endogenous FSAP on TFPI **(Fig. 4.7E).** Similarly, lungs from collagen/epinephrine treated $FSAP^{-/-}$mice showed significantly high TFPI (Fig. 4.9C). Ceruloplasmin and β actin were used as loading controls and respective plasma FSAP level was measured to confirm the lack of endogenous circulating FSAP in the knockout mice **(Fig. 4.7)**.

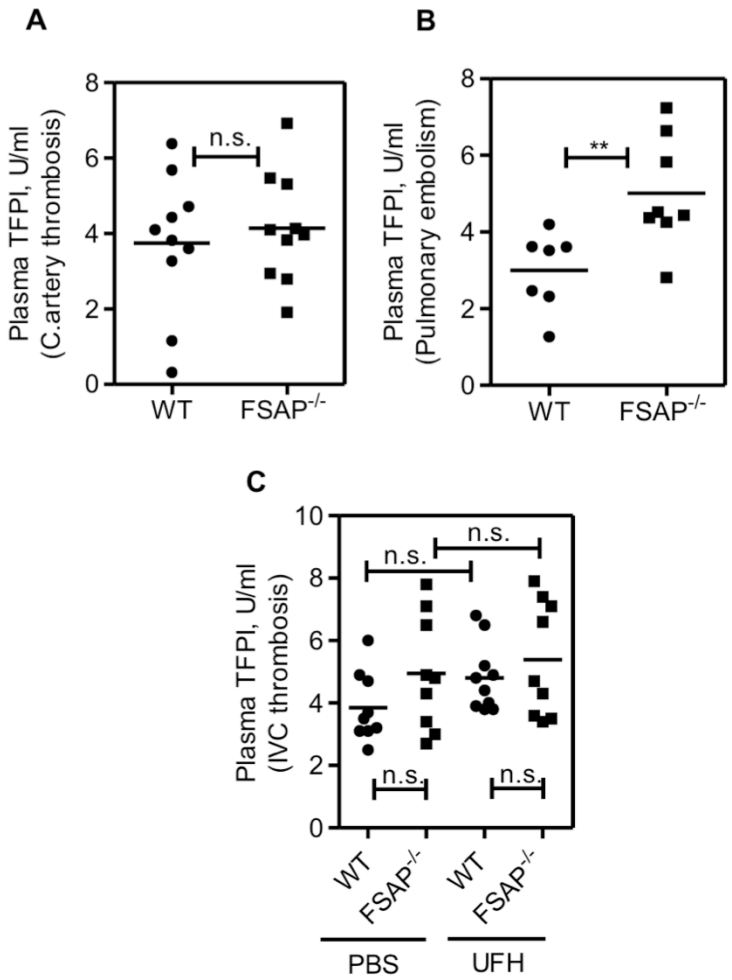


Figure 4.9: Status of TFPI in plasma of mice from the different thrombosis model: Mouse plasma from WT and FSAP$^{-/-}$ mice subjected to $FeCl_3$ induced carotid artery thrombosis, collagen/epinephrine induced pulmonary embolism and IVC thrombosis models were analyzed for TFPI by functional assay. Data were expressed in mean bars (n=7 to 10). *p<0.05, **p<0.01 and n.s. indicates not significant**.** Experiment performed by Michael Etscheid at Paul Erlich Institute, Germany. C.artery thrombosis: Carotid artery thrombosis; IVC: Inferior vena cava.

TFPI activity was also measured in the mice plasma using a functional assay that measures FXa generation in TFPI-deficient plasma in a TF-dependent manner. In the localized models of arterial **(Fig. 4.9A)** and venous thrombosis **(Fig. 4.9B)** there were no differences between TFPI levels but in the systemic thrombosis **(Fig. 4.9C)** model, TFPI levels were higher in FSAP$^{-/-}$ mice as shown by the functional assay **(Fig. 4.9A-C)** which correlated well with the Western blot analysis **(Fig. 4.7)**. Thus above study results represented that there was an association between lack of FSAP and higher circulating TFPI thereby showing that endogenous FSAP can regulate TFPI levels *in vivo*.

4.7.The role of active TFPI in the *in vivo* effects of endogenous FSAP

To further strengthen our hypothesis, active TFPI was also investigated by ligand blotting which relies on the binding of biotinylated FXa to active TFPI. The plasma from all the three mice thrombosis models were adopted for the ligand blotting to detect active circulating TFPI.

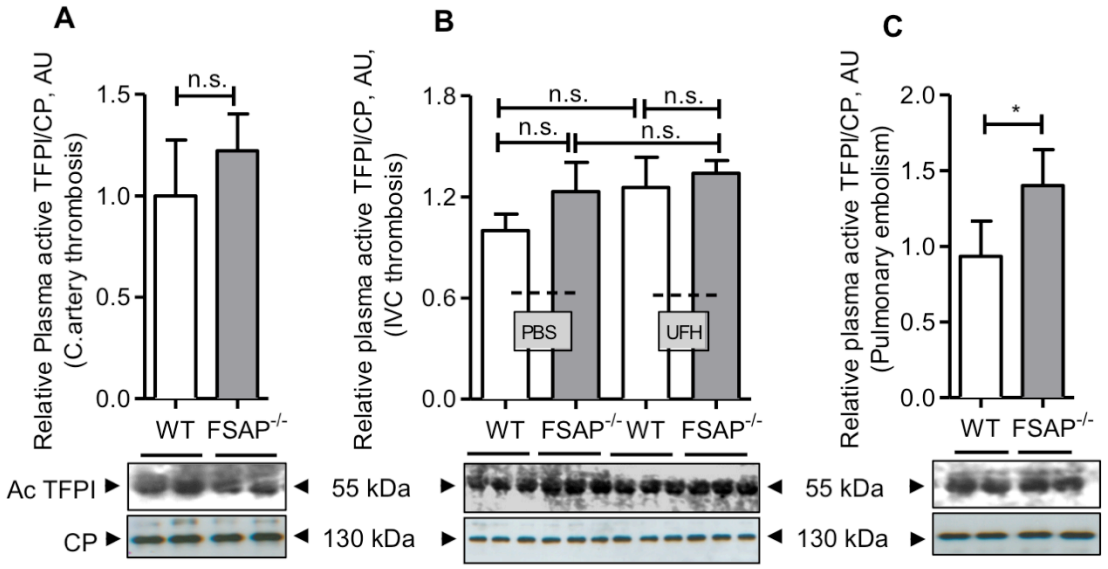


Figure 4.10: Endogenous active TFPI in FSAP$^{-/-}$ mice: Active TFPI in plasma of WT and FSAP$^{-/-}$ mice with **(A)** carotid artery thrombosis **(B)** venous thrombosis and **(C)** pulmonary thromboembolism were analyzed by western blotting (n=4). Plasma from WT and FSAP$^{-/-}$ mice was compared with respect to ceruloplasmin (CP) (loading control). Bands were quantified and the intensity of the TFPI band is represented in arbitrary units (AU) in relation to the CP bands. **(D)** mice of both strains were given PBS or unfractionated heparin (100 U/Kg body weight) 10 min before induction of thrombosis. Data were expressed as mean ± SD *p<0.05 and n.s. indicates not significant. C.artery thrombosis: Carotid artery thrombosis; IVC: Inferior vena cava.

In the localized models of $FeCl_3$ induced arterial **(Fig. 4.10A)** and venous thrombosis **(Fig. 4.10B)** there were no differences between TFPI levels but in the collagen/epinephrine induced systemic thrombosis **(Fig. 4.10C)** model, TFPI levels were higher in FSAP$^{-/-}$ mice as revealed by the functional assay method **(Fig. 4.9A-C)** which correlated well with the Western blot analysis **(Fig. 4.7)**. In summation, the data obtained from the present study, clearly provided evidence that there is an association between a lack of FSAP and higher circulating active TFPI levels *in vivo*.

4.8 The role of FVII activation in the *in vivo* effects of endogenous FSAP

While FVII was initially proposed to be a substrate for FSAP, we also investigated both FVII and FVIIa levels in the plasma **(Fig. 4.11)**. Since we were particularly interested in

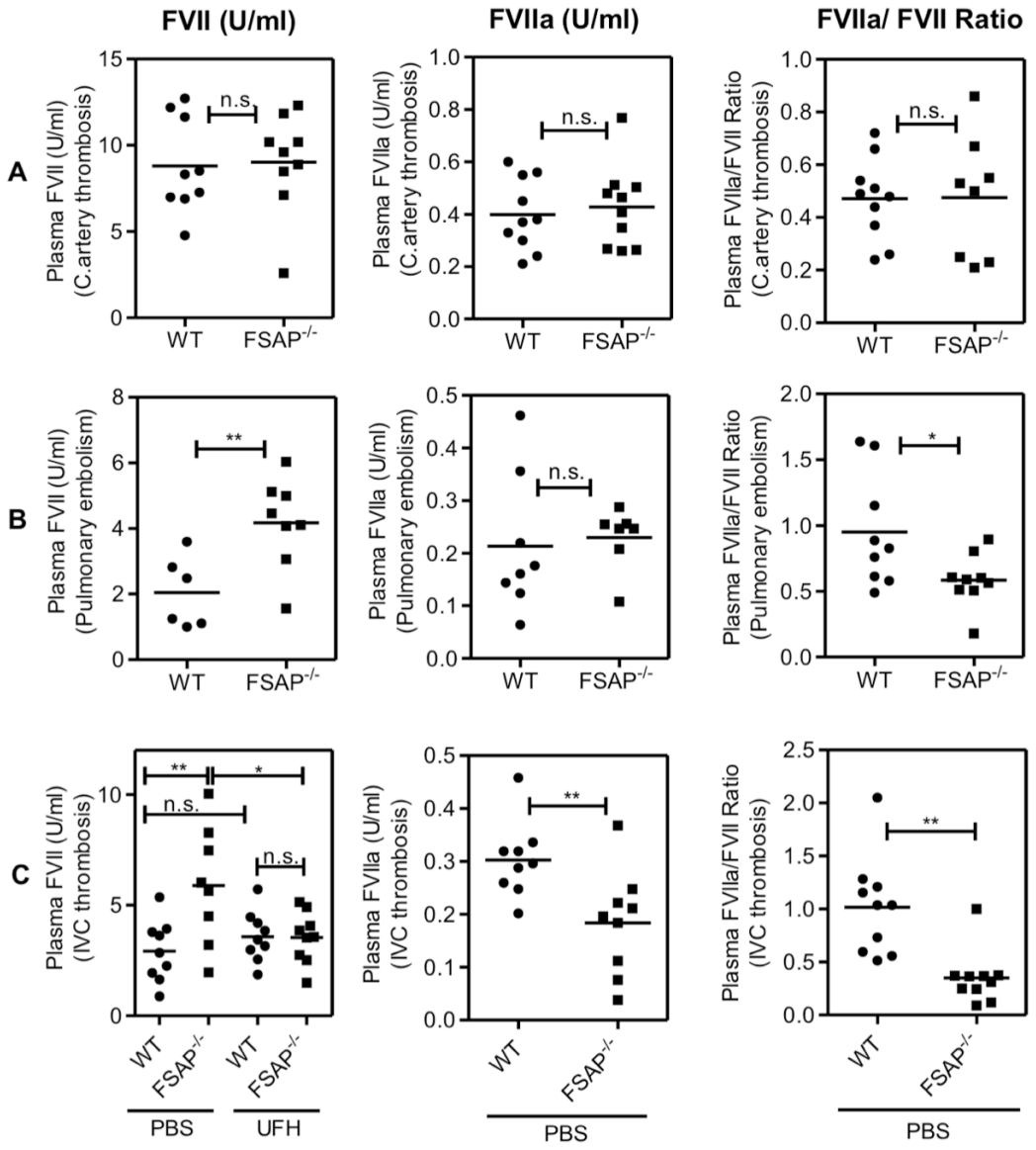


Figure 4.11: Status of extrinsic pathway factors in plasma of mice from the different thrombosis model: Mouse plasma from WT and $FSAP^{-/-}$ mice subjected to $FeCl_3$ induced carotid artery thrombosis **(Row A)**, collagen/epinephrine induced pulmonary embolism **(Row B)** and IVC thrombosis **(Row C)** were analyzed for TFPI, FVII, FVIIa and FVIIa/FVII ratio. Data were expressed in mean bars (n=7 to 10). *p<0.05, **p<0.01 and n.s. indicates not significant. Experiment performed by Michael Etscheid at Paul Erlich Institute, Germany. C.artery thrombosis: Carotid artery thrombosis; IVC: Inferior vena cava.

the question whether FSAP activates FVII, we also computed the ratio of FVIIa/ FVII to minimize the effect of deviations in the individual mice. In the venous thrombosis as well as the pulmonary thromboembolism model, $FSAP^{-/-}$ mice had lower levels of FVIIa/ FVII

(Fig. 4.11B &C). In the experiments where unfractionated heparin was given to the mice prior to the induction of venous thrombosis, no FVIIa levels could be measured due to the interference of heparin in the assay **(Fig. 4.11C)**. Hence, we could confirm that activation of endogenous FVII is a function of FSAP and this could be observed in the local thrombosis models as well as in the systemic thrombosis model.

4.9. Effect of endogenous FSAP on plasminogen and its activators

Romisch and colleagues have shown that FSAP could activate pro-uPA to uPA

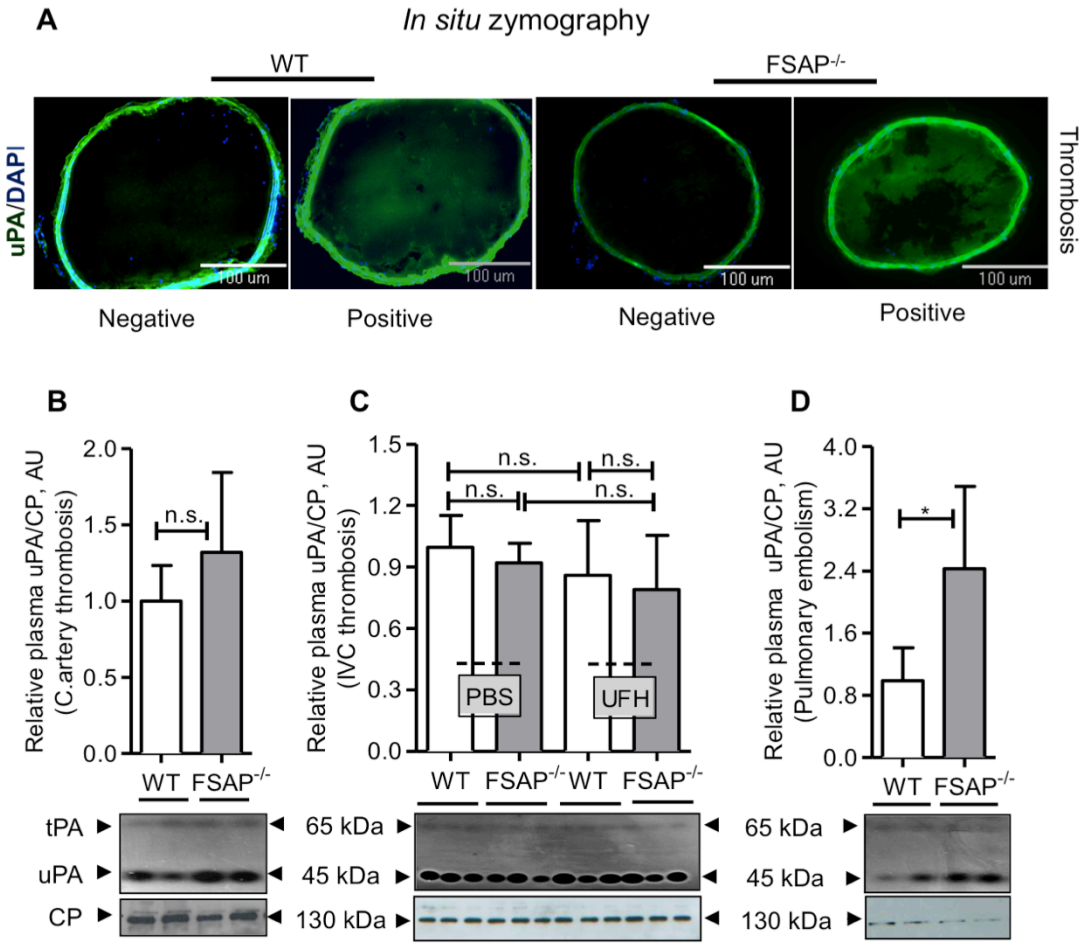


Figure 4.12: Endogenous uPA activity in FSAP$^{-/-}$ mice: Plasminogen activation was determined by *in situ* zymography. Fresh frozen unfixed sections of vessels with thrombi for WT and FSAP$^{-/-}$ mice were overlaid with a mixture containing plasminogen, and quenched fluorescent BODIPY FL casein E6638 and incubated at 37˚C. Activation of plasminogen was indicated by the development of green fluorescence. Plasminogen activation in plasma of mice with carotid artery thrombosis **(B)**, venous thrombosis **(C)** and pulmonary thromboembolism **(D)** were determined by gel-based casein zymography in the presence of plasminogen. Activation of plasminogen is indicated by dark lysis bands of either 45 kDa which is due to uPA or 65 kDa which is due to tPA. Both strains **(C)** of mice were given PBS or unfractionated heparin (100 U/Kg body weight) 10 min before induction of thrombosis. Plasma from WT and FSAP$^{-/-}$ mice was compared with respect to ceruloplasmin (CP) (loading control). Bands were quantified and the intensity of the TFPI band is represented in arbitrary units (AU) in relation to the CP bands. Data were expressed in mean ± SD (n=4). *p<0.05 and n.s. indicates not significant. C.artery thrombosis: Carotid artery thrombosis; IVC: Inferior vena cava

and thereby promote fibrinolysis *in vitro* (Romisch, 2002). So the differences in plasma fibrinolytic activity between WT and FSAP$^{-/-}$ mice were investigated using casein zymography. *In situ* zymography showed that there was higher plasminogen activity in thrombi of FSAP$^{-/-}$ mice compared to WT littermates **(Fig. 4.12A)**. Moreover, we also observed higher uPA levels in the thrombus of FSAP$^{-/-}$ mice. This activity was due to uPA since it was completely inhibited by the uPA inhibitor amiloride (Luikart et al., 2002). Plasma from WT and FSAP$^{-/-}$ mice showed no significant differences in uPA activity in the carotid and venous thrombosis models but was increased about 2.4-fold in the collagen/epinephrine induced pulmonary thromboembolism model. In the same experiment we did not observe any changes in tPA activity **(Fig. 4.12B-D)**. Plasma ceruloplasmin level was determined to calculate the normalized levels of uPA and tPA. Plasminogen levels, measured at the protein level by

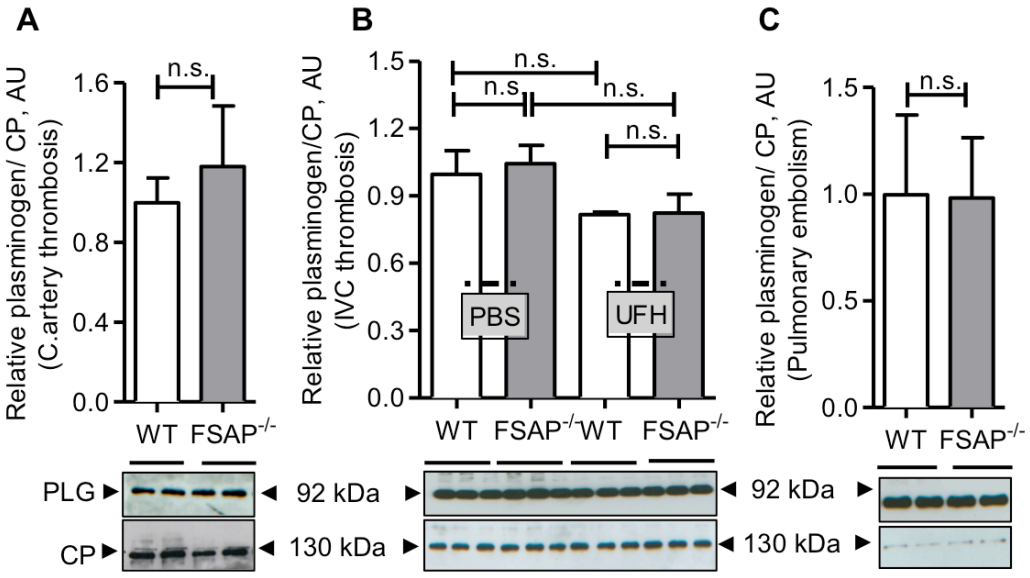


Figure 4.13: Plasminogen levels in FSAP$^{-/-}$ mice: Plasminogen in plasma of WT and FSAP$^{-/-}$ mice with **(A)** carotid artery thrombosis **(B)** venous thrombosis and **(C)** pulmonary thromboembolism were analyzed by Western blotting (n=4). Plasma from WT and FSAP$^{-/-}$ mice was compared with respect to ceruloplasmin (CP) (loading control). Bands were quantified and the intensity of the plasminogen band is represented in arbitrary units (AU) in relation to the CP bands. Data were expressed in mean ± SD. *p<0.05 and n.s. indicates not significant. C.artery thrombosis: Carotid artery thrombosis; IVC: Inferior vena cava.

Western blotting showed no differences in both strains of mice in all of the three mouse model experiments **(Fig. 4.13)**. Thus, contrary to our expectations we found that a lack of endogenous FSAP is associated with increased uPA activity, which in turn promotes fibrinolysis.

4.10. The effect of exogenously applied FSAP *in vivo*

While the lack of endogenous FSAP influenced the outcome of thrombosis and haemostasis, we also investigated the consequences of applying exogenous FSAP

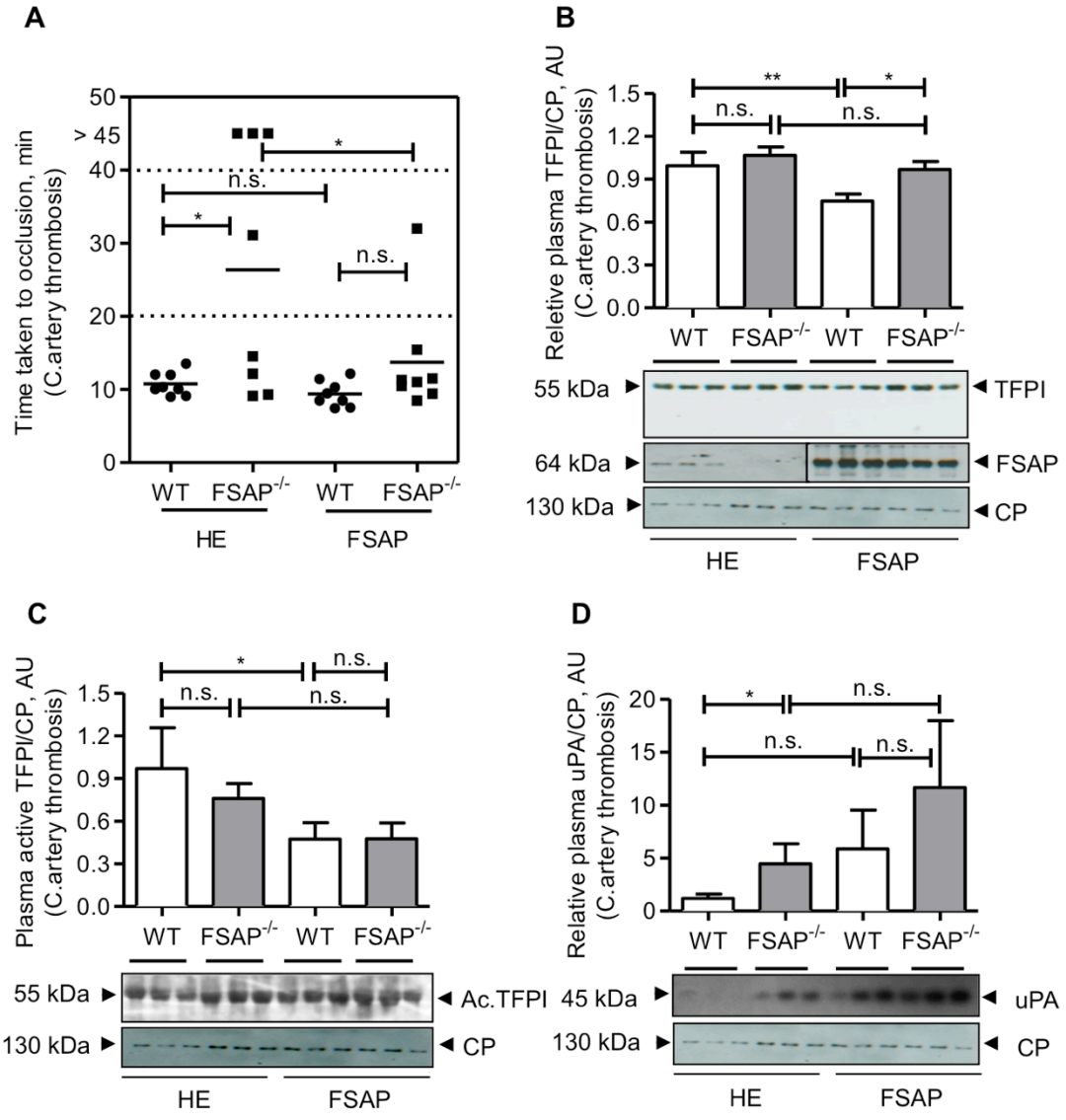


Figure 4.14: Thrombosis in FSAP$^{-/-}$ mice reconstituted with exogenous FSAP: (A) human FSAP (1.2 mg/Kg body weight) or HE (control buffer) was applied intravenously in WT and FSAP$^{-/-}$ mice before 15 min before $FeCl_3$-induced injury was performed. The scatter plot shows time to occlusion (TTO) in WT and FSAP$^{-/-}$ (n=8) mice. Data were represented as percentage blocks. (B) At the end of the experiment plasma was collected and analyzed by Western blotting for TFPI in WT (n=3) and FSAP$^{-/-}$ (n=3) mice. FSAP and CP were used as loading control as well as genotype control. (C) Active (ac) TFPI was measured by biotinylated FXa ligand blotting for active TFPI in WT (n=3) and FSAP$^{-/-}$ (n=3) mice. (D) Plasminogen activation in FSAP treated plasma was analyzed by gel based casein zymography in the presence of plasminogen. Activation of plasminogen is indicated by dark lysis uPA band at 45 kDa. Plasma from WT and FSAP$^{-/-}$ mice was compared with respective ceruloplasmin (CP) bands. Data were expressed in mean ± SD. *p<0.05, **p<0.01 and n.s. indicates not significant. C.artery thrombosis: Carotid artery thrombosis.

(1.2 µg/kg, intravenously) in mouse. Elevated plasma FSAP levels were reached in mice with exogenously applied FSAP **(Fig. 4.14A)** and

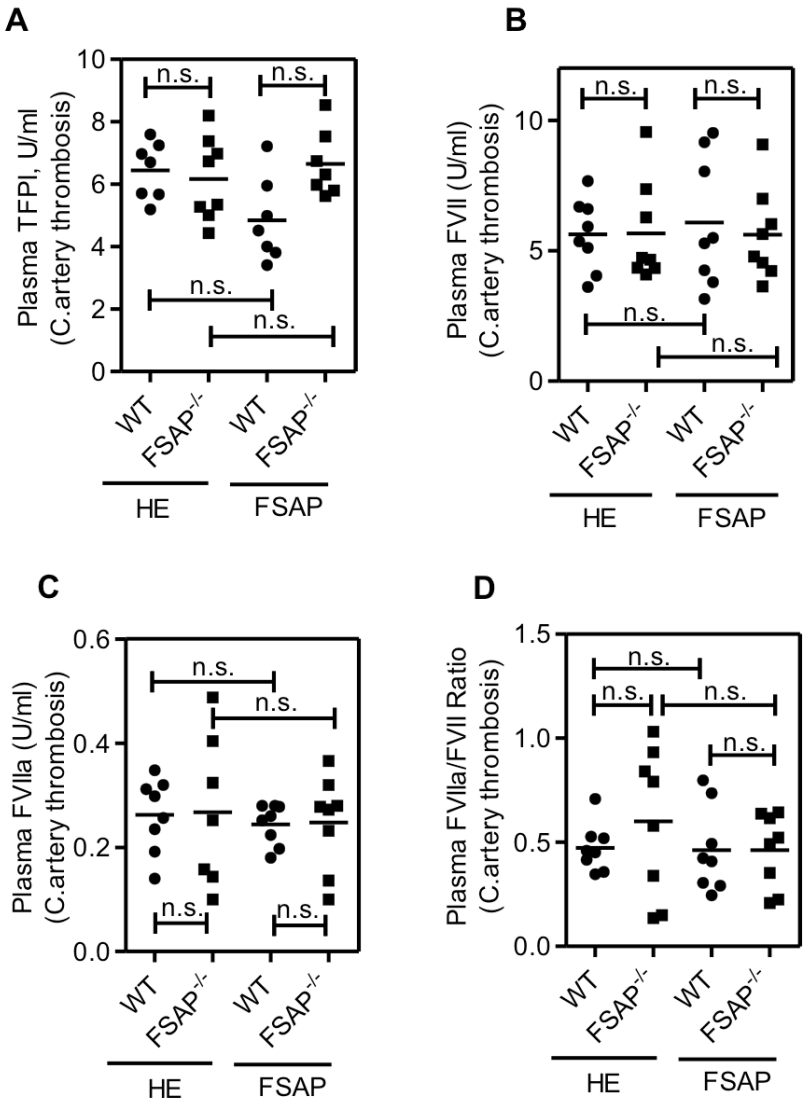


Figure 4.15: Status of extrinsic pathway factors in plasma of mice reconstituted with exogenous FSAP: Mouse plasma from WT and FSAP$^{-/-}$ mice subjected to $FeCl_3$-induced carotid artery thrombosis was analyzed for TFPI **(A)**, FVII **(B)**, FVIIa **(C)** and FVIIa/FVII ratio **(D)**. Data were expressed in mean bars (n=7 to 10). *p<0.05, n.s. indicates not significant. Experiment performed by Michael Etscheid at Paul Erlich Institute, Germany. C.artery thrombosis: Carotid artery thrombosis.

a 2-fold increase was seen when compared to normal WT levels in FSAP$^{-/-}$ mice and 3.5-fold in WT mice after application of exogenous FSAP **(Fig. 4.14 A)**. In WT mice the application of exogenous FSAP did not alter the occlusion times rather it shortened it but this was not statistically significant. In FSAP$^{-/-}$ mice there was no occlusion in 37% of HE

buffer treated mice whereas after intravenous FSAP application all mice showed occlusion **(Fig. 4.14A).**

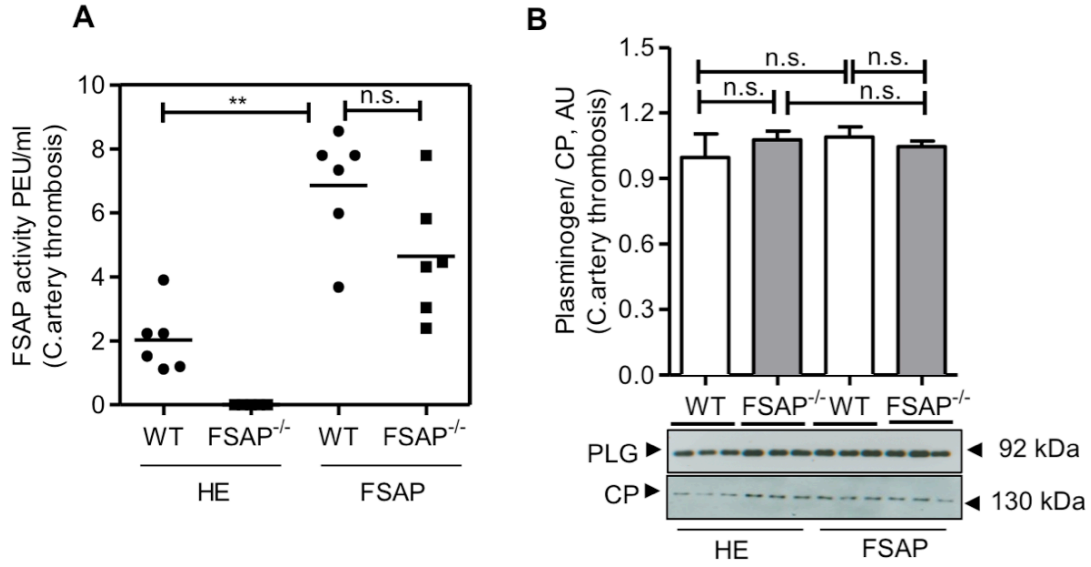


Figure 4.16: FSAP activity assay and Plasminogen level: FSAP activity **(A)** was measured in plasma samples from mice reconstituted with exogenous FSAP. Data were expressed in plasma equivalent units (PEU) whereby standard human plasma represent 1 PEU/ml. Data were represented as scatter plots with mean bars (n=5-7). **Plasminogen levels in FSAP$^{-/-}$ mice**: Plasminogen in plasma **(B)** of WT and FSAP$^{-/-}$ mice reconstituted with exogenous FSAP followed by carotid artery thrombosis was analyzed by Western blotting (n=3). Plasma from WT and FSAP$^{-/-}$ mice was compared with respect to ceruloplasmin (CP) (loading control). Bands were quantified and the intensity of the plasminogen band was represented in arbitrary units (AU) in relation to the CP bands. Data were expressed in mean ± SD and analyzed with one-way ANOVA followed by *Bonferroni Post hoc* test *p < 0.05, ** p<0.01 and n.s. indicates no significant.

Western blot analysis demonstrated that plasma TFPI decreased after application of exogenous FSAP and the decrease was more prominent in WT mice compared to FSAP$^{-/-}$ mice **(Fig. 4.14B).** Active TFPI was measured by ligand blotting which relies on the binding of biotinylated FXa to active TFPI by Western blotting. This quantitative analysis further confirmed that exogenous FSAP decreased TFPI in WT littermates but there were no significant differences between WT and FSAP$^{-/-}$ mice **(Fig. 4.14C).** Thus, exogenously applied FSAP also decreased TFPI activity in plasma in WT littermates but the decrease was not significant. We also measured FVII, FVIIa and the FVIIa/FVII ratio through functional assays in plasma. The FVII, FVIIa and the FVIIa/FVII ratio was not altered with exogenous FSAP **(Fig. 4.15A-D)** in WT littermates and FSAP$^{-/-}$ mice. uPA activity in the plasma of FSAP$^{-/-}$ mice was measured through casein zymography and it was high in FSAP treated groups compared to HE treated group. FSAP activity assay also performed in this model mouse plasma and showed the successful tail vein injection of FSAP **(Fig. 4.16A).** In addition, we measured the plasminogen level in FSAP treated

groups and we did not find any influence of exogenous FSAP on plasminogen level **(Fig. 4.16B)**. Thus, reconstitution of exogenous FSAP reversed the phenotype of the FSAP$^{-/-}$ mice and this could be associated with the decrease in TFPI but not activation of FVIIa or uPA.

4.11. Physiological status of coagulation factors in WT and FSAP$^{-/-}$ mice

In the above thrombosis experiments we have demonstrated that lack of endogenous FSAP leads to an alteration in thrombosis and haemostasis *in vivo*. Although FVII and

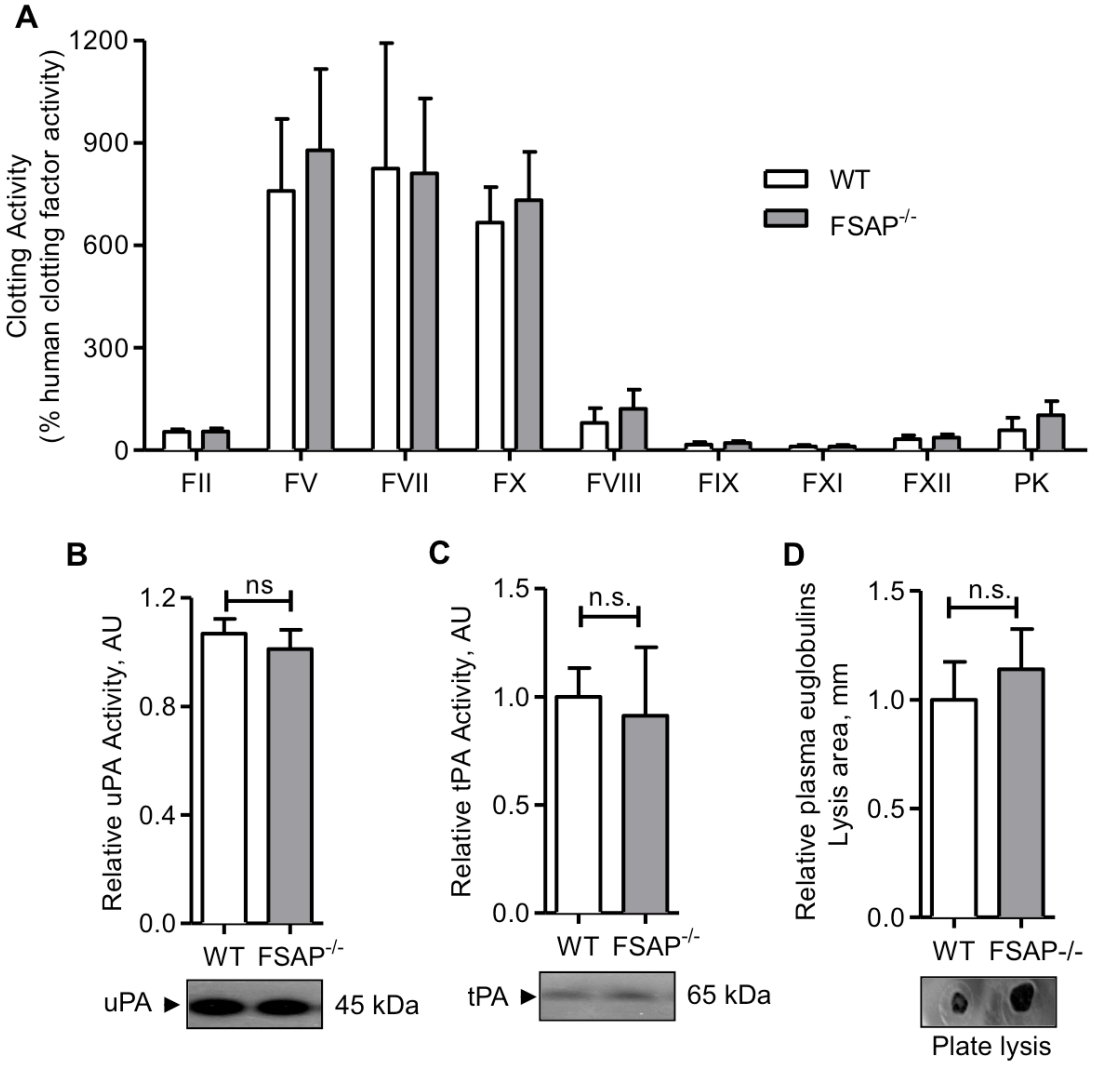


Figure 4.17: Normal plasma parameters in WT and FSAP$^{-/-}$ mice: (A) Coagulation factors were measured in the plasma of WT and FSAP$^{-/-}$ mice (n=10) Data were expressed in mean ± SD. *p<0.05 and n.s. indicates not significant. (Michael Etscheid at Paul Erlich Institute, Germany performed clotting assays).

TFPI have been considered as potential candidates that are regulated by FSAP we also screened for any other changes in whole blood, plasma and platelets that may account for the observed phenotype. Concentration of FII, FV, FVII, FX, FVIII, FIX, FXI, FXII and plasma kallikrein (Döttl K, 1936) was measured in mouse plasma using human deficient plasma and appropriate human standards. No differences were observed in these factors between the WT and FSAP$^{-/-}$ mice **(Fig. 4.17A)**.

5. Discussion

Vascular injury or damage results in the activation of plasma circulating factor VII, which in turn activates the extrinsic pathway system *via* interaction with cellular TF. Many reports suggest the source of TF to be damaged subendothelial cells and its activity is inhibited by TFPI. Loss of TFPI, TF, FVII or thrombin leads to embryonic lethality, which demonstrates their critical significance in thrombosis, haemostasis and embryonic development. Our results with the $FSAP^{-/-}$ mice indicate that endogenous FSAP is a subtle modifier of these coagulation pathways. Our study showed that FSAP functions through a mechanism, which is common to both thrombosis as well as haemostasis *in vivo*. The deletion of endogenous FSAP leads to comparatively higher levels of TFPI and. This increase in TFPI decelerates the extrinsic pathway. Thus the lack of FSAP prolongs clotting time and leads to the formation of unstable clots *in vivo*.

5.1. FSAP and haemostasis

The tail-bleeding assay provides a measure of the physiological "response to injury" coagulation *in vivo* (Kung et al., 1998) and is sensitive to different levels of coagulation factors (Dejana et al., 1979; Gailani et al., 1997). However, complete tail transection could lead to opening up of the both lateral veins as well as central artery which in turn may lead to variations in the bleeding time. However, it is unclear how the outcomes of the tail-bleeding assay can be extrapolated to humans (Mackman, 2004). Data suggests that, initial bleeding time in $FSAP^{-/-}$ mice is similar to that of WT littermates. However, some $FSAP^{-/-}$ mice were bleeding until 12 min. Thus in-comparison with WT littermates, re-bleeding incidence and total blood loss were high in these mice, thereby revealing that FSAP influences the normal primary haemostatic function and may lead to enhanced re-bleeding events by destabilization of clot.

5.2. Endogenous/exogenous FSAP and stability of thrombosis

Numerous experimental mouse models as well as different induction methods have been used so for by researchers to study thrombosis *in vivo* (Sachs and Nieswandt, 2007). One method to initiate endothelial damage is through photochemical substances like Rose Bengal (Westrick et al., 2007) and Hematoporphyrin (Rauova, 2012), which when injected into the body, rapidly accumulates on the endothelial surfaces. These accumulated photochemical substances are then exposed to green light (540 nm) which leads to a photochemical reaction at the targeted site resulting in the production of free

radicals/oxygen species that damage the endothelial cells around the site and induce thrombosis (Kikuchi et al., 1998). Another model utilizes lasers to target the vessel walls and damage it.

The procedure that has been most widely used to induce vascular injury is the direct $FeCl_3$ application on the arterial vessel surface to induce endothelial damage followed by the formation of a platelet rich thrombus. Although the exact mechanism by which thrombus initiation by $FeCl_3$ is not clear, it has been shown that the morphology of the thrombi is similar to those found in humans (Farrehi et al., 1998). The $FeCl_3$ induced vascular injury has been performed on major arteries and veins like carotid and femoral arteries, jugular veins, and minor arterioles and venules like mesenteric and cremasteric arterioles and venules in mice, rats, guinea pigs and rabbits (Chen et al., 2011; Eckly et al., 2011; Konstantinides et al., 2001; Leadley et al., 1999; Li et al., 2010; Marsh Lyle et al., 1998). Although this model offers many advantages, there are some limitations. The destructive oxidative stress induced by $FeCl_3$ may denature or alter circulating and membrane-bound proteins. Use of occlusion time as an endpoint of thrombosis experiments is somewhat crude (Ghosh et al., 2008; Owens et al., 2011). There are many reports on the use of 10% to 15% $FeCl_3$ for arterial thrombosis. In our present study, optimized concentration of $FeCl_3$ (10%) was used for experimental procedures. However, in the venous thrombosis model, an optimized model with 3.5% $FeCl_3$ was used to trigger the vascular injury, while retaining good sensitivity to heparin.

Although tissue factor (TF) initiates thrombosis in most of these models, the source of cellular TF varies depending on the nature of injury. If the integrity of the vessel wall is disturbed, blood comes in contact with the TF expressing sub-endothelium cells (Kretz et al., 2010). Regardless of the source of TF, thrombosis will occur provided there is sufficient TF to overcome the inhibitory effects of TFPI (van 't Veer and Mann, 1997). $FeCl_3$ causes denudation of the endothelium, (Wang and Xu, 2005) and exposes the sub-endothelium, including collagen (Kurz et al., 1990). Platelet accumulation after $FeCl_3$ exposure to the vascular wall is dependent on both collagen and thrombin. Collagen provides a TF independent mechanism of thrombus formation (Kretz et al., 2010). This model is simple and sensitive to both anticoagulant and anti-platelets drugs.

Three different groups, using different vascular beds, and methods of analysis, replicated the in vivo results with $FSAP^{-/-}$ mice independently. Although a heterogeneity response was seen in case of $FSAP^{-/-}$ mice, this cannot be attributed to the age or sex of

these mice. Moreover, the mice used were backcrossed for 10 generations with the C57BL/6J background and littermate controls from heterozygous crosses and maintained in the same room. Thus the obvious factors were excluded and the origin of this heterogeneity needs to be further investigated. Nevertheless, we have shown here that the mice reconstituted with human FSAP can reverse the phenotype in $FSAP^{-/-}$ mice. Through the activity assay, we observed that circulating FSAP level in human and mouse plasma are almost equal. Reconstitution of human FSAP in $FSAP^{-/-}$ mice followed by carotid artery thrombosis restored the arterial thrombosis to the pattern observed in WT mice. Furthermore, WT mice that received the human FSAP showed faster occlusion than the HE buffer treated WT mice. These results confirmed that FSAP support the procoagulation process.

Thrombus firmness mostly depends on the second wave mediators, including ADP and TXA_2. They, together with epinephrine, share the property of binding G-protein coupled receptors. These GPCRs are known to act in concert to achieve full platelet activation by cAMP suppression, increase calcium ions concentration and phospholipase activation (Pozgajova et al., 2006). Epinephrine alone is not enough to promote platelet structure change, aggregation, or $\alpha IIb\beta_3$ activation (Lanza and Cazenave, 1985; Nieswandt et al., 2001), but it potentiates different responses with collagen and thereby contributes to platelet activation *in vitro* as well as *in vivo* (Pozgajova et al., 2006). The activated platelets in turn increase TXA_2, leading to stronger platelet aggregation. In our study, $FSAP^{-/-}$ mice showed more resistance to collagen/epinephrine induced pulmonary thromboembolism compared to WT littermates *in vivo*. These finding implies that FSAP is required for stable platelet plug formation. This protective effect in $FSAP^{-/-}$ mice may be due to the high TFPI which disturbs the coagulation process *in vivo*.

5.3. Endogenous/exogenous FSAP and FVII activation

Factor VII is produced in the liver and circulates in the plasma where it binds to tissue factor (TF), an integral membrane protein, which is exposed to the blood circulation upon vessel injury. This TF:VIIa complex in turn activates FXa that leads to prothrombin activation. However, this complex is inhibited by TFPI. Factor VII activating protease was so named, because FVII was identified as the initial substrate (Romisch, 2002). Recent, studies on human plasma proposed that activation of FVII was much weaker than inactivation of TFPI (Kanse et al., 2012a). In order to analyze this

concept *in vivo*, we also measured circulating factor like FVII, FVIIa and the ratio of FVIIa/FVII. The results showed that the FVII antigen level in plasma from WT and $FSAP^{-/-}$ mice were normal. However, there was a consumption of FSAP in the thromboembolism model which resulted in increased residual FVII levels in $FSAP^{-/-}$ mice compared to WT mice. This higher the residual FVII were correlated with higher the TFPI and ultimately leads to lower the coagulation in $FSAP^{-/-}$ mice. Interestingly, addition of exogenous FSAP into the $FSAP^{-/-}$ mice did not alter the levels of FVII as well as FVIIa. This result is well correlating with the recent in vitro study that FVII might not be a direct physiologically relevant substrate for FSAP:

5.4.Endogenous/exogenous FSAP and TFPI

The primary inhibitor of the TF pathway is TFPI, which inactivates FXa and FVIIa immediately following their generation. Alternative splicing leads to the generation of 2 main isoforms called TFPIα and TFPIβ. TFPIα has all 3 *Kunitz* domains as well as the C-terminal heparin-binding region whereas TFPIβ has only the first 2 *Kunitz* domains and is linked to a glycosylphosphatidylinositol anchor that restricts it to the surface of the plasma membrane. TFPIα is produced by platelets and TFPIβ by endothelial cells (Mast, 2011). A large majority of the TFPI in an adult mouse is TFPIβ, which is more resistant to release by heparin. The activity of TFPI is regulated by a number of proteases such as plasmin, neutrophil elastase (Broze and Girard, 2012; Massberg et al., 2010) and FSAP (Kanse et al., 2012a), which cleave specific sites within the molecule. Our previous studies have shown that TFPIα is a better substrate than TFPIβ *in vitro* but on endothelial cells FSAP is capable of cleaving TFPIβ (Kanse et al., 2012a). Although the specific functions of different forms of TFPI are not completely determined, the full-length TFPI shows a strong anticoagulant effect than the truncated form (Dahm et al., 2006; Lindahl et al., 1991).

The assay of TFPI is complex, as *in vivo* the protein molecule is present in various intravascular pools and is heteregenous in its structure. Degree of lipidation and truncation influence the antigen detection as well as anticoagulant effect. Antigen assay and chromogenic assay are used to detect total TFPI. TFPI activity assay is based on the ability of TFPI in the sample to inhibit TF:FVIIa catalytic activity, in presence of FXa (Bendz et al., 2000). In this study the levels of TFPI were measured using three different methods and showed an excellent correlation confirming the reliability of these

conclusions. TFPI was measured using western blot; ligand blot with FXa, which binds active TFPI; and a TFPI activity assay based on measuring FXa generation. We also measured TFPI in all the models plasma as well as in thrombi by western blotting and immunofluorescence analysis.

In the collagen/epinephrine model of pulmonary thromboembolism, where large-scale systemic changes would be expected, higher levels of TFPI were observed in plasma of $FSAP^{-/-}$ mice using all three methods. Also the lung homogenates from this model showed significantly higher TFPI in $FSAP^{-/-}$ mice. In control mice as well as when thrombosis (induced locally with $FeCl_3$) vessels showed no differences in plasma TFPI. Although the immunostaining of TFPI showed higher staining in $FSAP^{-/-}$ mice carotid artery thrombi the same was not observed by western blotting, which is a more quantitative technique. Thus, our results confirm that endogenous FSAP is able to inhibit TFPI *in vivo* and thus promote the pro-coagulation through up regulation of the extrinsic pathway.

6. CONCLUSION AND FUTURE PROSPECTS

6.1. CONCLUSION

While deficiency of FSAP in human is unidentified so far, carriers of MI-SNP have lower proteolytic activity. A few clinical studies have proposed an association between MI-SNP and venous thrombosis (Ahmad-Nejad et al., 2012b; Hoppe et al., 2005). Whereas on the other hand, other studies did not find such an association (Franchi et al., 2006; Gulesserian et al., 2006; Pecheniuk et al., 2008; van Minkelen et al., 2005; Weisbach et al., 2007). In our thrombosis studies with $FSAP^{-/-}$ mice, we observe that the deficiency of FSAP activity or loss of FSAP activity, as like in the MI-SNP carriers, would lead to an anti-thrombotic phenotype due to the TFPI inhibition by FSAP and we speculate that there is no correlation with thrombosis and MI-SNP *in vivo*.

The findings of the present study are as follows:

1. Tail-bleeding assay model of haemostasis showed that $FSAP^{-/-}$ mice had significantly higher blood loss and re-bleeding incidence. It confirms that FSAP is required for the normal haemostasis process.

2. Thrombosis models ($FeCl_3$-induced carotid artery model, mesenteric arteriole model, venous thrombosis model and pulmonary embolism model) showed that $FSAP^{-/-}$ mice were protected against thrombosis *in vivo*.

3. Functional assays clearly showed that FVII might not be a physiologically relevant substrate for FSAP

4. Plasma and tissue samples were assessed for TFPI measurement using various methods (function assays, western blotting, ligand blotting, immuno-capturing) and the outcome clearly indicates that FSAP inhibits TFPI *in vivo.*

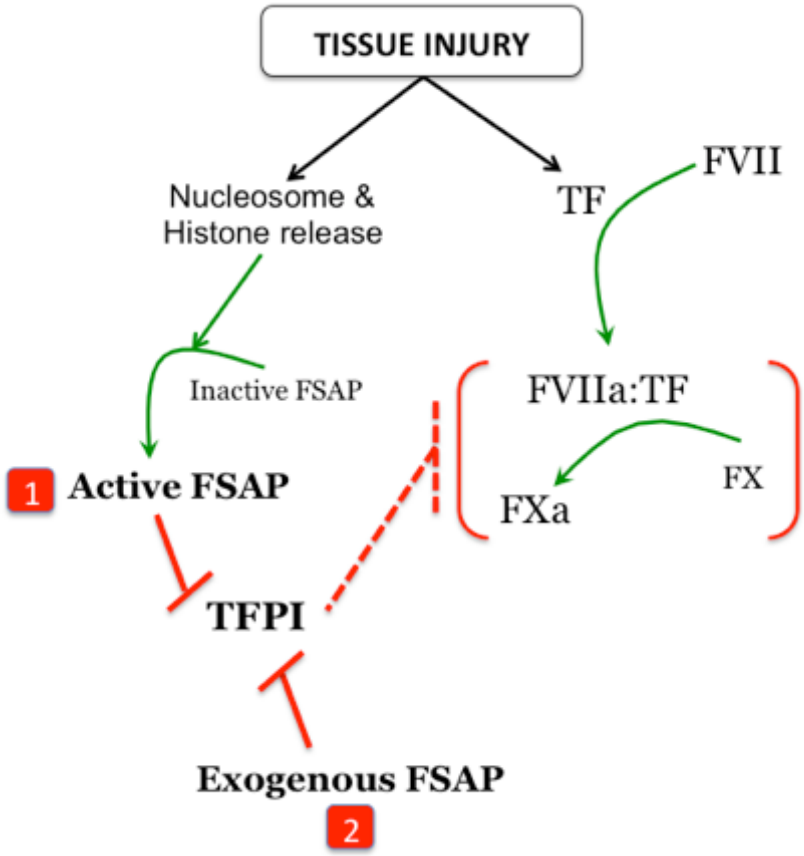


Figure 6.1: Role of exogenous human FSAP *in vivo (proposed model)*: FSAP activation *in vivo* is due to the release of nuecleosomes and histones in response to vascular injury. This activation leads to up-regulation of extrinsic pathway by inhibition of TFPI (1). Similarly, the exogenous FSAP (2) injection also showed that the FSAP inhibits TFPI.

In light of our results we predict that a loss of FSAP activity, as in carriers of MI-SNP, is likely to protect against thrombosis. However, species-specific differences in the actions of FSAP cannot be excluded at this stage. We suggest that FSAP activation *in vivo* is due to the release of nucleosomes and histones in response to vascular injury. This hypothesis mirrors well with the study that DNAse treatment or blocking histones reduces thrombus incidences (Esmon, 2012; Fuchs et al., 2012). Our mice models revealed that the endogenous FSAP plays a key role in extrinsic coagulation pathway *via* inhibition of TFPI *in vivo* **(Fig. 6.1)**. The delayed coagulation seen in our case ($FSAP^{-/-}$ mice) might be due to the influence of tissue factor pathway inhibitor (TFPI) on extrinsic system. Thus, FSAP could be a cofounding factor in thrombosis and haemostasis *in vivo*.

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SUMMARY

Factor VII activating protease (FSAP), a serine protease produced in the liver (plasma conc.12 μg/mL) has been associated with cardiovascular diseases. *In vitro* studies revealed that FSAP has a dual function in the coagulation system via inhibition of tissue factor pathway inhibitor (TFPI) and activation of pro-urokinase (pro-uPA). Even though functional polymorphism in the FSAP gene is linked with cardiovascular diseases, due to contradictory clinical observations as well as its dual role in the coagulation system *in vitro*, the real function and mechanistic aspects of FSAP is ambiguous. To identify the actual function of FSAP in thrombosis and haemostasis, we characterized the FSAP deficient mice using various *in vivo* and *ex-vivo* mouse models. Our results showed that in pulmonary thromboembolism model $FSAP^{-/-}$ mice had a significantly higher survival than WT littermates ($p < 0.01$). The carotid artery injury as well as mesenteric arteriole models revealed that the occlusion time was significantly increased in $FSAP^{-/-}$ mice ($p < 0.01$). Partial venous thrombosis (PVT) showed a trend in the reduction of total thrombus content and thrombus weight in $FSAP^{-/-}$ mice. Western blotting analysis of plasma samples as well as immunohistochemical analysis of thrombotic carotid arteries and lung tissue extracts showed a slightly increased TFPI expression in $FSAP^{-/-}$ mice when compared to the WT littermates. *In situ* and gel-based casein zymography showed that $FSAP^{-/-}$ mice had more uPA than the WT littermates. Plasma samples from all these models did not show any influence of plasminogen levels. *In vitro* analysis of FSAP on platelet TFPI demonstrated that FSAP could inhibit not only the circulating β form of TFPI but also the platelet derived α form of TFPI. Tail-bleeding assay revealed that more than 80% of $FSAP^{-/-}$mice showed re-bleeding incidences compared to WT littermate controls. The clinical relevance of Marburg-I polymorphism is not well understood because of contradictory observations in various studies. Interestingly, our mice models revealed that the endogenous FSAP plays a key role in extrinsic coagulation cascade *via* inhibition of TFPI.

ZUSAMMENFASUNG

Die Faktor VII aktivierende Protease (FSAP), eine in der Leber synthetisierte Serinprotease (Plasmakonzentration: 12 µg/ mL), spielt eine Rolle bei kardiovaskulären Erkrankungen. *In vitro* Studien zeigten, dass FSAP eine duale Rolle in der Hämostase innehat. Einerseits wirkt FSAP prokuagolatorisch durch Hemmung des Gewebsfaktor-Plasminogen Inhibitors(TFPI), andererseits fibrinolytisch durch Aktivierung der Prourokinase (pro-uPA). Obwohl der funktionale Polymorphismus des FSAP-Gens mit kardiovaskulären Erkrankungen verbunden ist, bleibt die Funktion und Wirkungsweise von FSAP aufgrund widersprüchlicher klinischer Untersuchungen wie auch seiner dualen Rolle im Hämostasesystem, unklar. Zur Aufklärung der Funktion von FSAP in Hinsicht auf Thrombose und Hämostase wurden FSAP-defiziente Mäuse in verschiedenen *in vivo* und *ex vivo* Modellen getestet. Unsere Ergebnisse zeigten, dass $FSAP^{-/-}$ Mäuse in einem Lungen-Thrombose-Embolie-Modell eine signifikant höhere Überlebensrate aufwiesen als WT-Mäuse ($p < 0.01$). Bei Verletzung der Karotis-Arterie oder der Mesenterialarteriole war die Okklusionszeit bei $FSAP^{-/-}$ Mäusen signifikant erhöht ($p < 0.01$). Eine partiale Venenthrombose (PVT) zeigte tendenziell eine Verringerung des Thrombus in Größe und Gewicht. Westernblot-Analysen von Plasmaproben wie auch immunohistochemische Untersuchungen an thrombotischen Karotis-Arterien und Lungenextrakten ergaben einen leichten Anstieg der TFPI-Expression bei $FSAP^{-/-}$ Mäusen verglichen mit WT-Mäusen. In der In-situ-Zymographie sowie Gel basierenden Kasein-Zymographie zeigten $FSAP^{-/-}$ Mäuse gegnüber WT-Mäusen höhere uPA-Spiegel. Plasmaproben aus allen Modellen zeigten keinen Einfluss von FSAP auf die Plasminogenkonzentration. *In vitro* Untersuchungen an Plättchen-TFPI zeigten, dass FSAP nicht nur die zirkulierende ß-Form von TFPI, sondern auch die platelet-derived α-Form von TFPI hemmt. Bei Messungen der Blutungszeit durch Verletzung des Schwanzes der Mäuse neigten mehr als 80% der $FSAP^{-/-}$ Mäuse zu wiederholtem Bluten gegenüber WT-Mäusen. Die klinische Relevanz des Maburg I Polymorphismus ist aufgrund der widersprüchlichen Beobachtungen an Venenthrombose-Modellen wenig verstanden. Unsere Mausmodelle zeigten, dass endogenes FSAP eine Schlüsselrolle bei der extrinsischen Blutgerinnungskaskade durch Hemmung von TFPI innehat.

DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communication. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Date:……

SUBRAMANIAM, SARAVANAN
(Surname, First name)

Article

- **Saravanan Subramaniam,** Thielmann I, Etscheid M, Morowski M, Pragst I, Nieswandt B, *Kanse, S* M. "Deficiency of factor VII activating protease (FSAP) attenuates thrombus formation in mice" – *Journal of thrombosis and haemostasis - IN PRESS.*

- **Saravanan Subramaniam** and Sandip Kanse. "Ferric chloride (FeCl3) - induced carotid arterial thrombosis in mice" – Current Protocols in Mouse Biology - *IN PRESS.*

Abstracts

- **Subramaniam, S** and Kanse, SM. *Role of Factor VII activating protease in Thrombosis and Atherosclerosis in vivo.* International Giessen Graduate School for Life science (GGL), 4th Annual conference, 21st -22nd September 2011, Germany. ***(Poster)***

- **Subramaniam, S**, Thielmann I, Michael Etscheid, Morowski M, Ingo Pragst, Bernhard Nieswandt, Kanse, SM. *Defective thrombus formation in mice lacking Factor VII Activating Protease*. International Giessen Graduate School for Life science (GGL), 5th Annual conference, 18th -19th September 2012, Germany. ***(Poster)***

- **Subramaniam, S** and Kanse, SM. *Role for FSAP in Thrombosis and Atherosclerosis*. DAAD - *IPID Doktorandentreffen,* Universität Köln, 2nd – 4th May 2013, Germany. ***(Talk)***

- **Subramaniam, S**, Thielmann I, Michael Etscheid, Morowski M, Ingo Pragst, Bernhard Nieswandt, Kanse, SM. *Thrombosis and Haemostasis in Mice Lacking Factor VII Activating Protease (FSAP).* XXIV congress of the International Society on Thrombosis and Haemostasis, 29th June -4th July 2013, The Netherlands ***(Talk)***

- **Subramaniam, S,** Thielmann I, Michael Etscheid, Morowski M, Ingo Pragst, Bernhard Nieswandt, Kanse, SM. *Thrombosis and Haemostasis in Mice Lacking Factor VII Activating Protease (FSAP).* International Giessen Graduate School for Life science (GGL), 6th Annual conference, 11th-12th September 2013, Germany. ***(Talk)***

REAGENTS

PRODUCT	COMPANY
2-Mercaptoethanol	Sigma-Aldrich Chemie, Munich, Germany
Acetone 99,8%	Merck, Darmstadt, Germany
ADP	Sigma (Deisenhofen, Germany)
Agarose	Sigma Life Science, USA
Amiloride	Sigma-Aldrich Chemie, Steinheim, Germany
APS	Roth (Karlsruhe, Germany)
Aqua ad iniectabilia (H_2O dd)	Baxter, Unterschleißheim, Germany
Arginine	Sigma-Aldrich Chemie, Munich, Germany
Beta-mercaptoethanol	Roth (Karlsruhe, Germany)
Bovine serum albumine (BSA)	Sigma-Aldrich Chemie, Munich, Germany
Calcium chloride	Sigma-Aldrich Chemie, Munich, Germany
Chromogenic substrate S1344	PNAPEP 1344, Haemochrom Diagnostica, France
Collagen (Horm)	NYCOMED, Germany
Convulxin	Alexis Biochemicals (San Diego, USA)
DAPI	Linaris, Wertheim, Germany
DQ-casein	Molecular Probes, Leiden, The Netherlands
Dylight 488	Pierce (Rockford, IL, USA)
Eosin Y Disoldium salt	Sigma-Aldrich Chemie, Munich, Germany
EDTA	Carl ROTH, Germany
Epinephrine	Adrenalin 1:100, JENAPHARM®, Germany
Erythrocyte lysis buffer	Biolegend, San Diego, CA, USA
Ethanol	Sigma-Aldrich, Germany
Ferric chloride	$FeCl_3$. $6H_2O$, Fluka Chemie, Buchs, Switzerland
Formalin solution (PFA) 3.7%	Carl ROTH, Karlsruhe, Germany
Glycerol	Sigma-Aldrich Chemie, Munich, Germany
Glycine	Carl ROTH, Germany

Hematoxylin solution	Merck, Darmstadt, Germany
Heparin	Ratiopharm, Ulm, Germany
Histofix 4 % (PFA)	Carl ROTH, Karlsruhe, Germany
Iso-propanol	Merck, Darmstadt, Germany
Lysine	Sigma-Aldrich Chemie, Munich, Germany
Methanol	Merck, Darmstadt, Germany
Penicillin / Streptomycin	PAA Laboratories, Pasching, Austria
Potassium chloride	Carl ROTH, Germany
Skim milk powder	Fluka Analytical, Buchs, Switzerland
Sodium acetate	Fluka Chemie, Buchs, Switzerland
Sodium azide	Sigma-Aldrich Chemie, Munich, Germany
Sodium chloride 0.9% (NaCl)	Carl ROTH, Germany
Sodium citrate (3.8%)	Carl ROTH, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl ROTH, Karlsruhe, Germany
TEMED	Carl ROTH, Karlsruhe, Germany
Thrombin	Roche Diagnostics (Mannheim)
Tissue factor	Siemens, Innovin
Triton X-100	Sigma-Aldrich Chemie, Munich, Germany
Tris	Carl ROTH, Karlsruhe,Germany
Trypan blue solution (0.4%)	Sigma-Aldrich Chemie, Munich, Germany
Tween 20	Sigma-Aldrich Chemie, Munich, Germany
U46619	Alexis Biochemicals (San Diego, USA)
Vectashield MM H 1000	Vector Laboratories, Burlington, CA, USA
Zinc chloride	Carl ROTH, Karlsruhe,Germany

DRUGS USED FOR ANESTHESIA

PRODUCT	COMPANY
Atropinsulfate-solution 0,5 mg/mL	Fresenius Kabi, Bad Homburg, Germany
Isoflurane	Baxter, Unterschleißheim, Germany
Ketamine 50 mg/mL	Inresa, Freiburg, Germany
Xylazine 2%	Bayer, Leverkusen, Germany

SURGERY MATERIALS

PRODUCT	COMPANY
Butterfly perfusion set Micro Flow 0,5 19 mm (24 Gau)	Meditop/surgyplus, MALAYSIA
Cover glass for slides	Menzel, Braunschweig, Germany
Parafilm®	American National Can™, Neenah, WI, USA
Pipettes 1000 / 200 / 100 / 10 / 2	Eppendorf, Wesseling-Betzdorf, Germany
Safe Seal Microcentrifuge Tubes 0.65 mL/2 mL	Sorenson Bioscience, Inc., Salt Lake City, UT, USA
Single-use syringe 1ml, Injekt F, Tuberkulin	B. Braun, Melsungen, Germany
Single-use syringe 5mL, Injekt Solo	B. Braun, Melsungen, Germany
Skin disinfection Softasept®	B. Braun, Melsungen, Germany
Sterile needle, 20 Gau, 0,9 70 mm	Terumo Euope, Leuven, Belgium
Sterile needle, 30 Gau, 0,3 13 mm	Terumo Euope, Leuven, Belgium
Sterile needle, BD Microlance ™ 26 Gau, 0,45 13mm	BD Drogheda, Ireland
Tissue Tek OCT Compound	Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands
Ultrasound gel	Aquasonic 100™, Parker Labs

ANTIBODIES: WESTERN BLOTTING

NAME	SPECIES	ISOTYPE	WORKING DILUTION	COMPANY
TFPI	Rabbit	Polyclonal	1:5000	Santa Cruz Biotech, Germany
Fibrin (ogen)	Rabbit/αHuman	Polyclonal	1:10,000	DAKO, Denmark
Plasminogen	Rabbit/αHuman	Polyclonal	1:10,000	DAKO, Denmark
PAI-1	Rabbit	Polyclonal	1:1000	Molecular Innovation, Germany
FSAP (ME)	Rabbit	Polyclonal	1:1000	In-house preparation, Dr. Michael Etscheid, Langen
Ceruloplasmin	Rabbit/αHuman	Polyclonal	1:10,000	DAKO, Denmark
B actin	Rabbit	Polyclonal	1:5,000	Cell Signaling, USA

ANTIBODIES: IMMUNOFLUORESCENCE

NAME	SPECIES	ISOTYPE	WORKING DILUTION	COMPANY
TFPI	Rabbit	Polyclonal	1:100	Santa Cruz Biotech, Germany
IgG	Rabbit	Polyclonal	1:100	Santa Cruz Biotech, Germany

ACTIVITY ASSAY: IMMUNOFLUORESCENCE

ASSAY	KIT	COMPANY
***In situ* zymography**	EnzChek® Protease Assay Kit BODIPY FL E6638, Green Fluorescence	Life Technologies, USA

RNA/cDNA/RTPCR KITS

PURPOSE	KIT	COMPANY
RNA isolation	GenElute, Miniprep Kit	Sigma-Alderch, Germany
cDNA synthesis	GeneAmp Thermostable rTth reverse transcriptase	Applied Biosystems, USA
q-PCR	SYBR-Green: SensiMix-HiROX	Bioline, USA

BUFFERS

Acid-citrate-dextrose (ACD) buffer pH 4.5

Trisodium citrate dehydrate	85 mM
Citric acid anhydrous	65 mM
Glucose anhydrous	110 mM

Blocking solution for immunoblotting

BSA or fat-free dry milk	5%
in PBS or washing buffer (TBST)	

Coomassie staining solution

Acetic acid	10%
Methanol	40%
Coomassie Brilliant blue	0.01%

Coomassie destaining solution

Glacial acetic acid	10%
Methanol	30%

Phosphate buffered saline (PBS), pH 7.14

NaCl	137 mM
KCl	2.7 mM
KH_2PO_4	1.5 mM
Na_2HPO_4	8 mM

SDS sample buffer, 2X

β Mercaptoethanol	10%
TRIS buffer (1.25 M), pH 6.8	10%
Glycerine	20%
SDS	4%
Bromophenolblue	0.02%

Stripping buffer, pH 6.8

β Mercaptoethanol	100 mM
SDS	2%
Tris HCI pH 6.7	62.5 mM

Tris-buffered saline (TBS) 10X, pH 7.3

Tris 250 mM	30 g
NaCl 1.5 M	80 g
KCl	2 g
d.d.a	1 L

Blotting buffer (Western blot)

Tris	0.02M	12,125g
Glycin	0.15M	56,250g
Methanol		1L
d.d.a		5L

Tyrode´s buffer, pH 7.3

NaCl	137 mM
KCl	2.7 mM
$NaHCO_3$	12 mM
NaH_2PO_4	0.43 mM
$CaCl_2$	1 mM
$MgCl_2$	1 mM
HEPES	5 mM
BSA	0.35%
Glucose	0.1%

Washing buffer (Western blot)

Tween 20 in TBS, pH 7.2	0.1%

Trenngel buffer, pH 8.8

Tris/HCl 1.5 M	90.82 g
10 % SDS (0.4%)	20 mL
d.d.a	500 mL

Sammelgel buffer, pH 6.8

Tris/HCl 1 M	24.2 g
10% SDS (0.4%)	8 mL
d.d.a	200 mL

FSAP activity: Coating buffer

Na_2CO_3	15 mM
$NaHCO_3$	35 mM
pH 9,0	

FSAP activity: Standard buffer

Sodium Citrate	20mM
$NaCl_2$	150mM

FSAP activity: Blocking buffer

Standard buffer	20 mM
BSA	2 %
Arginin	100 mM

FSAP activity: Dilution buffer

Standard buffer	20 mM
BSA	1%
Tween 80	0.1%
Heparin	1.57 mg/mL

FSAP activity: Assay buffer -I

TBS-T80	0.1%
$CaCl_2$	2 mM
Heparin	20 µg/mL

FSAP activity: Assay buffer -II

TBS-T80	0.1%
Pro uPA	10 µg/mL

Gelatin zymography: Washing buffer (4X)

1M Tris pH 7.6	30 mL
200 mM $CaCl_2$	30 mL
10 mM $ZnCl_2$	60 µL
Triton X100	15 g

Casein zymography: Washing buffer

Triton X100	2. 5%
d.d.a	

Casein zymography: Agarose

Noble agarose	2. 5%
d.d.a	

Gelatin zymography :Incubation buffer (4X)

1M Tris pH 7.6	20 mL
200 mM $CaCl_2$	20 mL
10 mM $ZnCl_2$	40 µL
Triton X100	4 g
10% NaN_3	4 mL

Gelatin zymography: Lower gel (4X)

Gelatin 2 %	3 mL
Water	11.55 mL
Trenngel buffer	7.5 mL
Acryl amide	7.95 mL
APS	0.2 mL
TEMED	0.02 mL

Western Blot: Trenngel gel (4X)

Trenngel buffer	7.5 mL
Acryl amide	10.05 mL
Water	12.45 mL
APS	0.2 mL
TEMED	0.02 mL

Western Blot: Sammel gel (4X)

Sammel buffer	2.5 mL
Acryl amide	1.65 mL
Water	5.72 mL
APS	0.1 mL
TEMED	0.01 mL

WITH THE BLESSINGS OF ALMIGHTY

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.