UNIVERSITÀ DEGLI STUDI DI MILANO

Graduate School in Pharmacological Sciences

Department of Pharmacological and Biomolecular Sciences

XXVIII CICLO

The role of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in a venous thrombosis mouse model

BIO/14

Eva Tarantino

Matricola R10117

TUTOR: Chiar.ma Prof.ssa Elena Tremoli

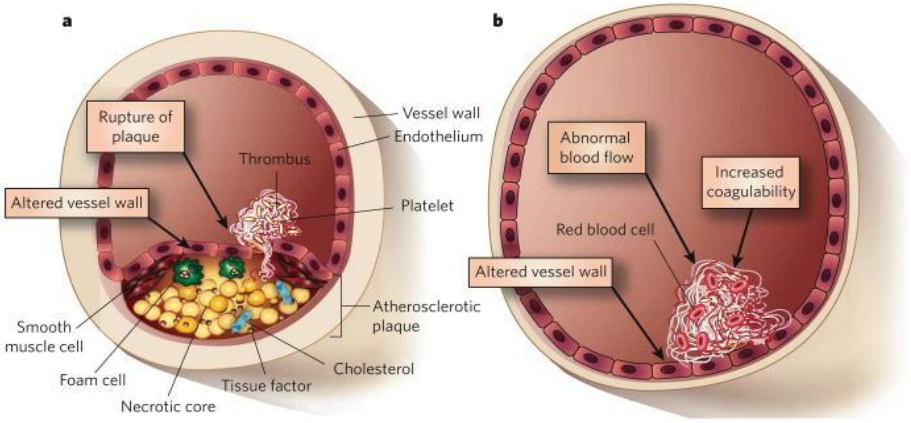
COORDINATORE: Chiar.mo Prof. Alberto Corsini

ANNO ACCADEMICO 2014-2015

I. *Introduction*

I. 1 ARTERIAL AND VENOUS THROMBOSIS

Arterial atherothrombotic disease (acute myocardial infarction, ischemic stroke and peripheral artery disease) and venous thromboembolism (deep venous thrombosis and pulmonary embolism) are usually considered as distinct entities from mechanistic and clinical points of view (Andrei MC, 2014). However, at the base of both diseases there is thrombosis, a disease process that consists in the formation of blood clots within the blood vessels, which affect or prevent the normal circulation of the blood (Heit JA, 2002). The pathophysiology of arterial thrombosis differs from that of venous thrombosis, as reflected by the different ways in which they are treated. Traditional medical teaching stressed the differences between arterial thrombosis and venous thrombosis. Thrombi are composed of platelets and fibrin. However, arterial thrombi tend to occur at sites of arterial plaque rupture where shear rates are high, and are predominantly platelet-rich, so called 'white thrombi'. In contrast, venous thrombi tend to occur at sites where the vein wall is often normal, but blood flow and shear rates are low, resulting in red cell-rich, and they are called 'red thrombi' (Fig. 1) (Franchini M, 2008) (Mackman N, 2008).



Mackman N., Nature, 2008.

Fig. 1: Arterial and venous thrombus. (a) Artery: the primary trigger of arterial thrombosis is rupture of an atherosclerotic plaque. This involves disruption of the endothelium and release of constituents of the plaque into the lumen of the blood vessel. **(b) Vein:** by contrast, in venous thrombosis, the endothelium remains intact but can be converted from a surface with anticoagulant properties to one with procoagulant properties.

I. 1.1 Venous Thromboembolism (VTE): Deep Vein Thrombosis (DVT) and Pulmonary Embolism (PE)

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are collectively referred to as venous thromboembolism (VTE), which is the third leading cause of cardiovascular-associated death, after myocardial infarction (MI) and stroke.

DVT occurs frequently with an estimated annual incidence of one per 1000 person in adult population (White RH, 2003) (Silverstein MD, 1998), creating considerable morbidity. Complications include post-phlebitic syndrome and chronic thromboembolic pulmonary hypertension.

VTE is predominantly a disease of older age (Klatsky AL, 2000) (Hooper WC, 2002); only rarely events occurred among young patients. Incidence rates increased markedly with age for both men and women and for both DVT and PE (Klatsky AL, 2000) (Hooper WC, 2002). As the average population age increases, the absolute number of VTE events will probably increase, and an increasing proportion of these events will be manifest as PE with its associated poor survival. These findings have serious implications for the future. DVT is an important complication of several inherited and acquired disorders, but may also occur spontaneously. Prevention of recurrent VTE, DVT and PE, is the main reason for accurate diagnosis and adequate treatment.

The pathophysiology of vein thrombosis involves three interrelated factors (“Virchow's triad”): damage to the vessel wall, slowing down of the blood flow, and increase in blood coagulability (Lensing WA, 1999). The first two components of Virchow's triad represent acquired conditions; in contrast, blood hypercoagulability has both intrinsic and extrinsic causes. Acquired risk factors for VTE include cancer, obesity and major surgery (Weinmenn EE, 1994). Other risk factors for vein thrombosis include age, immobilization, fractures, puerperium, paralysis, use of oral contraceptives, and the antiphospholipid syndrome (Salzman EW, 1993) (Carson JL, 1992) (Prandoni P, 1996) (Triplett DA, 1995) (WHO collaborative study of cardioscular, 1995).

Deep venous valve pockets are naturally susceptible to periods of blood stasis and constitute the primary location of thrombus formation in veins (Brooks EG, 2008). Venous valve pockets assist blood return to the right atrium of the heart by preventing backflow

of blood in response to gravity. Muscular contractions in the leg and thigh facilitate blood return to the heart by compressing the deep veins. In the absence of activity (e.g., immobility or bed rest), there are reduced blood flow and stasis in the deep valve pockets. Thus, an alteration in blood flow can result in the accumulation of numerous pro-thrombotic agents (Hamer JD, 1981). These states not only predispose apparently healthy people to thrombosis, but also are likely to trigger thrombosis in people with inherited thrombophilic abnormalities. Inherited thrombophilia is a genetically determined tendency to VTE. The well-established inherited pro-thrombotic abnormalities are deficiencies of antithrombin, protein C, protein S (Hirsh J, 1993) (Heijboer H, 1990), and FV Leiden, a mutation in coagulation FV (Arg 506→Gln), that results in resistance to activated protein C (Bertina RM, 1994) (Simioni P, 1997). Hyperhomocysteinaemia is also associated with the occurrence of vein thrombosis and may be the consequence of a hereditary defect in the enzymes involved in methionine metabolism or deficiencies of vitamins, such as cobalamin, folate, or pyridoxine (den Heijer M, 1996) (den Heijer M, 1992). Other less well established causes include dysfibrinogenemia, and abnormalities of the fibrinolytic system (Hirsh J, 1993) (Heijboer H, 1990). A mutation in coagulation FII (prothrombin 20210A) and increased levels of coagulation FVIII have also been recognised as risk factors of thrombophilic syndromes (Poort SR, 1996) (Koster T, 1995). One or more of these thromobophilic abnormalities can be found in 40–60% of patients with a first episode of vein thrombosis. Increased amounts of circulating tissue factor (TF), the initiator of the coagulation cascade, are recurrently considered the major trigger of venous thrombosis (Prandoni P, 1993) (Hirsh J, 1993) (Mackman N, 2008) (Tesselaar M, 2007).

I. 2. HEMOSTASIS AND THROMBOSIS

I. 2.1 Thrombus formation

Host defense mechanism that maintains the circulatory system at high pressure and prevents blood loss after vessel wall injury is **hemostasis**. Under physiological conditions, basal endothelial wall displays anti-adhesive and anti-thrombotic properties that prevent activation of the hemostatic system. At steady state, endothelial cells (ECs) express molecules such as the ecto-nucleotidase CD39 (apyrase), prostaglandin I_2 (PGI_2) or nitric oxide (NO) that prevent platelet activation and aggregation (Costa AF, 2004) (Radomsky MW, 1987). ECs can also express inhibitors of the coagulation cascade such as thrombomodulin (TM), tissue factor pathway inhibitor (TFPI) (Camerer E, 1996) (Østerud B, 1995) (Broze Jr GJ, 2003) and anti-thrombin III (AT III) (Jesty J, 1996). Disruption of the integrity of the vessel wall by mechanical or functional trauma allows circulating platelets to come in contact with the subendothelial matrix. This process involves the endothelial wall, circulating blood components such as red blood cells, platelets themselves, leukocytes, microparticles and circulating blood molecules, including blood coagulation proteins (Furie B, 1992). Hemostatic system is activated after a vascular injury, leading rapidly to thrombus formation generated by the aggregation of **platelets** (Furie B, 2008) and consequent activation of coagulation cascade (Fig.2).

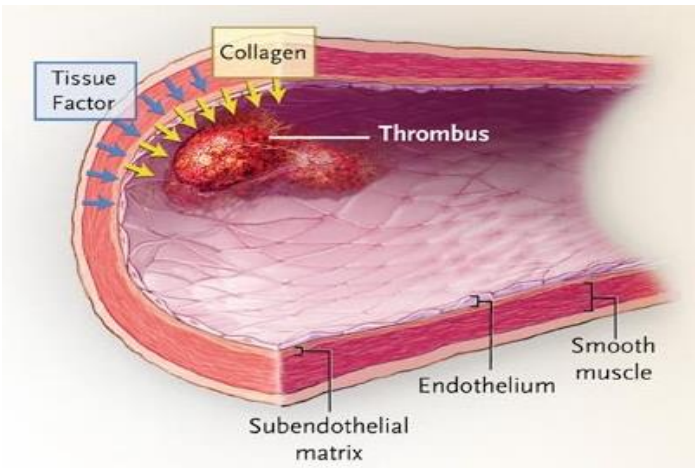


Fig. 2: Response to vascular injury. Collagen and tissue factor (TF) associated with the vessel wall provide a hemostatic barrier to maintain the high-pressure circulatory system. Collagen (yellow arrows), located in the subendothelial matrix beneath the endothelium, is not exposed to flowing blood under normal conditions. TF (blue arrows), located in the medial (smooth muscle) and adventitial layers of the vessel wall, is exposed to flowing blood when the vessel is disrupted or punctured. Both collagen and thrombin initiate thrombus formation. Collagen is a first line of defense, and TF a second line of defense.

I. 2.1.1 Role of platelets in thrombosis

Mammalian platelets are fragments of cytoplasm released into circulating blood by megakaryocytes, a precursor hematopoietic cell residing in the bone marrow (Shulze H, 2007). Without a nucleus, platelets have no control on gene expression but possess a translational machinery that can direct protein synthesis (Weirich AS, 2004). The physiological role of platelets consist in a fundamental contribution to hemostasis (Weiss HJ, 1975); indeed, platelets are central both to normal hemostasis, to limiting blood loss after injury, but act also in pathological conditions, such as DVT and arterial thrombosis (Denis CV, 2007).

Hemostasis and thrombosis are related aspects of the response to vascular injury, but the former protects from bleeding after trauma while the latter is a disease mechanism. Platelets and the hemostatic system, however, cannot discriminate between alterations of the vessel wall caused by traumatic wounds and those resulting from pathologic processes. Consequently, chronic vascular diseases can acutely induce massive platelet responses, usually initiated by the occurrence of destabilizing events such as the sudden rupture of an atherosclerotic plaque, which lead to the formation of intravascular occluding aggregates and fibrin clots, thrombi (Ruggeri ZM, 2002). A key functional aspect of platelets is their ability to circulate in a quiescent state surveying the integrity of the inner vascular surface, coupled to a prompt reaction wherever alterations are detected. In either situation, adhesive interactions mediated by specific membrane receptors support the initial attachment of single platelets to cellular and extracellular matrix constituents of the vessel wall and tissues. After injury, adherent platelets can rapidly recruit additional platelets to the site of injury, necessary to achieve hemostasis, or different types of leukocytes, which set off host defense responses. This selective recruitment is orchestrated by activation pathways (Petrich BG, 2007) stimulated by the initial adhesive interactions and by soluble agonists released or generated locally, that lead to the exposure on the platelet surface of different molecules capable to attract circulating cells. In many respects, therefore, platelet adhesion to vascular wall structures, to one another or to other blood cells are facets of the same fundamental biological process (Ruggeri ZM, 2009). To initiate thrombus formation at the site of vessel

wall damage, platelets need to be captured from the flowing blood onto the exposed sub-endothelium. Platelets are activated upon contact with sub-endothelial matrix proteins, including von Willebrand factor (VWF), collagen, and fibronectin (Broos K, 2011). Their activation leads to exposure of cell surface anionic phospholipids, which serve as a nidus for the assembly of procoagulant proteins.

I. 2.1.1.1 Platelets Receptors involved in thrombosis process

I. 2.1.1.1.1. Adhesion Receptor: GPIb-IX-V complex and Von Willebrand Factor

The thrombosis event is mediated by a major platelet receptor that is constitutively active and expressed on resting platelets: the glycoprotein (platelet glycoprotein [GP]) Ib-IX-V complex (Bermeier W, 2000) (Berndt MC, 2001) (Fig. 3). Four different genes encode this adhesion receptor: the α- and β-subunits of GPIb, GPIX, and GPV. Deletion of the individual subunits has helped to define their roles in the hemostatic/thrombotic response (Lopez JA, 1998) (Ware J, 2004). In particular, blocking GPIbα and particularly its ligand-binding site resulted in a virtual absence of single-platelet adhesion in a ligation-induced injury model of the carotid artery (Massberg S, 2003). In addition, recent studies in transgenic mice expressing a fusion protein made of the cytoplasmic and transmembrane portions of human GPIbα (to rescue platelet generation) linked to the interleukin-4 receptor α-chain (Kanaji T, 2002) support the absolute requirement for GPIbα in the process of platelet tethering and consequently in thrombosis (Bergmeier W, 2006) (Kostantinides S, 2006) (Denis CV, 2007).

Von Willebrand factor (VWF) is the main ligand of GPIbα. VWF is a large, multimeric plasma protein that undergoes a conformational change when bound to matrix or under high-shear conditions; this conformational change allows its binding to GPIbα (Ruggeri ZM, 2003). VWF is also stored in platelet α-granules and is released after platelet activation. The GPIbα-VWF interaction is able to withstand very high shear rate conditions and is characterized by fast association and dissociation rates, allowing slow platelet translocation on the vessel wall (Savage B, 1998). VWF deficient mice ($VWF^{-/-}$) exhibited delayed platelet adhesion and reduced thrombus formation after vascular ferric chloride

application (Denis CV, 1998) (Ni H, 2000). However, platelets in these mice can still adhere and form thrombi, suggested that VWF deficiency appeared less severe than the lack of functional GPIbα. Under high-shear conditions, as arterial compartment, GPIbα was required to initiate platelet adhesion (Chauhan AK, 2006). Similarly, under low-shear condition as venous thrombi, transgenic platelets lacking the extracellular domain VWF receptor GPIbα showed decreased adhesion to injured veins (Chauhan AK, 2006). The absence of VWF prevented thrombus growth and subsequent occlusion of the vessel (Ni H, 2000) (Denis CV, 2007), suggesting that VWF plays an essential role in occlusive thrombi formation at both venous (Chauhan AK, 2006) and arterial (Ni H, 2000) shear rates.

After the initial tethering and translocation of platelets on the exposed sub-endothelium, a number of agonists can initiate signaling events that will lead to platelet activation, followed by firm adhesion. A very important aspect of platelet activation is the transition of a low-affinity binding state of platelet integrin's to a high-affinity state.

I. 2.1.1.1.2 Collagen Receptors: Integrin $\alpha_2\beta_1$ and GPVI

Two collagen receptors have been identified on the platelet surface: the **$\alpha_2\beta_1$ integrin** and a member of the immunoglobulin superfamily, **GPVI** (Clemetson KJ, 2001) (Fig. 3). Defining the respective roles of these two receptors generated intense debate, and mice deficient in these receptors helped to clarify the issues.

To define the role of the **$\alpha_2\beta_1$ integrin** *in vivo*, a genetically engineered mouse in which expression of the $\alpha_2\beta_1$ integrin was completely delated, α_2 –deficient mice ($\alpha_2^{-/-}$) was generated (Chen J, 2002) (Holtkotter O, 2002). The $\alpha_2^{-/-}$ mice reported the formation of occlusive thrombi in thrombosis models (Gruner S, 2003) (He L, 2003), although occlusion time was delayed in one of those studies (Gruner S, 2003) (He L, 2003). Platelets from $\alpha_2^{-/-}$ mice failed to adhere to type I collagen under shear stress (Chen J, 2002) and showed decreased rate of aggregation (Holtkotter O, 2002). Although, in some cases, such as platelets and mammary epithelial cells, the null phenotype was consistent with predictions based on the expression pattern of the integrin and with the *in vitro* data. In

other instances, the *in vivo* findings suggest alternative mechanisms for biological processes, such as wound healing and reject the proposed obligatory role for the $\alpha_2\beta_1$ integrin (Chen J, 2002). Indeed, Gruner S. *et al.* (Gruner S, 2003), demonstrated that there were other receptors that mediate shear-resistant adhesion in the absence of $\alpha_2\beta_1$ functional and $\alpha_2\beta_3$ receptor. In particular, He L. *et al*., using a model of endothelial injury to the carotid artery, provided evidence that the $\alpha_2\beta_1$ integrin played a critical role in vascular thrombosis at the blood-vessel wall interface under flow conditions. In contrast, the $\alpha_2\beta_1$ integrin was not required for the formation of thrombi and pulmonary emboli following intravascular injection of collagen. This result was in accordance with the observation that $\alpha_2^{-/-}$ mice display normal bleeding times and supports the notion that $\alpha_2\beta_1$ was not required for platelet adhesion *in vivo.*

The second collagen receptor, **GPVI**, is associated with the signaling adapter Fc receptor-γ (FcRγ) chain. For a long time, GPVI was considered a very attractive target for the development of new antithrombotic drugs (Nieswandt B, 2003). A dramatic reduction of platelet tethering, translocation, and firm adhesion as well as a delay in occlusion time was observed using GPVI-deficient mice ($GPVI^{-/-}$) in ferric chloride–induced or mechanical vascular injury (Massberg S, 2003). $GPVI^{-/-}$ mice exhibited a very variable phenotype, with some mice behaving like their wild-type littermates while others presented a markedly abnormal thrombotic response (Kostantinides S, 2006). The explanation for this difference was not completely understood. However, it could be related to the extent of vascular injury and whether another activation pathway could compensate for the loss of GPVI. In fact, mice deficient in FcRγ and $GPVI^{-/-}$ displayed an abnormal thrombotic response only when an important collagen exposure was induced, such as ferric chloride injury model. However, $GPVI^{-/-}$ mice displayed slightly prolonged bleeding times. These observations suggest that GPVI plays a major role in pathological arterial thrombosis but not in physiological hemostasis (Kostantinides S, 2006). Nevertheless, the role of GPVI as an adhesion receptor is under debate. However, "seven single-nucleotide polymorphisms" (SNPs) of the GP6 gene, that encodes for the GPVI, showed a significant association with VTE (Kotulicova D, 2013). The GP6 polymorphisms were robustly identified as additional VTE-associated locus by genome-wide association studies (GWAS)

and a large-scale association study, focusing mainly on non-synonymous polymorphisms. It may supported the idea that variability of the GP6 gene may be associated with an increased risk of VTE in SNPs. However, few data exist about the correlation between this receptor -and its ligand- and VTE.

I. 2.1.1.1.3 G-protein-coupled Receptor: PAR family

In addition to GPVI, a number of other important activation receptors are present on the surface of platelets, such as members of the protease-activated receptor (PAR) family, the thrombin receptors. An important role for PARs in hemostasis and thrombosis is established in animal models, and studies in knockout mice and non-human primates raise the question of whether PAR inhibition might offer an appealing new approach to the prevention and treatment of thrombosis. In particular, PARs have been most intensely studied in the context of efforts to understand thrombin signaling.

Thrombin, most potent platelet activator, is rapidly generated at the site of vascular injury and leads to shape change, integrin activation, and granule secretion. Thrombin inhibition reduces thrombus formation in animal models (Dubois C, 2006) (Mangin P, 2006) (Coughlin SR, 2005) (Ni H, 2001). Indeed, studies of inhibitors of PAR function in human platelets and studies in PAR knockout mice suggested that PARs were necessary for platelet activation by thrombin (Coughlin SR, 2005). Such studies also revealed interesting species differences that were important to consider in attempting to extrapolate findings in mouse to human. While human platelets utilize PAR1 and PAR4 to respond to thrombin, mouse platelets utilize PAR3 and PAR4 (Kahn ML, 1998). PAR1 agonists, that are fully active on human, and mouse PAR1 in heterologous expression systems (Connolly AJ, 1996) activate human but not mouse platelets (Connolly TM, 1994) (Derian CK, 1996), and knockout of PAR1 had no effect on thrombin signaling in mouse platelets but did ablate thrombin signaling in mouse fibroblasts (Connolly AJ, 1996). It was these observations that triggered a search for other thrombin receptors in mouse platelets and led to identification of PAR3 (Ishihara H, 1997). Thus, mouse PAR3 appeared to be a good candidate for the 'missing' mouse platelet thrombin receptor. Indeed, knockout mouse studies revealed that PAR3 was necessary for activation of mouse platelets at low but not

high concentrations of thrombin (Kahn ML, 1998). Despite this, it seemed that mouse PAR3 was not itself mediator for transmembrane signaling, but instead worked as a cofactor that localizes thrombin to the surface of the mouse platelet to promote cleavage and activation of mouse PAR4 at low thrombin concentrations. The PAR4-deficient mice ($PAR4^{-/-}$) were protected against thromboplastin-induced pulmonary embolism and ferric chloride-induced thrombosis of mesenteric arterioles (Sambrano GR, 2001) (Weiss EJ, 2003).

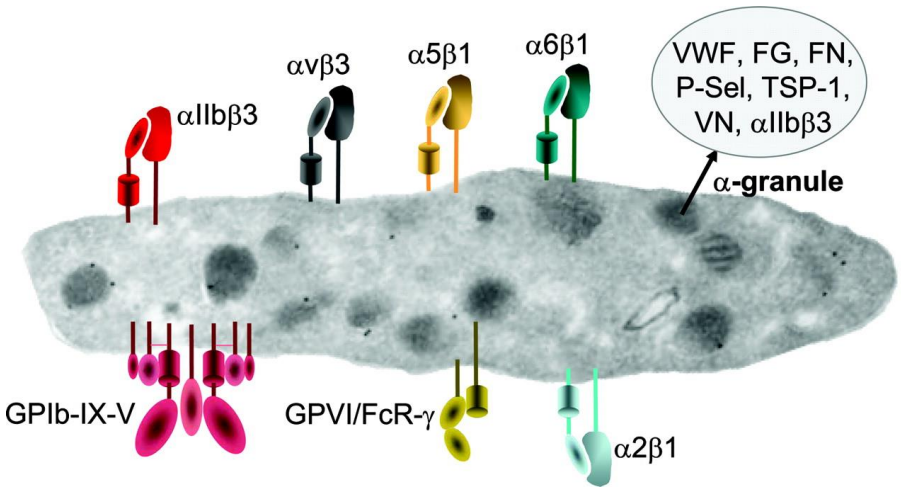
I. 2.1.1.1.4 ADP Receptors: $P2X_1$, $P2Y_1$ and $P2Y_{12}$

Hollopeter *et al.* (Hollopeter G, 2001) identified another important class of platelet receptors in 2001. Upon vascular injury, platelets are activated, leading to the formation of a platelet plug. This activation involves processes such as shape change, aggregation, secretion of granule contents, including adenosine diphosphate (ADP) and adenosine triphosphate (ATP), and generation of lipid mediators, such as Thromboxane A_2 (TXA_2) (Swamiinathan M, 2004). The receptors involved in this process are **$P2X_1$, $P2Y_1$ and $P2Y_{12}$ receptor**, a G Protein Coupled Receptor (GPCR). The $P2X_1$ receptor is a ligand-gated adenosine triphosphate (ATP) receptor that was originally mistaken for an ADP receptor. This calcium-influx-causing receptor mediates platelet shape change and plays an important role in thrombus formation in small arterioles (Swaminathan M, 2004). The $P2Y_1$ receptor, through activation of G_q and phospholipase C, is required for ADP-induced platelet shape change, fibrinogen receptor activation, and TXA_2 generation (Swaminathan M, 2004). The G_i-coupled $P2Y_{12}$ receptor plays an important role in platelet aggregation, potentiation of dense granule release, and TXA_2 generation (Swaminathan M, 2004). Each of these receptors has a specific function during platelet activation and aggregation, which has implications for their involvement in thrombosis. ADP is released from platelet dense granules and binding to $P2Y_1$ and $P2Y_{12}$ receptors on the platelet surface (Gachet C, 2006). ATP and a wide range of its triphosphate analogues behave as antagonists (Gachet C, 2005) (Kauffenstein G, 2004). ADP plays crucial roles in the physiological process of hemostasis and in the development and extension of arterial

thrombosis (Born GV, 1985) (Jones S, 2011). Extracellular Ca^{2+} modulates ADP-evoked aggregation through altered agonist degradation: implications for conditions used to study P2Y receptor activation (Jones S, 2011) and its action is mediated by $P2Y_1$ and $P2Y_{12}$ (Gachet C, 2006) (Hechler B, 2011). ADP is the best important molecular target of the antiplatelet drugs, such as clopidogrel and prasugrel of which the active metabolites formed in the liver covalently bind to the receptor (Savi P, 2006) (Algaier I, 2008). The pharmacological importance of this receptor in hemostasis and thrombosis was well recognized. Indeed, bleeding tendencies in patients with storage pool deficiencies or defective ADP receptors demonstrate the important role of ADP in the platelet activation processes. This is further substantiated by the clinical efficacy of ADP receptor antagonists in preventing and/or treating thrombotic conditions.

I. 2.1.1.1.5 Thromboxane Receptor: TP

Thromboxane A_2 (TXA_2) in human stimulates two subtypes of G protein-coupled TP receptor, TPα and TPβ, but its effects in platelets are mediated predominantly through α isoform (Swaminathan M, 2004). The two isoforms are known to differentially modulate the adenyl cyclase activity as the TPα isoform activates it, while the TPβ isoform inhibits it (Davì G, 1997). Generated TXA_2 amplifies the agonist-induced platelet activation by signaling via its surface receptor, activating and recruiting the surrounding platelets to the site of the growing thrombus (Roberts CK, 2002) (Riccioni G, 2007). Data obtained in mice lacking the TP receptor showed prolonged bleeding times and resistance to thromboembolism as compared with wild-type littermates mice (Myung SK, 2013). Treatment with SQ 29,548, a TP receptor antagonist, also resulted in prolongation of the bleeding time in wild-type mice (Myung SK, 2013). Furthermore, platelets from TP receptor-deficient mice ($TP^{-/-}$) showed impaired aggregation responses to TP receptor agonist, U46619 (Myung SK, 2013). These findings demonstrate the critical role of TXA_2 and the TP receptor in hemostasis.



Denis CV and Wagner D, Arteriosclerosis Thrombosis and Vascular Biology, 2007

Fig. 3: Major adhesion receptors expressed at the surface of resting platelets.

I. 2.1.2 Activation of coagulation cascade

The traditional description of coagulation pathway involved a cascade model of a stepwise sequence of proteolytic reactions of the coagulation factors, usually divided in the **extrinsic pathway**, or tissue factor (TF)-dependent pathway and the **intrinsic pathway** or a contact factor-dependent pathway (Rumbaut RE, 2010) (Fig. 4). As platelets, coagulation factors play essential roles in not only hemostasis but also thrombosis. The coagulation cascade represent a pathway that culminate in the activation of thrombin with consequent formation of fibrin. The newly synthesized mediators involved in coagulation cascade are synthesized when needed, and include the metabolites of arachidonic acid (AA), platelet-activating factor (PAF), reactive oxygen species (ROS), nitric oxide (NO) and cytokines.

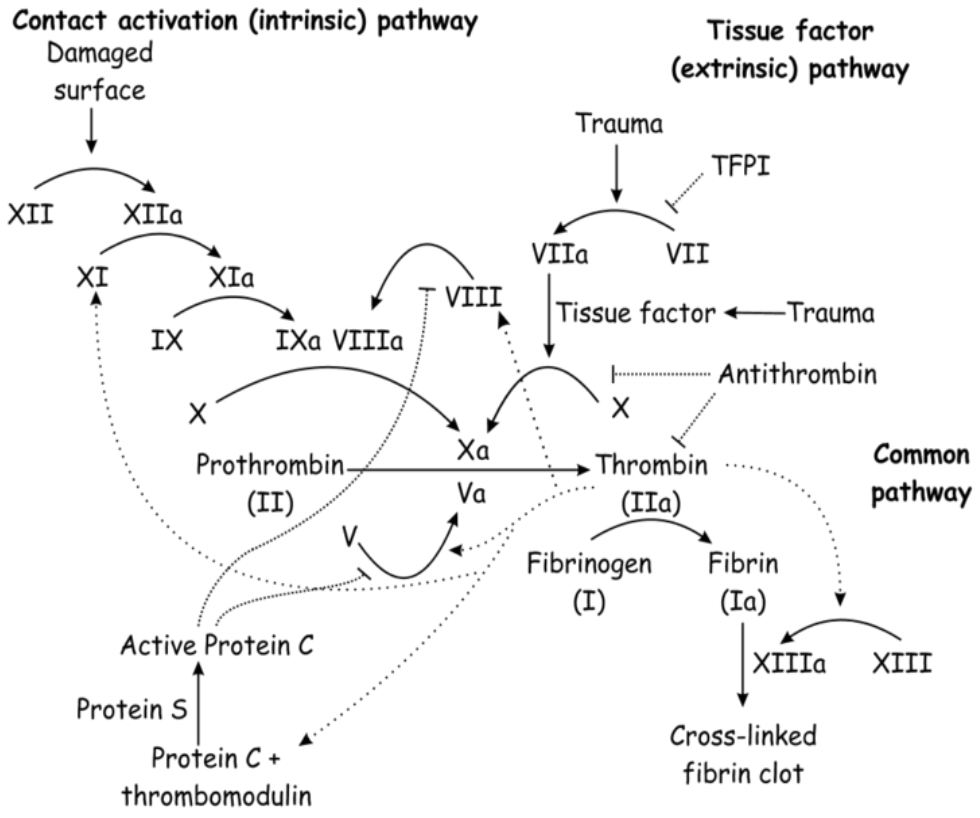


Fig.4: Coagulation cascade include two different pathway: intrinsic pathway (or contact factor-dependent) and extrinsic pathway (or tissue factor (TF)-dependent pathway).

The traditional regulation of blood coagulation view set the extrinsic pathway as triggers of the initiation phase, whereas amplification requires the intrinsic pathway (Mackman N, 2007).

Damage to blood vessel walls exposes Tissue Factor (TF)-containing cells from underlying cell layers to the bloodstream. Indeed, a low level of activity of the TF pathway probably occurs at all times in the extravascular space. The coagulation proteins leave the vasculature, percolate through the tissues, and are found in the lymph roughly in proportion to their molecular size (Le D, 1998) (Miller GJ, 2000). TF is then able to bind, in presence of calcium, Factor VII (FVII), which circulates at low levels in the bloodstream. The calcium forms a bridge between TF and FVII. In hemostasis models, fibrin formation at a wound site is triggered primarily, if not exclusively, by the FVIIa-TF complex (Baglia FA, 1998) (Oliver JA, 2002) (Li X, 1997) (Gailani D, 2007). The TF-FVIIa complex is traditionally referred to as the extrinsic pathway and is proposed to be the primary activator of the coagulation protease cascade *in vivo*. This sets off an extracellular cascade involving sequential serine protease activations and the propagation of the thrombus involves recruitment of additional platelets and amplification of the coagulation cascade by the intrinsic pathway of blood coagulation (Mackman N, 2007).

The intrinsic pathway is typically painted as a sequence of proteolytic reactions culminating in Factor IX (FIX) activation. The correctly functionality of FIX is crucial: deficiency of FIX or its cofactor, Factor VIII (FVIII), cause hemophilia B and hemophilia A, respectively, the severe forms of which are associated with crippling hemorrhage into joints and muscles, and soft tissue bleeding that can be life threatening (Lozier JN, 2005) (Gailani D, 2007). In this scenario, Factor XII (FXII), belonging to the intrinsic pathway, is not required for fibrin formation (Mackman N, 2007) (Morrissej JH, 1987) (Rapaport SI, 1964). In fact, FXII deficient mice ($FXII^{-/-}$) have a normal hemostatic capacity. However, thrombus formation in $FXII^{-/-}$ mice is defective in arterial thrombosis, stroke models, venous stasis and PE models (Renné T, 2012) (Renné T, 2005) (Cheng Q, 2010) (Muller F, 2009). In particular, FXII seems to be important for thrombus stability.

Although the relative contributions of the FVII-TF complex and FXII for FIX activation are uncertain. In many circumstances, activation of FIX by FVIIa-TF is the more important mechanism. In particular, FVII-TF complex is activated by auto-cleavage to FVIIa-TF that activates small amounts of FIX and Factor X (FX). Association between FXa and FactorV (FVa) leads to prothrombinase complexes on the TF-bearing cells (stromal fibroblasts and leukocytes) (Monroe DM, 1996). Indeed, FXa then associates with FVa, converts FII (prothrombin) to Factor II (FIIa) (thrombin), which converts fibrinogen to fibrin, leading to fibrin deposition and the activation of platelets to form blood clots (the activation of Factor XIII (FXIII) to FXIIIa stabilizes the fibrin clot by cross-linking it). The procoagulant Factor VIII (FVIII) plays an important role in the activation of thrombin and at last in the formation of a fibrin-rich thrombus (Gouse BM, 2014). The small amount of thrombin generated on TF-bearing cells has several functions. Thrombin is the final protease generated in the coagulation cascade, and it is the only factor capable of cleaving soluble fibrinogen into insoluble fibrin then promoting clot formation. Once generated from its inactive precursor prothrombin (FII) by prothrombinase complex (FXa-FVa-Ca^{2+}-membrane phospholipids), it can diffuse freely to encounter a large number of potential substrates both procoagulant and anticoagulant. Thrombin activates platelets through the cleavage of two protease platelet receptors, promoting platelets aggregation (Coughlin SR, 2005). Although platelets have already adhered at the site of injury and become partially activated, thrombin can induce a higher level of procoagulant activity than adhesive interactions alone (Alberio L, 1999). For instance, thrombin promotes positive feedback amplification of the coagulation pathway, leading to its own generation by proteolitically converting FXI to FXIa (a serine protease of the intrinsic pathway), as well as FVIII and FV (cofactors in the generation of FXa and thrombin, respectively) (Davie EW, 2006) (Baglia FA, 1998) (Oliver JA, 2002). The predisposition to VTE among subjects with FV Leiden (a congenital FV resistance to cleavage by activated protein C) or with the gain-of-function G20210A variant in the prothrombin gene (Dahlback B, 1997) (Mannucci PM, 2000), suggests that a fully functional system of vascular and blood-borne thrombo-resistance is important for venous patency.

Thrombin stimulates activation of ECs, including cell surface expression and secretion of cellular adhesion molecules as well as the production of growth factors and cytokines. Indeed, thrombin promotes cytokine elaboration by smooth muscle cells and stimulates the proliferation of both smooth muscle cells and fibroblasts. By the end of the amplification phase, the stage is set for large-scale thrombin generation in the propagation phase.

Elevated levels of different coagulation factors such as TF, FVIII and prothrombin have been linked to an increased thrombotic risk. In particular, the relationship between FVIII activity, platelet activity, and DVT is well described in adults with spinal cord injury (Faustino EV, 2015). In fact, risk factors for poor outcomes after a thrombotic event have been well defined in adults. Indeed, ipsilateral recurrent thrombosis has been associated with subsequent development of the post-thrombotic syndrome (Faustino EV, 2015), and multiple clinical and laboratory abnormalities, including elevated levels of FVIII (Kyrle PA, 2000) and D-dimer (Palareti G, 2002) (Eichinger S, 2003), increase the risk of recurrent thromboembolism.

I. 2.1.2.1 Tissue Factor: the first protein involved in a blood coagulation cascade

Tissue factor (TF) is a 47-kDa transmembrane glycoprotein that is the primary initiator of coagulation *in vivo* (Manly DA, 2011); it initiates coagulation following contact with FVII/FVIIa, as previously described. Recent experimental evidence, in particular from animal models, suggests an important role for circulating TF in thrombosis, but only few studies had investigated its role in venous thrombosis (Manly DA, 2011). TF is constitutively expressed in perivascular cells, in most non-vascular cells (Nemerson Y, 1988) (Mackman N, 2007) (Østerud B, 2006), in whole blood and in cell-free plasma, where functionally active TF is carried on cell-derived microparticles (Key NS, 2010), and it is essential for hemostasis.

Morawitz, in the early 20th century, in his classic four-component theory of blood coagulation, described that a tissue component, known as thrombokinase or Factor III (FIII), could lead to fibrinogen cleavage in the presence of calcium and prothrombin

(Boulton F, 2006). This tissue component, subsequently named Tissue Factor, is a transmembrane receptor for FVII/FVIIa. For several decades, the prevailing opinion was that TF is expressed exclusively in the extravascular space in healthy subjects and forms a hemostatic envelope around blood vessels (Drake TA, 1989). Actually, we know that TF is localized predominantly to the tunica media and tunica adventitia in blood vessels, which prevents inappropriate activation of coagulation in the absence of vascular injury. In addition, TF is expressed in the parenchyma of highly vascularized organs such as the placenta, brain, heart, kidneys, and lungs and protects these organs from excessive hemorrhage (Fleck RA, 1990) (Bouchard BA, 1997). In physiological condition, cells in direct contact with blood do not express TF (e.g. ECs) or express it at very low levels (e.g. subset of CD14-positive monocytes) (Egorina EM, 2005) (Fig. 5). However, *in vivo* endothelium is an important source of TF after injury, contributing to thrombosis in various diseases, and *in vitro* ECs express TF after stimulation (Parry GC, 1995) (Bevilacqua MP, 1986) (Colucci M, 1983). In addition, Drake and colleagues (Drake TA, 1991) using a highly sensitive immunohistochemical procedure showed that TF was present on ECs of the microvasculature of the spleen but not on ECs in other tissues. Moreover, more recently, TF protein was observed on ECs at branch points in the aorta of septic baboons (Lupu C, 2005). Nevertheless, TF antigen has also been observed on circulating ECs in patients with sickle cell disease and on ECs of the pulmonary vein in a mouse model of sickle cell disease (Solovey A, 1998) (Solovey A, 2004). Finally, TF was detected on ECs of cardiac vessels in rat models of angiotensin II-induced cardiac vasculopathy and cardiac allograft vasculopathy (Muller D, 2000) (Holschermann H, 1999).

In addition, expression of TF increased in peripheral blood monocytes in various disease states. For instance, exposure of monocytes to bacterial lipopolysaccharide (LPS) induces TF expression (Steinemann S, 1994) (Morrissey JH, 1987) (Virga GD, 1990) (Rapaport SI, 1964) (Chirwing JM, 1979) as a result of the transcriptional activation of the TF gene (Østerud B, 1998). Conversely, LPS does not induce TF expression in lymphocytes (Drake TA, 1989). Under debate is whether neutrophils express TF (Østerud B, 2004) (Nakamura S, 2004). Several studies demonstrated robust TF expression by neutrophils in different disease states (Higure A, 1996) (Todoroky H, 2000) (Maugeri N, 2006) (Ritis K, 2006), and

upon activation. TF expression has also been observed in human eosinophils (Moosbauer C, 2007). However, human and animal studies indicated that TF expression by leukocytes played an important role in thrombosis associated with a variety of diseases (Gailani D, 2007). Hathcock and Nemerson provided evidence that circulating TF was necessary for the propagation of the clotting reaction (Hathcock JJ, 2004). Indeed, TF was observed only at the edges of a hemostatic clot, whereas it was found throughout a thrombotic clot (Boles J, 2010). Circulating TF or blood-borne TF can contribute to thrombus formation *in vivo* (Giensen PL, 1999). In particular, circulating TF enhances the propagation of a thrombus triggered by vessel wall TF through its ability to sustain thrombin production at the clot surface. The majority of circulating TF is in the form of microparticles (MPs). These are small (<1-μM) membrane vesicles released from activated or apoptotic cells (Morel O, 2006). Monocytes, ECs, vascular smooth muscle cells, tumor cells, and possibly platelets (Mackman N, 2007) generate TF-positive MPs. Remarkable, elevated levels of TF-positive MPs have been observed in patients with a variety of diseases, including cancer, cardiovascular disease, sickle cell disease, and endotoxemia and VTE (Misumi K, 1998) (Tesselaar M, 2007) (Shet AS, 2003) (Aras O, 2004). In addition, hematopoietic cell–derived TF-positive MP were shown to play a central role in fibrin generation within a microvascular thrombus formed in response to laser injury (Chou J, 2004). TF-positive MPs are incorporated into a nascent thrombus; nevertheless, it is not provide that this TF is functional. In addition, it is very difficult to distinguish a role for circulating TF from the known role of the intrinsic pathway in the propagation of clotting *in vivo*. It has showed that an increase in MP restored hemostasis in a mouse model of hemophilia A, although the role of TF was not directly analyzed (Hrachovinovà I, 2003). In this study, the major challenge on circulating TF measurement in healthy individuals and in animal models is that this protein is present only in small amounts. However, some controversy remains regarding the role of blood-borne TF in hemostasis. Indeed, its measurement is a formidable challenge, and in murine models, the absolute requirement for blood-borne TF -as opposed to vessel wall TF- appears to be dependent on the model of vascular injury, as well as the size of the vessel that has been injured (Chou J, 2004) (Day SM, 2005) (Wang L, 2009) (Kretz CA, 2010).

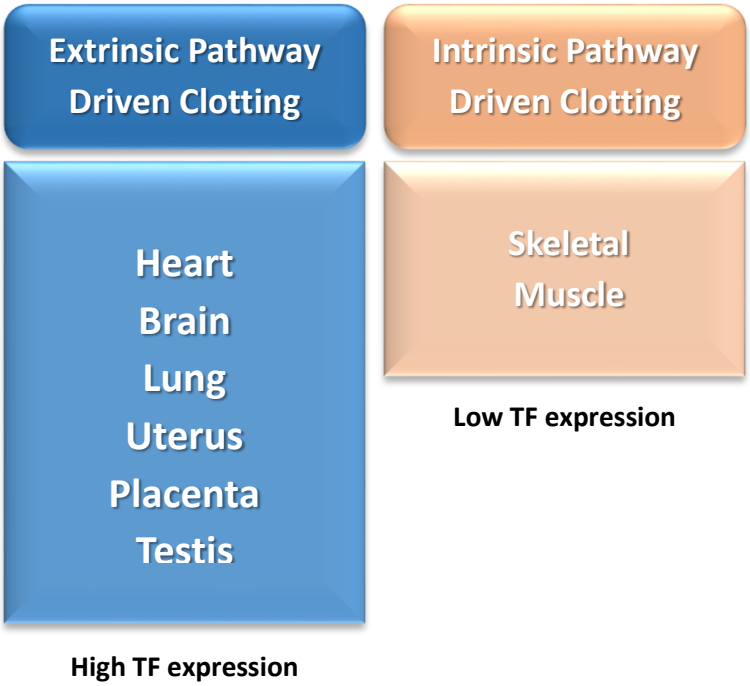
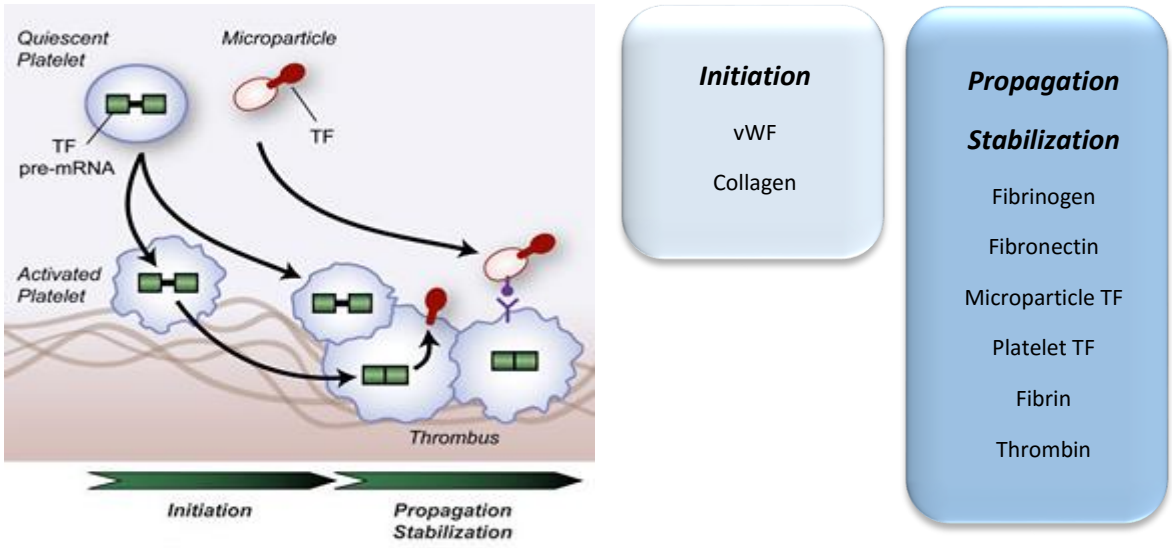


Fig. 5: Model for tissue-specific hemostasis. It is proposed that the extrinsic pathway mediates hemostasis in tissues that express high levels of tissue factor (TF), whereas the intrinsic pathway mediates hemostasis in tissues that express low levels of TF.

The concept that platelets, after simulation, express TF is still under debate. An early study by Engelmann and colleagues (Zillmann A, 2001) showed that platelets isolated from collagen-stimulated whole blood contained functional TF. This group also detected TF in α-granules of resting platelets that was exposed on the cell surface after platelet activation (Muller I, 2003). Another study found that TF associated with the platelet surface was inactive, but then released, TF was functionally active (Siddiqui FA, 2002). In contrast, Butenas and colleagues (Butenas S, 2005) founded no detectable TF antigen or activity on quiescent or ionophore-stimulated platelets. Similarly, Østerud and colleagues (Østerud B, 2006) failed to detect TF activity in collagen-activated platelets. Recent studies have helped to resolve the controversy of whether or not platelets express TF. These studies showed that platelets have the capacity to bind TF-positive MPs, stored TF in α-granules, and to synthesized TF *de novo* (Fig. 6). The fact that platelets can expressed TF dramatically changes our view of the regulation of blood coagulation, suggesting that

TF may contributed to both initiation and amplification of the clotting cascade. Recently, platelets were shown to express TF pre-mRNA, which can be spliced into mature mRNA on platelet activation, leading to the production of bioactive TF protein (Gould WR, 2005). However, the role of this platelet-derived TF in thrombus formation still needs to be addressed *in vivo* and further studies are needed to determine its physiological role in hemostasis and thrombosis.



Schwertz H, Journal of Experimental Medicine, 2006

Fig. 6: Proposed model by which platelet-derived tissue factor contributes to propagation and stabilization of a thrombus.

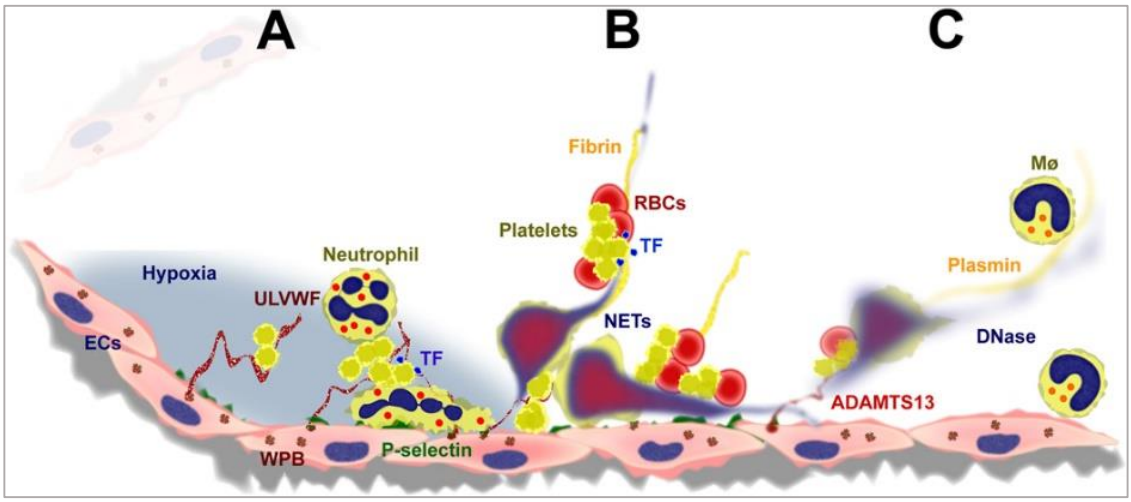
I. 2.1.3 Relevance of neutrophils in venous thrombosis

The first evidence of neutrophil involvement in thrombosis was the observation of the presence of these cells at the site of thrombus formation. This association between leukocytes and platelet in thrombus was described many years ago. In fact, Darbousset described that both platelets and leukocytes are recruited at the site of a vascular lesion in guinea pig mesentery using intravital microscopy in 1882 (Darbousset R, 2014). In 2005, Gross *et al.* showed that leukocyte recruitment into growing thrombi typically started at 2–3 minutes post-injury and increased over time (Gross PL, 2005). More

recently, von Brühl *et al*. showed, in a DVT model, that neutrophils are essential in the early phase of venous thrombosis (von Brühl ML, 2012). Neutrophils depletion with Ly6-G or Gr-1 antibodies significantly decreases both thrombus formation and fibrin generation, suggesting that neutrophils contain molecules required to support the onset of the coagulation cascade (Darbousset R, 2014) (von Brühl ML, 2012).

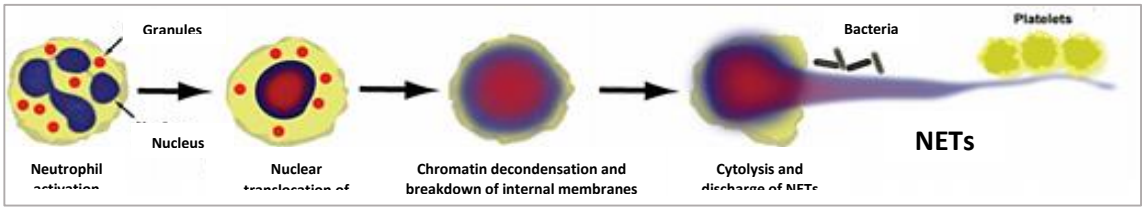
Neutrophils are the most abundant white blood cells in mammals. They represent 10 to 20% in mice, while represent 50 to 80% of circulating cells in adult humans. These cells, together with basophils and eosinophils, represent the polymorphonuclear cell family. Neutrophils are generated in the bone marrow (Rankin SM, 2010) where they also undergo maturation over approximately 14 days (Orkin SH, 2008) (Seely AJE, 2003). At steady state in humans, approximately 10^9 neutrophils per kilogram of body weight are formed each day (Dancey JT, 1976) and only 1% is released into the circulating blood (Semerad CL, 2002) while the others remain in the bone marrow for 4 to 6 days and constitute the neutrophil reserve pool. During infection or disease, the number of circulating neutrophils is drastically increased. In blood, the half-life of mature neutrophils is approximately 6–8 hours, and then these cells are cleared in the spleen, liver or bone marrow (Rankin SM, 2010). Neutrophils are also characterized by surface antigen expression of specific proteins. In humans, the key neutrophil marker is CD16, whereas in mouse they are Ly-6G and Ly-6C. Circulating neutrophils exist in several states: inactivated, resting, primed, and activated. Inactivated neutrophils are difficult to activate in comparison to primed neutrophils. Priming is a process in which the neutrophil response to an activating stimulus and is enhanced by prior exposure to small non-activating concentrations of this stimulus or other stimuli (Guthrie LA, 1984), including ATP (Naum CC, 1991), tumor necrosis factor alpha (TNF-alpha) (Onnmein K, 2008), interleukin 8 (IL-8) (Guichard C, 2005) and LPS (Bylund J, 2002). Upon activation, several changes occur in neutrophils, including an increase in intracellular calcium concentration (Carrillo C, 2011) (Shaff UY, 2010), changes in the expression of surface receptors (Videm V, 2004), exocytosis of granule contents (Lacy P, 2006), and modification in shape and F-actin reorganization (Howard TH, 1990). In association with these changes, two major processes result from neutrophil activation. Activated

neutrophils produce a large amount of reactive oxygen species following the activation of the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase system (Sheppard FR, 2005); moreover, upon stimulation, neutrophils are able to produce a web-like trap containing DNA/histones and material present in neutrophil granules such as anti-microbial peptides (Brinkmann V, 2004). This process is called NETosis (Brinkmann V, 2004). In brief, enzymes from granules translocate to the nucleus and facilitate chromatin decondensation (e.g., by the action of peptidylarginine deiminase 4 [PADI4] (Li P, 2010) (Alias S, 2013), internal break down membranes, and cytolysis, contributing to NET release. NETs are intact chromatin fibers containing histones and other proteins that form scaffolds, which can retain large quantities of microbes. The direct contact with antimicrobial proteins leads to fast elimination of infection (Brinkmann V, 2004) (Fuchs TA, 2012) (Fuchs TA, 2007). NETs are large structures that can contribute to thrombus formation and promote thrombus stability (Ni H, 2000) (Fig. 7 and 8). They are abundant in experimental deep-vein thrombi in baboons (Fuchs TA, 2010) and mice (von Brühl ML, 2012) (Brill A, 2012), where co-localize with vWF and fibrin (Brill A, 2012) (Fuchs TA, 2010). NETs cause platelet adhesion, activation, and aggregation, and are able to bind red blood cells (RBCs). RBCs may promote coagulation by exposing phosphatidylserine (PS) and altering blood viscosity (Andrews DA, 1999). *In vitro*, NETs stimulate the extrinsic pathway by cleaving TF pathway inhibitor (Fuchs TA, 2010) and stimulate the intrinsic coagulation pathway by binding FXII (Massberg S, 2010), thus promoting fibrin formation. In addition, NETs provide a tissue-type plasminogen activator (t-PA)–thrombolysis-resistant scaffold for blood clots (Fuchs TA, 2010). Recalcified blood was incubated with NETs releasing neutrophils. Only when blood was treated with the combination of t-PA, ADAMTS-13, and DNase complete clot lysis may occur. DNase- or t-PA-lysis alone led to partial thrombolysis of the blood clot. Blood clots treated with t-PA lacked fibrin but were held together by a scaffold of extracellular DNA (Fuchs TA, 2012). Treatment of mice with DNase1 (von Brühl ML, 2012) (Brill A, 2012) cleaved the NET scaffold and prevented thrombus formation underscoring the importance of NETs in DVT (Fuchs TA, 2010).



Fuchs T.A. et al., Artheriosclerosis Thrombosis and Vascular Biology, 2012

Fig 7: Neutrophil extracellular trap (NET) in the timeline of DVT. A: DVT is initiated by local hypoxia and activation of endothelial cells (EC) as a result of flow restriction/disturbances. Activated endothelium several factors (such as vWF and P-selectin) which mediate platelet and neutrophil adhesion. Activated platelets recruit TF containing MPs that enhance thrombin generation in the growing thrombus. B: Activated platelets and endothelium or other stimulus induce NET formation in adherent neutrophils. NETs provide an additional scaffold for platelet and RBCs adhesion, promote fibrin formation, and exacerbate platelet and endothelial activation. C: Plasmin, ADAMTS13 and DNase mediate thrombolysis by degrading fibrin, VWF and DNA, respectively. Monocytes/macrophages (MØ) release an additional source of DNase and generate plasmin and promote restoration of blood flow.



Fuchs T.A. et al., Artheriosclerosis, Thrombosis and Vascular Biology, 2012

Fig. 8: NET formation and function. Scheme of NET formation (NETosis). Enzymes from granules (red) translocate to the nucleus (blue) and facilitate chromatin decondensation. Internal membranes break down, and cytolysis releases NETs.

Despite the debate regarding the presence of TF in neutrophils, it is now clear that these cells express TF upon activation. Indeed, Maugeri *et al.* showed that, upon stimulation with P-selectin or N-formyl-methionyl-leucyl-Phenylalanine (fMLP), human neutrophils contain and express active TF on their surface (Maugeri N, 2006). Moreover, neutrophils stimulated with serum from patients with antiphospholipid syndrome (APS) express functionally active TF and TF mRNA (Ritis K, 2006). In this condition, the activation of C5a Receptor-Tissue Factor (C5a) attracts neutrophils to the site of inflammation and increases TF expression (Ritis K, 2006). More recently, Darbousset *et al.,* showed that purified mouse neutrophils may express TF (Darbousset R, 2012). In their study, they used a specific antibody to show that TF accumulated over time at the site of vascular damage. TF accumulation was strongly decreased by inhibiting neutrophil binding to vascular endothelium, suggesting that neutrophils represent the main source of TF in their laser-injury *in vivo* model. Moreover, in a chemically induced injury model, Massberg *et al*. showed that the neutrophil-derived proteases, such as Neutrophil Elastase and Cathepsin G, are required for thrombus formation. Indeed, these two enzymes play a crucial role in proteolytic inactivation of tissue factor pathway inhibitors, thereby promoting initiation of the coagulation cascade (Massberg S, 2010).

I. 2.1.4 Relevance of monocytes/macrophages in venous thrombosis

It is generally thought that the accumulation of macrophages is dependent on the recruitment of circulating monocytes, and direct visualization of these cells entering the thrombus has recently been shown. Circulating monocyte subsets can be distinguished based on their expression of surface receptors (Auffray C, 2009). Circulating inflammatory monocytes express Ly6C in the mouse and are recruited into tissue, where they undergo activation in a pathogen-dependent response (Auffray C, 2009) (Serbina NV, 2008). When monocytes migrate through tissue, they differentiate into macrophages (van Furth R, 1968) (Fig. 9). The contribution of $Ly6C^+$ monocytes in the formation and resolution of venous thrombosis has recently been shown. Indeed is known that monocytes are recruited in large numbers into maturing human and experimental

venous thrombi (McGuinness CL, 2001), and thrombus resolution does not occur if their recruitment is restricted.

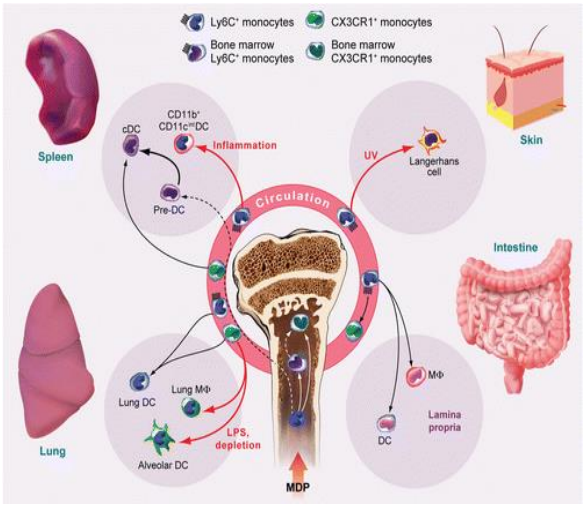


Fig. 9: Monocyte differentiation into dendritic cell (DCs) and tissue macrophages. Macrophage-DC progenitors (MDPs) give rise to Ly6C$^+$ bone marrow monocytes, which exit the bone marrow, in part guided by CCR2-dependent signals. Black arrows indicate differentiation steps into tissue DCs and macrophages that occur under homeostatic conditions. Red arrows indicate differentiation steps that occur under inflammatory conditions. Dashed arrows represent steps that remain uncertain.

Serbina V., Immunology, 2007

In thrombus formation process, the initial neutrophil infiltrate is replaced by monocyte-derived macrophages (Astrup T, 1967) (McGuinness CL, 2001) (Wakefiled TW, 1999) that have the capacity to express a host of chemotactic agents, proteases, and growth factors that orchestrate tissue remodeling and revascularization (Knighton DR, 1989). Evidence suggests that monocyte chemo-attractants, such as the cysteine-cysteine (CC) chemokine, monocyte chemotactic protein-1 (MCP1), are also expressed in the thrombus as it organizes, and treatment with exogenous MCP1 enhances resolution (Humphries J, 1999) (Waltham M, 2000). In particular, impaired resolution occurs in CC chemokine receptor type 2 (CCr2) knockout mice (CCr2$^{-/-}$) (Henke PK, 2006) (Ali T, 2006), indicating that CCr2 is required for the exit of Ly6C$^+$ monocytes from bone marrow (Serbina NV, 2006) (Fig. 9).

McGuinness *et al.* (McGuinness CL, 2001) provided evidence about the relevance of monocytes in venous thrombus resolution. They showed that endogenous and injected monocytes migrated into the thrombus during natural resolution, probably via the vein wall. Increasing the numbers of macrophages by direct injection

of fibrinolytically active peritoneal cells into the thrombus resulted in a large reduction in thrombus size and an increase in recanalization (Tahir A, 2006). These data suggest that increasing monocyte recruitment into the thrombus might improve its resolution and support the concept that the monocyte/macrophage are key mediator of venous thrombus resolution (McGuinness CL, 2001) (Singh I, 2003), supporting the evidence that the CC chemokine family and their receptors have an important role in the complex process that regulates monocytes/macrophages recruitment.

As previously described, the first step in venous thrombosis is activation of the endothelium and expression of the adhesion receptors P-selectin and E-selectin, as well as vWF. The activated endothelium then captures circulating leukocytes, TF-positive microvescicles (MVs), and platelets. Finally, induction of TF expressed by the bound leukocytes together with TF on MVs triggers thrombosis (Mackman N, 2012).

Activated monocytes and tumor cells are the primary sources of TF-positive MVs in the circulation (Owens AP, 2011) and data suggest that TF expression by leukocytes and possibly leukocyte-derived MVs initiated thrombosis in DVT mouse model (Von Brühl ML, 2012). Von Brühl *et al.* demonstrated that particularly monocytes contribute to TF-driven coagulation during mouse DVT. Indeed, TF expression by $Ly6G^{+}$ neutrophils was weak compared with monocytes, that revealed a strong TF signal (Von Brühl ML, 2012).

I. 2.2. Thrombus Stabilization

Many factors, including local calcium concentration, pH, and platelet numbers, affect clot stability (Celi A, 2003). In the last few years, it has become apparent that the primary activation and aggregation step is followed by a second wave of activation signals meant to prevent platelet aggregates from falling apart. Clot retraction is also an illustration of this process. Indeed, platelet-mediated clot retraction is necessary for the consolidation of a platelet thrombus by making it less susceptible to fibrinolysis. This event is virtually absent in patients or in mice lacking αIIbβ3, suggesting a major role for this receptor in stabilization of the thrombus, as previously described. The defect in stabilization of platelet thrombi in CD40L-deficient mice ($CD40L^{-/-}$) is a good example of the importance of stabilization process (Andre P, 2002) (Prasad KS, 2003). The thrombi formed in the arterioles of these mice looked lacelike rather than compacted, as seen in wild-type mice (Andre P, 2002). CD40L is a part of an autocrine loop, and it is released when primary platelet agonists activate platelets and it then further enhances platelet activation. Transmembrane proteins interacting with αIIbβ3 have also been implicated in thrombus stabilization (Lau LM, 2004) (Goschnick MW, 2006). The tetraspanin family members, CD151 (Lau LM, 2004) and TSSC6 (Goschnic MW, 2006), are able to regulate the outside-in αIIbβ3 signaling. TSSC6-deficient mice ($TSSC6^{-/-}$) display increased embolization and impaired thrombus stabilization after ferric chloride–induced injury (Goschnic MW, 2006).

The final pathway contributing to thrombus stability is the formation of the fibrin network deriving from the coagulation cascade. In a laser-injury thrombosis model, normal fibrin formation is dependent on the recruitment of blood-borne MPs TF to the thrombus, with a minimal contribution of vessel wall TF (Chou J, 2004). Mice deficient in either P-selectin or P-selectin glycoprotein ligand-1 (PSGL-1) develop platelet thrombi containing small amount of TF or of fibrin. This result complements the observation that high levels of soluble P-selectin are associated with a procoagulant state (Andre P, 2000). Finally, the hematopoietic cell–derived TF should help to promote thrombus stability until healing is completed.

For the preservation and the stability of clot more important is the role of Fibrinogen. Fibrinogen is a soluble 340-kDa protein that circulates in whole blood (Wolberg AS, 2007). It consists of two sets of three distinct disulfide-linked polypeptide chains (Aα, Bβ, and γ), whose synthetic programs are directed by three separate genes on chromosome 4. The major molecular target of fibrinogen is thrombin, which convert fibrinogen to fibrin monomers as thrombin removes N-terminal fibrinopeptides A and B. The resulting monomer is a disulfide-linked trinodular protein whose N- and C-termini converge at the E- and D-nodules, respectively. Assembly of fibrin fibers then proceeds in a stepwise fashion. After an initial lag phase, release of fibrinopeptide A encourages protofibril formation by the lateral aggregation of fibrin fibers, wherein the E domain of one homodimer interacts with the D domain of a second to generate a half-staggered, overlapping fibrillar pattern within the developing thrombus (Wolberg AS, 2007). Fibrin is cross-linked at lysine residues by FXIIIa and forms fibrillar aggregates, which, together with platelets and RBCs, provide structural integrity to the growing thrombus (Aleman NN, 2014). Turbidity and circulatory flow assist in fibrin polymerization and protofibril assembly by orienting the fibers as the growing thrombus forms (Gersch KC, 2010), (Campbell RA, 2010), (Neeves KB, 2010), (Blomback B, 1994), (Wolberg AS, 2002). Differences in the molecular structure of fibrin are believed to contribute to vascular thrombus persistence (Miniati M, 2010). Stability is also based partly found on fibrin fiber diameter, and the geometry of the fibrin network. The variables that affect fiber architecture are ultimately important for fibrinolysis, since both fiber size and arrangement impact tissue plasminogen activator (tPA) binding and rates of fibrinolysis (Carr ME, 1995), (Gabriel DA, 1992), (Longstaff C, 2011), (Collett JP, 2003). Local thrombin concentration also affects this process. Indeed, clot structure, as higher thrombin concentrations generate more stable clots (Wolberg AS, 2007), (Blomback B, 1994) (Wolberg AS, 2003).

Fragile clots are more susceptible to fibrinolysis and bleeding, whereas firm clots are more resistant, but may promote thrombosis (Undas A, 2011), (Cilia AL, 2011) (Ariens RA, 2013). Thrombus formation depends upon not only the total fibrinogen concentration, but also the isoform composition of the fibrinogen pool. Clot structure, therefore, reflects

the complex interplay of many factors ranging from polymorphisms in fibrinogen itself, to the efficiency of thrombin generation, the reactivity of associated cells, such as platelets, and the biochemical milieu (Rijken DC, 2009).

I. 2.3. Fibrinolysis

Fibrinolysis is a highly regulated enzymatic process that prevents unnecessary accumulation of intravascular fibrin and enables the removal of thrombi. Fibrin surfaces are key activation sites for fibrinolysis (Cesarman-Mau G, 2005). Activation of the fibrinolytic system is dependent on the conversion of the plasma zymogen, plasminogen, to the trypsin-like serine protease plasmin by the physiological activators urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). Because both fibrin and fibrinogen increase conversion of plasminogen to plasmin, they facilitate their own destruction (Doolittle RF, 2008) (Thorsen S, 1992). Plasminogen, the inactive precursor of the plasmin, is synthetized primary in the liver (Bohmfalck JF, 1980) (Saito H, 1983); however, other sources have been identified that include adrenal glands, kidney, brain, testis, heart, lung, uterus, spleen, thymus, and gut (Zhang L, 2002). When plasminogen (PG) becomes activated and is converted to plasmin, it unfolds a potent enzymatic domain that dissolves the fibrinogen fibers. These fibrinogen fibers entangle the blood cells in a blood clot. This process is called fibrinolysis. PG's relevance in the fibrinolysis process was identified also in *in vivo* experiments: mice with a total deficiency in PG ($PG^{-/-}$; homozygous-deficient mice) were utilized. $PG^{-/-}$ mice was not lethal and these mice survived well into adulthood. Pulmonary clot lysis in $PG^{-/-}$ mice was determined by measuring the spontaneous lysis of a radiolabeled plasma clot after its injection into the jugular vein. Relative to wild type mice, clot lysis was severely compromised in $PG^{-/-}$ mice, with an intermediate reduction of fibrinolytic capacity in $PG^{+/-}$ mice (heterozygous-deficient). When i.v. bolus of PG were administered to $PG^{-/-}$ mice, normal clot lysis and dissolution of spontaneous fibrin deposits in the liver were observed, indicating that *in vivo* clot lysis is dependent on the presence of PG. Additional studies have indicated that

polymorphonuclear leukocytes may compensate for a lack of PG in the degradation of fibrin(ogen) (Zhang B, 2002).

tPA and uPA are two primary serine proteases that activated from plasminogen to plasmin, the primary fibrinolysis. tPA is synthesized and released by ECs, and uPA is produced by monocytes, macrophages, and urinary epithelium. Binding of t-PA to ECs promotes their fibrinolytic activity and stimulates cell proliferation (Barnathan ES, 1988) (Welling TH, 1996). Both activators have exceedingly short half-lives in circulation (4–8 minutes) due to the presence of high concentrations of specific inhibitors, such as PAI-1. Compared to tPA, uPA has lower affinity for plasminogen, does not require fibrin as a cofactor, and, under normal conditions, appears to act mainly in extravascular locations. Both tPA and uPA are cleared by the liver after forming complexes with a low density lipoprotein (LDL)-receptor-like protein (Bu G, 1994). Because plasmin increases activator activity by converting single-chain tPA and uPA to their two-chain counterparts, plasminogen exerts positive feedback on its own activation (Cesarman-Maus G, 2005), (Hoylaerts M, 1982) (Tate KM, 1987).

Serine protease inhibitors or serpins neutralize circulating plasmin and plasminogen activators, which are present in excess concentrations (Travis J, 1983). Serpins form covalent complexes with their unique target enzymes that are subsequently cleared from the circulation. The three serpins most important in fibrinolysis are plasminogen activator inhibitor-1 and 2 (PAI-1 and PAI-2), and α2-antiplasmin (A2AP). Other non-serpin plasmin inhibitors include α2-macroglobulin, C1-esterase inhibitor, and members of the contact pathway of the coagulation cascade, which also play minor roles in plasmin inhibition.

PAI-1 is released into the circulation from ECs, platelets, and other cells (Sprengers ED, 1987). It is associated primarily with the extracellular matrix, resulting in stabilization of its activity (Levin EG, 1987), and it is upregulated by a large number of proinflammatory cytokines (Hajjar HA, 2014). Quiescent ECs express low or no PAI-1 (Cines DB, 1998), but after exposure to thrombin and inflammatory stimuli, the expression of PAI-1 is highly upregulated, which results in impaired fibrinolytic function (Loskutoff DJ, 1989) (Sawdey MS, 1991).

PAI-2 is more important in pregnancy, and its concentrations increase as the pregnancy progresses. Deficiencies in PAI-2 have been associated with adverse pregnancy outcomes (Cesarman-Maus G, 2006) (Coolman M, 2012).

Plasmin and A2AP bind with 1:1 stoichiometry, whereupon both become inactive. Plasmin is protected from inhibition by A2AP upon binding to fibrin, while initially fibrin-bound A2AP protects the clot from fibrinolysis (Plow EF, 1981) (Ichinose A, 1983) (Kimura S, 1986) (Schneider M, 2004). Binding of thrombin to thrombomodulin (TM) accelerates activation of thrombin-activatable fibrinolysis inhibitor (TAFI). Thrombomodulin is protein cofactor expressed on ECs that modifies the substrate specificity of thrombin, apparently by an allosteric mechanism (Sadler JE, 1997). By regulating the expression of TM, ECs decrease or increased the rate of intravascular fibrinolysis (Cines DB, 1998).

TAFI is a non-serpin fibrinolysis inhibitor that is activated by thrombomodulin-associated thrombin, and it is important to prevent excess unregulated plasmin or plasminogen activator activity. TAFI is a carboxypeptidase that removes C-terminal lysine and arginine residues on fibrin, thereby decreasing the number of available plasminogen binding sites, slowing plasmin generation, and stabilizing clots (Broze GJ, 1996) (Mosnier MO, 2001).

The contribution of ECs to fibrinolysis differs with their metabolic status (i.e., quiescent or activated), their vascular derivation, and the concentration of other hemostatically active molecules in the local plasma milieu (Cines DB, 1998). In a resting state, the ECs surface is profibrinolytic and helps maintain blood in its fluid state (Cines DB, 1998).

Annexin A_2 ($ANXA_2$), an important component of cell-based fibrinolysis, is a member of the annexin family of calcium-binding proteins that fulfill diverse physiologic functions (Rescher U, 2008) (Gerke V, 2005) (Fatimathas L, 2010) (Rescher U, 2004). The annexins represent a >50-member family of calcium-dependent, phospholipid-binding proteins of largely unknown functions (Gerke V, 2002) (Fig. 10). The canonical annexin "fold" allows these proteins to shuttle intracellularly between aqueous and membrane compartments in response to fluctuations in calcium concentration. $ANXA_2$ is a 36-kDa protein produced by ECs, monocytes, macrophages, trophoblast cells, and some tumor cells. It exists both free in the cytoplasm and in association with intracellular and plasma membrane surfaces (Gerke V, 2002) (Gerke V, 2005). When $ANXA_2$ is membrane

associated, the tightly packed, alpha-helical 33-kDa core domain forms a disk whose convex face is associated with membrane phospholipid and whose concave face is oriented away from the membrane. Membrane binding is mediated by at least two potential Ca^{2+} binding "annexin" repeats, features common to all annexin family proteins (Gerke V, 2002). The core specifies membrane-binding capability, whereas the tail possesses an invariant tyrosine 23, the pp60-c-src phosphorylation target (Isacke CM, 1986).

On the surface of ECs and monocytes, $ANXA_2$ forms a heterotetrameric complex with another protein, **S100A10 (also known as p11)**. As a member of the S100 family of proteins, S100A10 contains Ca^{2+}-binding helix-loop-helix motifs and confers increased phospholipid binding affinity on $ANXA_2$ (Hajjar KA, 1990). The C-terminal region of S100A10, particularly its hydrophobic residues within the C-terminal extension, contributes critical contact points for binding to $ANXA_2$ (Réty S, 1999).

The **$ANXA_2$-S100A10 complex** strongly promotes the tPA-dependent activation of plasmin independently of fibrin (Rescher U, 2008) (Waisman DM, 1995) (Flood EC, 2011). In particular, $ANXA_2$ is a co-receptor for the plasma-derived fibrinolytic zymogen, plasminogen, and tPA (Hajjar KA, 1996) (Hajjar KA, 1993) (Dassah M, 2009) (Flood EC, 2011) (Kwon M, 2005) (Madureira PA, 2011). Upon binding, the $ANXA_2$ tail peptide assumes α-helical conformation that presents key hydrophobic residues.

Translocation of $ANXA_2$ to the outer leaflet of the plasma membrane of the EC is a key regulatory step governing vascular fibrinolysis (Dassah M, 2009) (Flood EC, 2011), and it is initiated by several factors including heat stress, thrombin stimulation, and hypoxia (Peterson EA, 2003) (Deora AB, 2004) (Huang B, 2011). $ANXA_2$ translocation in ECs occurs within minutes, and requires the presence of adequate S100A10. Indeed, in the ECs, S100A10 is stabilized by $ANXA_2$, which, upon binding, masks a critical "degron," or polyubiquitination site on S100A10. In the absence of sufficient $ANXA_2$, S100A10 is polyubiquitinated and targeted to the proteasome for degradation (He KL, 2008). Recently, fibrinolysis was assessed in S100A10-null mice ($S100A10^{-/-}$), which displayed increased vascular fibrin, reduced clearance of thrombi, and impaired neovascularization of Matrigel thrombi (Surette AP, 2011).

Inhibition of the $ANXA_2$ complex's function may increase thrombosis risk by impairing fibrinolysis. Mouse deficient in $ANXA_2$ ($ANXA_2^{-/-}$) display normal development, fertility, and lifespan; fibrin accumulation is evident in both intravascular and extravascular locations within the lungs, spleen, small intestine, liver, and kidney (Ling Q, 2004). Microvascular ECs isolated from $ANXA_2^{-/-}$ mice, moreover, lack the ability to support tPA-dependent plasmin generation *in vitro*, and arterial injury *in vivo* leads to an increased rate and severity of vascular occlusion in the $ANXA_2^{-/-}$ mouse. In addition, $ANXA_2^{-/-}$ mice display fibrin accumulation within blood vessels and impaired clearance of injury induced thrombi (D. AB 2004). High-titer antibodies directed against $ANXA_2$ have been observed with increased frequency in patients with antiphospholipid syndrome and a history of thrombosis, and in a cohort of patients with cerebral venous thrombosis (Cesarman-Mau G, 2006) (Cesarman-Mau G, 2011). Polymorphisms in $ANXA_2$ have also been associated with vascular occlusion in patients with sickle cell disease (Cesarman-Mau G, 2006) (Flanagan JM, 2011). Conversely, high levels of $ANXA_2$ are expressed by blast cells in acute promyelocytic leukemia (APL) and seem to contribute to increased fibrinolysis and bleeding (Menell JS, 1999) (Stein E, 2009). On APL cells, $ANXA_2$ may increases fibrinolysis in concert with protein S100A10, which is also upregulated in an autonomous APL cell line (O'Connell PA, 2011). In human subjects, overexpression of $ANXA_2$ in acute promyelocytic leukemia leads to a hyperfibrinolytic bleeding diathesis reflective of excessive cell surface $ANXA_2$-dependent generation of plasmin (Menell JS, 1999).

Thus, surface expression of $ANXA_2$ may represent a crucial cellular defense mechanism against intravascular thrombosis following vascular injury or stress.

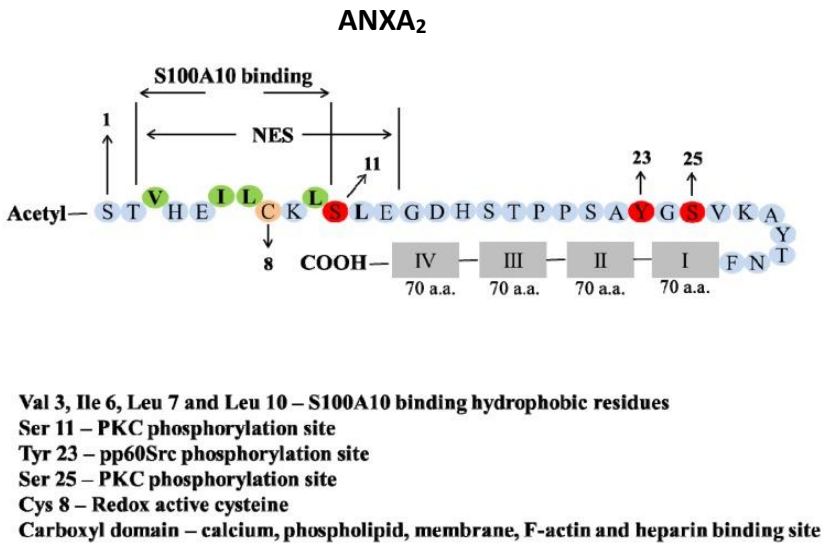


Fig. 10: Domain structure of annexinA_2 ($ANXA_2$). AnnexinA2 is composed to two domains—the amino-terminal domain and carboxyl-terminal domain. The S100A10 binding site is an amphipathic α-helix, with the hydrophobic residues, Val-3, Ile-6, Leu-7 and Leu-10 making contacts with S100A10.

Bharadwaj A. et al., International Journal of Molecular Science, 2013

Fibrin degradation products (FDPs) begin to form as plasminogen is activated and plasmin begins to degrade the thrombus. Multiple FDPs, including fibrinopeptide B and other fibrin degradation monomers and dimers are released (Baley K, 1951) (Baley K, 1955) (Latallo ZS, 1962). When fibrin polymers are cleaved by plasmin at the D fragment site, the resulting D-dimer fragment reflects the degree of thrombosis and plasmin activity. D-dimer assays have found predictive and prognostic value in a number of disease states, including disseminated intravascular coagulation (DIC), pulmonary embolism, DVT, and cancer-associated thrombosis (Khalafallah A, 2014) (van der Hulle T, 2013) (Gomes M, 2014). Individual FDPs may have immunomodulatory effects (Jennewein C, 2011). Fibrinopeptide B can serve as a chemoattractant for neutrophils, monocytes and macrophages (Senior RM, 1986) (Richardson DL, 1976). Some FDPs appear to have thromboregulatory properties in animal models. For example, synthetic peptides based on the degradation product fibrin B knob have been shown to impair fibrinolysis (Pandi L, 2009). Addition of FDPs to arterial canine blood or plasma *ex vivo* prolongs clot formation as assayed by multiple coagulation tests (Mischke R, 2004) (Mischke R, 2000). The mechanism of the anticoagulant effect of FDPs remains unclear (Ittyerah TR, 1979).

I. 3 CYCLOOXYGENASE AND PROSTANOIDS

In humans, arachidonic acid is metabolized into many potent bioactive compounds, such as prostaglandins (PGs) and thromboxanes (TXs), leukotrienes (LTs), and lipoxins (LXs) (Demetz E, 2014) (Smith WL, 2000).

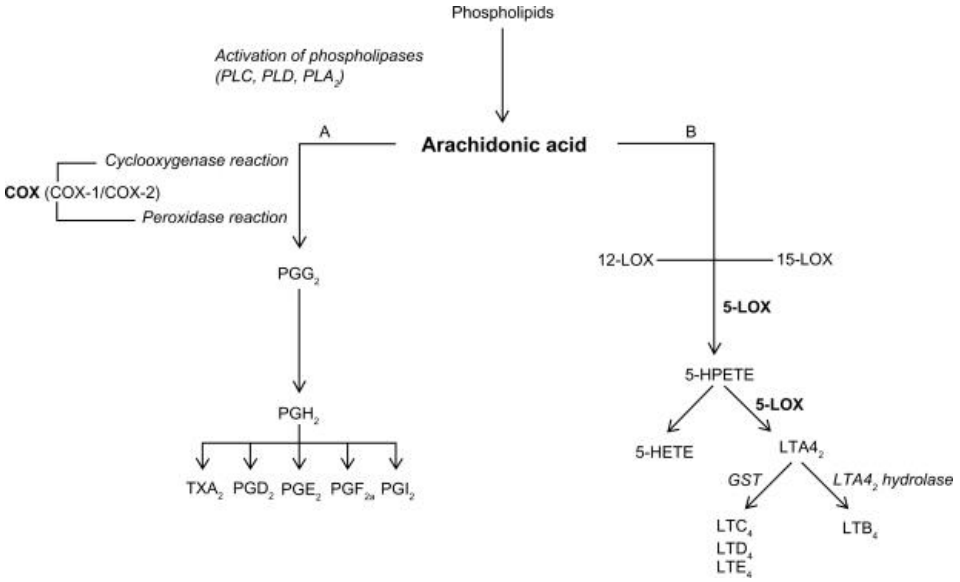
Arachidonic acid (AA or cis-,cis-,cis-,cis-5,8,11,14-eicosatetraenoic acid), an essential polyunsaturated fatty acid to 20 carbon atoms, is the main precursor of eicosanoids, biologically active lipids that have been implicated in cellular signaling cascades of physiological and pathophysiological relevance (Wang D, 2010).

The first step in the AA cascade is cleavage and release of AA from the phospholipid-bound form. It is suggested that this may be achieved with the assistance of at least one of the three different enzymes, namely phospholipase A_2 (PLA_2), phospholipase C (PLC) and phospholipase D (PLD) (Farooqui AA, 2005). Mammalian cells contain several isoforms of the enzyme PLA_2 (Murakami M, 1999), which receive their stimulatory signals from a vast range of inflammatory signals, cytokines, growth factors and hormones. The majority of AA metabolites can act both as pro- and anti-inflammatory mediators (Cabral GA, 2005), modulating gene expression, cytokine signaling and other immune regulatory factors.

AA, once released, is metabolized through three major pathway: 1) the way of prostaglandin H synthase (PGH synthase), called also cyclooxygenase way (COX pathway), which leads to the formation of prostanoids; 2) the way of lipoxygenase (5-, 12- and 15-lipoxygenase), that leads to the formation of hidroxyicosatetraenoic acid (HETE); 3) the way of leukotrienes (LOX pathway) (McMahon B, 2004) (Serhan CN, 2007). The metabolic pathways that have greater pharmacological interest in the context of cardiovascular disease is the COX pathway, from which are obtained prostaglandins, prostacyclins and thromboxanes.

I. 3.1 The COX pathway

In the early 90's it was discovered the existence of two principal isoforms of cyclooxygenase enzymes: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), but to date, another cyclooxygenase have been identified, cyclooxygenase-3 (COX-3) (Fig. 11).



Bhandary A.K. et al., Journal of Pain Research, 2010

Fig. 11: The cyclooxygenase (COX) pathway.

Although they differ in their pattern of expression and tissue distribution in human cells (Williams CS, 1999) (Chandrasekharan NV, 2002), collectively they are responsible for the stepwise conversion of AA to the three classes of prostanoids. COX-1 and COX-2 are present in different districts of the organism, are regulated in different manner and performing distinct biological functions.

The COX-1 enzyme (Fig. 12), also known as Prostaglandin H2 Synthase 1 (PTGS1), is a protein constitutively expressed by almost every cells in the human body, including platelets; it is involved in intercellular communication and tissue homeostasis; it is also responsible for the production of prostaglandins involved in gastric protection and in self-regulation of renal blood flow. The COX-1 gene is part of the house-keeping genes that

express a homodimeric protein integrated in cell membranes. The COX-1 is constituted by 599 amino acids and has a molecular weight of 72Kda, intervenes in the synthesis of prostanoids instant that occurs within a few minutes by stimulation with calcium. Actually, the COX-3 enzyme is a protein that derived from a different splicing of mRNA of COX-1 in which intron 1 is not deleted. It is an enzymatic protein present mainly in the central nervous system.

The COX-2 enzyme (Fig. 12), or PTGS2, is an inducible protein produced by macrophages, fibroblasts and ECs. It is normally absent from most cells but is highly induced by a variety of stimuli associated with inflammatory responses, such as cytokines at sites of inflammation and during tumor progression (Dubois RN, 1998) (Morita I, 2002) (Patrignani P, 2005). The production of prostanoids, which contributes to the vasodilation, edema and hyperalgesia characteristics of inflammatory processes, is mainly a result of the local induction of expression of COX-2 in inflammatory cells. COX-2 is an "immediate-early response gene" and its mRNA is highly induced in response to cellular transformation. The human COX-2 is a protein of 604 amino acids, which has been identified and cloned in 1991 (Ferreri NR, 1999). Near the C-terminus contains an insert of 18 amino acids that are not present in the COX-1, while, all the essential residues that form the channel hydrophobic binder the substrate (Yagamata K, 1993), the catalytic sites and residues immediately adjacent to it, are highly conserved in two isoforms. Although COX-1 and COX-2 have the same three-dimensional protein folds and share over 60% amino acid sequence identity, COX-2 displays a branched substrate-binding site, whereas COX-1 has a non-branched substrate-binding site and has conformational less flexible structure (Luong C, 1996) (Picot D, 1994). The crystallographic structure of COX-2 reveals a homodimer with each monodimer containing three structural domains, the Epidermal Growth Factor (EGF)-like, the membrane-binding and the catalytic domain (CD). The CD contains the active sites of both the cyclooxygenase and peroxidase activity. The cyclooxygenase active site is located at the end of a long hydrophobic channel, formed by residues Tyr385, Phe381, Phe518, Leu384 and Trp387. Substrate-binding requires hydrophobic interactions and hydrogen bonds to Arg120 and Tyr335 as well as a salt-bridge formation between residues Arg120 and Glu524 (Selinsky BS, 2001). Catalytic

activity is exerted by residue Tyr385, which, upon binding of AA, removes its 13-pro-S hydrogen to initiate Prostaglandin G_2 (PGG_2) formation (Rowlinson SW, 2003). The presence of the hydrophobic pocket in the COX-2 side increases the volume of the catalytic site of about 25%, making it capable of accommodation of bulky molecules, relevant in pharmacological treatment.

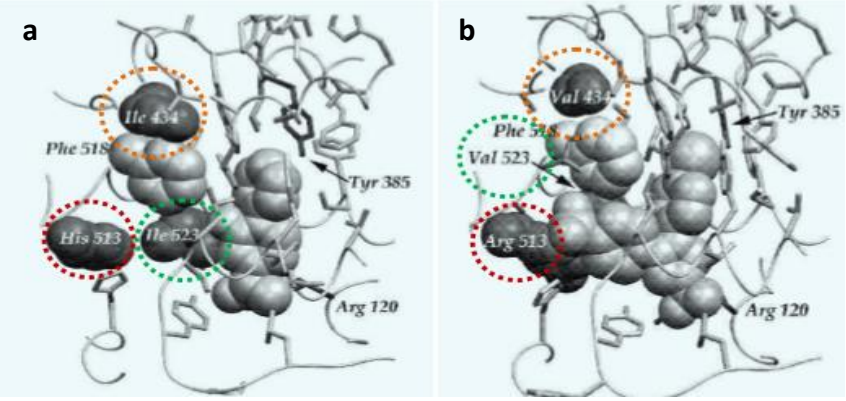


Fig. 12: Structure of COX-1 (a) and COX-2 (b) enzymes

The first step in the **COX metabolic pathway,** which leads to the formation of prostanoids, is oxygenation of AA by its cyclooxygenase activity to give PGG_2, followed by rapid conversion of PGG_2 by its peroxidase activity into Prostaglandin H_2 (PGH_2, the parent compound). PGH_2 is an unstable endoperoxide, catalyzed by a number of cell-specific isomerases that lead to the formation of the prostaglandins (PGs) prostacyclin D_2 (PGD_2), prostacyclin E_2 (PGE_2), prostacyclin $PGF_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin I_2 (PGI_2) and thromboxane A_2 (TXA_2) (Urade Y, 1995).

Prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$) are produced by leukocytes, platelets and ECs. They are of biogenic amines that exert a biological effect in an autocrine or paracrine manner by binding to their cell surface receptors, which belong to the G protein-coupled receptor (GPCR) family. These receptors are designated DP (also known as PTGDR) and GPR44 for prostaglandin D_2 (PGD_2); EP1, EP2, EP3 and EP4 (also known as PTGER1, PTGER2, PTGER2 and PTGER4, respectively) for PGE_2; FP (also known as PTGFR) for $PGF_{2\alpha}$; IP (also known as PTGIR) for PGI_2; and TP (also known as TBXA2R) for TXA_2 (Wang D, 2010). However,

prostaglandins and their metabolites can also bind nuclear receptors such as PPARs. For example, 15-deoxy-Δ12,14-PGJ_2 ($15dPGJ_2$), is a natural ligand for PPARγ. PGE_2 can also indirectly activate PPARδ in some contexts (Wang D, 2004). The prostaglandins act in different tissues (eg. intestinal, bronchial, uterine and stomach); more important function is developed by PGE_2 that protect the mucosa of the gastrointestinal lining: PGE_2 inhibits the production of HCl in the stomach and favors the production of mucus and bicarbonate secretion, which counteracts the gastric acidity. Moreover, prostaglandins stimulate platelet in maintaining normal homeostasis, preserving the resistance to thrombotic process acting on the surface of vascular ECs. In addition, they are involved in pain and fever.

Prostacyclin or prostaglandin I, PGI_2, is a major product of COX-2 catalyzed metabolism of AA in the endothelium. PGI_2 is generated by prostacyclin synthase enzyme mainly in the intima and other layers of the vessel wall (Moncada S, 1977). It is a potent vasodilator, and inhibitor of platelets aggregation (Higgs GA, 1977) (Bayer BL, 1979) (Bourgain RH, 1979) (Gryglewski RJ, 1978), and for these characteristics, is considered the physiological antagonist of thromboxane. PGI_2 protects against atherothrombosis (Arehart E, 2007) and myocardial ischemia (Ogletree ML, 1979) (Jugdutt B, 1979). These and other studies have support the hypothesis that PGI_2 may have clinical value in the management of specific cardiovascular diseases. Moreover, PGI_2 knockout mice ($PGI_2^{-/-}$) exhibit increased atherosclerosis, enhanced thrombosis (Wang D, 2005) (Psaty BM, 2005) and greater proliferative response to carotid vascular injury with increased intima to media ratios (Arehart E, 2007).

Recently, it has been shown that PGI_2 activates PPARδ and NAD^+-dependent class III histone deacetylase sirtuin-1 (SIRT1), and decreased TF expression and activity, whereas inhibition of COX-2 has the opposite effect (Ghosh M, 2007) (Barbieri SS, 2012). In particular, in our publication, we show that PGIS may regulate SIRT1 and TF expression via both the IP receptor and PPARδ activation (Barbieri SS, 2012). Indeed, PGIS inhibition suppresses PPARδ activity (Ghosh M, 2007) and PPARδ plays a role in transcriptional regulation of SIRT1 (Okazaki M, 2010) and TF. These findings provide a potential mechanism for the increased thrombosis associated with COX-2 inhibition.

Thromboxane is produced mainly in platelets by the thromboxane synthase; is the main AA metabolite in human platelets and exhibits two major activities: stimulation of platelet function, including secretion of platelet-derived storage products, and vasoconstriction (Schror K, 1992). Once formed, thromboxane which has a very short half-life as thromboxane A_2 (TXA_2), is rapidly broken down by hydrolysis to the inactive thromboxane B_2 (TXB_2) (Katugampola SD, 2009) (Reilly M, 1993) (Sachinidis A, 1995). Thromboxane is produced locally by platelets, macrophages (Reilly M, 1993), vascular smooth muscle cells of arteries and veins (Serneri N, 1983), ECs (Mehta JL, 1983) (Sung C, 1989) and human cardiac atrial tissue (Mehta JL, 1985). The distinguishing feature of thromboxane is a 6-membered ether-containing ring. TXA_2 is a potent vasoconstrictor and promotes platelet aggregation. Moreover, it act also as stimulator of vascular smooth muscle cell growth (Sachinidis A, 1995) and is a positive inotropic mediator in the heart (Sakuma I, 1989). It is in homeostatic balance in the circulatory system with prostacyclin, a related compound. Increased production (approximately 10 ng ml^{-1}, compared with 1 – 2pg ml^{-1} in normal healthy plasma) of thromboxane has been implicated in cardiac pathology, including ischaemic heart disease (Serneri N, 1981), pulmonary hypertension (Fuse S, 1994) and heart failure (Gresele P, 1991). Additionally, increased production of thromboxane is also associated in vascular pathology, particularly with atherosclerosis of coronary artery disease, and accelerated atherosclerosis of saphenous vein graft (Mehta JL, 1988). The human thromboxane receptor is TP (Narumiya S, 1999). Molecular biology and pharmacological studies have identified mRNA encoding the TP receptor and TP receptor protein in animal and human tissues in culture (Haluscka PV, 1989). In humans, TP receptor exists in two isoforms, TPα and TPβ, which arise from alternative splicing after the seventh transmembrane domain and have different intracellular C-terminal intracytoplasmic edges (Narumiya S, 1999) (Ting HJ, 2012). Both TP receptor mRNA isoforms have been identified in several cells and tissues (Miggin SM, 1998), but TPα is the dominant isoform translated in platelets (Habib A, 1999) and vascular smooth muscle cells (SMCs), whereas the TPβ isoform is present in ECs (Kent KC, 1993) and vascular SMCs (Wikstrom K, 2008). In particular, radio-ligand binding studies have characterized

TP receptor protein in human kidney (Brown GP, 1999) and human SMCs in culture (Morinelli TA, 1990). However, TP receptor distribution and density in native human cardiovascular tissue has not been determined and it is unknown if receptor density is altered with cardiovascular disease in native human tissue.

Under normal conditions, TP receptor, activated by endothelium-derived vasoactive factors, participates to the control of vascular tone. The inflammatory response is initiated by the adhesion and migration of monocytes into the vascular adventitia promoted by ECs-TP receptor activation (Cayatte AJ, 2000) (Wilson SJ, 2009). Subsequently, the activation of TP receptor on platelets, monocytes/macrophages, ECs, and SMCs plays an important role in regulating platelet activation and vascular tone and in the pathogenesis of thrombosis and vascular inflammation. Indeed, TP receptor that mediates these functions, have a key role and particular relevance in cardiovascular diseases, in which TP receptor expression and TXA_2 are elevated (Katugampola SD, 2001). In particular, activation of the platelet TP receptor triggers platelet activation, secretion, and aggregation, which play important roles in the formation of both hemostatic plugs and pathological thrombi, particularly at high arterial wall shear rate (Roald HE, 1994). A normal response to TP receptor activation supports normal hemostasis. However, increased platelet TP receptor activation is frequently observed in platelet hyper-reactivity states, particularly in patients with type 2 diabetes mellitus (DM) and acute coronary syndromes (ACS) (Eto K, 1998) (Ferroni P, 2004). This may be at least in part due to the increased formation of prostanoids and isoprostanes, which increases the risk of atherothrombosis and promotes vascular inflammation through TP receptor activation (Davì G, 1999) (Szuldrzynski K, 2010). In addition, SMC-TP receptor activation triggers vasoconstriction, increasing the arterial blood pressure, which is one of the major risk factors for atherothrombosis (Capra V, 2014).

4. PHARMACOLOGICAL TREATMENT OF VENOUS THROMBOSIS:

Antithrombotic drugs

The available antithrombotic drugs are effective at reducing arterial thrombosis and venous thrombosis in patients with cardiovascular disease. This should be considered when evaluating -and discussing with the patient- secondary prevention with antithrombotic therapies. Antithrombotic are used to treat a wide variety of conditions that involve arterial or venous thrombosis, including prevention of VTE and long-term prevention of ischemic stroke in patients with atrial fibrillation.

The goal of prophylactic therapy in patients with risk factors for DVT is to prevent both its occurrence and its consequences, PE and the post-phlebitic syndrome. Of the patients who will eventually die of PE, two thirds survive less than 30 minutes after the event, not long enough for most forms of treatment to be effective (Donaldson GA, 1963). Preventing DVT in patients at risk is clearly preferable to treating the condition after it has appeared, a view that is supported by cost-effectiveness analysis (Salzman EW, 1980) (Hull RD, 1982) (Bergqvist D, 1990) (Paiement GD, 1991). The presence of clinical risk factors identifies patients with the most to gain from prophylactic measures (Kakkar VV, 1990), as well as patients who should receive antithrombotic prophylaxis during periods of increased susceptibility, such as post-operatively or post-partum. The blood concentration of certain hemostatic elements (e.g., platelets, AT III, protein C, protein S, vWF, and d-dimer) and other features such as age and euglobulin lysis time have been correlated with the subsequent development of DVT and have provided the basis for a predictive index. However, reliance on laboratory tests to select patients for antithrombotic prophylaxis has not been more accurate and certainly is not more economical than the use of clinically defined risk factors for this purpose.

In the pharmacological treatment for the prevention of DVT, are included a number of antithrombotic agents with important differences in efficacy and in the gravity and frequency of their principal side effect, bleeding.

Anticoagulants drugs are an important class of antithrombotic drugs. Anticoagulants slow down clotting, thereby reducing fibrin formation and preventing clots from forming and growing. Which targets are best for anticoagulant therapy and whether the anticoagulant drugs under development will have better therapeutic windows than the existing drugs are topics of intense debate (Weitz JI, 2007) (Hirsh J, 2007).

The two main classes of anticoagulant drug are vitamin K antagonists and heparins, which target multiple proteases in the coagulation cascade (Fig. 13).

Although sometimes eschewed because of fear of hemorrhagic side effects, the vitamin K antagonists, such as warfarin, remain valuable, especially in high-risk patients (Landefeld CS, 1989), such as those with malignant disease. An advantage of warfarin is that the dose recommended to prevent DVT is adequate to treat an established but undetected thrombus, which is not uncommon in such patients (Hyers TM, 1992) (Hirsh J, 1992). Extending treatment with vitamin K antagonists reduces the risk of recurrence while treatment continues (Prandoni P, 2007) (Boutitie F, 2011) (Agnelli G, 2001) (Kearon C, 1999) (Heit JA, 2000) (Agnelli G, 2003) (Eikelboom JW, 2007), but is associated with an increased risk of bleeding and the inconvenience of laboratory monitoring and dose adjustment (Linkins LA, 2003). Several studies have evaluated the efficacy of new oral anticoagulants, such as dabigatran and rivaroxaban, for the prevention of recurrent VTE as part of initial or extended treatment (Investigators, 2010) (Schulman S, 2009) (Schulman S, 2013) (Agnelli G, 2013). They have been shown to be effective alternatives to warfarin, but still carry a risk of bleeding and are more expensive.

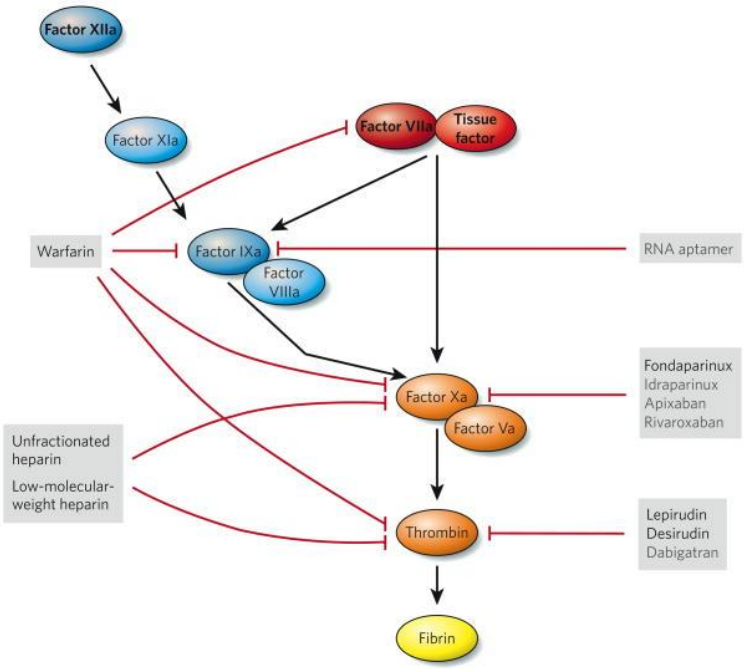
In the initial VTE treatment, is requires therapeutic dosages of unfractionated heparin (intravenous, subcutaneous monitored, or subcutaneous fixed dose) and low-molecular-weight heparin (LMWH) (Kearon C, 2012) (Buller HR, 2012). Small subcutaneous doses of heparin prevent DVT in patients at moderate risk as a result of general, mainly abdominal, surgery (Collins R, 1988) (Clagett GP, 1988). Experiments in animals suggested that heparin species below 7kDa have fewer hemorrhagic side effects than conventional heparin (Carter CJ, 1982), thus providing the impetus for the interest in preparations of LMWH that are less active against platelets (Salzman EW, 1980). Another important side effect of heparin is an allergic thrombocytopenia, “heparin-induced thrombocytopenia”,

which is sometimes complicated by thromboembolism (King DJ, 1984). Boshkov *et al*., in 1993, demonstrated a highly significant association between concomitant cardiovascular complications and the occurrence of an arterial thrombosis and with postoperative venous thrombosis in patients with heparin-induced thrombocytopenia (Boshkov LK, 1993). Heparin-induced thrombocytopenia has recently been reviewed critically (Schmitt BP, 1993). The combination of heparin with a purified concentrate of AT III results more efficient in preventing DVT after orthopedic operations than heparin alone (Francis CW, 1989). In particular, the combination could compensate for the decline in plasma concentrations of AT III that has been observed after hip arthroplasty and other major operations. Data from the RECORD (REgulation of Coagulation in ORthopedic Surgery to Prevent Deep Venous Thrombosis and Pulmonary Embolism) clinical trial show that the anticoagulant "Rivaroxaban" holds promise. When targeting factors in the coagulation cascade, it is important to consider that the sequential activation of factors by proteolytic cleavage results in an amplification of each step. Despite the possibility that the risk of bleeding is lower after inhibition of components of the intrinsic pathway than of the common coagulation pathway, most pharmaceutical companies have chosen to focus on inhibition of FXa (FXa, a component of the coagulation cascade) (Weitz JI, 2002) (Turpie A, 2007). The inhibition of the intrinsic pathway is expected to have less impact on ongoing thrombosis than would inhibition of the downstream proteases. Rivaroxaban is an orally available inhibitor of activated FX, and it reduced the incidence of VTE events in patients undergoing total hip replacement of 70% without an increase in bleeding. The reduction is from 3.7% in those administered a LMWH (enoxaparin) to 1.1% (Eriksson B, 2007). Although this, LMWH is effective and easily administered, making it the preferred anticoagulant irrespective of VTE treatment in the outpatient or inpatient setting.

Since the usual venous thrombus is a fibrin-rich clot formed within the circulation in dead waters, recirculating eddies, valve sinuses, and other areas of relative stasis (Sevitt S, 1974) it is not surprising that inhibition of thrombin generation and fibrin formation can prevent DVT. Platelets might also be involved, especially if there is direct trauma to the vein (Stamatakis JD, 1977). The different role played by platelets and fibrin in arterial and venous thrombosis contributes to the concept of these diseases as distinct entities.

However, venous thrombosis and PE remain an important cause of morbidity and mortality both in surgical patients and in immobilized medical patients (Dalen JE, 1986) (Thromboembolic Risk Factors (THRIFT) Consensus Group, 1992) (European Consesus Statement, 1992). Various thromboprophylactic treatments have therefore been devised to prevent or limit VTE (Statement, European Consensus, 1992) (Thromboembolic Risk Factors (THRIFT) Consensus Group, 1992) (Consensus Development Conference Report, 1986) (Kakkar V, 1990) (Clagett GP, 1988).

In particular the treatment with antiplatelet drugs was been reconsidered.



Mackman N., Nature 2010

Fig 13: Targets of anticoagulant drugs

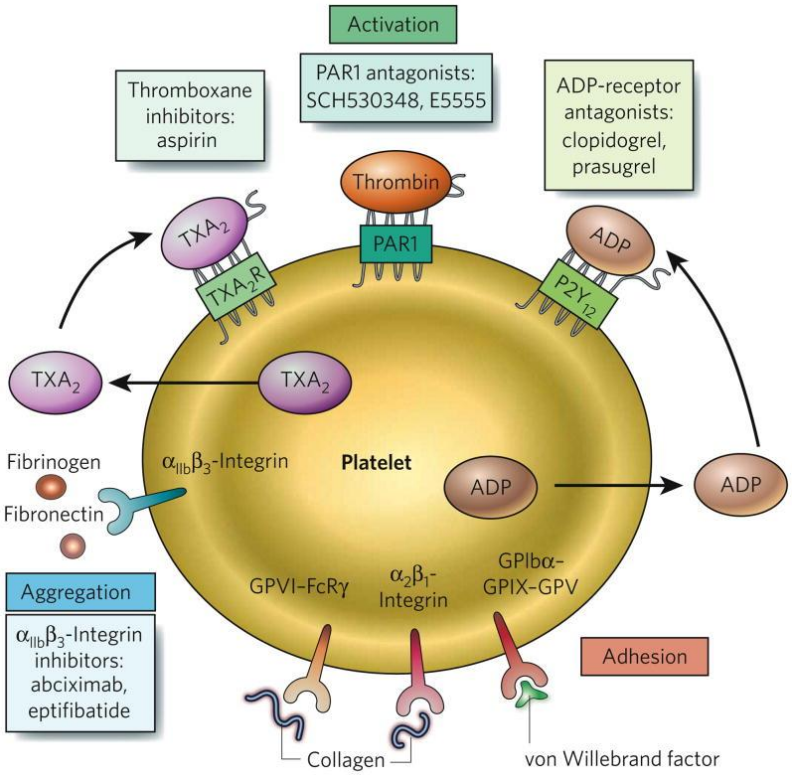
Antiplatelet drugs prevent platelets from clumping and prevent clots from forming and growing. Likewise, it is still stated that antiplatelet agents are more effective in arterial thrombosis and anticoagulants in venous thrombosis. In broad terms, arterial thrombosis is treated with drugs that target platelets (Fig. 14), and venous thrombosis is normally treated with drugs that target proteins of the coagulation cascade. Indeed, in inherited venous thrombotic disease, there can be increased activity or abundance of proteins that promote coagulation and/or decreased abundance of proteins that inhibit coagulation (i.e. Factor V Leiden, Haemophilia A and B, prothrombin mutations, etc).

Currently, the role of antiplatelet agents, in particular of COX-inhibitors, in venous thrombus initiation or propagation, is still discussed. Recent studies highlight a possible link between arterial diseases and venous thrombosis (Prandoni P, 2003): new perspectives exist for the role of antiplatelet drugs in the management of VTE.

It had previously been hypothesized that antiplatelet therapy did not influence VTE, and many surgeons and physicians do not use it routinely for thromboprophylaxis, even for patients who are at substantial risk of DVT or PE. Some previously direct randomized comparisons were available between the effects of aspirin and another antiplatelet regimen, or between one dose of aspirin and another, on DVT or PE, but these trials were generally too small to be reliable. In these direct comparisons, aspirin plus dipyridamole appeared to be more effective than aspirin alone at preventing DVT.

An overview of randomized trials of perioperative subcutaneous heparin (Collins R, 1988) showed that among surgical patients such treatment can roughly halve the risk not only of DVT but, more importantly, of PE. Subcutaneous heparin is now widely recommended for surgical or medical patients at high risk of venous occlusion, but antiplatelet therapy still is not (Thromboembolic Risk Factors (THRIFT) Consensus Group, 1992) (European Consesus Statement, 1992) (Consensus Development Conference Report, 1986) (Clagett GP, 1988) (Goldhaber SZ, 1992).

The “Collaborative overview of randomized trials of antiplatelet therapy” (Collaborative overview of randomised trials of antiplatelet therapy – III, 1994) provides an overview of the results of the randomized trials of antiplatelet therapy in which DVT was to be assessed systematically. Most such trials entailed a few weeks of perioperative treatment in general or orthopaedic surgery, but some entailed a few months of treatment for long stay medical patients at high risk of VTE (for example, those immobilized by a stroke or myocardial infarction or who suffer recurrent venous thrombosis). This study indicates that antiplatelet therapy - either alone or, for greater effect, in addition to other proved forms of thromboprophylaxis (such as subcutaneous heparin) - should be considered to reduce DVT and PE.



Mackman N., Nature 2010

Fig 14: Targets of antiplatelet drugs

I. 5. COX inhibitors in VTE

In the past decade, more progress has been made in understanding the role of cyclooxygenase enzymes (COXs) in biology and disease than at any other time in history. Two cyclooxygenase isoforms have been identified and are referred to as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Under many circumstances the COX-1 enzyme is produced constitutively (i.e., gastric mucosa) whereas COX-2 is inducible (i.e., sites of inflammation). Nonsteroidal anti-inflammatory drugs (NSAIDs) are active on both enzymes.

COX-1 inhibitors: Aspirin (acetylsalicylic acid or ASA) is probably the best studied NSAIDs. It is used by millions of people worldwide to reduce, in particular, pain and inflammation. On a molecular level, aspirin exert its pharmacologic effect by inhibiting the COXs enzymes. It inhibits the COX activity of prostaglandin (PG) G/H synthases (PGHS), by acetylating serine 529 in COX-1 (Cannon CP, 2012) (Cheng Y, 2002). Aspirin first binds to arginine 120 and blocks the access of arachidonic acid (AA) through a narrow hydrophobic channel to the catalytic site at tyrosine 385, leading to suppression of the generation of the precursor of thromboxane A2 (TXA2), that is PGH2 (Bombardier C, 2000). Aspirin inhibits COX-2 by acetylating serine 516; however, the affinity of COX-2 is 170 times lower than that of COX-1 (Patrono C, 2004).

Aspirin is actually used in the treatment of acute myocardial infarction (MI) and in the secondary prevention of cardiovascular disease (CVD) among both men and women (Antithrombotic Trial Collaboration, 2002). To date, five randomized trials have evaluated aspirin in the primary prevention of CVD (Physician Health Group, 1989) (No authors, 1998) (Hansson L, 1998). These trials indicate that, as compared with placebo, aspirin therapy was associated with a significant reduction in the risk of MI (32%), but the data on the risk of stroke and death from CVD remain inconclusive (Eidelmen RS, 2003). Experts believe that the protective benefits of aspirin for cardiovascular disease and cancer occur at doses of 75 to 100 milligrams a day. The same dose of 100 milligrams is use in patients that were followed up for recurrent VTE, arterial ischemic events (MI and stroke), bleeding, once daily. Indeed, patients with unprovoked VTE remain at high risk of recurrence following discontinuation of vitamin K antagonist therapy, with about a 10%

risk within the first year and 5% per year thereafter (Prandoni P, 2007) (Boutitie F, 2011) (Agnelli G, 2001) (Kearon C, 1999) (Heit JA, 2000) (Agnelli G, 2003). Aspirin has been studied extensively as prophylaxis against DVT, and its use has given rise to intense controversy (Clagett G, 1992) (Patrono C, 1994). In a large meta-analysis (Antiplatelet Trialist Collaboration, 1994), prophylactic aspirin reduced both proximal and distal DVT by 30 to 40 percent and PE by 60 percent in patients undergoing general surgical, orthopedic, and medical procedures. The physician's familiarity with the drug is an asset, but aspirin appears to provide less protection than can be achieved safely with more modern programs of anticoagulation.

Subsequently, aspirin treatment has been evaluated in the WARFASA (The Warfarin and Aspirin trial) and ASPIRE (Aspirin to prevent recurrent venous thromboembolism) trials (Becattini C, 2012) (Brighton TA, 2012), where it reduced the risk of the second event of VTE. The ASPIRE and WARFASA studies were designed to examine the efficacy and safety of low-dose aspirin in the extended treatment of VTE. Eligible patients in these trials were those with a first episode of unprovoked VTE, defined as proximal DVT or PE, who had completed initial treatment with heparin and warfarin or an equivalent anticoagulant regimen. During the follow-up period, VTE occurred in 18.4% patients assigned to placebo 13.1% assigned to aspirin. This was a 32% relative reduction in VTE. Aspirin reduced the rate of recurrent DVT without symptomatic PE by 34% and PE with or without symptomatic DVT by 34% (Simes J, 2015). The rate of bleeding was low and did not differ significantly between the aspirin and placebo groups. The net clinical benefit (symptomatic VTE, MI, stroke, all-cause mortality and major bleeding) was improved with aspirin by 33% (Simes J, 2015).

COX-2 inhibitors: The inhibition by NSAIDs, however, can be harmful when the reduced synthesis of PGs causes a deterioration of the normal function of the gastrointestinal mucosa, with formation of more or less serious injuries. Therefore, it was tried to produce anti-inflammatory drugs that would act only on COX-2, in order to block the pain and inflammation, leaving intact the PGs in the stomach, useful for its protection. It seems obvious, that cardiovascular safety and gastrointestinal risks are undoubtedly connected

by an interaction with PGs (Chen Y, 2002), as a result of the varying mechanisms of action of different COX-inhibiting drugs.

Thus were born the so-called COX2-selective drugs (which block the COX-2 but not the COX-1), known as **COXIBs**. The COXIBs, introduced into clinical practice in United States in late 1998, were considered such as a pharmacological alternative approach to NSAIDs. They were developed as NSAIDs with an improved gastrointestinal side effect profile (Laine L, 2002) (Hunt RH, 2003). COX-2 inhibitors not only lacked the antiplatelet effects of aspirin; by inhibiting the production of prostacyclin (also called prostaglandin I2 or PGI_2), they were also disable one of the primary defenses of the endothelium against platelet aggregation, hypertension, and atherosclerosis. COX-2 inhibitors also promoted an imbalance in favor of vasoconstriction. The first COXIB put on the market was celecoxib, commercially CELEBREX (all drugs in this category end up precisely with the word COXIB). A short distance away was the rofecoxib (VIOXX commercially) and gradually others such as valdecoxib and etoricoxib (ARCOXIA commercially).

However, approximately six years later the COXIBs were approved for use in the US, the results of three randomized, placebo-controlled trials provided new evidence about the cardiovascular risks of rofecoxib, celecoxib, and valdecoxib (Bresalier RS, 2005) (Solomon SD, 2005) (Nussmeier NA, 2005). These results have prompted the need for subsequent investigations, such as the APPROVe (Adenomatous Polyp Prevention on Vioxx) trial (Bresalier RS, 2005), which have resulted in the withdrawal of rofecoxib from the global market in September 2004. Indeed, APPROVe trial includes patients with a history of colorectal adenomas, where treatment was stopped early because rofecoxib doubled the risk of major cardiovascular events (Psaty BM, 2005). These findings confirmed the increased risk of MI previously seen in 1999 in the VIGOR (Vioxx Gastrointestinal Outcomes Research) trial (Bombardier C, 2000): in November 1999, 79 patients out of 4,000 taking Vioxx had serious heart problems or have died, compared with 41 patients taking naproxen, a NSAIDs. These results, prompted scientists to review the cardiovascular-safety results of a similar trial, the APC (Adenoma Prevention with Celecoxib) study (Solomon SD, 2005). At either 200 or 400 mg twice a day, celecoxib in the APC trial was associated with a tripling of the risk of cardiovascular events. Moreover,

the effect of COX-2 inhibitor reported in some trial (Nusmeier NA, 2005) showed that the short-term use of valdecoxib and its prodrug parecoxib was associated with increased cardiovascular risk in patients undergoing coronary bypass surgery. The APC trials, taken together with data from the APPROVe trial (McAdam BF, 1999) and various observational studies (Villalba ML, 2005) (Tergum SL, 2001), suggested that cardiovascular risks seen with celecoxib may also extend to other NSAIDs and COXIBs (e.g., etoricoxib and diclofenac). Indeed, not all studies had found just selective COX-2 inhibitors to be associated with greater cardiovascular risk (Solomon SD, 2004) (Arber N, 2006), which has resulted in the current controversy around the safety profile and application of COX-targeting drugs for the treatment of inflammatory conditions. CLASS trial (Celecoxib Long-term Arthritis Safety Study) involved a comparison of celecoxib with two traditional NSAIDs, diclofenac and ibuprofen. A year later its publication (Silverstein FE, 2000), the FDA posted the data on its website in the process of considering an appeal for labeling that would distinguish celebrex from other traditional NSAIDs.

The recently raised COX-2-dependent cardiovascular effects seem to depend on a number of variables such as dosing, half-life and dosing intervals.

Few data are available on the clinical association between COXIBs and VTE. Schmidt *et al*. (Schmidt M, 2011) hypothesized that prothrombotic drugs such as non-aspirin NSAIDs increase the risk of VTE. They conducted their study on population that take on non-aspirin NSAIDs (ibuprofen, naproxen, ketoprofen, dexibuprofen, piroxicam, tolfenamic acid, and indomethacin), “older” COXIBs, introduced into clinical practice in 1998, and were developed as NSAIDs with an improved gastrointestinal side effect profile (Laine L, 2002), and “newer” COXIBs (Capone ML, 2007). They found that the use of non-selective NSAIDs or COXIBs was associated with an increased risk of VTE. This study was one of the first to examine the association between COXIBs and VTE: case reports had previously associated celecoxib with DVT (Chan AL, 2005) and valdecoxib with PE (Westgate EJ, 2005). In a murine model, rofecoxib has also been associated with VTE (Nagai N, 2008) and the risk increase was related to both DVT and PE (Huerta C, 2007). In their study, they found an association for both new and long-term use of non-selective NSAIDs, older COXIBs, and newer COXIBs (Huerta C, 2007).

II. *Aim of the Study*

Venous thrombosis is one of the major cause of mortality and morbidity in developed countries, more than one per 1000 persons each year in Western populations (Heit JA*,* 2004*) (*Kyrle PA*,* 2005*).* Venous thrombosis occurs mainly in the deep vessels of the lower limbs and is associated with serious complications such as pulmonary embolism (PE), deep vein thrombosis (DVT) and post-thrombotic syndrome (PTS) (Heit JA*, 2006)* (Kyrle PA*,* 2005).

A venous thrombus occurs when blood clots within the vein. Arterial thrombi usually occur when endothelium damaged lead to the activation of the coagulation cascade (López JA, 2009), in contrast, most of venous thrombi occur where vessel wall is intact (Martinelli I, 2010), but blood flow and shear rates are low. In these conditions, tissue factor (TF) triggers the clotting cascade, leading to fibrin generation/deposition (Bovill EG, 2011) that entraps red blood cells and leukocytes (Martinelli I, 2010).

Classical pharmacological treatment of VTE are anticoagulant drugs and, recently, thrombolytic agents. However, the use of these drugs has several limitations: wide variability dose/response relationship between patients and in the same patient, multiple interactions with other drugs/foods, variability of daily doses, need of periodic withdrawals of blood during therapy, problems of overdosing (Konstantinides S, 2014) (Klok FA, 2014) (Levy JH, 2014).

Anticoagulant therapy is the mainstay for the treatment of VTE, but recent studies suggested that antiplatelet therapy provided some protection against VTE. Indeed, it has been suggested that Aspirin may be useful in the prevention of VTE (Antiplatelet Trialists' Collaboration, 1994*)*. Aspirin inhibits the COX-activity of prostaglandin (PG) G/H synthases (PGHS), acetylating serine 529 in COX-1 (Patrono C*,* 2009) (Patrono C*, 2004*), with consequent suppression of the generation of prostanoids including thromboxane A_2 (TXA_2), that is PGH_2 (Patrono C*,* 2004*).* Aspirin inhibits also the isoform COX-2; however, the affinity of COX-2 is 170 times lower than that of COX-1 (Patrono C*, 2004).*

Specific cyclooxygenase-2-selective inhibitors (COXIBs) aimed to directly targets COX-2, enzyme responsible of inflammation and pain (Pedersen AK, 1984), were developed. Few

data are available about the effect of COX-2 inhibitors in venous thrombosis, nevertheless their clinical use seem associated with increased risk of venous thrombosis (Armstrong PC, 2011).

The aim of this study was to highlight the different implication of COX-1 and COX-2 enzymes in venous thrombosis and to identify the molecular mechanisms responsible for this effect, in order to developed new therapeutic strategies to prevent venous thrombosis. In particular, we focused on the impact of inhibition of COX-pathway on leukocyte activation, important regulators of formation and propagation of venous thrombus.

To reach these goals, venous thrombosis was induced in WT mice treated with Aspirin and in COX-2 knockout (COX-2KO) by inferior vena cava (IVC) ligation and thrombi characterized 48 hours after surgery. Remarkable, human venous thrombi are very similar in terms of composition and structure to those obtained with the experimental muse model here used.

III.1 *Results*

III. 1 Effect of deletion of COX-1 enzyme in a venous thrombosis mouse model

III. 1.1 ASA administration reduces urinary levels of Thromboxane A_2 in IVC mouse model

To investigate the effects of the inhibition of cyclooxygenase-1 (COX-1) enzyme on venous thrombosis, wild type (WT) mice were treated with ASA (Aspirin, Acetylsalicylic acid, 3mg/Kg) or with vehicle (ethanol) once a day for 3 days. After 24 hours of treatment, mice were subjected to ligation of inferior vena cava (IVC) to induce venous thrombosis. Urine has been collected at baseline and once every day throughout the study to measure 2,3-dinor Thromboxane B_2 (TXB-M), stable metabolite of Thromboxane A_2 (TXA_2). In WT control mice, IVC ligation, markedly affected the levels of urinary excretion of TXB-M (Fig. 1A). In particular, 24 hours post ligation, urinary TXB-M levels were significantly higher in control operated mice (16.30 ± 2.27 ng/mg creatinine) compare to the levels of TXB-M before IVC ligation and compare to baseline (8.14 ± 0.84 ng/mg creatinine and 6.0 ± 1.16 ng/mg creatinine, respectively). TXB-M levels slightly, but not significantly, decreased at 48 hours after stenosis in control mice (12.67 ± 0.97 ng/mg creatinine, $p<0.05$) (Fig. 1A). Administration of ASA, as expected (Lopez LR, 2014), reduced urinary TXB-M levels in ASA-treated mice compared to vehicle-treated mice (5.40 ± 0.35 ng/mg creatinine), and prevented the excretion of TXB-M induced by IVC ligation both at 24 and 48 hours (6.96 ± 0.45 ng/mg creatinine and 6.98 ± 0.73 ng/mg creatinine, respectively; Fig. 1A).

III. 1.2 Thrombus weight results decrease after ASA and SQ 29,548 treatment

Fourth-eight hours after IVC ligation, mice were sacrificed, and thrombi were dissected and measured. In ASA treated-mice, thrombus weight was significantly reduced by about 28% compare to control mice (control: 8.59±0.63 mg/thrombus; ASA: 6.17±0.71 mg/thrombus) (Fig. 1B). Moreover, a positive correlation between thrombus size and TXB-M levels in urine was found at 48 hours after IVC ligation ($Y = 0.9301*X + 3.459$; r^2: 0.5013; $p<0.001$) (Fig. 1C).

To investigate the involvement of TXB_2/TP pathway in this contest, another group of mice was treated with SQ 29,548 (1 mg/Kg), a selective thromboxane receptor antagonist, or with vehicle. SQ 29,548 reduced thrombus weight compare to control mice (control: 11.5±1.07 mg/thrombus; SQ 29,548: 7.06±0.81 mg/thrombus). In particular, thrombus size was reduced by about 38% in SQ 29,548 treated-mice (Fig. 1D).

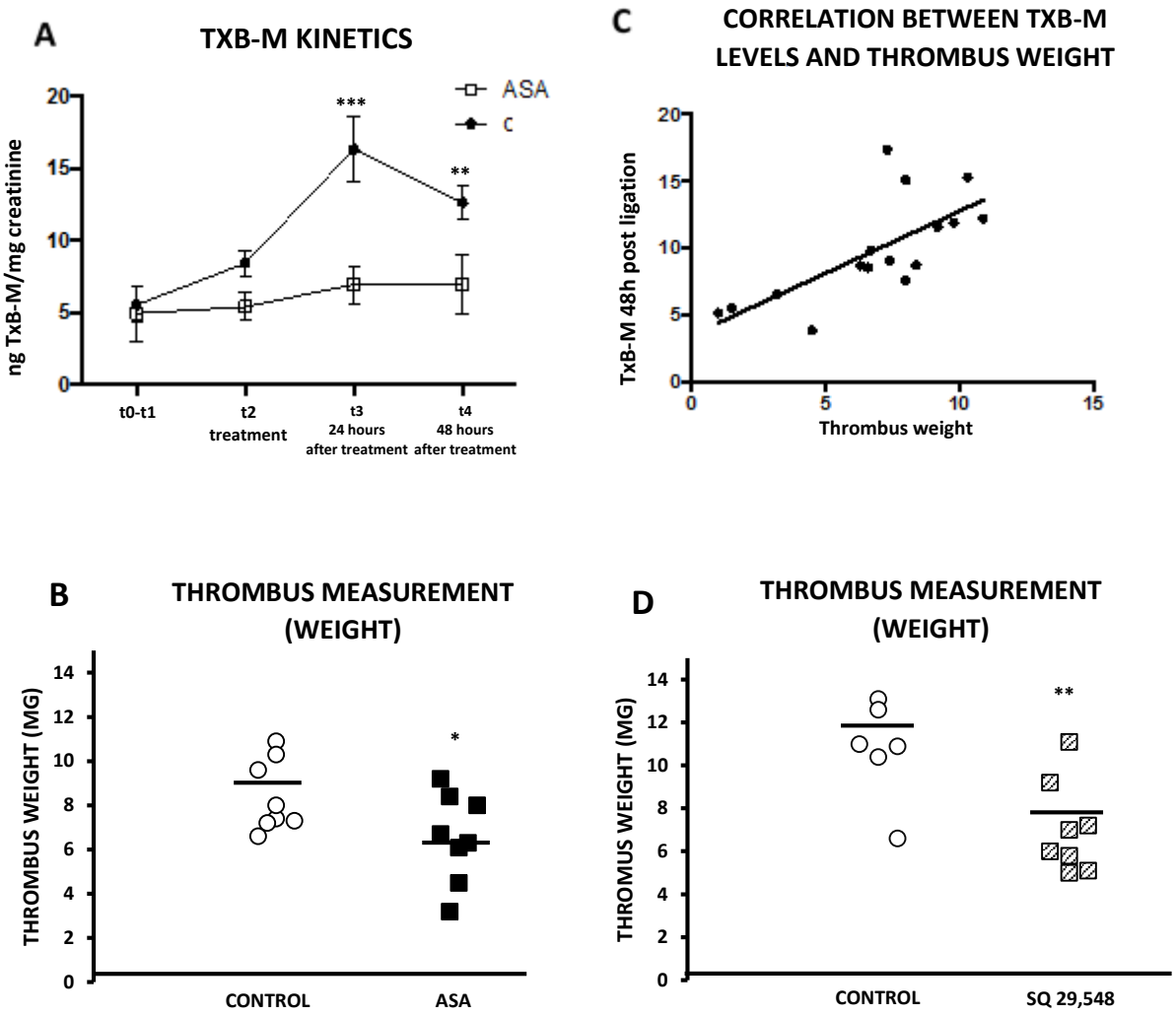


Fig. 1: Effect of ASA and SQ 29,548 on urinary levels of 2,3-dinor Thromboxane B_2 (TXB-M) and thrombus size after IVC ligation. Mice were treated with vehicle, ASA **(A-C)** or with SQ 29,548 **(D)** before IVC ligation. **(A)** Kinetics of urinary TXB-M levels and **(B)** thrombus weight 48 hours after ligation of IVC in ASA-treated mice. **(C)** Correlation between urinary TXB-M levels and thrombus weight at 48 hours after ligation of IVC. **(D)** Thrombus weight at 48 hours after ligation of IVC in SQ 29,548-treated mice (* $p<0.05$; ** $p<0.01$; *** $p<0.001$ versus control; n= 8 mice/group). Data shown are mean ± SEM.

III. 1.3 ASA and SQ 29,548 treatment affect thrombus composition

The effects of ASA and SQ 29,548 administration on thrombus composition were examined using histological analysis (Fig. 2). In particular, Picro-Mallory staining on progressive section of thrombi was used to visualize fibrin content within the thrombus. Treatment with ASA or with SQ 29,548 significantly decrease of total fibrin infiltration compare to control mice (control: 40.50 ± 5.18% fibrin/thrombus; ASA: 32.87 ± 2.72% fibrin/thrombus, $p<0.5$; SQ 29,548: 30.38 ± 2.97% fibrin/thrombus, $p<0.5$) (Fig. 2A, B).

Comparable results were obtained using Martius Scarlet and Blue staining, that recognize three different stages of fibrin maturation: yellow-orange for fresh fibrin, red for mature fibrin and blue for old fibrin. In particular, thrombi from control mice showed 35.30 ± 1.01% of old fibrin infiltration (blue), whereas the percentage of old fibrin decrease in thrombi from ASA and SQ 29,548-treated mice at 20.91 ± 1 .23% and 29.15 ± 1.62% ($p<0.001$; $p<0.01$ respectively) (Fig. 2C, D), respectively.

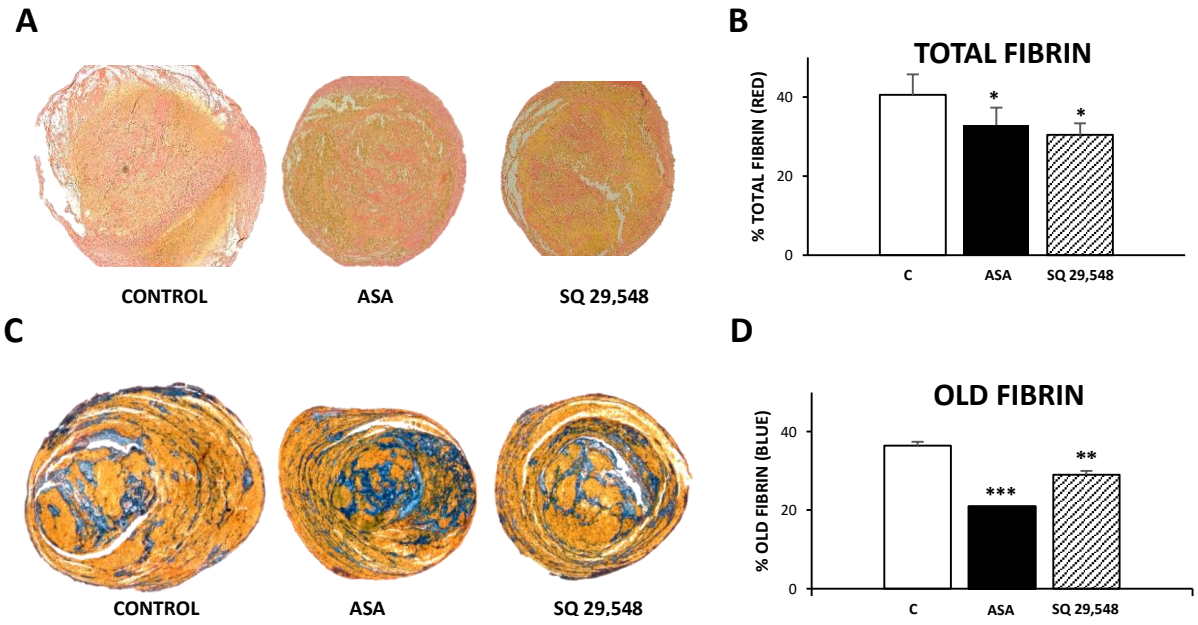


Fig. 2: Fibrin content in thrombi at 48 hours after ligation of IVC. Representative images of **(A) Picro-Mallory staining** and **(C) Martius Scarlet Blue staining (MSB). (B), (D):** representative sections of thrombi from control, ASA and SQ 29,548-treated mice; **(B)** percentage of total fibrin infiltration (red stain); **(D)** percentage of old fibrin within the thrombus (*$p<0.05$; **<p0.01; ***$p<0.001$ versus control; n= 8 mice/group). Data shown are mean ± SEM.

Then, the number of nucleated cells infiltrated in the thrombi was analyzed. Hematoxylin/eosin (H/E) staining showed that both ASA and SQ 29,548 treatment decreased by about 20% the number of nucleated cells infiltrated within the thrombi compared to control animals group (control: 167.7±2.38 cells/field, ASA: 137.8±9.9 cells/field, SQ 29,548: 142.98±5.17 cells/field. Fig. 3A). Immunohistochemical analyses were performed to identify the type of cells present in the thrombi. The cells recruited in the venous thrombi of control mice were almost exclusively represented by leukocytes. Indeed, about 99% were CD45 positive ($CD45^+$) cells, as previously showed by von Brühl et al. (von Brühl ML, 2012). In addition, as expected, neutrophils ($Lys6G^+$ cells) were about 70% of total leukocytes, while monocytes ($F4/80^+$ cells) were the remaining cells (Fig. 3C, D). ASA and SQ 29,548 treatment significantly decreased infiltration of $CD45^+$ cells compare to control (control: 145.80±1.81 $CD45^+$ cells/field, ASA: 111.28±3.36 $CD45^+$ cells/field, SQ 29,548: 105.18±1.05 $CD45^+$ cells/field) (Fig. 3B). In particular, both the number of $Lys6G^+$ cells and of $F4/80^+$ cells in thrombi were significantly reduced by both pharmacological treatment (control: 111.34±5.02 $Lys6G^+$ cells/field, ASA: 108.4±5.18 $Lys6G^+$ cells/field, SQ 29,548: 77.64±0.60 $Lys6G^+$ cells/field; control: 42.8±1.28 $F4/80^+$ cells/field, ASA: 25.5±1.78 $F4/80^+$ cells/field, SQ 29,548: 27.5±1.44 $F4/80^+$ cells/field) (Fig. 3C,D).

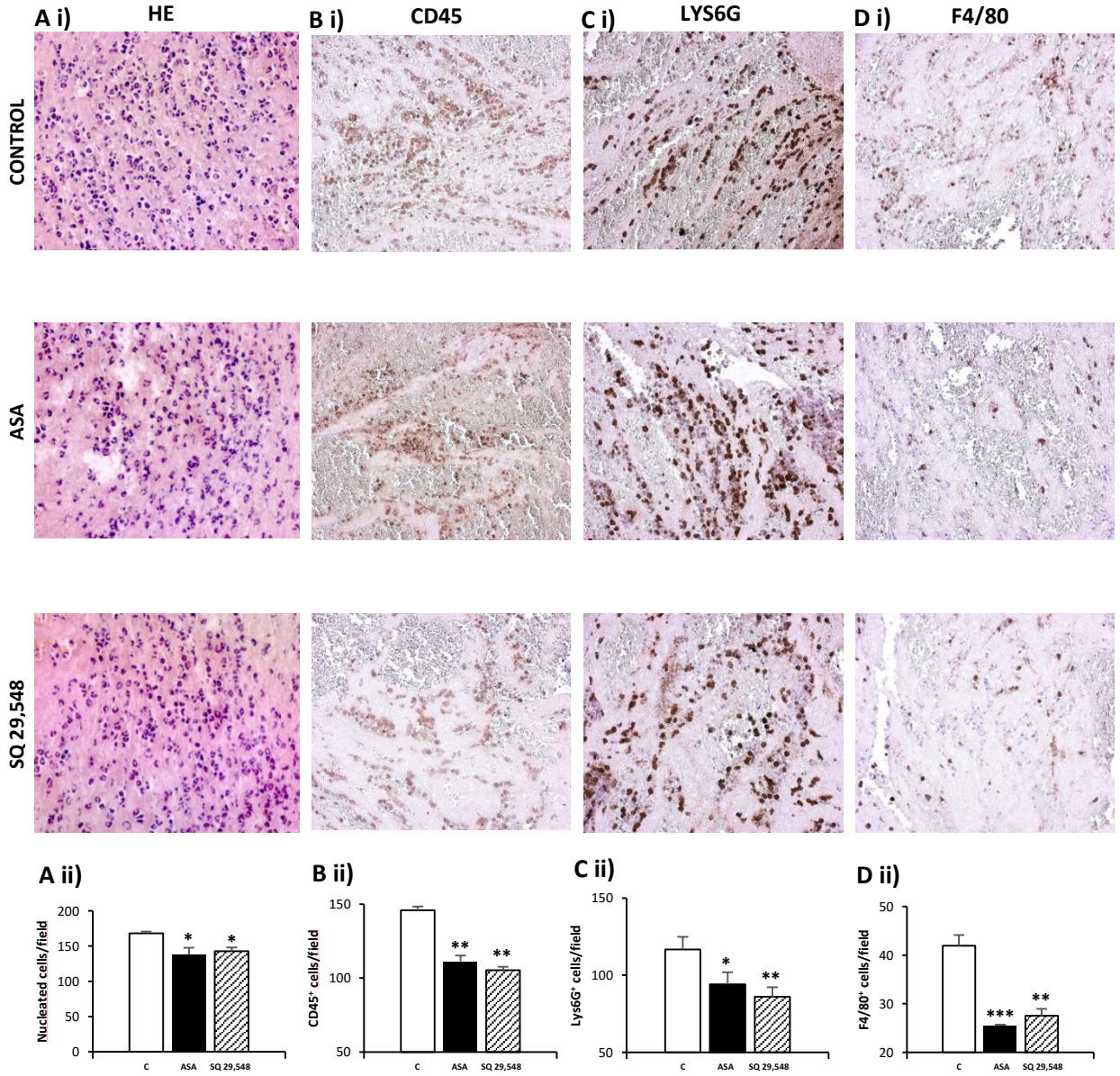
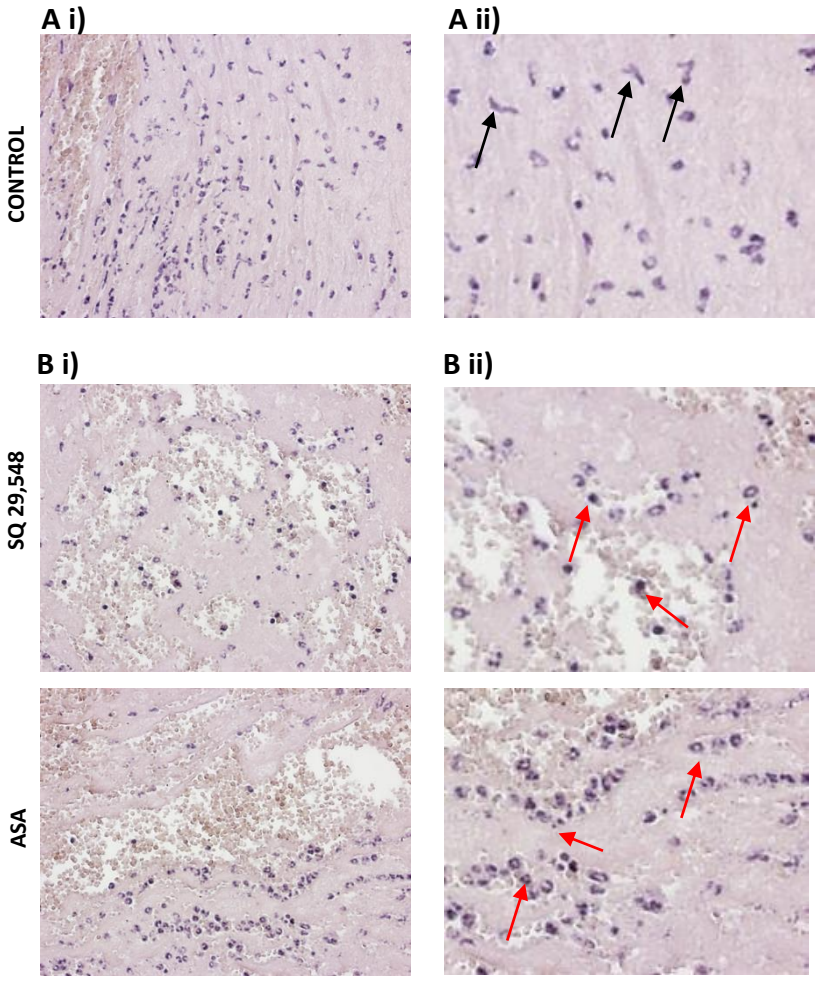


Fig. 3: Characterization of venous thrombi isolated at 48 hours after IVC ligation showed different cellular infiltration in control mice compare to ASA or SQ 29,548-treated mice. Nucleated cells infiltration results significantly decrease after ASA and SQ 29,548 administration. Leukocytes resulted affect by ASA and SQ 29,548 administration, and in particular, neutrophils (Lys6G positive cells) and monocytes/macrophages (F4/80 positive cells) results decrease after treatment. **(A i)** hematoxylin/eosin staining and quantification of panels **(A ii)** expressed as nucleated cells per field. Immunohistological analysis of CD45 **(B i)**, Lys6G **(C i)**, F4/80 **(D i)**. **(A i)**, **(B i)**, **(C i)** and **(D i)** expressed as positive cells per field (20X magnification). **(A ii), (B ii), (C ii), (D ii):** quantification of panels (*p<0.05; **p<0.01; ***p<0.001 versus control; n=6 mice/group). Data shown are mean ± SEM.

III. 1.3.1 ASA and SQ 29,548 reduced NETs formation *in vivo*

Recently, the emerging role of Neutrophil Extracellular Traps (NETs) in thrombosis was shown. NETs bind erythrocytes and platelets (Fuchs TA et al., 2010) and promote coagulation by degradation of Tissue Factor Pathway Inhibitor (TFPI) (Massberg S et al., 2010). Hematoxylin/eosin staining on sections of control thrombi developed after 48 hours of stenosis showed a dotted nuclear staining and a diffuse extracellular DNA (hematoxylin staining; Fig. 4A; black arrow). In contrast, both ASA and SQ 29,548 thrombi (Fig. 4B; red arrow) had dense nuclei. In addition, lower levels of citrullinated histone H3 (H3Cit), a NET marker that recognized residues 2, 8 and 17, were found thrombi from ASA and SQ 29,548-treated mice compared to control (Fig. 4C).



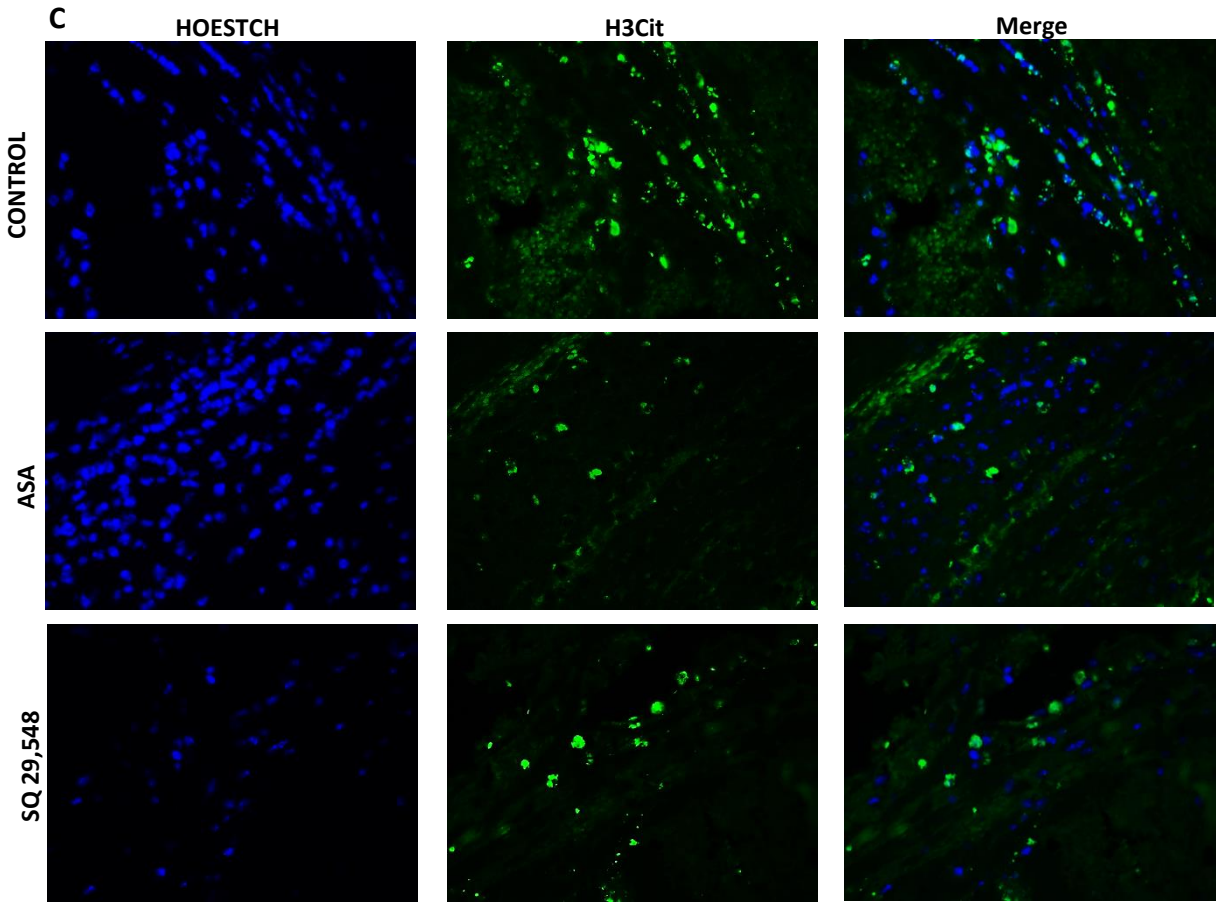


Fig. 4: Effect of ASA and SQ 29,548 treatment on NETs formation in 48 hours venous thrombi. (A i) Hematoxylin/eosin (20X magnification), **(A ii)** (40X magnification) showed normal NETs formation in control venous thrombi. **(B i)** (20X magnification), **(B ii)** (40X magnification) demonstrated a significantly inhibition of NETs formation after ASA and SQ 29,548 treatment. **(C)**: Immunofluorescence staining of hypercitrullinated histone H3 (H3Cit, green) and Hoechst (nuclear marker, blue), as NETs marker confirmed these data (40X magnification).

III. 1.3.2 ASA and SQ 29,548 affects Tissue Factor activity and expression

Recent studies suggested that Thromboxane A_2 (TXA_2) could be added into the list of stimuli that induce TF expression in different type of cells, including monocytes (Eligini S, 2006; Del Turco S, 2014).

IVC ligation increased TF-positive MPs (MP-TF) (Figure 5A), as previously showed in a different animal model (Himber J, 2003). Administration of both ASA and SQ 29,548 markedly reduced MP-TF activity compare to control group, bringing it back to basal level (Fig. 5A). Similarly, TF immunohistochemical staining on section of thrombi showed that pharmacological treatments decreased the number of TF-positive cells (control: 106.59±6.95 TF^+ cells/field; ASA: 76.03±3.71 TF^+ cells/field; SQ 29,548: 90.76±5.19 TF^+ cells/field) (Fig. 5B).

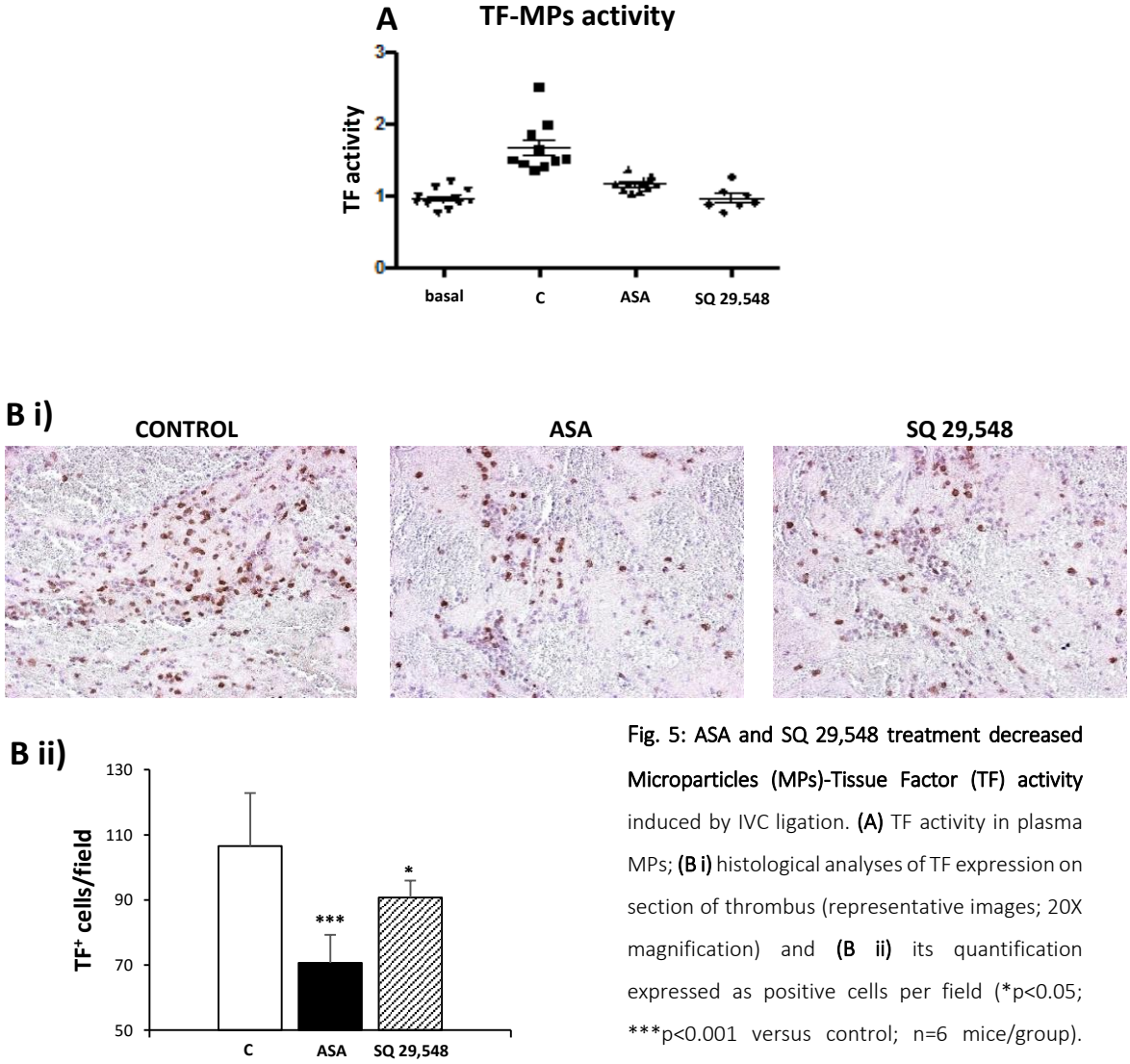


Fig. 5: ASA and SQ 29,548 treatment decreased Microparticles (MPs)-Tissue Factor (TF) activity induced by IVC ligation. **(A)** TF activity in plasma MPs; **(B i)** histological analyses of TF expression on section of thrombus (representative images; 20X magnification) and **(B ii)** its quantification expressed as positive cells per field (*p<0.05; ***p<0.001 versus control; n=6 mice/group). Data shown are mean ± SEM.

III. 1.4 Key role of TP-non-platelet-receptor in venous thrombosis

To investigate the possible redundant role of TP-receptor activation on platelet in a venous thrombosis contest, experiments of platelets transfusion were performed. WT mice were selectively depleted of platelets and then treated with SQ 29,548 or with vehicle. Twenty-four hours later, platelet-deficient mice were transfused with fresh platelets isolated from untreated WT mice, and immediately underwent to ligation of IVC to induce venous thrombosis. Forty-height hours after ligation mice were sacrificed and thrombi were collected. Remarkably, despite the infusion of fresh platelets, SQ 29,548 treatment significantly reduced thrombus weight compare to control (control: 8.4±0.66 mg/thrombus; SQ 29,548: 1.58±1.78 mg/thrombus).

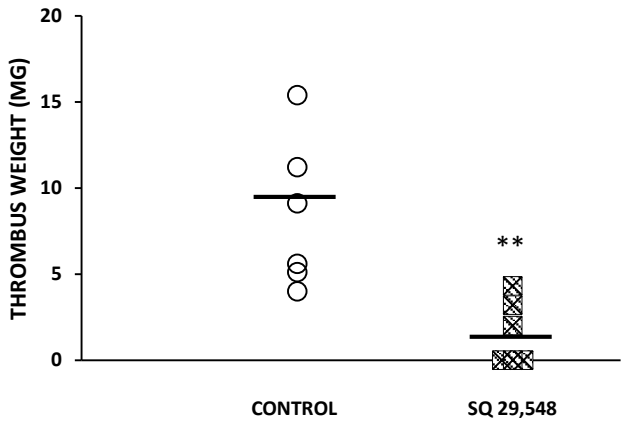


Fig. 6: Reinfusion of new platelets in WT mice with a depletion of platelets and treated with SQ 29,548 caused a reduction in thrombus weight at 48 hours after IVC ligation compare to control. (**p<0.01 versus control; n=6 mice/group). Data shown are mean ± SEM.

III. 1.5 Inhibition of TXA_2/TP receptor pathway prevents NETs formation in peritoneal neutrophils stimulated by supernatant of activated platelets (APS) *in vitro*

To assess the ability of TXA_2 to induce NETs formation, neutrophils were treated for 2 hours with medium alone, with medium plus collagen, with supernatant of platelet incubated with or without collagen (stimulated – APS- and un-stimulated platelets –N/APS-, respectively) under stirring for 10 minutes, or with I-BOP (2μM). When incubated in medium alone, medium plus collagen or with supernatant of stirred platelets, neutrophils did not spontaneously extrude extracellular traps, as showed by the absence of staining for extracellular DNA and Histone H3 (H3Cit) (Fig. 7A; Fig. 8). In contrast, supernatant of stimulated platelets (APS) or I-BOP induced extracellular traps formation, as showed by release of Hoechst-positive strand-like material and by staining for H3Cit in neutrophils (Fig. 7B, C; Fig. 8). Interesting, incubation of neutrophils with SQ 29,548 (10μM) significantly decreased NETs formation induced by both APS and I-BOP (Fig.7D; Fig. 8).

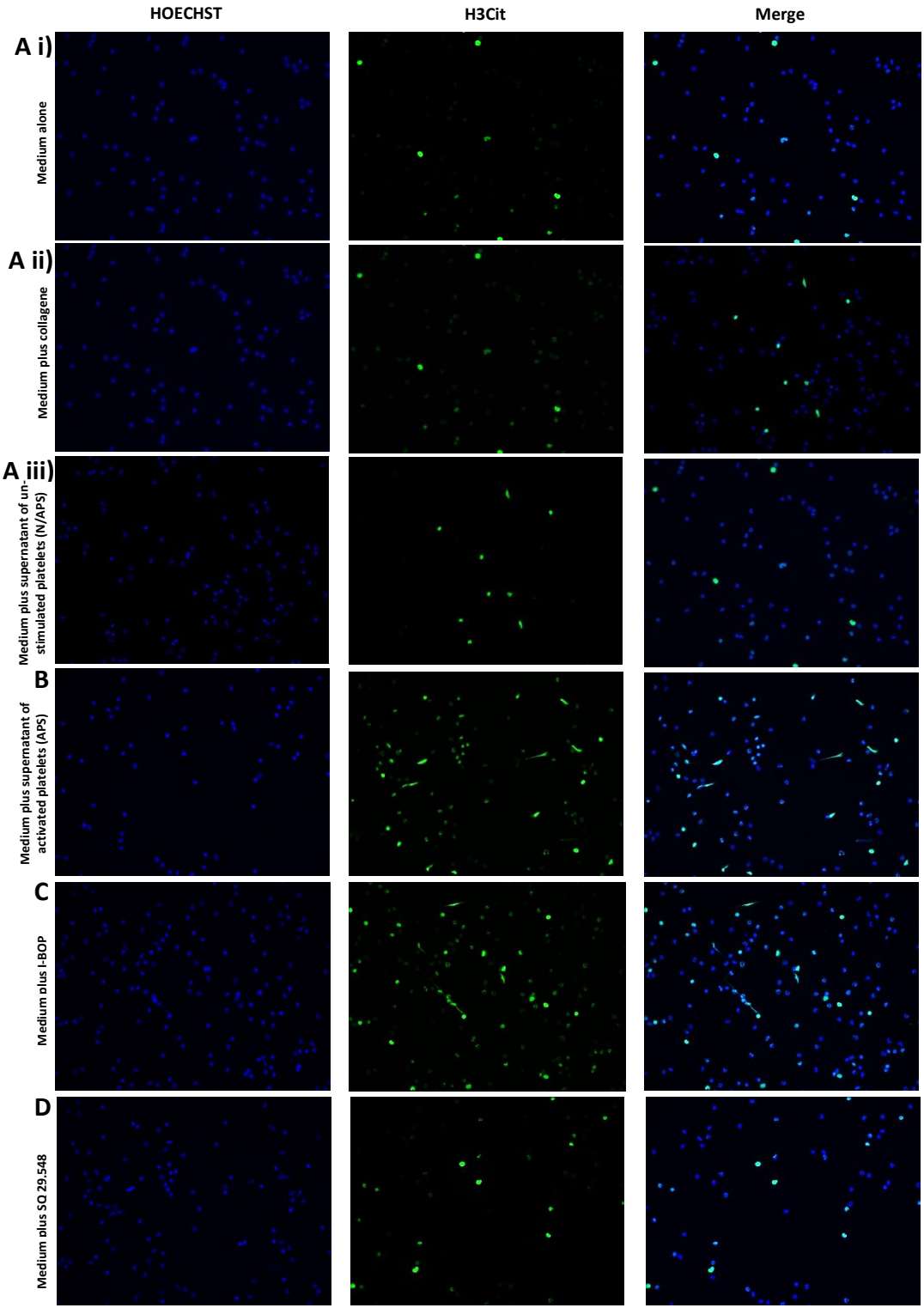


Fig. 7: Immunofluorescence of neutrophils NETs (H3Cit) formation after incubation for 2 hours with. **(A i)** medium alone; **(A ii)** medium plus collagen; **(A iii)** supernatant of un-stimulated (N/APS)**; (B)** stimulated platelets (APS); **(C)** I-BOP; **(D)** APS plus SQ 29,548, as indicated. Representative image of 6 independent experiments. Data shown are mean ± SEM.

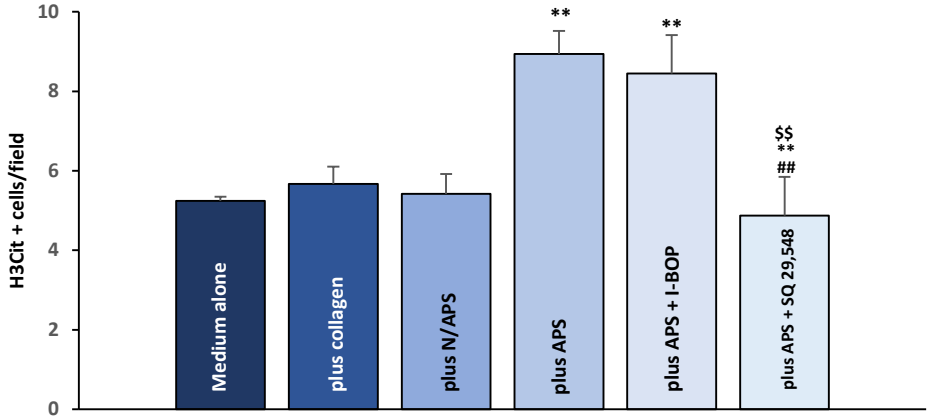


Fig. 8: Quantization of Neutrophils NETs (H3Cit) formation after incubation for 2 hours with medium alone, medium with collagen, supernatant of un-stimulated and stimulated platelets (N/APS, APS), and I-BOP with or without SQ 29,548 as indicated. (n=6 independent experiment; **p<0.01 versus medium alone; ##p<0.01 versus medium plus APS; $$p<0.01 versus medium plus I-BOP). Data shown are mean ± SEM.

III. 1.6 TF activity and expression was modulated after SQ 29,548 incubation: *ex vivo* and *in vitro* experiments

To sustain the hypothesis of ability of TxA_2 platelet-released to modulated expression/activation of TF in neutrophils and monocyte/macrophages, *in vitro* experiments were carried out.

In particular, neutrophils and mouse peritoneal macrophages (MPMs) were incubated for 4 hours with medium alone (control cells), medium plus collagen (data not shown), I-BOP (2µM) or supernatant of un-stimulated and stimulated platelets (N/APS, APS), in presence or absence of SQ 29,548 (10µM). Then TF expression and/or activity was analysed. The incubation of neutrophils and MPMs with I-BOP or with APS induced a significantly increased of TF activity compared to control cells (Fig. 9A, B). The treatment with SQ 29,548 completely prevented TF activity induced by APS (Fig. 9A, B) in both neutrophils and monocytes/macrophages. In addition, western blot analysis in monocytes/macrophages showed that incubation of MPMs with SQ 29,548 completely inhibited TF expression induced by APS (Fig. 9C).

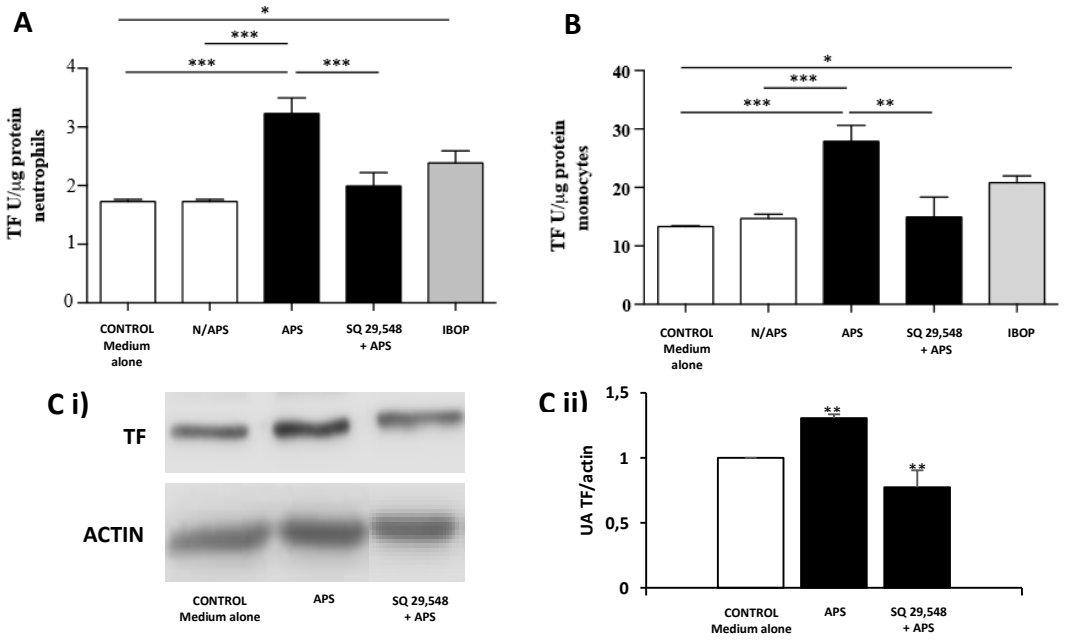


Fig. 9 TXA_2 released from stimulated platelets increased activity and expression of Tissue Factor (TF) in neutrophils and macrophages. Neutrophils **(A)** and mouse peritoneal macrophages **(B)** were incubated for 4 hours with medium alone, medium plus collagen (data not shown), supernatant of un-stimulated (N/APS) and stimulated platelets (APS), with or without (not shown) SQ 29,548 plus APS and with I-BOP, as indicated. Activity **(B)** and expression **(C i) (C ii)** of TF was assessed in peritoneal macrophages (*p<0.05; **p<0.01; ***p<0.001). Data shown are mean ± SEM.

III.2 *Results*

III. 2 Effect of deletion of COX-2 enzyme in a venous thrombosis mouse model

III. 2.1 COX-2 deletion affected venous thrombus formation

To assess whether COX-2 deletion might concur to venous thrombosis, wild type (WT) and COX-2 knockout (COX-2KO) males' mice of 10-12 weeks were subjected to ligation of inferior vena cava (IVC) and 48 hours post ligation mice were subjected to vascular ultrasonography analysis (US). Through the use of this technique we can monitor thrombus formation and propagation every day throughout the experiment (Fig. 1, 2).

Both at 24 hours and 48 hours after ligation, COX-2KO mice developed bigger thrombi than WT mice (24 hours: WT: 0.4±0.14 mm^2/thrombus; COX-2KO: 1.34±0.28 mm^2/thrombus; $p<0.05$. Fig. 1A, B; 48 hours: WT: 1.26±0.29 mm^2/thrombus; COX-2KO: 2.47±0.74 mm^2/thrombus; $p<0.05$. Fig. 2A, B). In particular, at 48 hours after ligation, thrombi from COX-2KO mice were bigger compared to those measured in both groups of animals at 24 hours (Fig. 3).

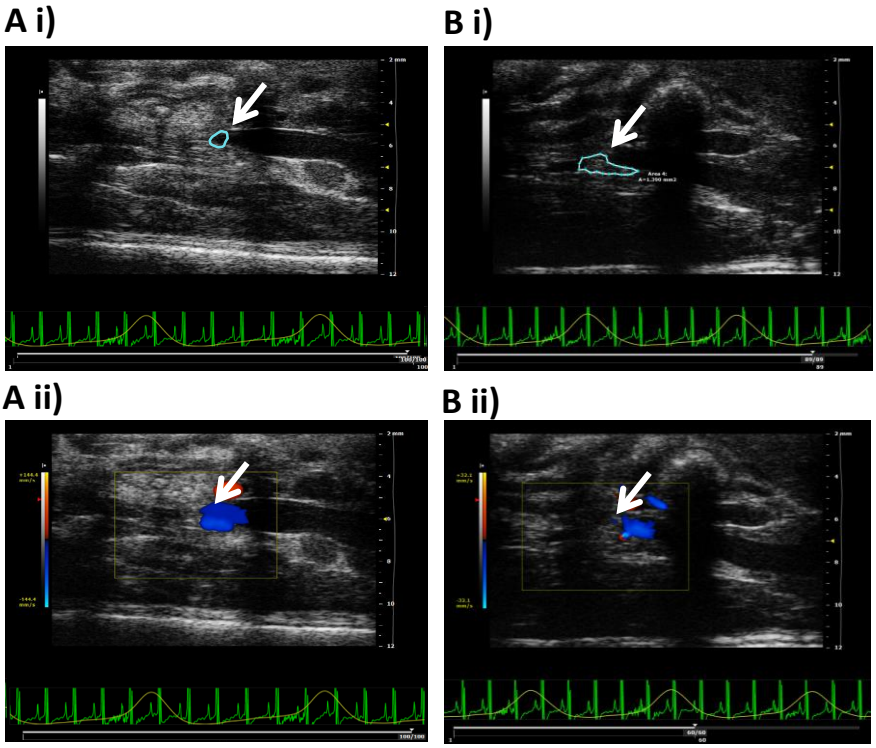


Fig. 1: Vascular ultrasonography (US) imaging of thrombus formation in WT and COX-2KO mice 24 hours after IVC ligation. Representative image of longitudinal vision of **(A)** WT and **(B)** COX-2KO inferior vena cava (IVC) with or without color-doppler function **(A i) (A ii) (B i) (B ii)**. White arrows indicated the presence of thrombi. n=7-8 mice/group.

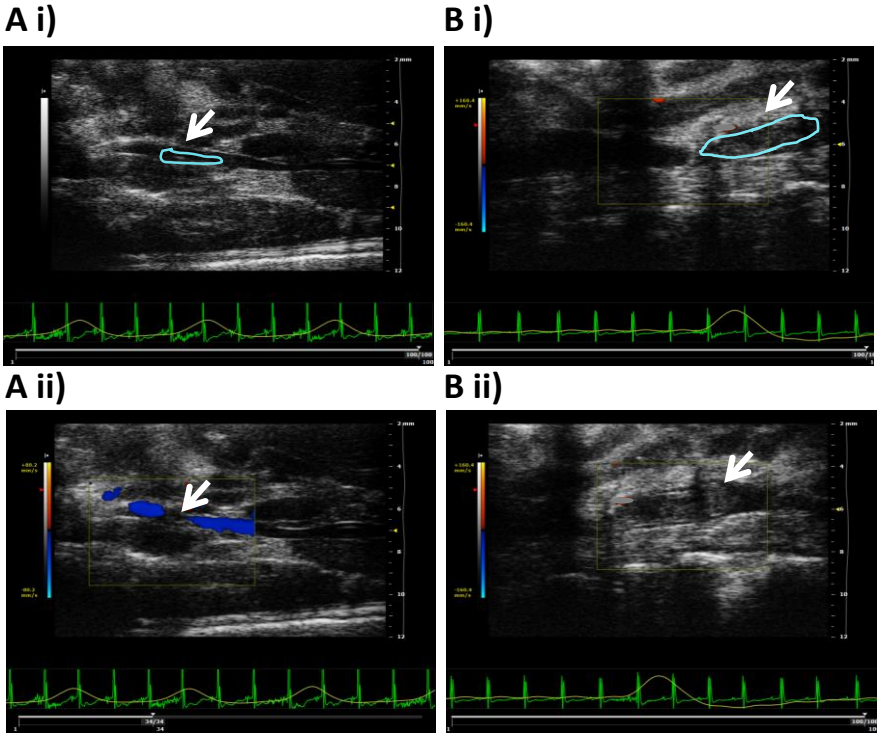


Fig. 2: Vascular ultrasonography (US) imaging of thrombus formation in WT and COX-2KO mice 48 hours after IVC ligation. Representative image of longitudinal vision of **(A)** WT and **(B)** COX-2KO inferior vena cava (IVC) with or without color-doppler function **(A i) (A ii) (B i) (B ii)**. White arrows indicated the presence of thrombi. n=7-8 mice/group.

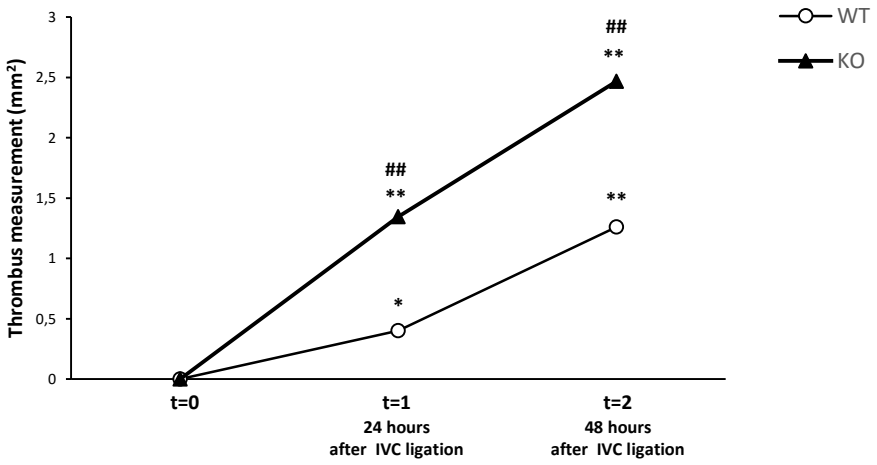


Fig. 3: Deletion of COX-2 enzyme significantly increased thrombus size. Measurement of thrombus area obtained with vascular ultrasonography (US): 24 hours after IVC ligation (t=1) and 48 hours after IVC ligation (t=2) versus basal (t=0) (*p<0.05 WT vs basal at 24 hours after IVC ligation; **p<0.001 WT and COX-2KO vs basal at 24 and 48 hours after IVC ligation; ##p<0.01 vs WT at 24 and 48 hours after IVC ligation; n=7-8 mice/group). Data shown are mean ± SEM.

Thrombus measurements obtained pathologically at sacrifice of animals confirmed the data obtained by US. In particular, COX-2KO thrombus weight, during acute thrombogenesis (48 hours after IVC ligation), increased of 1.3 fold compared to thrombus detected in WT mice (WT: 11.54 ± 2.67 mg/thrombus; COX-2KO: 22.42 ± 2.13 mg/thrombus) (Fig. 4).

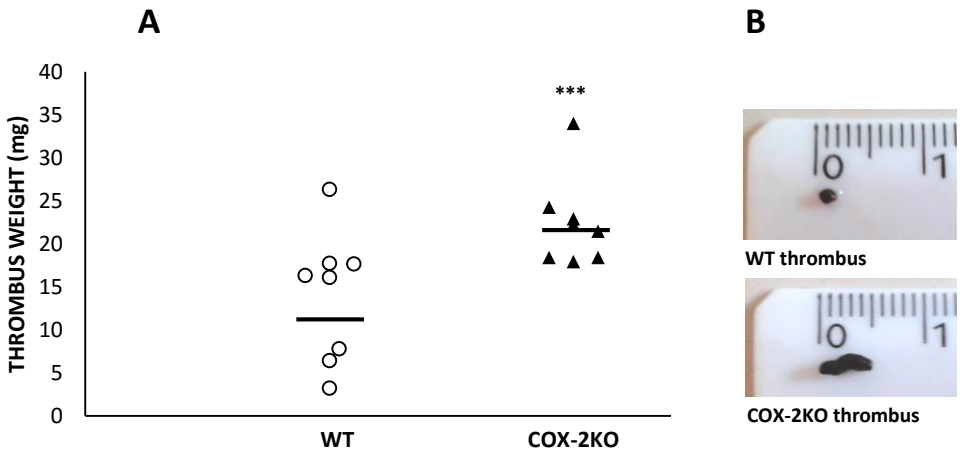


Fig. 4: COX-2 deletion increased thrombus weight induced after IVC ligation compared to WT mice. (A) Forty-eight hours after IVC ligation thrombi from WT mice (open circles) and COX-2KO mice (black triangle) were removed and weighed (***p<0.001 vs WT mice; n=7-8 mice/group). **(B)** Macroscopic appearance of venous thrombi of WT and COX-2KO mice after IVC ligation. Representative results are shown here. Data shown are mean ± SEM.

III. 2.2 COX-2KO deletion affects thrombus composition

Composition of WT and COX-2KO trombi were examined in terms of fibrin content and infiltrated cell types. Deletion of COX-2 caused a greater fibrin deposition compare to WT (WT: 43% fibrin/thrombus section; COX-2KO: 68% fibrin/thrombus section; $p<0.05$), detected with Picro-Mallory stain (Fig. 5). In particular, an increased in old fibrin content was detected with Martius Scarlet Blue stain in thrombi from COX-2KO mice than from WT mice (WT: 36 % old fibrin/thrombus section; COX-2KO: 58 % old fibrin/thrombus section; $p<0.05$) (Fig. 6).

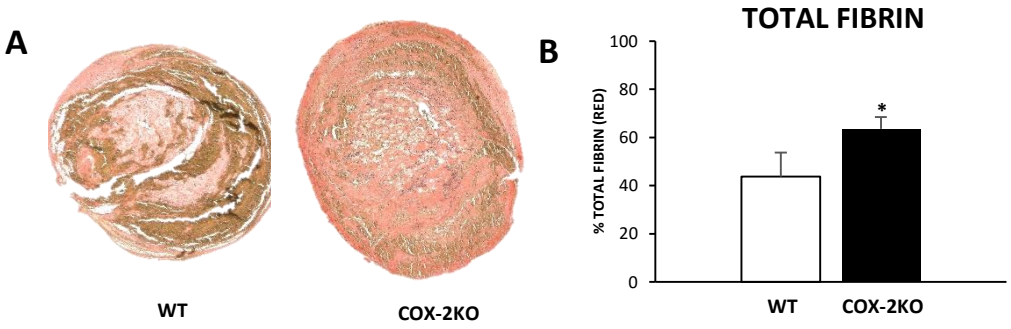


Fig. 5: Picro-Mallory stain on progressive section of WT and COX-2KO thrombus: (A) fibrin infiltration (red stain; 10X magnification) within the thrombus 48 hours after ligation of IVC in WT mice and COX-2KO mice and **(B)** their quantization (*$p<0.05$ vs WT; n=6 mice/group). Data shown are mean ± SEM.

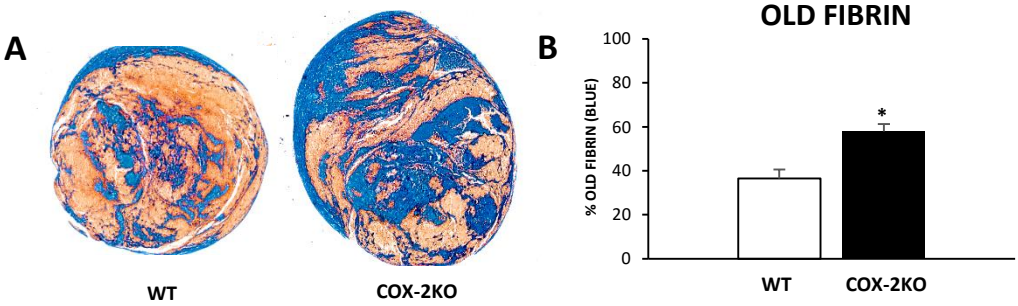


Fig. 6: COX-2KO mice, through Martius Scarlet Blue stain (MSB), showed an increased in old fibrin. (A) Old fibrin infiltration (blue= old fibrin; 10X magnification) within the thrombus 48 hours after ligation of IVC in WT mice and COX-2KO mice and **(B)** their quantization (*$p<0.05$ vs WT; n=6 mice/group). Data shown are mean ± SEM.

In addition, the number of nucleated cells infiltrated in venous thrombi was significantly reduced in COX-2KO thrombi than in WT thrombi (WT: 170.76±21.52 nucleated cells/field; COX-2KO: 128.5±8.96 nucleated cells/field; $p<0.05$) (Fig. 7A). In particular, the total number of leukocytes, identified as CD45 positive cells (Fig. 7B), was significantly decreased in COX-2KO thrombi compare to WT (WT: 129.5±2.34 $CD45^+$ cells/field; COX-2KO: 119.06±1.08 $CD45^+$ cells/field; $p<0.01$) (Fig. 7B). Neutrophils, identified as Lys6G positive cells (Fig. 7C), and monocytes, as F4/80 positive cells (Fig. 7D), were then analyzed. COX-2KO mice showed a slightly reduction in the total number of neutrophils compare to WT mice (WT: 130.53±5.89 $Lys6G^+$ cells/field; COX-2KO: 109.47±3.42 $Lys6G^+$ cells/field; $p<0.05$) (Fig. 7C). However, the percentage of neutrophils compared to total cell number infiltrated in thrombi was similar in two groups (WT: 78 % $Lys6G^+$; COX-2KO: 75 % $Lys6G^+$; p=ns) (Fig. 7C). In contrast, total number of monocytes resulted slightly increased in COX-2KO thrombi (WT: 64.70±0.3 $F4/80^+$ cells/field; COX-2KO: 71.73±1.88 $F4/80^+$ cells/field; $p<0.05$) (Fig. 7D), whereas the percentage was significantly increased than WT thrombi (WT: 40 % $F4/80^+$; COX-2KO: 51 % $F4/80^+$; $p<0.01$) (Fig. 7D).

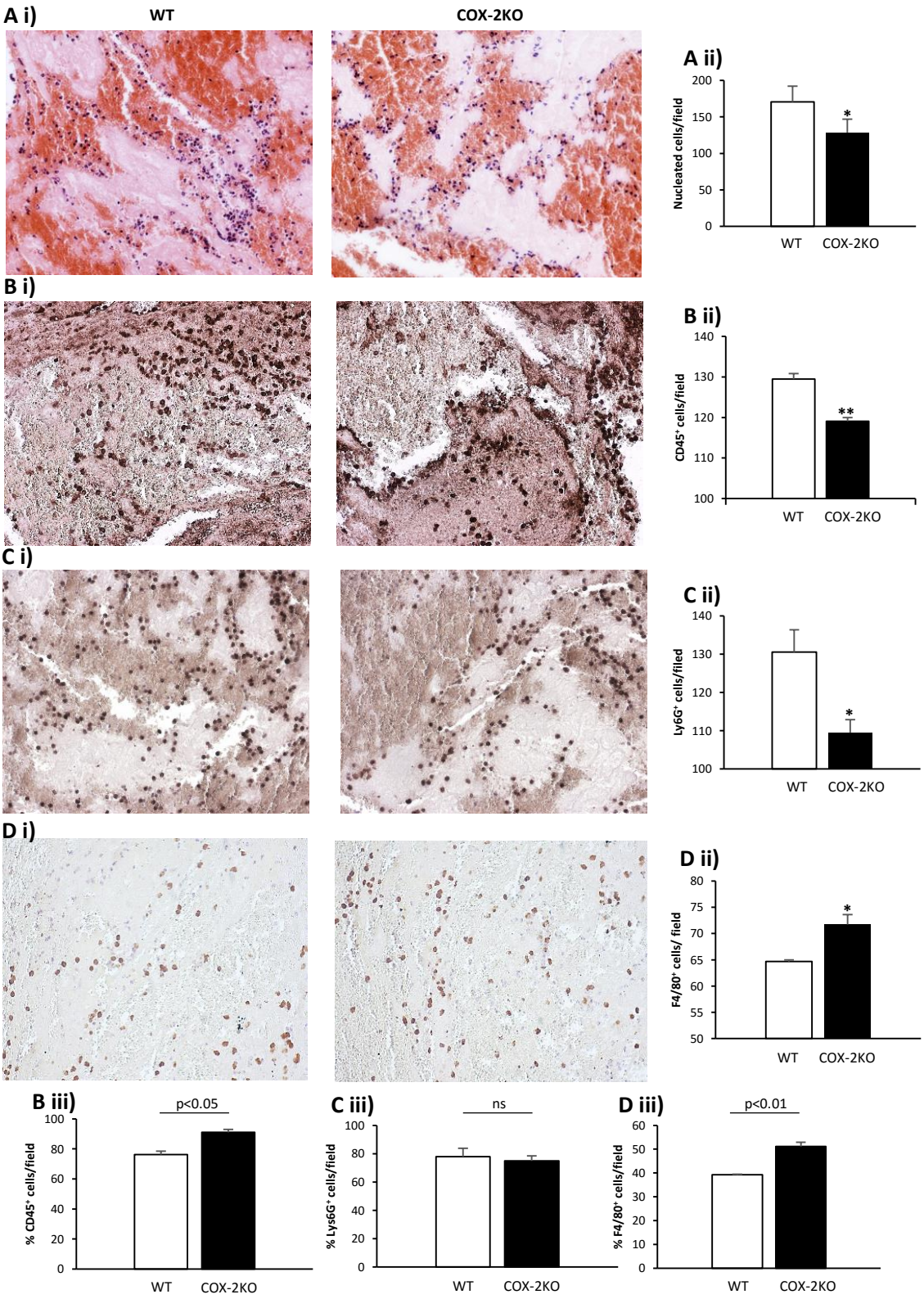


Fig. 7: Immunohistochemical analysis on sections of WT and COX-2KO thrombi showed different cellular infiltration within the thrombi. **(A i) (A ii)** Hematoxylin/eosin showed reduction in cellular infiltration within the COX-2KO thrombi than WT, and **(B i) (B ii)** histological analyses showed that also leukocytes infiltration (CD45), **(C i) (C ii)** neutrophils infiltration (Lys6G) and **(D i) (D ii)** monocytes/macrophages infiltration (F4/80) were significantly decreased in COX-2KO thrombi. **(A iii) (B iii) (C iii) (D iii)** represented percentage quantization of cellular infiltration expressed as positive cells per field (20X magnification) (*p<0.05; **p<0.01 vs WT; n=6 mice/group). Data shown are mean ± SEM.

III. 2.3 COX-2 deletion affects Tissue Factor (TF), AnnexinA2 (ANXA2) and S100A10 expression in thrombi

Tissue Factor (TF) expressed by vessel wall or by circulating cells plays a key role in both arterial and venous thrombosis (Furie B, 2007) (Manly DA, 2011) (von Brühl ML, 2012). We previously showed that TF activity was substantially increased in circulating plasma microparticles and in total leukocytes from COX-2KO mice compared to WT mice (Barbieri SS, 2012). Here we showed that thrombi from COX-2KO mice have a greater number of TF-positive cells compare to thrombi obtained from WT mice (WT: 52.20±6.27 TF^+ cells/field; COX-2KO: 82.75±5.10 TF^+ cells/field; $p<0.01$) (Fig. 8).

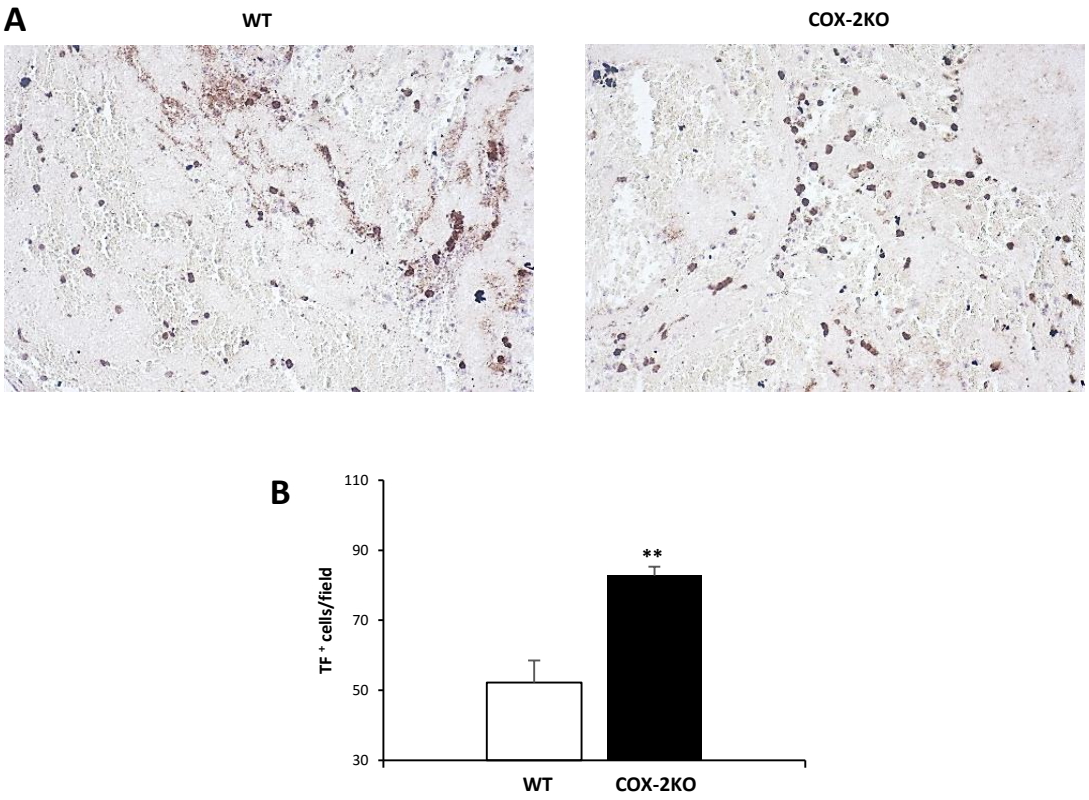


Fig. 8: COX-2 deletion caused a significantly decrease of Tissue Factor expression in venous thrombi. (A) Histological analyses of TF expression on section of thrombus from WT and COX-2KO mice 48 hours IVC ligation (20X magnification) and **(B)** quantization of positive cells per field. (**p<0.01 vs WT; n=6 mice/group). Data shown are mean ± SEM.

In addition, in view of their potential role in the fibrinolytic process and in the TF modulation (Laumonnier Y, 2006), immunohistochemistry analysis of AnnexinA2 (ANXA2) and of its ligand S100A10 have been performed on sections of WT and COX-2KO thrombi. Remarkably, a higher number of ANXA2-positive cells (Fig. 9) concomitantly with a lower number of S100A10-positive cells (Fig. 10) were detected in thrombi from COX-2KO than WT mice (WT: 64.23±11.11 $ANXA2^+$ cells/field and COX-2KO: 113.81±3.21 $ANXA2^+$ cells/field; $p<0.05$; WT: 55.72±1.86 $S100A10^+$ cells/field and COX-2KO: 34.63±4.31 $S100A10^+$ cells/field; $p<0.001$).

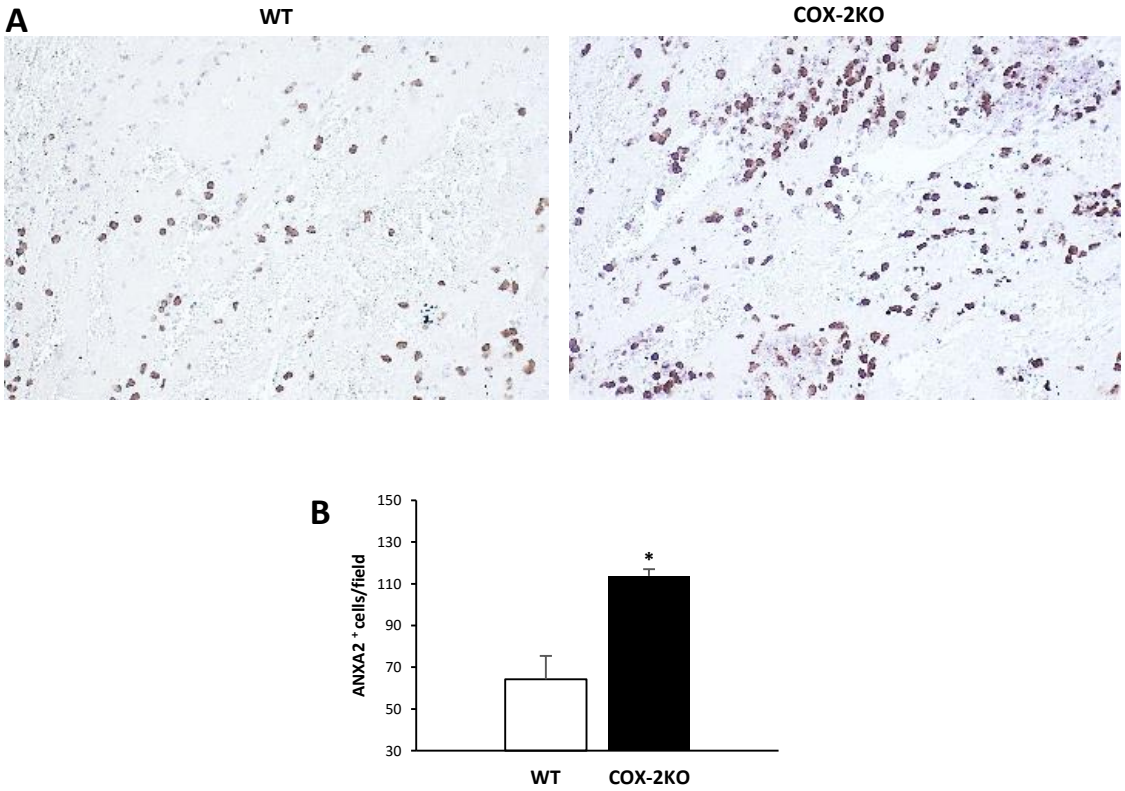


Fig 9: ANXA2 expression in venous thrombi was increased in COX-2KO mice. (A) Representative images of immunohistochemical analysis (20X magnification), and **(B)** quantization of ANXA2 in WT and COX-2KO thrombi expressed as positive cells per field (*$p<0.05$ vs WT; n=6 mice/group). Data shown are mean ± SEM.

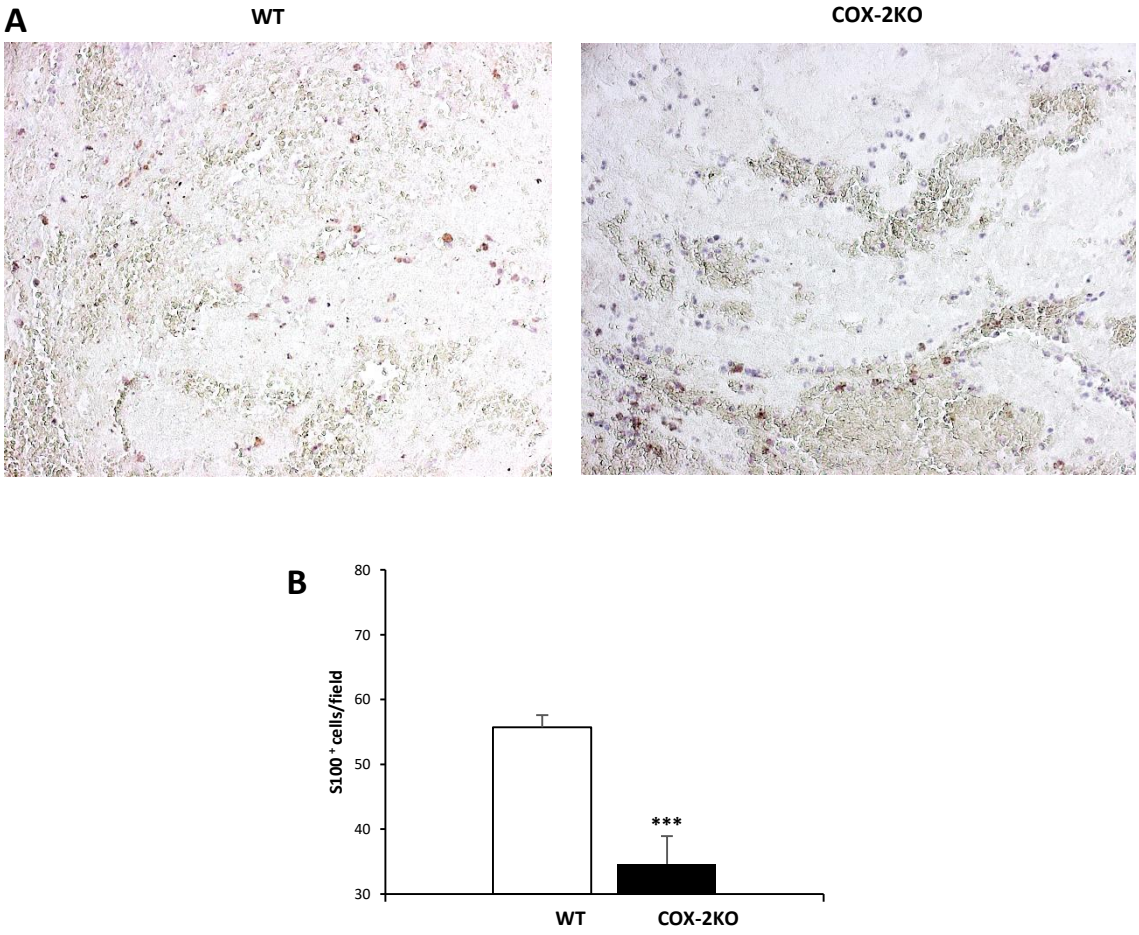


Fig. 10: S100A10 expression in venous thrombi was decreased in COX-2KO mice. (A) Representative images of immunohistochemical analysis (20X magnification), and **(B)** quantization of S100A10 in WT and COX-2KO thrombi expressed as positive cells per field (***p<0.001 vs WT; n=6 mice/group). Data shown are mean ± SEM.

III. 2.3.1 Inhibition of COX-2 increased TF via ANXA2

In vitro studies were performed to assess the potential relationship between COX-2, TF, ANXA2 and S100A10.

Peritoneal macrophages from COX-2KO mice showed greater levels of TF and in ANXA2 expression, and lower levels of S100A10 than WT (Fig. 11). Similar results about ANXA2 expression were obtained when RAW 264.7 cells, a mouse leukemic monocyte macrophage cell line, were transfected with specific COX-2 siRNA and compared to cells transfected with non-specific siRNA (Fig. 12).

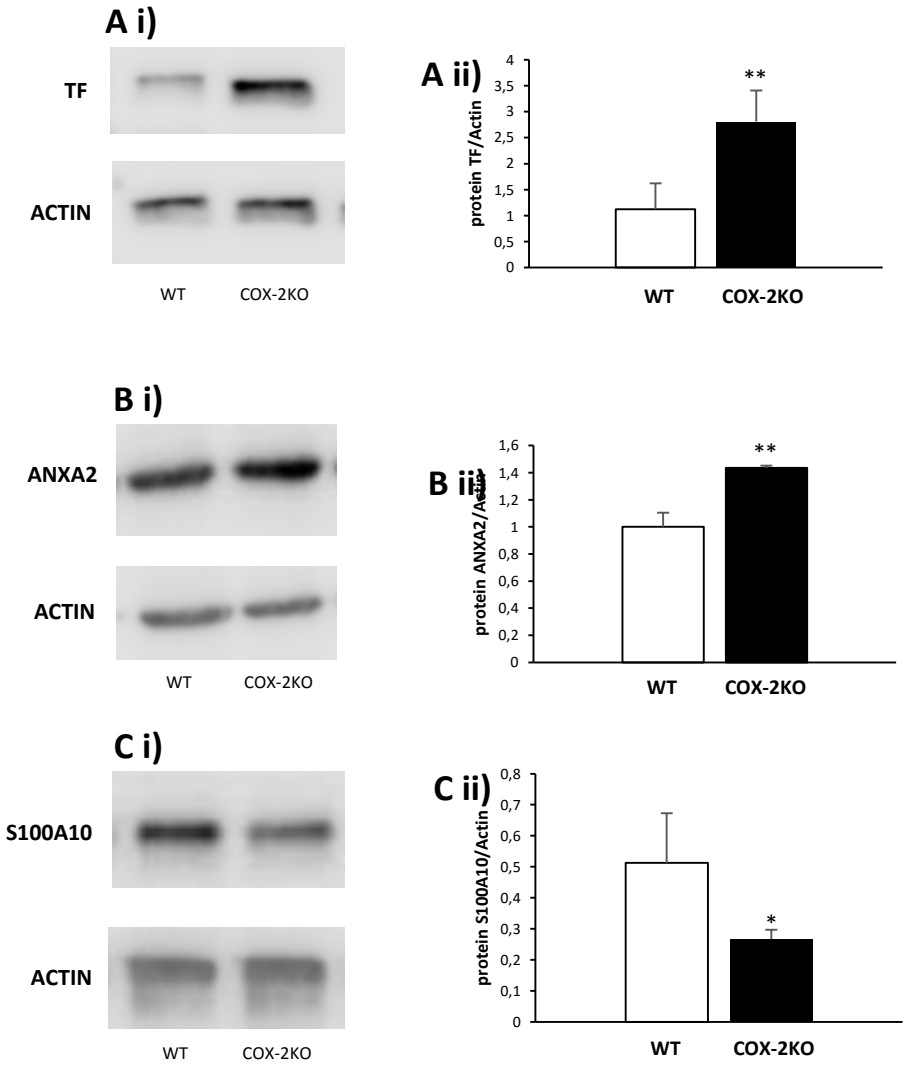


Fig. 11: Peritoneal macrophages from COX-2KO mice showed **(A i)** greater levels of expression of TF, **(B i)** ANXA2 and **(C i)** S100A10 than WT cells. **(A i) (B i) (C i)** representative images of Western Blot; **(A ii) (B ii) (C ii)** protein content normalized to actin expression of TF, ANXA2 and S100A10 (*p<0.05; **p<0.01 vs WT cells). Data shown are mean ± SEM of 6 independent experiments.

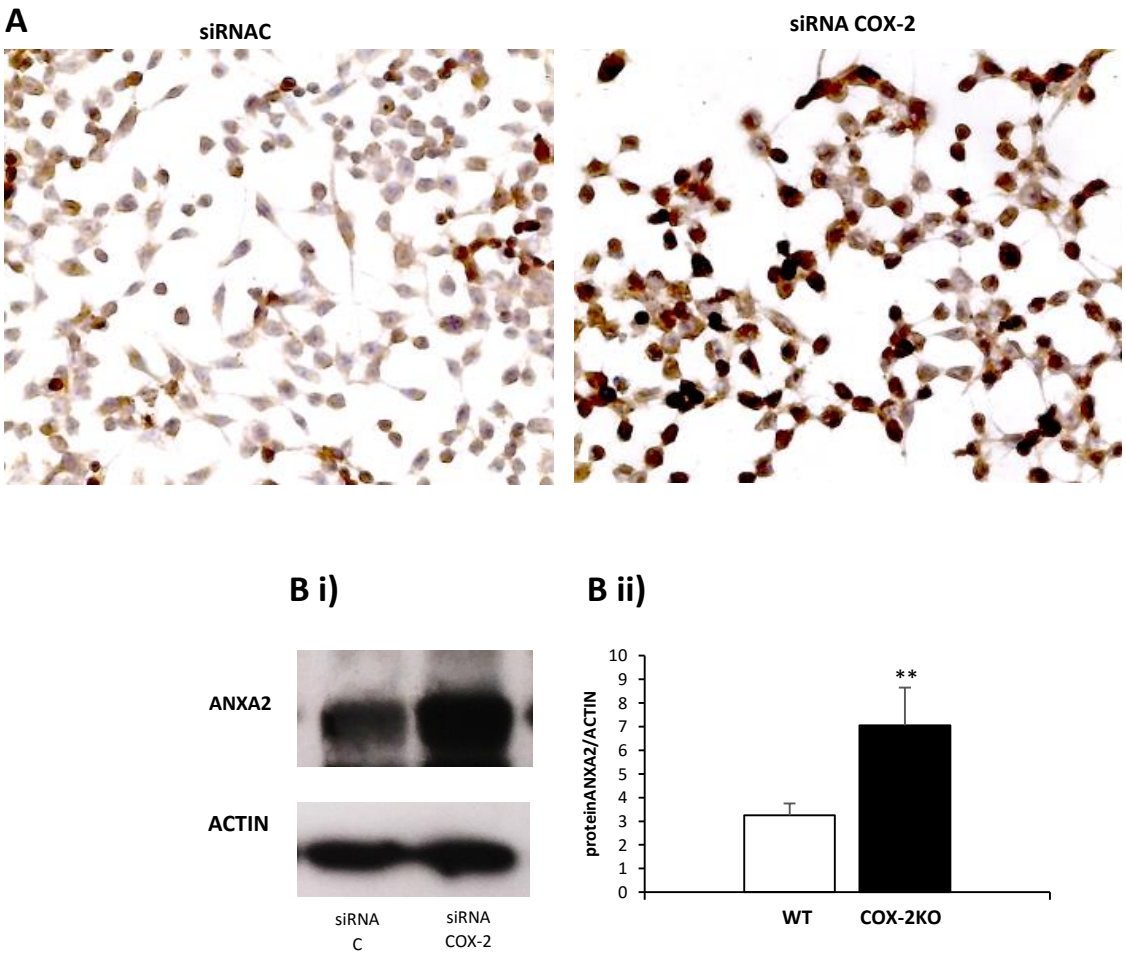


Fig. 12: Silencing of COX-2-enzyme increased ANXA2 expression in RAW 267.4 cells. Immunohistochemical analysis of ANXA2 was performed on RAW 267.4 cells transfected with siRNA control cells or siRNA COX-2 cells **(A)**. ANXA2 **(Bi) (Bii)** was analyzed also by western blotting analyses. Representative images of 4 independent experiments.

Localization of ANXA2 in peritoneal monocytes/macrophages collected from WT and COX-2KO mice, analyzed with western blot analysis of total lysed and of cytosol, membrane and nuclear fractions, showed that ANXA2 mainly localized at the nuclear level in COX-2KO mice, while at the membranes levels in WT mice (Fig. 13).

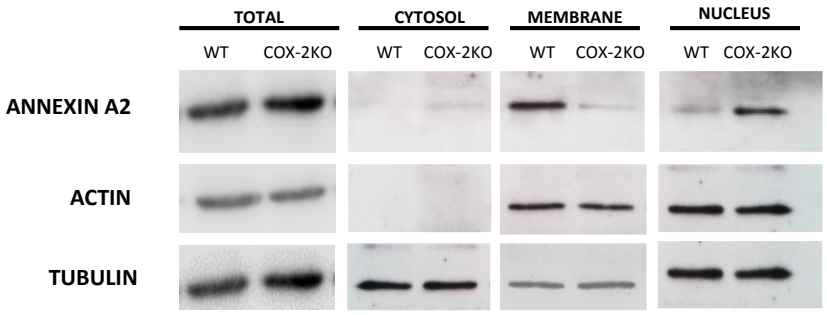


Fig.13: ANXA2 localized in different manner in COX-2KO peritoneal macrophages cells. Western blot of ANXA2 on fractions (cytosol, membrane and nucleus) of peritoneal macrophages cells from WT and COX-2KO mice (n=6 mice/group). Data shown are mean ± SEM of 6 independent experiments.

IV.1 *Discussion*

IV. 1 Role of COX-1 in venous thrombosis

In this study, we provide evidence that the inhibition of cyclooxygenase-1 (COX-1) is responsible for a significantly decrease in venous thrombus size in mice. Indeed, low-dose of aspirin is sufficient to decrease TF activity in plasma microparticles (MP-TF), through a TXA_2/TP-receptor mechanism. In particular, we showed that thromboxane platelet-produced induces monocytes/macrophages and neutrophils activation with consequent increased of TF expression and neutrophil extracellular traps (NETs) formation, factors that cooperate in the occurrence of venous thrombus formation.

The increased levels of TXB-M and the higher circulating levels of DNA, as surrogate marker of NETs, have been observed in pathological conditions including DVT and/or VTE (Diaz JA, 2013) (Klotz TA, 1984) (Patrignani P, 2008). In contrast, while the relevance of TF in venous thrombus formation is mostly accepted (von Brühl ML, 2012), contradicting results have been obtained about the circulating levels of TF in the DVT (Kooiman J, 2015) (Thaler J, 2014) (Walenga JM, 2014) (Ye R, 2012). Accordingly with our data, TF activity on MPs increased in mice that developed venous thrombi after IVC ligation (Ramacciotti E, 2009). This suggests that MP-TF activity might represent an early circulating marker of venous thrombus formation, and sustains the concept of the important role of MP-TF in venous thrombus amplification. Indeed, the correlation observed between TF and the number of MPs, associate with the higher venous thrombus weight in mice injected with MPs or TF^+ microvescicles than mice exposed only to IVC ligation (Ramacciotti E, 2009) (Geddings JE, 2015), well supports our data concerning the correlation of TF activity on MPs and thrombus weight. In addition, ASA treatment, affecting both MP-TF activity and thrombus size induced by IVC ligation, strengthen this concept.

ASA, producing an irreversible acetylation of cyclooxygenase (COX), leads to suppression of prostaglandin H_2 generation, a precursor of TXA_2. Low-dose ASA, sufficient to completely inhibit platelet COX-1 activity, is traditionally used in primary and secondary prevention of artery diseases (Ganjehei L, 2014), and now emerging data suggests that it reduces also the risk of second event of VTE (Becattini C, 2012) (Bringhton TA, 2012).

Many molecular mechanisms by which ASA affect venous thrombosis have been hypothesised, focusing on its effect on platelet inhibition. Indeed, the interaction

between platelets, leukocytes and vascular endothelium is a critical step in the development of venous thrombus (Fuchs TA, 2012). Recently, von Brühl *et al.* founded that platelets, via glycoprotein Ib, contribute to DVT progression by promoting leukocytes recruitment and stimulating TF coagulation pathway (von Brühl ML, 2012). Here, we showed that platelets support venous thrombus formation also by TXA_2 production. Indeed, the inhibition of TXA_2 /TP pathway by ASA and SQ 29,548 treatment, reduced the presence of neutrophils and monocytes/macrophages as well as NETs and TF in venous thrombi, and prevented thrombus growing.

It was established that both neutrophils and monocytes/macrophages expressed TF in venous thrombi. In particular, monocytes/macrophages, but not vessel wall, are the major contributor of TF expression during venous thrombosis in undamaged vessel wall condition (von Brühl ML, 2012). All monocytes/macrophages present in venous thrombus strongly express TF whether only a subset of neutrophils is TF^+ (von Brühl ML, 2012). Although the critical role of neutrophils in venous thrombus formation is well documented (Fuchs TA, 2012), with an inhibition of thrombus growth after neutrophils depletion (von Brühl ML, 2012), the question whether the subset of TF^+ neutrophils produces also NETs remains to be elucidated. Nevertheless, it is plausible to believe that inhibition of TXA_2 signalling pathway improving vessel wall rheology leads to reduction in the thrombus growth. The experiments of platelets transplantation allow to exclude a redundant effect of TXA_2 platelet-produced on platelet activation and to emphasize the impact of TXA_2 on leukocyte activation in the development of DVT. Indeed, we showed that fresh platelets infused in mice treated with SQ 29,548 were unable to restore the thrombus size detected in control mice or in vehicle-treated mice. However, platelets activation was required for venous thrombus development. In fact, depletion or inactivation of platelet by clopidogrel completely prevented venous thrombus formation (data not shown) (von Brühl ML, 2012) (Geddings JE, 2015).

Our hypothesis was strongly supported by *in vitro* data. In fact, activation of platelet with PAR-1 induces NETosis, and inhibition of TP receptor prevents this process (Caudrillier A, 2012). Moreover, activation of TP receptor contributes to induce TF expression and activity in monocytes and endothelial cells (Del Turco S, 2014) (Eligini S, 2006). In addition,

we found that neutrophils and/or monocytes exposed to supernatant of stimulated platelet (ASP) with collagen or to IBOP, a stable analogue of TXA_2, were equally efficient to promote NETs formation and TF activity, respectively. Again, SQ 29,548 decreased NETs formation and TF activity induced by ASP, providing the critical role of TXA_2 in this context.

Of note, ASA-induced acetylation of fibrinogen increases clot permeability favouring fibrinolysis (He S, 2009) (Williams S, 1995). However, the similar results obtained with ASA and with the selective TP receptor antagonist suggest that the reduced amount of fibrin detected in the thrombi is marginally linked to direct effect of ASA on fibrin structure and stability.

In conclusion, these results provide a new link between thromboxane platelet-produced and the occurrence of venous thrombosis. However, it will be to evaluate whether the treatment with TP receptor antagonists prevents venous thrombus development and VTE also in human.

IV.2 *Discussion*

IV. 2 Role of COX-2 in venous thrombosis

The use of both "old and new" COXIBs were associated with a two-fold or more risk of VTE (Schmidt M, 2011). However, the molecular mechanisms by which COX-2 inhibition affects VTE are not fully understood.

We showed that deletion of COX-2 increased expression and activity of TF and altered distribution of Annexin-A2 (ANXA2) in monocytes/macrophages, events that could predispose to earlier venous thrombus formation here described. In particular, take advantage of vascular ultrasonography analysis (US) that allow to monitor the development of venous thrombus from the early stages of thrombus formation (6-24 hours) to maximum amplification (48 hours) of thrombi, we provide evidence that COX-2KO mice developed venous thrombi faster than WT mice and that these thrombi were also bigger.

The enhanced platelet reactivity, the higher level of TXA_2, associated with the increased of TF activity in plasma microparticles (MP-TF) and in vessel wall, previously observed in COX-2KO mice by our group (Barbieri SS, 2012) (Barbieri SS, 2015), can alone explain the propensity of COX-2KO mice to develop venous thrombi.

In particular, platelets play a key role in DVT propagation, supporting accumulation of leukocytes and promoting NETs formation. NETs produced by leukocytes provide an additional scaffold for platelet and red blood cells adhesion, promote fibrin formation, and exacerbate platelet and endothelial activation. In addition, activated platelets recruit MPs-TF that enhance thrombin generation in the growing thrombus (Ramacciotti E, 2009), as previously extensively explained (discussion 1, page 92). In agreement with this concept, in the first part of thesis, we showed that inhibition of TXA_2 platelet-produced suffices to reduce venous thrombus development. The major cell sources of TF, responsible of generation and propagation of venous thrombus, is under debate. Some studies suggest that thrombus formation in the venous vasculature is primarily driven by TF from the vessel wall (Day SM, 2005) (Zhou J, 2009). In contrast, recently von Brühl *et al.* (von Brühl ML, 2012) provide evidence that leukocytes and not vessel wall are the major contributor to TF production. In particular, these authors showed that neutrophils and monocytes are the first cells recruited after the reduction of blood flow.

Recruited leukocytes start fibrin formation via blood cell-derived TF, which is the final trigger of massive fibrin deposition, the major characteristic of DVT (von Brühl ML, 2012). Indeed, is known that monocytes, and to a lesser extent neutrophils, expressed TF in venous thrombi, suggesting that both subsets, particularly monocytes, contribute to TF-driven coagulation during mouse DVT (von Brühl ML, 2012).

Interestingly, COX-2KO mice have a lower number of total leukocytes but a higher number of monocytes and of TF positive cells, associated with higher levels of old fibrin in the venous thrombi compared to WT mice.

Many studies have implicated leukocytes not only in thrombus formation, but also in DVT resolution. In particular, the role of neutrophils in the process of formation/growth of the venous thrombus is complex. The impact of neutrophils reduction on venous thrombus size is unclear. Indeed, the absence of neutrophils leads to the formation of largest thrombi in rat, (Varma MR, 2003), in contrast, prevent venous thrombus formation in mouse (Saha P, 2011) (von Brühl ML, 2012).

Similarly, monocytes/macrophages contribute to TF production and release an additional source of DNase, to generate plasmin and promote restoration of blood flow.

Despite this, the role of leukocytes in DVT development and the precise contribution of different leukocytes subsets to DVT induction/resolution is unclear. In addition, the heterogeneity of neutrophil/monocyte population makes it difficult to interpret their presence in the venous thrombus. It is possible that while a population of neutrophil/monocyte contribute to thrombus formation, another plays a more important role in its resolution.

The imbalance in the subset of neutrophils and/or monocytes may support the early thrombus growth and impair venous thrombus resolution observed in COX-2KO mice. In particular, higher PAI-1 levels (Riehl TE, 2011) associated with lower tPA levels (unpublished data) may result in the reduced fibrinolysis in these mice.

Remarkably, ANXA2, which orchestrates different biological processes including ffibrinolysis (Dassah M, 2009), was over-expressed in COX-2KO venous thrombi. However, the different distribution of ANXA2, preferentially at membrane levels in WT and in the cytosol/nucleus in COX-2KO macrophages, associated with a reduced levels of

its binding partner S100A10, may explain the reduced fibrinolysis and the increased of fibrin deposition observed in knockout mice. Unfortunately, no information are available about the distribution/function of ANXA2 in COX-2KO endothelial cells.

Indeed, ANXA2 translocation on the cells surface, which is dependent on both expression of S100A10 protein and tyrosine phosphorylation of ANXA2, is a key regulatory step governing vascular fibrinolysis (Dassah M, 2009) (Flood EC, 2011), increasing plasmin generation (Deora AB, 2004). The inhibition/alteration of the $ANXA_2$-S100A10 complex's function may increase thrombosis risk by impairing fibrinolysis (Hedhli N, 2012). Moreover, fibrinolysis assessed in S100A10-null mice ($S100A10^{-/-}$), which displayed increased vascular fibrin, reduced clearance of thrombi, and impaired neovascularization of Matrigel thrombi, confirmed the relevance of this protein in thrombotic disorders (Surette AP, 2011).

In contrast, the role of ANXA2 in the nucleus is not well understood. However, it is recently showed that intracellular ANXA2 regulates NF-kB signaling faciliting nuclear translocation of p50 subunit (Jung H, 2015), suggesting that ANXA2 can promote the transcription of different gene including also TF. In accordance to Zhou H *et al*., (Zhou H, 2009), we showed that deletion of ANXA2 by specific siRNA reduced expression and activity of TF in monocytes/macrophages. All these findings support our hypothesis that the increased expression of ANXA2 and its accumulation into the nucleus can up-regulate TF expression in monocytes and promote thrombus growth in COX-2KO mice.

Nevertheless, future study will be performed to investigate the contribution of vessel wall in this context. In effect, COX-2KO mice had increased plasma levels of asymmetric dimethylarginine (ADMA) and reduced endothelial nitric oxide responses (Ahmetaj-Shala B, 2015), factors that may affect venous (Wang G, 2013).

In conclusion, we can hypothesize a new possible mechanism ANXA2-dependent in DVT induction/resolution. An alteration in the balance of ANXA2 and S100A10, accompanied with a different localization of ANXA2 in cells, can influences the fibrinolysis process and TF expression. This new described mechanism can be useful in the prevention of DVT, however additional studies need to be performed.

V. *Conclusion*

Data here obtained show that the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in a venous thrombosis mouse model could lead to opposite effect on the thrombus development, stabilization and resolution.

In particular, COX-1 inhibition is responsible of an impairment development and growth of venous thrombus, with a mechanism most likely dependent of TXA_2 /TP pathway.
In contrast, COX-2 inhibition caused an increased in thrombus development, growing accompanied with reduction in the thrombus resolution. These finding seem linked to the abnormal ANXA2 accumulation into the cytosol and the nucleus in cells from COX-2KO mice.

All data obtained support evidences that both COX-1 and COX-2 play a key role in DVT, opening the way to novel therapeutic approaches.

VI. *Materials & Methods*

VI. 1 Animal model and *in vivo* procedures

COX-2 heterozygote mice, obtained from The Jackson Laboratory (Bar Harbor, ME) on a hybrid C57BL/6:129 strain background (B6,129-*Ptgs2*^{tm1Jed}/J), and were crossed with FVB/J wild-type mice for 10 generations to introduce the targeted *Cox-2* allele onto an FVB background. FVB-*Cox-2* null mice were healthier than those on the original B6:129 background, with less morbidity and increased life spans. For the other experiments, wild type (WT) mice were used.

All animals were kept in isolators under standard ambient conditions (temperature of 22-25 ° C, humidity 40-70%) with photoperiodic cycle light/dark 12/12h. Food and water were provided *ad libitum*. COX-2 wild type mice (WT), and COX-2 knockout (COX-2KO) were generated by the coupling of heterozygous mice and offspring, weaned at about 3 weeks of age, were genotyped by PCR analysis of DNA genomic obtained by caudal biopsy.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved by the Animal Care and Committee on Ethics of the University of Milan (No. 12/2009). All surgery was performed under anesthesia, and all efforts were made to minimize suffering of animals.

VI. 1.1 DNA extraction and genotyping of animals: Genotypes were determined by PCR analysis of genomic DNA prepared from tail tips. The DNA concentration was measured. Mice were genotyped by PCR amplification using the following primers:

Mouse 546 5'-ATCTCAGCACTGCATCCTGC-3'

Mouse 547 5'-CACCATAGAATCCAGTCCGG-3'

Mouse 013 5'-CCTGGGTGGAGAGGCTATTC-3'

VI. 1.2 Animal model of venous thrombosis: Inferior Vena Cava Ligation (IVC): WT and COX-2KO mice, weighing 20 to 30 gr and between 12 to 14 weeks in age, were used. WT and COX-2KO mice, subjected or not to pharmacological treatment with vehicle, ASA or SQ 29,548, were subjected to surgery. Before surgery, mice underwent general anesthesia by injection of ketamine hydrochloride (75 mg/kg; Intervet) and medetomidine (1 mg/kg; Virbac). A ventral midline incision (2 cm) was made through the skin and abdominal wall exposing the abdomen. A sterile PBS1X soaked 2x2 in gauze was used to reflect the intestines to the animal's left side

allowing the visualization of the inferior vena cava (IVC). The IVC was exposed and separated from the aorta at the level of the renal veins. 4-0 polypropylene was used to ligated IVC below the renal veins. Control animals from each experimental group undergo the above dissection used for the surgical approach, except the IVC was not ligated (sham animals). After inducing thrombosis, the abdomen was closed. Mice were then recovered in an individual mouse cage, observed post-operatively (45 minutes to 2 hours) under a heating lamp, then returned to their original housing units. Mice subjected to the above thrombosis-induction models were sacrificed at 48 hours after ligation under anesthesia. IVCs were dissected and excised. Thrombi were collected, weighted, washed in 10% phosphate-buffered saline (PBS 10%), and soaked overnight in 4% paraformaldehyde.

VI. 1.3 Pharmacological treatment of animals: WT mice were subjected to pharmacological treatment and randomized in different groups: treated with vehicle (ethanol/physiological solution, gavage/i.p.; n= 8), with ASA (3 mg/Kg, gavage; n= 8) once a day for 3 days, and with SQ 29,548 (1 mg/Kg, i.p.; n= 8) for 1 day. Twenty-four hours after the first treatment with vehicle, mice treated with ASA and SQ 29,548 underwent to surgery for the ligation of inferior vena cava. Forty-height hours after ligation, all animals were sacrificed and thrombi were collected.

VI. 1.4 Ultrasonography analysis (US): Ultrasonography imaging was performed at 24 and 48 hours after ligation of IVC, in WT and COX-2KO mice to monitor thrombus formation and growth. All IVC images were acquired with a Vevo2100 micro imaging system (Visualsonics, Toronto, Canada) equipped with high resolution a 256 elements linear probe of center frequency of 30 MHz. Two different image modes were used for this study: the so-called “B mode” is a grayscale image of eco-density useful precise for anatomic localization of IVC; the “color Doppler mode”, which is able to detect blood flow provided that flow direction is not perpendicular to the probe axis. Therefore, during acquisition, particular care has been taken to the direction of the IVC relative to the probe in order to exclude this eventuality. The flow is color-coded: red means blood flowing toward the probe, blue blood flowing away from probe. The transducer was able to acquire images 15.36 x 12 mm either for the B mode either for color Doppler images. In all experiments anesthesia was induced with 2% Isoflurane in 100% O_2, then, when the animal was deeply sedated, the Isoflurane was reduced to 1%. Heart rate of the animal was monitored and kept at 500 beats per minutes. Temperature was controlled using a heating pad to 37±0.5°C. The abdomen of the mouse was shaved and warm ultrasound transmission gel was applied to enable visualization and optimize image quality. The IVC was searched at first on an axial B mode image,

then the probe was rotated by 90 degrees to a sagittal view where the clot was visible then the instrument was switched to color Doppler mode to confirm the absence of blood flow after the ligation point. Finally, again in B mode, the image was frozen on an optimal view and clot cross-sectional area measurement were recorded using the VEVO software

VI. 1.5 Urine collection: During the experiments, WT mice were housed in metabolic cages and fed mouse chow ad libitum to assess urinary excretion of fluid and to measured TXB_2 levels after pharmacological treatment. Urine collections (24 and 48 hours pool) were performed during an adaptation period of 3 days on a standard diet. Urine were collected and were stored at -20°C.

VI. 1.6 Platelets depletion and repletion:

VI. 1.6.1 *In vivo* Carboplatinum depletion of platelets: To deplete platelets, a 150-µl volume of Carboplatinum (Pfizer, 150mg/15ml), a chemotherapeutic agent that causes thrombocytopenia, at the nonlethal dose of 125 mg/kg. It was intraperitoneal injected in WT mice 7 days before SQ 29,548 treatment (Barbieri SS, 2012). Platelets depletion was measured in the peripheral blood by cell count, and the procedure resulted in a > 90 percentage depletion of platelets.

VI. 1.6.2 Platelet sorting and repletion into $pls^{-/-}$ mice: Washed platelets obtained from platelet rich plasma (PRP) of WT mice were counted and $\approx 1\times10^9$ washed platelets per animal were intravenously reinfused. Immediately after platelets injection, mice underwent to ligation of IVC as previously described.

VI. 2. Blood collection

VI. 2.1 Measurement of Tissue Factor activity in plasma samples: For plasma preparation, blood samples were collected into 3.8% sodium citrate (1 part sodium citrate/ 9 blood) by cardiac venipuncture from anesthesized (ketamine chlorhydrate 75 mg/Kg / medetomidine 1 mg/Kg in physiologic water) WT and COX-2KO mice. Blood was centrifugated at 3000 rpm for 20 minutes at 4°C and immediately stored at -80°C until further analysis. Microparticle-associated TF (MP-TF) activity in plasma was measured using MP-TF kits from Hyphen BioMed according to the manufacturer's instructions.

VI. 2.2 Platelets-poor plasma (PPP) collection: Blood samples were diluted 1:1 with Hepes-Tyrode's buffer and centrifuged at 100 g for 10 minutes. Platelet rich plasma (PRP) was gently removed with a plastic pipette. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at 2000 g for 20 minutes.

VI. 2.3 Washed platelets (WPs): washed platelets were obtained by serial centrifugations of PRP with addiction of 1.2 µM PGI_2 and 0.01 mg/L apyrase. Washed platelets were subsequently stimulated with collagen 1µg/ml for 10 minutes at 37°C under stirring and used subsequently.

VI. 3. *Ex vivo* procedures

VI. 3.1 Urine collection for 2, 3-dinor-TXB_2 measurement: the determination of urinary 2,3-dinor-TXB_2 was performed using a modified LC-MS/MS method previously described (Cavalca V, 2010). Briefly, mice urine (1 mL) were acidified with 3% formic acid (500 µL) before being loaded onto SPE cartridges (Oasis® HLB 1cc, 30 mg, Waters, Milford, MA, USA) that were conditioned with methanol and water. Cartridges were then washed with 1% formic acid, 5% acetonitrile, hexane and the analyte was eluted with 100% acetonitrile.

The eluted fractions were evaporated to dryness using a vacuum centrifuge at room temperature (SpeedVac Savant SC210, Thermo Scientific, MA, USA) and reconstituted with 100 µL of 10% acetonitrile before the LC-MS/MS analysis.

We used an Accela HPLC System (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a triple quadrupole mass spectrometer TSQ Quantum Access (Thermo Fisher Scientific) outfitted with electrospray ionization (ESI) source. The Xcalibur® software, version 2.0 (Thermo Fisher Scientific) was used for system control, data acquisition and processing.

The chromatographic separation was performed using an XBridge C_{18} column (100 mm x 2,1 mm, 3.5 µm; Waters Milford, MA, USA) and the mobile phase was composed by two solvents: solvent A (Water with 0.1% ammonium hydroxide) and solvent B (Methanol:Acetonitrile 50:50 v/v with 0.1% ammonium hydroxide). The selected reaction-monitoring (SRM) was performed in negative mode by monitoring the transitions *m/z* 341.1 → *m/z* 141.02+166.93.

VI. 3.2 Collection, processing and inclusion of thrombi: Forty-eight hours after the IVC ligation, the mice were euthanized and gently perfused with PBS and 4% paraformaldehyde

(PFA). Inferior vena cava was dissected and the thrombi collected were immediately measured and weighed, and then fixed over-night in 4% PFA.

The following day the thrombi were washed twice in PBS 1X buffer solution for one hour at room temperature and dehydrated in solutions of increasing alcohols (from 50% Ethanol to 100% Ethanol). For a complete dehydration of the samples, the thrombi were placed in xylene and subsequently embedded in paraffin. Sections about 3µm were mounted on SuperFrost Plus microscope slides, and thrombi were visualized with different staining.

VI. 3.2.1 Histopathology and immunohistochemical analysis:

VI. 3.2.1.1 Hematoxylin and eosin: The sections of thrombi were first deparaffinated and subsequently rehydrated through a decreasing scale of alcohols. Then, sections were immersed in a hematoxylin solution for nuclear counterstaining and in eosin solution for cytoplasmic staining. Then, sections were washed in H_2O for a few seconds and subjected to a dehydration process by steps in increasing alcohol solutions and a last step in xylene. The slides were finally mounted with specific mounting (Fast Drying Mounting Medium, Bio-Optica) to be subsequently photographed by light microscopy.

The images for cell counts were obtained by "ZEUS" camera and analyzed with ImageJ software. For each thrombus were analyzed 5 sections and for each of them were taken 5 random pictures of different areas (20X). The total of images obtained for each sample is 25.

VI. 3.2.1.2 Picro-Mallory stain: The sections of thrombi were first deparaffinated and subsequently rehydrated through a decreasing scale of alcohols. Slides were rinsed in distillated water and then were incubated for 10 minutes at room temperature with mix solution to stain nuclei with an acid resistant nuclear stain (Weigert's iron hematoxylin), washed in distillated water and subsequently in *fontis* water for 10 minutes. Sections were placed in yellow mordant (Picric acid, saturated in 80% ethanol, Orange G, Lissamine yellow) for 2-3 minutes, washed in distilled water until only erythrocytes are yellow. Then, were placed in the red stain (Acid fuchsin, glacial acetic acid, distillated water) for 5-10 minutes, rinsed with 1% aqueous acetic acid and differentiate with the red differentiator (Stock differentiator, ethanol 95%, distillated water) until fibrin is prominent microscopically. Subsequently samples were rinsed well in distilled water and placed in the blue stain (Methyl blue, glacial acetic acid, distillated water) for 5 minutes. Then were rinsed briefly with 1% aqueous acetic acid, placed in the blue differentiator (Stock differentiator, distillated water) for 1-2 minutes and rinsed with 1% aqueous acetic acid. Finally,

sections were dehydrated with ethanol, cleared with xylene and mount with a resinous medium. With ZEUS camera, all section of each thrombus were analyzed with ImageJ software to identify collagen component within the thrombi.

VI. 3.2.1.3 Martius Scarlet and Blue stain (MSB): The sections of thrombi were first deparaffinated and subsequently rehydrated through a decreasing scale of alcohols. Sections were incubated for 1 hour at 60°C in Bouin's Solution and incubated with Harris' hematoxylin, differentiated in acid alcohol (1% hydrochloric acid plus 70% alcohol), rinsed in 95% alcohol and excess of alcohol was shacked from the slides, stained in Martius Yellow solution (0.5 gr of Martius Yellow, 100 ml alcohol 95%, 2 gr of phosphotungstinic acid) for 3 minutes. Slides were rinsed in distillated water, and incubated with Crystal Ponceau solution (1% Crystal Ponceau 6R in 2% acetic acid). After rinsing in distillated water, sections were differentiated in 1% phosphotungstic acid, washed in distillated water, stained in methyl blue solution (0.5% methyl blue in 1% acetic acid), rinsed in distillated water, dehydrated, cleared and mounted with specific mounting. With ZEUS camera, all section of each thrombus were analyzed with ImageJ software to identify collagen component within the thrombi.

VI. 3.2.1.4 Immunohistological analysis:

VI. 3.2.1.4.1 Immunohistochemical analysis: were performed according to the standard StreptAvidin Biotin method. Briefly, tissue sections were deparaffinated, then rehydrated in a graded series of ethanol as previously described. Inhibition of endogenous peroxidases was performed in 3% H_2O_2 for 10 min and antigen retrieval was recovered by use of bathwater. Sections were heated (95–98°C) in 0.01M sodium citrate buffer at pH 6.0 for 15 min or EDTA buffer (0,25mM or 1mM) pH8 for 15 or 30 minutes in according to the antibody used. PBS-triton 1% washing buffer was used for permeabilization, and aspecific sites are blocked in 1% Bovine Serum Albumin for 30 min at room temperature. Then, sections were incubated overnight with the primary antibody at 4°C. After incubation with corresponding biotinylated secondary antibody (Polyclonal Goat Anti-Rabbit Immunoglobulins/Biotinylated Code E0432; Polyclonal Rabbit Anti-Goat Immunoglobulins/Biotinylated Code E0466, Dako California; Polyclonal Goat Anti-Rat Immunoglobulins/Biotinylated, serotec), sections were incubated with streptavidin/HRP (code P0397; Dako California) and then stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Nuclear counterstaining was performed using Carazzi' Hematoxylin. Sections were rehydrated as previously described, and mounted. For negative controls, sections were incubated in the absence of primary antibodies. We obtained 10 visual field images from

every section for optical microscopy. The image for cellular count of positive cells for each antibody in each visual field images were obtained with Zeus camera. For each thrombus were analyzed five sections 20x and for each section was calculated the number of cells in 5 random field/section.

Primary Antibody	Isotype	Dilution	Antigen retrieval
Anti-CD45 BD Pharmingen™ Clone OX-1 (RUO)	Rat-polyclonal	1:50	30 min EDTA buffer pH8
Anti-F4/80 antibody [CI:A3-1]	Rat monoclonal	1:100	15 min EDTA buffer pH8
Biotin anti-mouse Ly-6 (clone 1A8)	Rat-polyclonal	1:1000	30 min citrate buffer pH6
Anti-AnnexinA2 antibody	Rabbit polyclonal	1:2000	15 min citrate buffer pH6
Anti-Tissue Factor [EPR8986]	Rabbit monoclonal	1: 200	15 min citrate buffer pH6
Anti-S100A10 (11250-1-AP)	Rabbit polyclonal	1:100	15 min citrate buffer pH6

VI. 3.2.1.4.2 Immunofluorescence of HistoneH3 on formalin-fixed-paraffin-embedded (FFPE) sections of thrombi: For immunofluorescence staining, sections of 3µm thickness of thrombi were used. Sections were deparaffinated and rehydrated. Antigen retrieval was performed in according to the antibodies used. Sections were washed in PBS, permeabilized in PBS-TRITON 0.1% and blocked with PBS+BSA 5% + TRITON 0.1% for 3 hours at room temperature. Then, were incubated with primary antibodies (Anti-Histone H3 antibody citrulline R2 + R8 + R17) diluted in PBS+BSA 5% + TRITON 0.1% overnight at 4°C. Subsequently, were washed in PBS and next, Alexa Fluor secondary antibody (Alexa Fluor anti-Rabbit 488) was used for detection. DNA was stained with 1µg/ml Hoechst 33342 (Invitrogen), and a coverslip was placed by fluorescence mounting medium. Images were acquired using a fluorescence microscope with an AxioCam and processed by AxioVision 4.6 software.

Primary Antibody	Isotype	Dilution	Secondary Antibody
Anti-Histone H3 (citrulline R2 + R8 + R17) antibody - ChIP Grade (ab5103)	Rabbit polyclonal	1:250	Anti-rabbit Alexa Fluor 488 1:500

VI. 3.2.1.4.3 Immunofluorescence of HistoneH3 on neutrophils cells: For immunofluorescence staining, plated cells were washed in PBS 1X, fixed in PFA 4%, permeabilized in PBS-TRITON 0,1% and blocked with PBS+BSA5% + TRITON 0,1% for 3 hours at room temperature. Then, were incubated with primary antibodies (Anti-Histone H3 antibody citrulline R2 + R8 + R17) diluted in PBS+BSA 5% + TRITON 0.1% overnight at 4°C. Subsequently, were washed in PBS and next, Alexa Fluor secondary antibody (Alexa Fluor anti-Rabbit 488) was used for detection. DNA was stained with 1µg/ml Hoechst 33342 (Invitrogen), and a coverslip was placed by fluorescence mounting medium. Images were acquired using a fluorescence microscope with an AxioCam and processed by AxioVision 4.6 software.

VI. 3.2.1.5 NETs immunofluorescence quantification: For quantification of NETs, serial cross sections were stained with Hoechst and the number of NETs was counted with a 40X objective in four fields of view (176 × 131 µm). The sections of thrombi originated from the proximal, middle, and distal part of the IVC.

VI. 3.2.1.6 Double Immunofluorescence of F4/80 and TF on formalin-fixed-paraffin-embedded (FFPE) sections of thrombi: For double immunofluorescence staining, sections of 3µm thickness of thrombi were used. Sections were deparaffinated, rehydrated and permeabilized with PBS-TWEEN 0.1%. Antigen retrieval was performed in according to the antibodies used. Sections were washed in PBS, and the first blocking step with PBS1X/GOAT SERUM 3%/TWEEN 0.1% for 1 hours at room temperature was performed. Then, were incubated with the first primary antibody (Anti-F4/80; clone BM8, 1:100) diluted in PBS1X/GOAT SERUM 3%/TWEEN 0.1% overnight at 4°C. Subsequently, decanted the first primary antibody solution, sections were washed in PBS 1X and then were incubated with secondary biotinylated antibody. Alexa Fluor streptavidin (Alexa Fluor 555) was used for detection of signal. For TF detection, a second blocking step with PBS1X/GOAT SERUM 10%/TWEEN 0.1% for 1 hours at room temperature was performed. Then sections were incubated with the second primary antibody (Anti-Tissue Factor; ab151748, 1:100) overnight at 4°C. Next day, sections were rinsed and incubated with secondary biotinylated antibody. Alexa Fluor streptavidin (Alexa Fluor 488) was used to the detection of TF signal. DNA was counterstaining with 1µg/ml Hoechst 33342 (Invitrogen), and a coverslip was placed by fluorescence mounting medium. Images were acquired using a fluorescence microscope with an AxioCam and processed by AxioVision 4.6 software.

VI. 4. *In vitro* experiments: cell studies

VI. 4.1 Peritoneal macrophages isolation: Peritoneal macrophages were isolated from WT mice as Zhang et al. protocol [14]. In particular, Mice were injected intraperitoneally with 3 ml of 4% thioglycollate medium (Sigma-Aldrich). After 72 hours of injection, the mice were sacrificed by CO_2 inhalation and the macrophages were harvested by peritoneal lavage recovered by injecting 5 ml of PBS with a syringe needle 20-G. The exudate thus obtained was centrifuged and pellet suspended in a solution of ACK for lysate the red blood cells potentially present. After a new

centrifugation, the pellet was suspended in Dulbecco's modified Eagle's medium containing 10% serum and plated in this same medium. The cells thus obtained were counted and plated in the same medium for 2 hours. Adherent cells were treated in serum free medium with or without stimuli.

VI. 4.1.1 Measurement of Tissue Factor activity: the adherent peritoneal macrophages were incubated with different stimulus, as supernatants of activated platelets (sPLTs) for 6 hours in the ratio 20:1 with macrophages, with I-BOP (2µM, Cayman Chemical, Ann Arbor MI USA) that was a stable TXB_2 analogue, and with SQ 29,548 (10µM, Cayman Chemical, Ann Arbor MI USA), a selective thromboxane receptor antagonist. Macrophages cells were incubated for 4 hours with the stimulus. Subsequently, TF activity was determined on these lysed cells by one-stage plasma recalcification assay. Samples were lysed with 15 mmol/L n-Octyl-B-D-glucopyranoside lysis, and total protein concentrations were determined. Each sample was mixed with citrated pooled WT mouse plasma and $CaCl_2$, and procoagulant activity was quantified by a 1-stage plasma recalcification time assay.

VI. 4.1.1.1 Procoagulant Activity (PCA) assay: Procoagulant activity was measured by recalcification time test of citrated mouse plasma pool at 37°C. To 40µl of sample, pre-incubated at 37°C in temperature bath for 1 minute, were added 40µl of PPP and 40µl of CaCl2 15mM. recalcification times were converted in arbitrary units of tissue factor (TF) by using a calibration curve of thromboplatin extracted from human placenta (Behring).

VI. 4.2 Peritoneal neutrophils isolation: Peritoneal neutrophils were isolated from WT mice. Mice were injected intraperitoneally with 3 ml of 4% thioglycollate medium (Sigma-Aldrich). After 4 hours of injection, mice were sacrificed by CO_2 inhalation and neutrophils were harvested by peritoneal lavage recovered by injecting 5 ml of PBS with a syringe needle 20-G. The exudate thus obtained was centrifuged and pellet suspended in a solution of ACK for lysate the red blood cells potentially present. After a new centrifugation, the pellet was suspended in Dulbecco's modified Eagle's medium containing 10% serum and plated in this same medium. The cells thus obtained were counted and plated in the same medium for 2 hours. Adherent cells were treated in serum free medium with or without stimuli [IBOP (Cayman Chemical), supernatant of stimulated platelets (APS)].

VI. 4.3 Cell culture of RAW 264.7 cells: Mouse leukemic monocyte macrophage cell line (RAW 264.7 cells) were cultured in a sterile flask and maintained in DMEM + GlutaMAX medium (Invitrogen) supplemented with HEPES (1mM, Invitrogen), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 10% FBS , at 37°C in 5% CO_2/95% air atmosphere. RAW 264.7 cells were stimulated with IBOP (2µM) for 4 hours vs control cells.

VI. 5. Protein preparation, assay and immunoblotting: Peritoneal macrophages were used for immunoblotting analysis, to verify the expression of TF protein. After incubation with different stimulus, the cells were lysed in cold RIPA buffer (25mM TRIS HCL, 100Mm NaCl, 2.5Mm EDTA, 1% Triton-X-100, 0,1% SDS, 1Mm Na3VO4, 1mM PMSF, 10mM Na-pyrophosphate, 10mM NaF pH 8.0 and protease inhibitor cocktail). The total protein in cell lysates were quantified by the Micro BCA protein assay kit (Thermo Scientific).

For separation of membrane/cytosol/ nucleus, cells were suspended in hypotonic buffer A (10mmol/l HEPES, pH 7.6, 10mmol/l KCl, 0.1mmol/l MgCl2, 0.1mmol/l DTT, 0.1mmol/l EDTA, 0.5mmol/l PMSF, and 1mmol/l each of pepstatin, aprotinin, and leupeptin) for 10 min in ice and vortexed every 10 seconds. After centrifugation, supernatants were removed as "cytosol/membrane fraction" and pellets, containing nuclei, were washed with buffer A before suspension in buffer C (10mmol/l HEPES, pH 7.6, 400mmol/l NaCl, 1.5mmol/l MgCl2, 0.1mmol/l EDTA, 0.1mmol/l DTT, 0.5mmol/l PMSF, and 1mmol/l pepstatin, aprotinin, and leupeptin and 5% glycerol) for 30 minutes in ice. The supernatants, designed "nuclear fraction", was collected by centrifugation at 12.000 g for 20 minutes. Cytosol/membrane fraction was then ultracentrifugated (10^5 g for 30 min at 4°C), the supernatants were removed as "cytosol fraction" and membranes were washed in buffer A and then dissolved in Laemmli sample buffer.

The protein concentration was obtained by interpolation of a standard curve obtained with increasing concentrations of albumin. Thirty µg samples were prepared with Laemmli method, and equivalent amounts of protein were separated on 12 or 15% SDS-PAGE gels, transferred to nitrocellulose membrane and bands of interest detected using antibodies anti- TF (rabbit anti-Tissue Factor, 1:1000, ab151748, Abcam), anti-Annexi2 (rabbit anti-Annexin-2, 1:1000, ab41803, Abcam), anti-S100A10 (rabbit anti-S100A10, 1:1000, Proteintech) and anti-tubulin (1:10000; Sigma Aldrich) as a loading control. The membranes were then incubated with the peroxidase-conjugated secondary antibody and the band of interest was captured and analyzed by the system ODYSSEY Fc (LI-COR).

VI. 6. Statistical Analysis

Statistical analyses were performed with GraphPad Prism6.0. Data were analyzed by the Mann-Whitney test, Dunn's test or ANOVA with repeated measures for main effects of treatment and time, followed by a Bonferroni post hoc analysis as appropriate. The Spearman coefficient (r) was calculated to quantify correlation between variables. Linear regression analysis was performed. Values of $p<0.05$ were considered statistically significant. Data are expressed as mean ± SEM.

VII. *References*

References

A, Breitenstein. "Sirt1 inhibition promotes in vivo arterial thrombosis and tissue factor expression in stimulated cells." *Cardiovascular Research*, 2011: 464–472.

A, Brill. "Neutrophil extracellular traps promote deep vein thrombosis in mice." *Journal of Thrombosis and Haemostasis*, 2012: 136–144.

A, Caudrillier. "Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury." *Journal of Clinical Investigation*, 2012: 2661-2671.

A, Celi. "Thrombus formation: direct real-time observation and digital analysis of thrombus assembly in a living mouse by confocal and widefield intravital microscopy." *Journal of Thrombosis and Haemostasis*, 2003: 60-68.

A, FitzGerald G. " Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists." *American Journal of Cardiology*, 1991: 11B-15B.

A, Habib. "Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets." *The Journal of Biological Chemistry*, 1999: 2645-2651.

A, Higure. "Macrophages and neutrophils infiltrating into the liver are responsible for tissue factor expression in a rabbit model of acute obstructive cholangitis." *Thorombosis and Haemostasis*, 1996: 791–795.

A, Ichinose. "Factor XIII-mediated cross-linking of NH2-terminal peptide of alpha 2-plasmin inhibitor to fibrin." *FEBS Letters*, 1983: 369–371.

A, Khalafallah. "Evaluation of the innovance d-dimer assay for the diagnosis of disseminated intravascular coagulopathy in different clinical settings." *Clinical and Applied Thrombosis/Hemostasis*, 2014: 91–97.

A, Packham M. "Role of platelets in thrombosis and hemostasis." *Canadian Journal of Physiology and Pharmacology* , 1994: 278-284.

A, Sachinidis. "Thromboxane A2 and vascular smooth muscle cell proliferation." *Hypertension*, 1995: 771–780.

A, Salminen. "SIRT1 longevity factor suppresses NF-kappaB -driven immune responses: regulation of aging via NF-kappaB acetylation?" *Bioessays*, 2008: 939-942.

A, Solovey. "Endothelial Cell Expression of Tissue Factor in Sickle Mice is Augmented by Hypoxia/Reoxygenation and Inhibited by Lovastatin." *Blood*, 2004: 840–846.

A, Solovey. "Tissue factor expression by endothelial cells in sickle cell anemia." *Journal of Clinical Investigation*, 1998: 1899–1904.

A, Tahir. "Monocyte recruitment in venous thrombus resolution." *Journal of Vascular Surgery*, 2006: 601–608.

A, Turpie. "Oral, direct factor Xa inhibitors in development for the prevention and treatment of thromboembolic diseases." *Arteriosclerosis Thrombosis and Vascular Biology*, 2007: 1238-1247.

A, Undas. "Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases." *Arteriosclerosis Thrombosis and Vascular Biology*, 2011: 88–99.

A, Zillmann. "Platelet-associated tissue factor contributes to the collagen-triggered activation of blood coagulation." *Biochemical and Biophysical Research Communications* , 2001: 603–609.

AA, Farooqui. "Signaling and interplay mediated by phospholipase A2, C, and D in LA-N-1 cell nuclei." *Reproduction Nutrition Development*, 2005: 613–631.

AB, Deora. "An annexin 2 phosphorylation switch mediates p11-dependent translocation of annexin 2 to the cell surface." *The Journal of Biological Chemistry*, 2004: 43411-43418.

AB, Holley. "Different finite durations of anticoagulation and outcomes following idiopathic venous thromboembolism: a meta-analysis." *Thrombosis*, 2010.

AF, Costa. "Intravenous apyrase administration reduces arterial thrombosis in a rabbit model of endothelial denudation in vivo ." *Blood Coagulation and Fibrinolysis*, 2004: 545-551.

AJ, Cayatte. "The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis." *Arteriosclerosis Thrombosis and Vascular Biology*, 2000: 1724-1728.

AJ, Connolly. "Role of the thrombin receptor in development and evidence for a second receptor." *Nature*, 1996: 516–519.

AJE, Seely. "Science review: cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance." *Critical Care*, 2003: 291–307.

AK, Chauhan. "von Willebrand factor and factor VIII are independently required to form stable occlusive thrombi in injured veins." *Blood*, 2006: 2424-2429.

AL, Chan. "Celecoxib-induced deep vein thrombosis." *Annals of Pharmacotherapy*, 2005: 1138.

AL, Cilia. "Role of fibrin structure in thrombosis and vascular disease." *Advances in Protein Chemistry and Structural Biology*, 2011: 75–127.

AL, Klatsky. "Risk of pulmonary embolism and/or deep venous thrombosis in Asian-Americans." *American Journal of Cardiology*, 2000: 1334-1337.

AP, McDonald. "Aging is associated with impaired thrombus resolution in a mouse model of stasis induced thrombosis." *Journal of Thrombosis Research*, 2010: 72-78.

AP, Owens. "Microparticles in hemostasis and thrombosis." *Clinical Research*, 2011: 1284–1297.

AP, Surette. "Regulation of fibrinolysis by S100A10 in vivo,." *Blood*, 2011: 3172–3181.

AS, Shet. "Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes." *Blood*, 2003: 2678–2683.

AS, Weyrich. "Change in protein phenotype without a nucleus: Traslational control in platelets." *Seminaries of Thrombosis and Haemostasis*, 2004: 491-498.

AS, Wolberg. "Analyzing fibrin clot structure using a microplate reader." *Blood Coagulation and Fibrinolysis*, 2002: 533–539.

AS, Wolberg. "Elevated prothrombin results in clots with an altered fiber structure: a possible mechanism of the increased thrombotic risk." *Blood*, 2003: 3008–3013.

AS, Wolberg. "Thrombin generation and fibrin clot structure." *Blood Reviews*, 2007: 131–142.

AT, Sands. "The master mammal." *Nature Biotechnology*, 2003: 31-32.

AW, Tsai. "Cardiovascular risk factors and venous thromboembolism incidence: the longitudinal investigation of thromboembolism etiology." *Archives of Internal Medicine*, 2002: 1182-1189.

B, Ahmetaj-Shala. "Reply to letter regarding article "Evidence that links loss of cyclooxygenase-2 with increased asymmetric dimethylarginine: novel explanation of cardiovascular side effects associated with anti-inflammatory drugs"." *Circulation*, 2015: 213-214.

B, Blombäck. "Fibrin in human plasma: gel architectures governed by rate and nature of fibrinogen activation." *Thrombosis Research*, 1994: 521–538.

B, Blombäck. "Fibrin in human plasma: gel architectures governed by rate and nature of fibrinogen activation." *Thrombosis Research*, 1994: 521–538.

B, Dahlback. "Resistance to activated protein C as a risk factor for thrombosis: molecular mechanisms, laboratory investigation, and clinical management." *Seminars in Hematology*, 1997: 217–234.

B, Eriksson. "Oral rivaroxaban compared with subcutaneous enoxaparin for extended thromboprohylaxis after total hip arthroplasty." *Blood*, 2007: abstract.

B, Furie. "Mechanisms of thrombus formation." *The New England Journal of Medicine*, 2008: 938–949.

B, Furie. "Molecular and cellular biology of blood coagulation." *The New England Journal of Medicine*, 1992: 800–806.

B, Hechler. "P2 receptors and platelet function." *Purinergic Signalling*, 2011: 293–303.

B, Huang. "Hypoxia-inducible factor-1 drives annexin A2 system-mediated perivascular fibrin clearance in oxygen-induced retinopathy in mice." *Blood*, 2011: 2918-2929.

B, Jugdutt. "Infarct size reduction by prostacyclin after coronary occlusion in conscious dogs." *Clinical Research*, 1979: 177.

B, McMahon. "Lipoxins: endogenous regulators of inflammation." *American Journal of Physiology*, 2004: 189-201.

B, Nieswandt. "Platelet-collagen interaction: is GPVI the central receptor?" *Blood*, 2003: 449–461.

B, Østerud. "Sites of tissue factor pathway inhibitor (TFPI) and tissue factor expression under physiologic and pathologic conditions." *ISTH Thrombosis and Haemostasis*, 1995: 873-875.

B, Østerud. "Sources of tissue factor." *Seminaris of Thrombosis and Hemostasis*, 2006: 11-23.

B, Østerud. "Tissue factor in neutrophils: no." *Journal of Thrombosis and Haemostasis*, 2004: 218–220.

B, Savage. "Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow." *Cell*, 1998: 657-666.

B, Zeng. "Influence of plasminogen deficiency on the contribution of polymorphonuclear leucocytes to fibrinogenolysis: studies in plasminogen knock-out mice." *Thrombosis and Haemostasis*, 2002: 805-810.

BA, Bouchard. "Human brain pericytes differentially regulate expression of procoagulant enzyme complexes comprising the extrinsic pathway of blood coagulation." *Arteriosclerosis Thrombosis and Vascular Biology*, 1997: 1–9.

BF, McAdam. "Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2." *Proceedings of the National Academy of Sciences*, 1999: 272-277.

BG, Petrich. "Talin is required for integrin-mediated platelet function in hemostasis and thrombosis." *The Journal of Experimental Medicine*, 2007: 3103-3111.

BL, Bayer. "Anti-aggregatory effect of prostacyclin (PGI2) in vivo." *British Journal of Pharmacology*, 1979: 10-12.

BM, Gouse. "New thrombotic events in ischemic stroke patients with elevated Factor VIII." *Thrmombosis* , 2014.

BM, Gouse. "New Thrombotic Events in Ischemic Stroke Patients with Elevated Factor VIII." *Thrombosis*, 2014.

BM, Psaty. "COX-2 Inhibitors — Lessons in Drug Safety." *New England Journal of Medicine*, 2005: 1133-1135.

BM, Psaty. "COX-2 inhibitors—lessons in drug safety." *The New England Journal of Medicine*, 2005: 1133–1135.

BM, Wojcik. "Interleukin-6: A potential target for post-thrombotic syndrome." *Annals of Vascular Surgery*, 2011: 229-239.

Boles, J. "Role of tissue factor in thrombosis in antiphospholipid antibody syndrome." *Lupus*, 2010: 370-378.

BP, Schmitt. "Heparin-associated thrombocytopenia: a critical review and pooled analysis." *American Journal of Medicine Science*, 1993: 208-215.

BS, Selinsky. "Structural analysis of NSAID binding by prostaglandin H2 synthase." *Biochemistry*, 2001: 5172–5180.

C, Auffray. "Blood monocytes: development, heterogeneity, and relationship with dendritic cells." *Annual Review of Immunology*, 2009: 669–692.

C, Becattini. "WARFASA Investigators. Aspirin." *New England Journal of Medicine*, 2012: 1959-1967.

C, Bombardier. "Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group." *New England Journal of Medicine*, 2000: 1520-1528.

C, Carrillo. "Activation of human neutrophils by oleic acid involves the production of reactive oxygen species and a rise in cytosolic calcium concentration: a comparison with N-6 polyunsaturated fatty acids." *Cellular Physiology and Biochemistry* , 2011: 329–338.

C, Denis. "A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis." *Proceedings of the National Academy of Sciences*, 1998: 9524-9529.

C, Dubois. "Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo." *Blood*, 2006: 3902–3906.

C, Gachet. "Regulation of platelet functions by P2 receptors." *Annual Review of Pharmacology and Toxicology*, 2006: 277-300.

C, Gachet. "The platelet P2 receptors in thrombosis." *Seminars in Thrombosis and Hemostasis*, 2005: 162-167.

C, Guichard. "Interleukin-8-induced priming of neutrophil oxidative burst requires sequential recruitment of NADPH oxidase components into lipid rafts." *The Journal of Biological Chemistry*, 2005: 37021–37032.

C, Huerta. "Risk factors and short-term mortality of venous thromboembolism diagnosed in the primary care setting in the United Kingdom." *Archives of Internal Medicine*, 2007: 935-943.

C, Jennewein. "Novel aspects of fibrin(ogen) fragments during inflammation." *Molecular Medicine*, 2011: 568–573.

C, Kearon. " A comparison of three months of anticoagulation with extended anticoagulation for a first episode of idiopathic venous thromboembolism." *New England Journal of Medicine*, 1999: 901-907.

C, Kearon. "Antithrombotic therapy for VTE disease: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines." *Chest*, 2012: 419-494.

C, Longstaff. "The interplay between tissue plasminogen activator domains and fibrin structures in the regulation of fibrinolysis: kinetic and microscopic studies." *Blood*, 2011: 661–668.

C, Luong. "Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2." *Nature Structural & Molecular Biology*, 1996: 927–933.

C, Lupu. "Tissue Factor-Dependent Coagulation Is Preferentially Up-Regulated within Arterial Branching Areas in a Baboon Model of Escherichia coli Sepsis." *American Journal of Pathology*, 2005: 1161–1172.

C, Moosbauer. "Eosinophils are a major intravascular location for tissue factor storage and exposure." *Blood*, 2007: 995–1002.

C, Patrono. "Aspirin as an antiplatelet drug." *New England Journal of Medicine*, 1994: 1287-1294.

C, Patrono. "Platelet-Active Drugs: the relationships among dose effectiveness and side effects." *Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy*, 2004: 234-264.

C, Sung. "Endothelial thromboxane receptors: biochemical characterisation and functional implications." *Biochemical and Biophysical Research Communications*, 1989: 326–333.

CA, Kretz. "Tissue Factor and Thrombosis Models." *Arteriosclerosis, Thrombosis, and Vascular Biology*, 2010: 900-908.

Carson, JL. "The clinical course of pulmonary embolism." *The New England Journal of Medicine*, 1992: 1240-1245.

CC, Naum. "Platelets and ATP prime neutrophils for enhanced O2-generation at low concentrations but inhibit O2-generation at high concentrations." *Journal of Leukocyte Biology*, 1991: 83–89.

Chen, Jianchun. "The α2 Integrin Subunit-Deficient Mouse." *American Journal of Pathology*, 2002: 337–344.

CJ, Carter. "The relationship between the hemorrhagic and antithrombotic properties of low molecular weight heparin in rabbits." *Blood*, 1982: 1239-1245.

CK, Derian. "Species differences in platelet responses to thrombin and SFLLRN. Receptor-mediated calcium mobilization and aggregation and regulation by protein kinases." *Thrombosis Research*, 1996: 505–519.

CK, Roberts. "Effect of diet and exercise intervention on blood pressure, insulin, oxidative stress, and nitric oxide availability." *Circulation*, 2002: 2530-2532.

CL, McGuinness. "Recruitment of labelled monocytes by experimental venous thrombi." *Journal of thrombosis and Haemostasis*, 2001: 1018-1024.

CL, Semerad. "G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood." *Immunity*, 2002: 413–423.

CM, Isacke. "Modulation of p36 phosphorylation in human cells: studies using anti-p36 monoclonal antibodies." *Molecular and Cellular Biology*, 1986: 2745-2751.

CN, Serhan. "Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways." *American Journal of Physiology*, 2007: 101-137.

Collaboration, Antiplatelet Trialists'. "Collaborative overview of randomised trials of antiplatelet therapy: III. Reduction in venous thrombosis and pulmnoary embolism by antiplatelet prophylaxis among surgical and medical patients." *British Journal of Medicine*, 1994: 235-246.

Collaboration, Antithrombotic Trialists'. "Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients." *British Medical Journal*, 2002: 141.

"Collaborative overview of randomised trials of antiplatelet therapy - III: Reduction in venous thrombosis and pulmonary embolism by antiplatelet prophylaxis among surgical and medical patients." *British Medical Journal* , 1994.

CP, Cannon. "COX-2 Inhibitors and Cardiovascular Risk ." *Physiology*, 2012: 1386-1387.

CS, Landefeld. "Major bleeding in outpatients treated with warfarin: incidence and prediction by factors known at the start of outpatient therapy." *American Journal Medicine*, 1989: 144-152.

CS, Williams. "The role of cyclooxygenases in inflammation, cancer and development." *Oncogene*, 1999: 7908–7916.

CV, Denis. "Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen." *Journal of Clinical Investigation*, 2000: 385-392.

CV, Denis. "Platelet adhesion receptors and their ligands in mouse models of thrombosis." *Arteriosclerosis Thrombosis and Vascular Biology*, 2007: 728-739.

CW, Francis. "Antithrombin III prophylaxis of venous thromboembolic disease after total hip or total knee replacement." *American Journal of Medicine*, 1989: 61S.

D, Bergqvist. "The cost-effectiveness of prevention of post-operative thromboembolism." *Acta chirurgica Scandinavica*, 1990: 36-41.

D, Gailani. "Intrinsic Pathway of Coagulation and Arterial Thrombosis." *Arteriosclerosis Thrombosis and Vascular Biology*, 2007: 2507-2513.

D, Kotuličová. "Variability of GP6 gene in patients with sticky platelet syndrome and deep venous thrombosis and/or pulmonary embolism." *Blood Coagulation and Fibrinolysis*, 2013: 543-547.

D, Le. "Hemostatic factors in rabbit limb lymph: relationship to mechanisms regulating extravascular coagulation." *American Journal of Physiology*, 1998: H769–H776.

D, Muller. "Angiotensin II (AT1) receptor blockade reduces vascular tissue factor in angiotensin II-induced cardiac vasculopathy." *American Journal of Pathology*, 2000: 111–122.

D, Picot. "The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1." *Nature*, 1994: 243–249.

D, Wang. "Cardiovascular hazard and non-steroidal anti-inflammatory drugs." *Current Opinion in Pharmacology*, 2005: 556.

D, Wang. "Eicosanoids and cancer." *Nature Reviews Cancer*, 2010: 181-193.

D, Wang. "Prostaglandin E2 promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta." *Cancer Cell*, 2004: 285–295.

DA, Andrews. "Role of red blood cells in thrombosis." *Current Opinion in Hematology*, 1999: 76–82.

DA, Gabriel. "The effect of fibrin structure on fibrinolysis." *The Journal of Biological Chemistry*, 1992: 24259–24263.

DA, Manly. "Role of Tissue Factor in Venous Thrombosis." *Annual Review of Physiology* , 2011: 515-525.

DA, Trégouët. "Common susceptibility alleles are unlikely to contribute as strongly as the FV and ABO loci to VTE risk: results from GWAS approach." *Blood*, 2009: 5298–5303.

DA, Triplett. "Antiphospholipid-protein antibodies: laboratory detection and clinical relevance." *Thrombosis Research*, 1995: 1-31.

DA, Tsakiris. "Hemostasis in the mouse (Mus musculus): a review." *Journal of Thrombosis and Haemostasis*, 1999: 177-188.

DB, Cines. "Endothelial cells in physiology and in the pathophysiology of vascular disorders." *Blood*, 1998: 3527–3561.

DC, Rijken. "New insights into the molecular mechanisms of the fibrinolytic system." *Journal of Thrombosis and Haemostasis*, 2009: 4–13.

DD, Myers. "P-selectin and leukocyte microparticles are associated with venous thrombogenesis." *Journal of Vascular Surgery*, 2003: 1075-1089.

DJ, King. "Heparin-associated thrombocytopenia." *Annuals of International Medicine*, 1984: 535-540.

DJ, Loskutoff. "Type 1 plasminogen activator inhibitor." *International Society on Thrombosis and Haemostasis*, 1989: 87–115.

DL, Richardson. "Chemotaxis for human monocytes by fibrinogen-derived peptides." *British Journal of Haematology*, 1976: 507–513.

DM, Monroe. "Transmission of a procoagulant signal from tissue factor-bearing cell to platelets." *Blood of Coagulation and Fibrinolysis*, 1996: 459–464.

DM, Monroe. "Transmission of a procoagulant signal from tissue factor-bearing cell to platelets." *Blood Coagulation and Fibrinolysis*, 1996: 459–464.

DM, Waisman. "Annexin II, tetramer: structure and function." *Molecular and Cellular Biochemistry*, 1995: 301–322.

DR, Knighton. "Macrophage-derived growth factors in wound healing(regulation of growth factor production by the oxygen microenvironment)." *American Review of Respiratory Disorders*, 1108–1111: 1989.

DR, Knighton. "The macrophages(effector cell wound repair)." *Clinical and Biological Research*, 1989: 217–226.

E, Arehart. "Prostacyclin, atherothrombosis, and cardiovascular disease." *Current Medicinal Chemistry*, 2007: 2161-2169.

E, Camerer. "Cell biology of tissue factor, the principal initiator of blood coagulation ." *Thrombosis Research*, 1996: 1-41.

E, Demetz. "The Arachidonic Acid Metabolome Serves as a Conserved Regulator of Cholesterol Metabolism." *Cellular Metabolism*, 2014: 787–798.

E, Ramacciotti. "Leukocytes- and platelet-derived microparticles correlated with thrombus weight and tissue factor activity in an experimental mouse model of venous thrombosis." *Thrombosis and Haemostasis*, 2009: 748-754.

E, Rolando. "Platelet-Vessel Wall Interactions in Hemostasis and Thrombosis." *Morgan & Claypool Life Sciences*, 2010.

E, Stein. "The coagulopathy of acute promyelocytic leukaemia revisited." *Best Practice & Research Clinical Haematology* , 2009: 153–163.

EA, Peterson. "Thrombin induces endothelial cell-surface exposure of the plasminogen receptor annexin 2." *Journal of Cell Science*, 2003: 2399-2408.

EC, Flood. "The annexin A2 system and vascular homeostasis." *Vascular Pharmacology*, 2011: 59–67.

EC, Flood. "The annexin A2 system and vascularhomeostasis." *Vascular Pharmacology* , 2011: 59-67.

ED, Sprengers. "Plasminogen activator inhibitors." *Blood*, 1987: 381–387.

EE, Weinmann. "Deep-Vein Thrombosis." *New England Journal of Medicine*, 1994: 1630-1641.

EF, Plow. "The presence and release of alpha 2-antiplasmin from human platelets." *Blood*, 1981: 1069–1074.

EG, Brooks. "Valves of the deep venous system: an overlooked risk factor." *Blood*, 2008: 367–369.

EG, Levin. "Association of a plasminogen activator inhibitor (PAI-1) with the growth substratum and membrane of human endothelial cells." *The Journal of Cell Biology*, 1987: 2543–2549.

EJ, Weiss. "Protection against thrombosis in mice lacking PAR3." *Blood*, 2003: 3240-3244.

EJ, Westgate. "Pulmonary embolism in a woman taking oral contraceptives and valdecoxib." *PLoS Medicine*, 2005: 197.

EM, Egorina. "Intracellular and surface distribution of monocyte tissue factor: application to intersubject variability." *Arteriosclerosis Thrombosis and Vascular Biology*, 2005: 1493-1498.

ES, Barnathan. "Tissue-type plasminogen activator binding to human endothelial cells: evidence for two distinct binding sites." *The Journal of Biological Chemistry*, 1988: 7792–7799.

EV, Faustino. "Factor VIII May Predict Catheter-Related Thrombosis in Critically Ill Children: A Preliminary Study." *Pediatric Critical Care Medicine*, 2015: 497-504.

EV, Faustino. "Factor VIII May Predict Catheter-Related Thrombosis in Critically Ill Children: A Preliminary Study." *Pediatric Critical Care Medicine*, 2015: 497-504.

EW, Davie. "An overview of the structure and function of thrombin." *Seminars in Thrombosis and Hemostasis*, 2006: 3-15.

EW, Salzman. "Effect of heparin and heparin fractions on platelet aggregation." *Journal of Clinical Investigation*, 1980: 64-73.

EW, Salzman. "Prophylaxis of venous thromboembolism: analysis of cost effectiveness." *Annals of Surgery*, 1980: 207-218.

EW, Salzman. "The epidemiology, pathogenesis, and natural history of venous thrombosis." *Hemostasis and thrombosis: basic principles and clinical practice*, 1993: 1275-1298.

F, Boulton. "A hundred years of cascading - started by Paul Morawitz (1879-1936), a pioneer of haemostasis and of transfusion." *Transfusion Medicine*, 2006: 1-10.

F, Boutitie. "Influence of preceding length of anticoagulant treatment and initial presentation of venous thromboembolism on risk of recurrence after stopping treatment: analysis of individual participants data from seven trials ." *British Medical Journal*, 2011: 3036.

F, Müller. "Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo." *Cell*, 2009: 1143–1156.

F, Öner Erkekol. "High plasma levels of factor VIII: an important risk factor for isolated pulmonary embolism." *Respirology*, 2006: 70-74.

FA, Baglia. "Prothrombin is a cofactor for the binding of factor XI to the platelet surface and for platelet-mediated factor XI activation by thrombin." *Biochemistry*, 1998: 2271–2281.

FA, Baglia. "rothrombin is a cofactor for the binding of factor XI to the platelet surface and for platelet-mediated factor XI activation by thrombin." *Biochemistry*, 1998: 2271–2281.

FA, Siddiqui. "The presence and release of tissue factor from human platelets." *Platelets*, 2002: 247–253.

FE, Silverstein. "Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. Celecoxib Long-term Arthritis Safety Study." *JAMA*, 2000: 1247-1255.

FR, Sheppard. "Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation." *Journal of Leukocyte Biology*, 2005: 1025–1042.

G, Agnelli. " Warfarin Optimal Duration Italian Trial Investigators. Extended oral anticoagulant therapy after a first episode of pulmonary embolism." *Annals of Internal Medicine*, 2003: 19-25.

G, Agnelli. "AMPLIFY Investigators. Oral apixaban for the treatment of acute venous thromboembolism." *New England JOurnal of Medicine*, 2013: 799-808.

G, Agnelli. "PLIFY-EXT Investigators. Apixaban for extended treatment of venous thromboembolism." *New England Journal of Medicine*, 2013: 699-708.

G, Agnelli. "Warfarin Optimal Duration Italian Trial Investigators. Three months versus one year of oral anticoagulant therapy for idiopathic deep vein thrombosis." *New England Journal Medicine*, 2001: 165-169.

G, Bu. "Cellular receptors for the plasminogen activators." *Blood*, 1994: 3427–3436.

G, Cesarman-Mau. "Molecular mechanisms of fibrinolysis." *British Journal of Haematology* , 2005: 307–321.

G, Cesarman-Maus. "Autoantibodies against the fibrinolytic receptor, annexin 2, in antiphospholipid syndrome." *Blood*, 2006: 4375–4382.

G, Cesarman-Maus. "Autoantibodies against the fibrinolytic receptor, annexin A2, in cerebral venous thrombosis." *Stroke*, 2011: 501–503.

G, Cesarman-Maus. "Molecular mechanisms of fibrinolysis." *British Journal of Haematology*, 2005: 307–321.

G, Clagett. "Prevention of venous thromboembolism." *Chest*, 1992: 391-407.

G, Davì. "Diabetes mellitus, hypercholesterolemia, and hypertension but not vascular disease per se are associated with persistent platelet activation in vivo. Evidence derived from the study of peripheral arterial disease." *Circulation*, 1997: 69-75.

G, Davì. "In vivo formation of 8-iso-prostaglandin f2alpha and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation." *Circulation*, 1999: 224-229.

G, Hollopeter. "Identification of the platelet ADP receptor targeted by antithrombotic drugs." *Nature*, 2001: 202-207.

G, Hron. "Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer." *Thrombosis and Haemostasis*, 2007: 119–123 .

G, Kauffenstein. "Adenine triphosphate nucleotides are antagonists at the P2Y receptor." *Journal of Thrombosis and Haemostasis*, 2004: 1980-1988.

G, Palareti. "Risk of venous thromboembolism recurrence: high negative predictive value of D-dimer performed after oral anticoagulation is stopped." *Journal of Thrombosis and Haemostasis*, 2002: 7-12.

G, Riccioni. "The role of the antioxidant vitamin supplementation in the prevention of cardiovascular diseases." *Expert Opinion on Investigational Drugs*, 2007: 25-32.

GA, Cabral. "Lipids as bioeffectors in the immune system." *Life Science*, 2005: 1699–1710.

GA, Donaldson. "A reappraisal of the application of the Trendelenburg operation to massive fatal embolism: report of a successful pulmonary-artery thrombectomy using a cardiopulmonary bypass." *New England Journal of Medicine*, 1963: 171-174.

GA, FitzGerald. "COX-2 and beyond: approaches to prostaglandin inhibition in human disease." *Nature Reviews Drug Discovery*, 2003: 879–890.

GA, FitzGerald. "The coxibs, selective inhibitors of cyclooxygenase-2." *New England Journal of Medicine*, 2001: 433–442.

GA, Higgs. "Prostacyclin (PGI2) inhibits the formation of platelet thrombi induced by adenosine diphosphate (ADP) in vivo [proceedings]." *British Journal of Pharmacology*, 1977: 137.

GC, Parry. "Transcriptional regulation of tissue factor expression in human endothelial cells." *Arteriosclerosis, Thrombosis, and Vascular Biology*, 1995: 612–621.

GD, Paiement. "Cost-effectiveness of prophylaxis in total hip replacement." *American Journal of Surgery*, 1991: 519-524.

GD, Virca. "Simplified Northern blot hybridization using 5% sodium dodecyl sulfate." *Biotechniques*, 1990: 370-371.

GJ, Broze. "Coagulation-dependent inhibition of fibrinolysis: role of carboxypeptidase-U and the premature lysis of clots from hemophilic plasma." *Blood*, 1996: 3815–3823.

GJ, Broze Jr. "The rediscovery and isolation of TFPI." *Journal of Thrombosis and Haemostasis*, 2003: 1671–1675.

GJ, Miller. "Haemostatic factors in human peripheral afferent lymph." *Journal of Thrombosis and Haemostasis*, 2000: 427–432.

GP, Brown. "Thromboxane receptors in human kidney tissue." *Prostaglandins & Other Lipid Mediators*, 1999: 179–188.

GP, Clagett. "Prevention of venous thromboembolism in general surgical patients: results of meta-analysis." *Annals of Surgery*, 1988: 227-240.

GR, Sambrano. "Role of thrombin signalling in platelets in haemostasis and thrombosis." *Nature*, 2001: 74–78.

Group, Physicians' Health Study Research. "Final Report on the Aspirin Component of the Ongoing Physicians' Health Study." *New England Journal of Medicine*, 1989: 129-135.

Group, Pulmonary Embolism Prevention (PEP) Trial Collaborative. "Prevention of pulmonary embolism and deep vein thrombosis with low dose aspirin: Pulmnoary Embolism Prevention (PEE) Trial." *Lancet*, 2000: 1295-1302.

Group, Thromboembolic Risk Factors (THRIFT) Consensus. "Risk of and prophylaxis for venous thromboembolism in hospital patients." *British Medical Journal*, 1992: 567-574.

GV, Born. "Adenosine diphosphate as a mediator of platelet aggregation in vivo: an editorial view." *Circulation*, 1985: 741-746.

H, Austin. "New gene variants associated with venous thrombosis: a replication study in United States whites and blacks." *Journal of Thrombosis and Haemostasis*, 2011: 489–495.

H, Heijboer. "Deficiencies of coagulation-inhibiting and fibrinolytic proteins in outpatients with deep-vein thrombosis." *The New England Journal of Medicine*, 1990: 1512–1515.

H, Hölschermann. "In situ detection of tissue factor within the coronary intima in rat cardiac allograft vasculopathy." *American Journal of Pathology*, 1999: 211–220.

H, Ishihara. "Protease-activated receptor 3 is a second thrombin receptor in humans." *Nature*, 1997: 502–506.

H, Jung. "Intracellular Annexin A2 regulates NF-kB signaling by binding to the p50 subunit: implications for gemcitabine resistence in pancreatic cancer." *Cell Death & Disease*, 2015.

H, Ni. "Increased thrombogenesis and embolus formation in mice lacking glycoprotein V." *Blood*, 2001: 368–373.

H, Ni. "Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen." *Journal of Clinical Investigation*, 2000: 385-392.

H, Ni. "Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen." *The Journal of Clinical Investigation*, 2000: 385–392.

H, Saito. "Synthesis and release of Hageman factor (Factor XII) by the isolated perfused rat liver." *Journal of Clinical Investigation*, 1983: 948–954.

H, Shulze. "Dynamic visualization of thrombopoiesis within bone marrow." *Science*, 2007: 1767-1770.

H, Todoroki. "Neutrophils express tissue factor in a monkey model of sepsis." *Surgery*, 2000: 209–216.

HA, Hajjar. "The molecular basis of fibrinolysis." *Nathan and Orkin's Hematology of Infancy and Childhood (8th ed.)*, 2014.

HE, Roald. "HN-11500–a novel thromboxane A2 receptor antagonist with antithrombotic activity in humans at arterial blood flow conditions." *Journal of Thrombosis and Haemostasis*, 1994: 103-109.

Health, Yale New Haven. "Glycoprotein IIb/IIIa inhibitors for heart attack and unstable angina." 2003.

HF, Dvorak. "Abnormalities of hemostasis in malignant disease." *Hemostasis and thrombosis: basic principles and clinical practice. 3rd ed. Philadelphia*, 1994: 1238-1254.

HJ, Ting. "Thromboxane A2 receptor: biology and function of a peculiar receptor that remains resistant for therapeutic targeting." *Journal of Cardiovascular Pharmacology and Therapeutics*, 2012: 248–259.

HJ, Weiss. "Platlets physiology and abnormalities of platelet function (secondo of two parts)." *The New England Journal of Medicine*, 1975: 580-588.

HL, Cheng. "Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice." *Proceedings of the National Academy of Sciences*, 2010: 10794-10799.

hormone, WHO collaborative study of cardiovascular disease and steroid. "Venous thromboembolic disease and combined oral contraceptives: results of international multicentre case-control study." *Lancet*, 1995: 1575–1582.

HR, Büller. "EINSTEIN–PE Investigators. Oral rivaroxaban for the treatment of symptomatic pulmonary embolism." *New England Journal of Medicine*, 2012: 1287-1297.

I, Algaier. "Interaction of the active metabolite of prasugrel, R-138727, with cysteine 97 and cysteine 175 of the human P2Y12 receptor." *Journal of Thrombosis and Haemostasis*, 2008: 1908-1914.

I, Hrachovinová. "Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A." *Nature Medicine*, 2003: 1020-1025.

I, Morita. "Distinct functions of COX-1 and COX-2." *Prostaglandins & Other Lipid Mediators*, 2002: 165–175.

I, Muller. "Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets." *FASEB Journal*, 2003: 476–478.

I, Sakuma. "Positive inotropic effect of the thromboxane analogue U-46619 on guinea pig left atrium: mediation by specific receptors and association with increased phosphoinositide turnover." *Canadian Journal of Physiology and Pharmacology*, 1989: 943–949.

I, Singh. "Failure of thrombus to resolve in urokinase-type plasminogen activator gene-knockout mice(rescue by normal bone marrow-derived cells) ." *Circulation*, 2003: 869–875.

IA, Naess. "Incidence and mortality of venous thrombosis: a population-based study." *Journal of Thrombosis and Haemostasis*, 2007: 692-699.

ID, Bezemer. "Gene variants associated with deep vein thrombosis." *Jama*, 2008: 1306–1314.

Investigators, The EINSTEIN. "Oral rivaroxaban for symptomatic venous thromboembolism." *New England Medicine*, 2010: 2499-2510.

J, Bylund. "Lipopolysaccharide-induced granule mobilization and priming of the neutrophil response to Helicobacter pylori peptide Hp(2-20), which activates formyl peptide receptor-like 1." *Infection and Immunity* , 2002: 2908–2914.

J, Chen. "The α(2) integrin subunit-deficient mouse: a multifaceted phenotype including defects of branching morphogenesis and hemostasis. ." *American Journal of Pathology*, 2002: 337–344.

J, Chou. "Hematopoietic cell-derived microparticle tissue factor contributes." *Hemostasis Thrombosis and Vascular Biology*, 2004: 3190-3197.

J, Hirsh. "Approach to the thrombophilic patient for hemostasis and thrombosis: basic princples and clinical practice." *Hemostasis and thrombosis: basic principles and clinical practice, JB Lippincott, Philadelphia*, 1993: 1543–1561.

J, Hirsh. "Beyond unfractionated heparin and warfarin: current and future advances." *Circulation*, 2007: 552–560.

J, Hirsh. "Heparin." *New England Journal of Medicine*, 1991: 1565-1574.

J, Hirsh. "Oral anticoagulants: mechanisms of action, clinical effectiveness, and optimal therapeutic range." *Chest*, 1992: 312-326.

J, Hirsh. "Prognosis in acute pulmonary embolism." *Lancet*, 1999: 1375-1376.

J, Humphries. "Monocyte chemotactic protein-1 (MCP-1) accelerates the organization and resolution of venous thrombi." *Journal of Vascular Surgery*, 1999: 894–899.

J, Jesty. "Initiation of the tissue factor pathway of coagulation in the presence of heparin: control by antithrombin III and tissue factor pathway inhibitor." *Blood*, 1996: 2301–2307.

J, Kooiman. "The HAS-BLED Score identifies patients with acute venous thromboembolism at high risk of major bleeding complications during the first six mounths of anticoagultant treatment." *PloS One*, 2015: 1-11.

J, Polgar. "The P-selectin, tissue factor, coagulation triad." *Thrombosis and Haemostasis*, 2005: 1590–1596 .

J, Simes. "Aspirin for the Prevention of Recurrent Venous Thromboembolism: The INSPIRE Collaboration ." *Circulation*, 2015.

J, Thaler. "Microparticle-associated tissue factor activity in patients with acute unprovoked deep vein thrombosis and during the course of one year." *Thrombosis Reasearch*, 2014: 1093-1096.

J, Travis. "Human plasma proteinase inhibitors." *Annual Review of Biochemistry* , 1983: 655–709.

J, Ware. "Dysfunctional platelet membrane receptors: from humans to mice." *Thrombosis and Haemostasis*, 2004: 478-485.

J, Zhou. "Inferior vena cava ligation rapidly induces tissue factor expression and venous thrombosis in rats." *Arteriosclerosis Thrombosis and Vascular Biology*, 2009: 863-869.

JA, Diaz. "Impaired fibrinolytic system in apoe gene-deleted mice with hyperlipidemia augments deep vein thrombosis." *Journal of Vascular Surgery*, 2011.

JA, Heit. " Predictors of recurrence after deep vein thrombosis and pulmonary embolism." *JAMA International Medicine*, 2000: 761-768.

JA, Heit. "The epidemiology of venous thromboembolism in the community: implications for prevention and management." *Journal of Thrombosis and Thrombolysis*, 2006: 23-29.

JA, Heit. "Venous thromboembolism epidemiology: implications for prevention and management." *Seminars in Thrombosis and Hemostasis* , 2002: 3-14.

JA, Heit. "Venous thromboembolism: disease burden, outcomes and risk factors." *Journal of Thrombosis and Hemostasis*, 2007: 1611-1617.

JA, Lopez. "Dysfunctional platelet membrane receptors: from humans to mice." *Blood*, 1998: 4397-4418.

JA, Oliver. "Activated protein C cleaves factor Va more efficiently on endothelium than on platelet surfaces." *Blood*, 2002: 539–546.

JA, Oliver. "Activated protein C cleaves factor Va more efficiently on endothelium than on platelet surfaces." *Blood*, 2002: 539–546.

JD, Hamer. "The PO2 in venous valve pockets: its possible bearing on throm-bogenesis." *British Journal of Surgery*, 1981: 166–170.

JD, Stamatakis. "Femoral vein thrombosis and total hip replacement." *British Molecular Journal*, 1977: 223-225.

JE, Dalen. "Venous thromboembolism: scope of the problem." *Chest*, 1986: S370-S373.

JE, Geddings. "Tissue Factor-positive tumor microvesicles activate platelets and ehnance thrombosis in mice." *Journal of Thrombosis and Hemostasis*, 2015.

JE, Sadler. "Thrombomodulin structure and function." *Journal of Thrombosis and Haemostasis*, 1997: 392-395.

JF, Bohmfalk. "Plaminogen is synthesized by primary cultures of rat hepatocytes." *Science*, 1980: 408-410.

JH, Lee. "Overexpression of SIRT1 protects pancreatic beta-cells against cytokine toxicity by suppressing the nuclear factor-kappaB signaling pathway." *Diabetes*, 2009: 344-351.

JH, Morrissey. " Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade." *Cell*, 1987: 129-135.

JI, Weitz. "Beyond heparin and warfarin: the new generation of anticoagulants." *Expert Opinion on Investigational Drugs*, 2007: 271–282 .

JI, Weitz. "Direct thrombin inhibitors in acute coronary syndromes: present and future." *Circulation*, 2002: 1004-1011.

JJ, Hathcock. "Platelet deposition inhibits tissue factor activity: in vitro clots are impermeable to factor Xa." *Blood*, 2004: 123-127.

JL, Carson. "The clinical course of pulmonary embolism." *New England Journal of Medicine*, 1992: 1240-1245.

JL, Mehta. "Human vascular tissues produce thromboxane as well as prostacyclin." *American Journal of Physiology*, 1983: 839–444.

JL, Mehta. "Increased prostacyclin and thromboxane biosynthesis in atherosclerosis." *Proceedings of the National Academy of Sciences*, 1988: 4511–4515.

JL, Mehta. "Prostacyclin and thromboxane A2 production by human cardiac atrial tissues." *American Heart Journal*, 1985: 1-3.

JL, Wallace. "The therapeutic potential of NO-NSAIDs." *Fundamental & Clinical Pharmacology*, 2003: 11–20.

JL, Williams. "Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) alter the kinetics of human colon cancer cell lines more effectively than traditional NSAIDs: implications for colon cancer chemoprevention." *Cancer Research*, 2001: 3285–3289.

JM, Chirgwin. "Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease." *Biochemistry*, 1979: 5294-5299.

JM, Flanagan. "Genetic predictors for stroke in children with sickle cell anemia." *Blood*, 2011: 6681–6684.

JM, Herbert. "Importance of platelets in experimental venous thrombosis in the rat." *Blood*, 1992: 2281-2286.

JM, Walenga. "Sustained release of tissue factor following thrombosis of lower limb trauma." *Clinical and Applied Thrombosis/Hemostasis*, 2014: 678-686.

JN, Lozier. "Clinical aspects and therapy of hemophilia, in Hoffman R, Benz E, Shattil S, Furie B, Cohen H, Silberstein L, McGlave P (eds)." *Hematology: Basic Principles and Practice*, 2005: 2047.

JP, Collet. "Dynamic changes of fibrin architecture during fibrin formation and intrinsic fibrinolysis of fibrin-rich clots." *The Journal of Biological Chemistry*, 2003: 21331–21335.

Jr, Myers D. "Selectins influence thrombosis in a mouse model of experimental deep venous thrombosis." *Journal of Surgery Research*, 2002: 212-221.

JS, Bennett. "Novel platelet inhibitors." *Annual Review of Medicine*, 2001: 161-184.

JS, Menell. "Annexin II and bleeding in acute promyelocytic leukemia." *The New England Journal of Medicine*, 1999: 994–1004.

JT, Dancey. "Neutrophil kinetics in man." *Journal of Clinical Investigation*, 1976: 705–715.

JW, Eikelboom. "Anticoagulation for venous thromboembolism." *British Medical Journal*, 2007: 645.

K, Bailey. "Action of thrombin in the clotting of fibrinogen ." *Nature*, 1951: 233–234.

K, Bailey. "The clotting of fibrinogen. I. The liberation of peptide material." *Biochimica et Biophysica Acta (BBA)*, 1955: 495–503.

K, Broos. "Platelets at work in primary hemostasis." *Blood Reviews*, 2011: 155–167.

K, Eto. "Platelet aggregation in acute coronary syndromes: use of a new aggregometer with laser light scattering to assess platelet aggregability." *Cardiovascular Research* , 1998: 223-229.

K, Misumi. "Comparison of plasma tissue factor levels in unstable and stable angina pectoris." *American Journal of Pathology*, 1998: 22–26.

K, Onnheim. "Tumour necrosis factor (TNF)-alpha primes murine neutrophils when triggered via formyl peptide receptor-related sequence 2, the murine orthologue of human formyl peptide receptor-like 1, through a process involving the type I TNF receptor and subcellular ." *Immunology*, 2008: 591–600.

K, Ritis. "A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways." *The Journal of Immunology*, 2006: 4794–4802.

K, Ritis. "A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways." *Journal of Immunology*, 2006: 4794–4802.

K, Schrör. "Thromboxane A2 and prevention of cardiovascular diseases." *Z Kardiol*, 1992: 185-189.

K, Szuldrzynski. "Elevated levels of 8-iso-prostaglandin F2alpha in acute coronary syndromes are associated with systemic and local platelet activation." *Polskie Archiwum Medycyny Wewnętrznej* , 2010: 19-24.

K, Takahara. " The response to thromboxane A2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems." *The Journal of Biological Chemistry*, 1990: 6836-6844.

K, Wikstrom. "Differential regulation of RhoA-mediated signaling by the TPalpha and TPbeta isoforms of the human thromboxane A2 receptor: independent modulation of TPalpha signaling by prostacyclin and nitric oxide." *Cellular Signalling*, 2008: 1497-1512.

K, Yamagata. "Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids." *Neuron - Cell*, 1993: 371-386.

KA, Hajjar. "Homocysteine-induced modulation of tissue plasminogen activator binding to its endothelial cell membrane receptor." *Journal of Clinical Investigation*, 1993: 2873-2879.

KA, Hajjar. "Identification and characterization of human endothelial cell membrane binding sites for tissue plasminogen activator and urokinase." *The Journal of Biological Chemistry*, 1990: 2908–2916.

KA, Hajjar. "Interaction of the fibrinolytic receptor, annexin II, with the endothelial cell surface. Essential role of endonexin repeat 2." *The Journal of Biological Chemistry*, 1996: 21652-21659.

KA, Hogan. "Mouse models in coagulation." *Journal of Thrombosis and Haemostasis*, 2002: 563-574.

KB, Neeves. "Thrombin flux and wall shear rate regulate fibrin fiber deposition state during polymerization under flow." *Biophysical Journal - Cell*, 2010: 1344–1352.

KC, Gersh. "Flow rate and fibrin fiber alignment." *Journal of Thrombosis and Haemostasis* , 2010: 2826–2828.

KC, Kent. "Identification of functional PGH2/TxA2 receptors on human endothelial cells." *Circulation Research*, 1993: 958–965.

KJ, Clemetson. "Platelet collagen receptors." *Thrombosis and Haemostasis*, 2001: 189-197.

KL, He. "Endothelial cell annexin A2 regulates polyubiquitination and degradation of its binding partner S100A10/p11." *The Journal of Biological Chemistry*, 2008: 19192-19200.

KM, Tate. "Functional role of proteolytic cleavage at arginine-275 of human tissue plasminogen activator as assessed by site-directed mutagenesis." *Biochemistry*, 1987: 338–343.

KS, Prasad. "Soluble CD40 ligand induces β3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling." *Proceedings of the National Academy of Sciences*, 2003: 12367–1237.

L, Alberio. "Review article: platelet-collagen interactions: membrane receptors and intracellular signalling pathways." *European Journal of Clinical Investigation*, 1999: 1066–1076.

L, Fatimathas. "Annexins as disease modifiers." *Histology and Histopathology*, 2010: 527–532.

L, Ganjehei. "S T elevation myocardial infarction: recent advances and updates." *Future Cardiology*, 2014: 633-666.

L, Hansson. "Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group." *Lancet*, 1998: 1755-1762.

L, He. "The contributions of the α2β1 integrin to vascular thrombosis in vivo." *Blood*, 2003: 3652–3657.

L, Laine. "The gastrointestinal effects of nonselective NSAIDs and COX-2-selective inhibitors." *Seminars in Arthritis and Rheumatism* , 2002: 25-32.

L, Pandi. "Two families of synthetic peptides that enhance fibrin turbidity and delay fibrinolysis by different mechanisms." *Biochemistry*, 2009: 7201–7208.

L, Wang. "Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury." *Blood*, 2009: 705-713.

L, Zhang. "Plasminogen has a broad extrahepatic distribution." *Thrombosis and Haemostasis*, 2002: 493-501.

LA, Guthrie. "Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme." *Journal of Experimental Medicine*, 1984: 1656–1671.

LA, Linkins. "Clinical impact of bleeding in patients taking oral anticoagulant therapy for venous thromboembolism: a meta-analysis." *Annuals of International Medicine*, 2003: 893-900.

listed, No authors. "Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. The Medical Research Council's General Practice Research ." *Lancet*, 1998: 233-241.

LK, Boshkov. "Heparin-induced thrombocytopenia and thrombosis: clinical and laboratory studies." *British Journal of Haematology*, 1993: 322-328.

LM, Lau. "The tetraspanin superfamily member CD151 regulates outside-in integrin αIIbbeta3 signaling and platelet function." *Blood*, 2004: 2368–2375.

LR, Lopez. "Platelet thromboxane (11-dehydro-Thromboxane B2) and aspirin response in patients with diabetes and coronary artery disease." *World Journal of Diabetes*, 2014: 115-127.

M, Cattaneo. "ADP receptors and clinical bleeding disorders." *Arteriosclerosis of Thrombosis and Vascular Biology*, 1999: 2281-2285.

M, Colucci. "Cultured human endothelial cells generate tissue factor in response to endotoxin." *Journal of Clinical Investigation*, 1983: 1893–1896.

M, Coolman. "Angiogenic and fibrinolytic factors in blood during the first half of pregnancy and adverse pregnancy outcomes." *Obstetrics & Gynecology*, 2012: 1190–1200.

M, Coolman. "Concentrations of plasminogen activators and their inhibitors in blood preconceptionally, during and after pregnancy." *European Journal of Obstetrics & Gynecology*, 2006: 22–28.

M, Dassah. "The endothelial cell annexin A2 system and vascular fibrinolysis." *General Physiology and Biophysics*, 2009: 20-28.

M, den Heijer. "Hyperhomocysteinemia as a risk factor for deep-vein thrombosis." *The New England Journal of Medicine*, 1996: 759–762.

M, den Heijer. "Is hyperhomocysteinaemia a risk factor for recurrent venous thrombosis?" *Lancet*, 1992: 882-885.

M, Dougald. "What Does It Take to Make the Perfect Clot?" *Arteriosclerosis Thrombosis and Vascular Biology*, 2006: 41-48.

M, Franchini. "Venous and arterial thrombosis: different sides of the same coin?" *European Journal of International Medicine*, 2008: 476-481.

M, Ghosh. "COX-2 suppresses tissue factor expression via endocannabinoid-directed PPARdelta activation." *Journal of Experimental Medicine*, 2007: 2053–2061.

M, Gomes. "Risk assessment for thrombosis in cancer." *Seminars in Thrombosis and Hemostasis*, 2014: 319–324.

M, Hoylaerts. "Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin." *The Journal of Biological Chemistry*, 1982: 2912–2919.

M, Kwon. "S100A10, annexin A2, and annexin A2 heterotetramer as candidate plasminogen receptors." *Frontiers in Bioscience*, 2005: 300-325.

M, Miniati. "Fibrin resistance to lysis in patients with pulmonary hypertension other than thromboembolic." *American Journal of Respiratory and Critical Care Medicine*, 2010: 992–996.

M, Murakami. "Different functional aspects of the group II subfamily (types IIA and V) and type X secretory phospholipase A(2)s in regulating arachidonic acid release and prostaglandin generation." *The Journal of Biological Chemistry*, 1999: 31435–31444.

M, Nordström. "A prospective study of the incidence of deep-vein thrombosis within a defined urban population." *Journal of International Medicine*, 1992: 155-160.

M, Okazaki. "PPARbeta/delta regulates the human SIRT1 gene transcription via Sp1." *Endocrine Journal*, 2010: 403–413.

M, Reilly. "Cellular activation by thromboxane A2 and other eicosanoids." *European Heart Journal*, 1993: 88-93.

M, Schmidt. "Non-steroidal anti-inflammatory drug use and risk of venous thromboembolism." *Journal of Thrombosis and Haemostasis*, 2011: 1326-1333.

M, Schneider. "A study of the protection of plasmin from antiplasmin inhibition within an intact fibrin clot during the course of clot lysis." *The Journal of Biological Chemistry*, 2004: 13333–13339.

M, Swaminathan. "Platelet Receptors for Adenine Nucleotides and Thromboxane A2." *Seminars in Thrombosis and Hemostasis*, 2004: 411-418.

M, Tesselaar. "Microparticle-associated tissue factor activity: a link between cancer and thrombosis?" *Thrombosis and Haemostasis*, 2007: 520–527 .

M, Waltham. "Vascular endothelial growth factor and basic fibroblast growth factor are found in resolving venous thrombi." *Journal of Vascular Surgery*, 2000: 988–996.

Mackman, Nigel. "Triggers, targets and treatments for thrombosis." *Nature*, 2008: 914-918.

Maria-Cristina, Andrei. "Is there a link between Atherothrombosis and Deep Venous Thrombosis?" *Journal of Clinical Medicine*, 2014: 94-97.

MC, Andrei. "Is there a Link Between Atherothrombosis and Deep Venous Thrombosis?" *Maedica (Buchar)*, 2014: 94-97.

MC, Berndt. "The vascular biology of the glycoprotein Ib-IX-V complex." *Journal of Thrombosis and Haemostasis*, 2001: 178-188.

MD, Silverstein. "Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study." *Archives of International Medicine* , 1998: 585-593.

ME, Carr. "Effect of fibrin structure on plasmin-mediated dissolution of plasma clots." *Blood Coagulation and Fibrinolysis*, 1995: 567–573.

ML, Brühl. "Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo." *Journal of Experimental Medicine*, 2012: 819–835.

ML, Capone. "Pharmacodynamic of cyclooxygenase inhibitors in humans." *Prostaglandins Other Lipid Mediators*, 2007: 85-94.

ML, Kahn. "A dual thrombin receptor system for platelet activation." *Nature*, 1998: 690-694.

ML, Ogletree. "Studies on the protective effect of prostacyclin in acute myocardial ischemia." *European Journal of Pharmacology*, 1979: 95-103.

ML, Villalba. "FDA medical officer review of VIOXX (rofecoxib), NDA 21-042 (capsules) and NDA 21-052 (oral solution)." *http://www.fda.gov/cder/foi/nda/index.htm*, 2005.

MN, Aghourian. "In vivo monitoring of venous thrombosis in mice ." *Journal of Thromobsis and Haemostasis*, 2012: 447-452.

MO, Mosnier. "The defective down regulation of fibrinolysis in haemophilia A can be restored by increasing the TAFI plasma concentration." *Journal of Thrombosis and Haemostasis*, 2001: 1035–1039.

MP, Bevilacqua. "Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1." *Proceedings of the National Academy of Sciences*, 1986: 4533–4537.

MR, Varma. "Neutropenia impairs venous thrombosis resolution in the rat." *Journal of Vascular Surgery*, 2003: 1090-1098.

MS, Sawdey. "Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo: tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-α, and transforming growth factor-β." *Journal of Clinical Investigation*, 1991: 1346–1353.

MW, Goschnick. "Impaired “outside-in” integrin alphaIIbβ3 signaling and thrombus stability in TSSC6-deficient mice." *Blood*, 2006: 1911–1918.

MW, Radomski. "The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide." *British Journal of Pharmacology*, 1987: 639-646.

N, Arber. "Celecoxib for the prevention of colorectal adenomatous polyps." *New England Journal of Medicine*, 2006: 885–895.

N, Cushman. "Epidemiology and risk factors for venous thrombosis." *Seminars in Thrombosis and Hemostasis*, 2007: 62-69.

N, Mackman. "New insights into the mechanisms of venous thrombosis." *Journal of Clinical Investigation*, 2012: 2331-2336.

N, Mackman. "Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis." *Arteriosclerosis Thrombosis and Vascular Biology*, 2007: 1687-1693.

N, Mackman. "Triggers, targets and treatments for thrombosis." *Nature*, 2008: 914-918.

N, Mackmann. "Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis." *Arteriosclerosis Thrombosis and Vascular Biology*, 2007: 1687-1693.

N, Maugeri. "Human polymorphomuclear leukocytes produce and express functional tissue factor upon stimulation." *JOurnal of Thrombosis and Haemostasis*, 2006: 1323–1330.

N, Maugeri. "Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation." *Journal of Thrombosis and Haemostasis*, 2006: 1323–1330.

N, Nagai. "Prothrombotic effect of Rofecoxib in a murine venous thrombosis model." *Thrombosis Research*, 2008: 668-673.

N, Serneri. " TxA2 production by human arteries and veins." *Prostaglandins*, 1983: 753–766.

N, Serneri. "Increased fibrinopeptide A formation and thromboxane A2 production in patients with ischemic heart disease: relationships to coronary pathoanatomy, risk factors and clinical manifestations." *American Heart Journal*, 1981: 185–194.

NA, Nussmeier. "Complications of the COX-2 inhibitors parecoxib and valdecoxib after cardiac surgery." *New England Journal of Medicine*, 2005: 1081-1091.

NN, Aleman. "Fibrinogen and red blood cells in venous thrombosis." *Thrombosis Research*, 2014: S38–S40.

NR, Ferreri. "Cyclooxygenase-2 expression and function in the medullary thick ascending limb." *American Journal of Physiology*, 1999: 360-368.

NS, Key. "Tissue factor and its measurement in whole blood, plasma, and microparticles." *Seminaris of Thrombosis and Haemostasis*, 2010: 865-875.

NV, Chandrasekharan. "COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic / antipyretic drugs: cloning, structure and expression." *Proceedings of the National Academy of Sciences*, 2002: 13926–13931.

NV, Serbina. "Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2." *Nature Immunology*, 2006: 311–317.

NV, Serbina. "Monocyte-mediated defense against microbial pathogens." *Annual Review of Immunology*, 2008: 421–452.

O, Aras. " Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia." *Blood*, 2004: 4545–4553.

O, Holtkotter. "Integrin α2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen. ." *Journa of Biological Chemistry*, 2002: 10789–10794.

O, Morel. "Procoagulant microparticles: disrupting the vascular homeostasis equation? ." *Arteriosclerosis Thrombosis and Vascular Biology*, 2006: 2594–2604.

Østerud, B. "Tissue factor expression by monocytes: regulation and." *Blood Coagulation and Fibrinolysis*, 1998: 9-14.

P, Andre. "CD40L stabilizes arterial thrombi by a β3 integrin–dependent mechanism." *Nature Medicine*, 2002: 247–252.

P, Andre. "Procoagulant state resulting from high levels of soluble P-selectin in blood." *Proceedings of the National Academy of Sciences*, 2000: 13835–13840.

P, Didisheim. "Animal models useful in the study of thrombosis and antithrombotic agents." *Prog Hemost Thromb journal*, 1972: 165-197.

P, Ferroni. "Platelet activation in type 2 diabetes mellitus." *Journal of Thrombosis and Haemostasis*, 2004: 1282-1291.

P, Gresele. "Thromboxane synthase inhibitors, thromboxane receptor antagonist and dual blockers in thrombotic disorders ." *Trends in Pharmacological Sciences*, 1991: 158–163.

P, Lacy. "Mechanisms of degranulation in neutrophils." *Allergy, Asthma & Clinical Immunology*, 2006: 98–108.

P, Li. "PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps." *Journal of Experimental Medicine*, 2010: 1853–1862.

P, Mangin. "Thrombin overcomes the thrombosis defect associated with platelet GPVI/FcRγ deficiency." *Blood*, 2006: 4346–4353.

P, Patrignani. "New insights into COX-2 biology and inhibition." *Brain Research Reviews*, 2005: 352–359.

P, Prandoni. "A simple ultrasound approach for detection of recurrent proximal-vein thrombosis." *Circulation*, 1993: 1730-1735.

P, Prandoni. "An association between atherosclerosis and venous thrombosis." *New England Journal of Medicine*, 2003: 1435-1441.

P, Prandoni. "Long-term outcomes after deep venous thrombosis of the lower extremities." *Vascular Medicine*, 1998: 57-60.

P, Prandoni. "The long-term clinical course of acute deep venous thrombosis." *Annals of International Medicine*, 1996: 1-7.

P, Prandoni. "The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism: a prospective cohort study in 1,626 patients." *Haematologica*, 2007: 199-205.

P, Prandoni. "Venous thromboembolism and the risk of subsequent symptomatic atherosclerosis." *Journal of Thrombosis and Haemostasis*, 2003: 1891-1896.

P, Saha. "Leukocytes and the natural history of deep vein thrombosis: current concepts and future directions." *Arteriosclerosis, Thrombosis and Vascular Biology*, 2011: 506-512.

P, Savi. "The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts." *Proceedings of the National Academy of Sciences*, 2006: 11069-11074.

P, Simioni. "The risk of recurrent venous thromboembolism in patients with an Arg506→Gln mutation in the gene for factor V." *The New England Journal of Medicine*, 1997: 399–403.

P, Ungprasert. "Non-steroidal anti-inflammatory drugs and risk of venous thromboembolism: a systematic review and meta-analysis." *Rheumatology (Oxford).*, 2015: 736-742.

PA, Kyrle. "Deep vein thrombosis." *Lancet*, 2005: 1163-1174.

PA, Kyrle. "High plasma levels of factor VIII and the risk of recurrent venous thromboembolism." *New England Journal of Medicine*, 2000: 457-462.

PA, Madureira. "The role of the annexin A2 heterotetramer in vascular fibrinolysis." *Blood* , 2011: 4789-4797.

PA, O'Connell. "Regulation of S100A10 by the PML-RAR-α oncoprotein." *Blood*, 2011: 4095–4105.

PD, Stein. "Estimated case fatality rate of pulmonary embolism." *American Journal of Cardiology*, 2004: 1979–1998.

PF, Fedullo. "Chronic thromboembolic pulmonary hypertension." *Clinics in Chest Medicine*, 2001: 561-581.

PK, Henke. "Deep vein thrombosis resolution is modulated by monocyte CXCR2-mediated activity in a mouse model." *Arteriosclerosis Thrombosis and Vascular Biology*, 2004: 1130-1137.

PK, Henke. "Targeted deletion of CCR2 impairs deep vein thrombosis resolution in a mouse model." *Journal of Immunology*, 2006: 3388-3397.

PL, Giesen. "Blood-borne tissue factor: another view of thrombosis." *Proceedings of the National Academy of Sciences*, 1999: 2311–2315.

PL, Gross. "Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development." *Journal of Leukocyte Biology*, 2005: 1318–1326.

PM, Farrehi. "Regulation of arterial thrombolysis by plasminogen activator inhibitor-1 in mice." *Circulation*, 1998: 1002-1008.

PM, Kearney. "Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Metaanalysis of randomised trials." *British Medical Journal*, 2006: 1302-1308.

PM, Mannucci. "The molecular basis of inherited thrombophilia." *Vox Sanguinis*, 2000: 39–45.

PV, Halushka. "Thromboxane, prostaglandin and leukotriene receptors." *Annual Review of Pharmacology and Toxicology* , 1989: 213–239.

Q, Cheng. "A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo." *Blood*, 2010: 3981–3989.

Q, Ling. "Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo." *The Journal of Clinical Investigation*, 2004: 38–48.

R, Collins. "Reduction in fatal pulmonary embolism and venous thrombosis by perioperative administration of subcutaneous heparin: overview of results of randomized trials in general, orthopedic, and urologic surgery." *New England Journal Medicine*, 1988: 1162-1173.

R, Darbousset. "Implication des neutrophiles dans la formation d'un thrombus in vivo chez la souris." *Pathologie Biologie*, 2014: 1-9.

R, Darbousset. "Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation." *Blood*, 2012: 2133–2143.

R, Mischke. "Detection of anticoagulant activities of isolated canine fibrinogen degradation products X, Y, D and E using resonance thrombography." *Blood Coagulation and Fibrinolysis*, 2004: 81–88.

R, Mischke. "Influence of fibrinogen degradation products on thrombin time, activated partial thromboplastin time and prothrombin time of canine plasma." *Haemostasis*, 2000: 123–130.

R, van Furth. "The origin and kinetics of mononuclear phagocytes." *JOurnal of Experimental Medicine*, 1968: 415-435.

R, Virchow. "Thrombosis and Emboli." *Book Review*, 1846.

R, Ye. "Circulating tissue factor positive microparticles in patients with acute recurrent deep venous thrombosis." *Thrombosis Research*, 2012: 253-258.

RA, Ariëns. "Fibrin(ogen) and thrombotic disease." *Journal of Thrombosis and Haemostasis*, 2013: 294–305.

RA, Campbell. "Flow profoundly influences fibrin network structure: implications for fibrin formation and clot stability in haemostasis." *Thrombosis and Haemostasis*, 2010: 1281–1284.

RA, Copeland. "Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase." *Proceedings of the National Academy of Sciences*, 1994: 11202–11206.

RA, Fleck. "Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal antihuman tissue factor antibody." *Thrombosis Research*, 1990: 421–437.

RA, Kraaijenhagen. "High plasma concentration of factor VIIIc is a major risk factor for venous thromboembolism." *Thrombosis and Haemostasis*, 2000: 5-9.

RD, Hull. "Cost-effectiveness of primary and secondary prevention of fatal pulmonary embolism in high-risk surgical patients." *Canadian Medical Association Journal*, 1982: 990-995.

RE, Rumbaut. "Platelet-vessel wall interactions in hemostasis and thrombosis." *Morgan & Claypool Life Sciences*, 2010.

Report, Consensus Development Conference. "Prevention of venous thrombosis and pulmonary embolism." *JAMA*, 1986: 744-749.

RF, Doolittle. "Searching for differences between fibrinogen and fibrin that affect the initiation of fibrinolysis." *Cardiovascular & Hematological Agents in Medicinal Chemistry*, 2008: 181–189.

RH, Bourgain. "The inhibitory effect of PGI2 (prostacyclin) on white platelet arterial thrombus formation." *Haemostasis*, 1979: 117-119.

RH, Hunt. "The gastrointestinal safety of the COX-2 selective inhibitor etoricoxib assessed by both endoscopy and analysis of upper gastrointestinal events." *The American Journal of Gastroenterology* , 2003: 1725–1733.

RH, White. "The epidemiology of venous thromboembolism." *Circulation*, 2003: I4-8.

RJ, Goldberg. "Occult malignant neoplasm in patients with deep venous thrombosis." *Archives of Internal Medicine*, 1987: 251-253.

RJ, Gryglewski. "Reversal of platelet aggregation by prostacyclin." *Pharmacological Research Communications*, 1978: 185-189.

RM, Bertina. "Mutation in blood coagulation factor V associated with resistance to activated protein C." *Nature,* , 1994: 64–67.

RM, Senior. "Effects of fibrinogen derivatives upon the inflammatory response. Studies with human fibrinopeptide B." *Journal of Clinical Investigation*, 1986: 1014–1019.

RN, Dubois. "Cyclooxygenase in biology and disease." *FASEB Journal*, 1998: 1063–1073 .

RS, Bresalier. "Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial." *New England Journal of Medicine*, 2005: 1092-1102.

RS, Eidelman. "An update on aspirin in the primary prevention of cardiovascular disease." *Archives of Internal Medicine*, 2003: 2006-2010.

RW, Colman. "The epidemiology, pathogenesis, and natural history of venous thrombosis." *Hemostasis and thrombosis: basic principles and clinical practice*, 1993: 1275-1298.

S, Alias. "Coagulation and the vessel wall in pulmonary embolism." *Pulmonary Circulation*, 2013.

S, Butenas. "Tissue factor activity in whole blood." *Blood*, 2005: 2764–2770.

S, Dawson. "Genetic variation at the plasminogen activator inhibitor-1 locus is associated with altered levels of plasma plasminogen activator inhibitor-1 activity." *Arteriosclerosis and Thrombosis*, 1991: 183-190.

S, Del Turco. "Procoagulant activity of circulating microparticles is associated with the presence of moderate calcified plaque burden detected by multislice computed tomography." *Journal of Geriatric Cardiology*, 2014: 13-19.

S, Eichinger. "D-dimer levels and risk of recurrent venous thromboembolism." *Jama*, 2003: 1071-1074.

S, Eligini. "Indobufen inhibits tissue factor in human monocytes through a thromboxane-mediated mechanism." *Cardiovascular Research*, 2006: 218-226.

S, Fuse. "Plasma thromboxane B2 concentrations in pulmonary hypertension associated with congenital heart disease." *Circulation*, 1994: 2952–2955.

S, Gruner. "Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo." *Blood*, 2003: 4021–4027.

S, Jones. "Extracellular Ca2+ modulates ADP-evoked aggregation through altered agonist degradation: implications for conditions used to study P2Y receptor activation." *British Journal of Haematology*, 2011: 83-91.

S, Kimura. "Cross-linking site in fibrinogen for alpha 2-plasmin inhibitor." *The Journal of Biological Chemistry*, 1986: 15591–15595.

S, Konstantinides. "Distinct antithrombotic consequences of platelet glycoprotein Ib and VI deficiency in a mouse model of arterial thrombosis." *Journal of Thrombosis and Haemostasis*, 2006: 2014-2021.

S, Lavu. "Sirtuins–novel therapeutic targets to treat age-associated diseases." *Nature Reviews Drug Discovery*, 2008: 841-853.

S, M.Day. "Murine thrombosis models." 2004.

S, Massberg. "A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo." *Journal of WExperimental Medicine*, 2003: 41-49.

S, Massberg. "Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases." *Nature Medicine*, 2010: 887–896.

S, Moncada. "An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation." *Nature*, 1976: 663-665.

S, Moncada. "Human arterial and venous tissues generate prostacyclin (prostaglandin x), a potent inhibitor of platelet aggregation." *Lancet*, 1977: 18-20.

S, Nakamura. "Tissue factor in neutrophils: yes." *Journal of Thrombosis and Haemostasis*, 2004: 214–217.

S, Narumiya. "Prostanoid receptors: structure, properties and functions." *Physiological Reviews*, 1999: 1193–1226.

S, Réty. "The crystal structure of a complex of p11 with the annexin II N-terminal peptide." *Nature Structural & Molecular Biology*, 1999: 89-95.

S, Schulman. " RE-MEDY Trial Investigators; RE-SONATE Trial Investigators. Extended use of dabigatran, warfarin, or placebo in venous thromboembolism." *New England Journal of Medicine*, 2013: 709-718.

S, Schulman. "Dabigatran versus warfarin in the treatment of acute venous thromboembolism." *New England Journal of Medicine*, 2009: 2342-2352.

S, Sevitt. "The structure and growth of valve-pocket thrombi in femoral veins." *Journal of Clinical Pathology*, 1974: 517-528.

S, Steinemann. "Role of the lipopolysaccharide (LPS)-binding protein/CD14 pathway in LPS induction of tissue factor expression in monocytic cells." *Arteriosclerosis and Thrombosis*, 1994: 1202-1209.

S, Thorsen. "The mechanism of plasminogen activation and the variability of the fibrin effector during tissue-type plasminogen activator-mediated fibrinolysis." *Annals of the New York Academy of Sciences*, 1992: 52–63.

SD, Katugampola. "Thromboxane receptor density is increased in human cardiovascular disease with evidence for inhibition at therapeutic concentrations by the AT(1) receptor antagonist losartan." *British Journal of Pharmacology*, 2001: 1385–1392.

SD, Katugampola. "Thromboxane receptor density is increased in human cardiovascular disease with evidence for inhibition at therapeutic concentrations by the AT1 receptor antagonist losartan." *British Journal of Pharmacology*, 2009: 1385–1392.

SD, Solomon. "Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention." *New England Journal of Medicine*, 2005: 1071-1080.

SD, Solomon. "Relationship between selective cyclooxygenase-2 inhibitors and acute myocardial infarction in older adults." *Circulation*, 2004: 2068–2073.

SH, Orkin. "Hematopoiesis. An evolving paradigm for stem cell biology." *Cell*, 2008: 631–644.

SI, Rapaport. "Pseudomonas septicemia with intravascular clotting leading to the generalized Shwartzman." *The New England Journal of Medicine*, 1964: 80-84.

SJ, Wilson. "Activation-dependent stabilization of the human thromboxane receptor: role of reactive oxygen species." *Journal of Lipids Research*, 2009: 1047-1056.

SK, Myung. "Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials." *British Medical Journal*, 2013: 10.

SL, Targum. "Consultation on NDA 21-042, S-007: review of cardiovascular safety database (on Vioxx or rofecoxib)." *http://www.fda.gov/ohrms/dockets/ac/01/briefing/3677b2_06_cardio.doc.*, 2001.

SM, Day. "Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall." *Blood*, 2005: 192-198.

SM, Miggin. "Expression and tissue distribution of the mRNAs encoding the human thromboxane A2 receptor (TP) alpha and beta isoforms." *Biochimica et Biophysica Acta*, 1998: 543–559.

SM, Rankin. "The bone marrow: a site of neutrophil clearance." *Journal of Leukocyte Biology*, 2010: 241–251.

SR, Coughlin. "Protease-activated receptors in hemostasis, thrombosis and vascular biology." *Journal of Thrombosis and Haemostasis*, 2005: 1800–1814.

SR, Coughlin. "Protease-activated receptors in hemostasis, thrombosis and vascular biology." *ournal of Thrombosis and Haemostasis*, 2005: 1800-1814.

SR, Coughlin. "Protease-activated receptors in hemostasis, thrombosis and vascular biology." *Journal of Thrombosis and Haemostasis*, 2005: 1800-1814.

SR, Poort. "A common genetic variation in the 3-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis." *Blood*, 1996: 3689–3703.

SR, Yang. "Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kappaB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging." *American Journal of Physiology*, 2007: L567–L576.

SS, Barbieri. "Abnormal megakaryopoiesis and platelet function in cyclooxygenase-2-deficient mice." *Thrombosis and Haemostasis*, 2015: 1218-1229.

SS, Barbieri. "Cyclooxygenase-2-Derived Prostacyclin Regulates Arterial Thrombus Formation by Suppressing Tissue Factor in a SIRT1-Dependent-Manner ." *Circulation*, 2012.

Statement, European Consensus. "Prevention of venous thromboembolism." *London: Med-Orion Publishing Co*, 1992.

SW, Rowlinson. "A novel mechanism of cyclooxygenase-2 inhibition involving interactions with Ser530 and Tyr385." *The Journal of Biological Chemistry*, 2003: 45763–45769.

SY, Yildiz. "Functional Stability of Plasminogen Activator Inhibitor-1." *The Scientific World Journal*, 2014: 1-11.

SZ, Goldhaber. "Cancer and venous thromboembolism." *Archives of Internal Medicine*, 1987: 216-216.

SZ, Goldhaber. "Report of the WHO/International Society and Federation of Cardiology Task Force." *JAMA*, 1992: 1727-1733.

T, Ali. "Monocyte recruitment in venous thrombus resolution." *Journal of Vascular Surgery*, 2006: 601-608.

T, Astrup. "Protease content and fibrinolytic activity of human leukocytes." *Blood*, 1967: 134–138.

T, Hirata. "Two thromboxane A2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation." *Journal of Clinical Investigation*, 1996: 949-956.

T, Kanaji. "Amelioration of the macrothrombocytopenia associated with the murine Bernard-Soulier syndrome." *Blood*, 2002: 2102-2107.

T, Koster. "Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis." *Lancet*, 1995: 152–155.

T, Renné. "Defective thrombus formation in mice lacking coagulation factor XII." *Journal of Experimental Medicine*, 2005: 271–281.

T, Renné. "In vivo roles of factor XII." *Blood*, 2012: 4296–4303.

T, van der Hulle. "Selective D-dimer testing for the diagnosis of acute deep vein thrombosis: a validation study." *Journal of Thrombosis and Haemostasis*, 2013: 2184–2186.

T, van der Hulle. "Variable D-dimer thresholds for diagnosis of clinically suspected acute pulmonary embolism." *Journal of Thrombosis and Haemostasis*, 2013: 1986–1992.

TA, Brighton. "ASPIRE Investigators. Low-dose aspirin for preventing." *New England Journal of Medicine*, 2012: 1979-1987.

TA, Drake. "Immunohistochemical assessment of tissue factor and thrombomodulin expression in tissues of baboons with lethal e. coli sepsis." *FASEB Journal*, 1991: A1437.

TA, Drake. "Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis." *American Journal of Pathology*, 1989: 1087-1097 .

TA, Drake. "Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis." *American Journal of Pathology*, 1989: 1087–1097.

TA, Drake. "Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis." *American Journal of Pathology*, 1989: 1087–1097.

TA, Fuchs. "Extracellular DNA traps promote thrombosis." *Proceedings of the National Academy of Sciences*, 2010: 15880–15885.

TA, Fuchs. "Neutrophil extracellular trap (NET) impact on deep vein thrombosis." *Arteriosclerosis Thrombosis and Vascular Biology* , 2012: 1777–1783.

TA, Fuchs. "Novel cell death program leads to neutrophil extracellular traps." *The Journal of Cell Biology*, 2007: 231–241.

TA, Morinelli. "Characterisation of thromboxane A2/prostaglandin H2 receptors in human vascular smooth muscle cells." *Life Science*, 1990: 1765–1772.

TH, Howard. "Lipopolysaccharide modulates chemotactic peptide-induced actin polymerization in neutrophils." *Journal of Leukocyte Biology*, 1990: 13–24.

TH, Welling. "Tissue plasminogen activator increases canine endothelial cell proliferation rate through a plasmin-independent, receptor-mediated mechanism." *Journal of Surgery Research*, 1996: 36–42.

TM, Connolly. "Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides." *Journal of Thrombosis and Haemostasis*, 1994: 627–633.

TM, Hyers. "Antithrombotic therapy for venous thromboembolic disease." *Chest*, 1992: 1636.

TR, Ittyerah. "Effect of fibrin degradation products and thrombin on fibrinogen synthesis." *British Journal of Haematology*, 1979: 661–668.

TW, Wakefield. "Neovascularization during venous thrombosis organization(a preliminary study)." *Journal of Vascular Surgery*, 1999: 885–892.

U, Rescher. "Annexins—unique membrane binding proteins with diverse functions." *Journal of Cell Science*, 2004: 2631–2639.

U, Rescher. "S100A10/p11: family, friends and functions." *European Journal of Physiology*, 2008: 575–582.

UY, Schaff. "Orai1 regulates intracellular calcium, arrest, and shape polarization during neutrophil recruitment in shear flow." *Blood*, 2010: 657–666.

V, Brinkmann. "Neutrophil extracellular traps kill bacteria." *Science*, 2004: 1532–1535.

V, Capra. "Impact of vascular thromboxane prostanoid receptor activation on hemostasis, thrombosis, oxidative stress, and inflammation." *Journal of Thrombosis and Haemostasis*, 2014: 126-137.

V, Cavalca. "Simultaneous quantification of 8-iso-prostaglandin-F(2alpha) and 11-dehydro thromboxane B(2) in human urine by liquid chromatography-tandem mass spectrometry." *Analytical Biochemistry*, 2010: 168-174.

V, Cécile. "Platelet Adhesion Receptors and Their Ligands in Mouse Models of Thrombosis." *Arteriosclerosis Thrombosis and Vascular Biology*, 2007: 728-739.

V, Gerke. "Annexins: from structure to function." *Physiological Reviews*, 2002: 331-371.

V, Gerke. "Annexins: linking Ca2 + signalling to membrane dynamics." *Nature Reviews Molecular Cell Biology*, 2005: 449–461.

V, Kakkar. "Prevention of venous thrombosis and pulmonary embolism." *American Journal of Cardiology*, 1990: 50-54.

V, Videm. "Changes in neutrophil surface-receptor expression after stimulation with FMLP, endotoxin, interleukin-8 and activated complement compared to degranulation." *Scandinavian Journal of Immunology*, 2004: 25–33.

VV, Kakkar. " Prophylaxis of venous thromboembolism." *World Journal of Surgery* , 1990: 670-678.

VV, Kakkar. "Prophylaxis of venous thromboembolism." *World Journal of Surgery*, 1990: 670-678.

W, Bergmeier. "Structural and functional characterization of the mouse von Willebrand factor receptor GPIb-IX with novel monoclonal antibodies." *Blood*, 2000: 886-893.

W, Bergmeier. "The role of platelet adhesion receptor GPIb far exceeds that of its main ligand, von Willebrand factor, in arterial thrombosis." *Proocedings of the National Academy of Science*, 2006: 16900-16905.

W, Thomas D. "Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2." *Journal of Clinical Investigation*, 1998: 1994-2001.

WA, Lensing. "Deep-vein thrombosis." *Lancet*, 1999: 479-485.

WC, Hooper. "Venous thromboembolism hospitalizations among American Indians and Alaska Natives." *Thrombosis Research*, 2002: 273-278.

White, Richard H. "The Epidemiology of Venous Thromboembolism." *Circulation*, 2003: 4-8.

WL, Smith. "Cyclooxygenases: structural, cellular, and molecular biology." *Annual Review of Biochemistry* , 2000: 145-182.

WP, Jeske. "A survey of venous thrombosis models." *Methods in Molecular Medicine*, 2004: 221-237.

WR, Gould. "Gas6 receptors Axl, Sky and Mer enhance platelet activation and regulate thrombotic response." *Journal of Thrombosis and Haemostasis*, 2005: 733-741.

WR, Gould. "Megakaryocytes endocytose and subsequently modify human factor V in vivo to form the entire pool of a unique platelet-derived cofactor." *Journal of Thrombosis and Haemostasis*, 2005: 450–456.

X, Li. "The physical exchange of factor VIII (FVIII) between von Willebrand factor and activated platelets and the effect of the FVIII B-domain on platelet binding." *Biochemistry*, 1997: 10760–10767.

X, Wang. "Effects of factor XI deficiency on ferric chloride-induced vena cava thrombosis in mice." *Journal of Thrombosis and Haemostasis*, 2006: 1982-1988.

X, Wang. "Murine model of ferric chloride-induced vena cava thrombosis: evidence for effect of potato carboxypeptidase inhibitor." *Journal of Thrombosis and Haemostasis*, 2006: 403-410.

XD, Yang. "Functional interplay between acetylation and methylation of the RelA subunit of NF-kappaB." *Molecular and Cellular Biology*, 2010: 2170-2180.

Y, Cheng. "Role of Prostacyclin in the Cardiovascular Response to Thromboxane A2." *Science*, 2002: 539.

Y, Laumonnier. "Identification of the annexin A2 heterotetramer as a receptor for the plasmin-induced signaling in human peripheral monocytes." *Blood*, 2006: 3342-3349.

Y, Nemerson. "Tissue factor and hemostasis." *Blood*, 1988: 1-8.

Y, Urade. "Prostaglandin D, E, and F synthases." *Journal of Lipid Mediators and Cell Signalling*, 1995: 257–273.

ZM, Ruggeri. "Platelet Adhesion under Flow ." *Microcirculation*, 2009: 58-83.

ZM, Ruggeri. "Platelets in atherothrombosis." *Nature Medicine*, 2002: 1227-1234.

ZM, Ruggeri. "Von Willebrand factor." *Current Opinion in Hematology*, 2003: 142-149.

ZS, Latallo. "Inhibition of fibrin polymerization by fibrinogen proteolysis products." *American Journal of Physiology*, 1962: 681–686.

Ringraziamenti

Ed eccomi qui, finalmente anche questo traguardo è stato raggiunto. Beh, le persone che desidero ringraziare sono davvero tantissime, ma prima di tutto sento di dover ringraziare la mia famiglia, che mi ha permesso di raggiungere questo obiettivo standomi accanto anche nei momenti più difficili: la mia mamma, con le nostre lunghe telefonate serali e sempre pronta a sostenermi, babbo con i suoi consigli molto preziosi, e mio fratello, che per me, nonostante l'età, rimarrà sempre "il mio fratellino"!

Ed insieme alla mia famiglia desidero ringraziare quella che ora, da un anno e mezzo, rappresenta la mia "nuova famiglia", ossia mio marito. Grazie per avermi sopportata e supportata! So che non è sempre stato facile starmi accanto nei miei momenti deliranti! L'averti vicino, con i tuoi consigli e la tua positività, mi ha aiutata davvero molto. Grazie marito!

Subito dopo viene quella che ho conosciuto come una collega, ma che presto è diventata anche una grande amica… la mia Pathway, detta anche Gigia (sorvolo sul soprannome datoti dal Manz!). Quante risate Pathway, quante emozioni abbiamo vissuto insieme e quante volte ci siamo sollevate a vicenda. Le nostre confidenze, i nostri momenti di delirio, le nostre risate fino alle lacrime… ormai è già da qualche mese che non lavoro più con te. Ho dovuto fare una scelta molto difficile andando via da quel laboratorio, Ma neanche per un secondo ho pensato che qualcosa sarebbe cambiato tra noi. Ti voglio troppo bene! Sei una persona speciale…

Così come non cambierà e non passerà l'amicizia con te Casty! Anche se non lavoriamo più insieme continuiamo a sentirci ogni tanto e a rivederci con i nostri aperitivi intrisi di confidenze, consigli ed aggiornamenti! Quegli aperitivi non mancheranno mai! Anche in te ho trovato una persona dolcissima e sempre pronta a dispensare un consiglio, pronta anche ad accettare confidenze non sempre condivise, senza però dare mai un giudizio negativo. Grazie Casty.

E con te Casty, colgo l'occasione per citare Paolino, che col suo modo di vestire allucinante con accostamenti di colore improponibili (Paolino, ti prego, datti una regolata!) mi ha dato modo di commentare quotidianamente il suo outfit! E come non menzionare Gigi, o meglio "m…..a Gigi"! scusa non ho resistito!

Ovviamente non posso non ringraziare te Annina, che mi hai fatto conoscere tutte queste persone mettendomi in contatto con quello che poi è stato "il mio lab" per questi anni. Sempre amiche, da più di 10 anni!

E i compagni di pranzo dove li mettiamo??? Tu "Church" che mi hai fatto da seconda mamma con i tuoi consigli e il tuo modo di fare sempre così rassicurante. Una donna di una dolcezza incredibile, che anche se ogni tanto si abbatte a causa della sua profonda sensibilità, è sempre in grado di rialzarsi e sfoggiare un sorriso meraviglioso. Donna da ammirare, senza dubbio. Cinzia, con la quale c'era sempre stato qualcosa di cui parlare. Giuly e Fede, che non posso che vedere come due sorelline minori, anche se in realtà la differenza d'età non è poi chissà quanta! Eppure le vedo così, con dolcezza! Meglio sorvolare sui nostri argomenti… Manz… che dire! Unico! Davvero unico! A volte insopportabile con il suo essere cinico, ma al tempo stesso sorprendentemente e pazzescamente divertentissimo! Ancora adesso, sentirti anche solo per messaggi, mi fa piangere dal ridere!

E poi tante altre persone che ho avuto il piacere di conoscere in questo percorso: la Manzoni! Quante volte ti ho fatto venire un infarto urlando il tuo nome a squarciagola nei corridoi! Silvia Casty, "scoperta" forse troppo tardi purtroppo e compagna di tragitto. Gerry, con le sue poesie dolcissime e i suoi complimenti che mi hanno sempre strappato un sorriso anche nei momenti difficili. Mary e Gianca, sempre disposti a scambiare due chiacchere. E tutti coloro che non ho menzionato ma che comunque sanno di occupare un posticino nel mio cuore e nei miei pensieri.

Infine un ringraziamento dovuto va a Silvia, che mi ha seguita in tutto questo percorso dandomi fiducia e portandomi a raggiungere questo obiettivo.

Un grazie di cuore a tutti.